NEUROENDOCRINE CONTROL OF FEEDING BEHAVIOR

EDITED BY: Serge H. Luquet, Riccarda Granata and Hubert Vaudry
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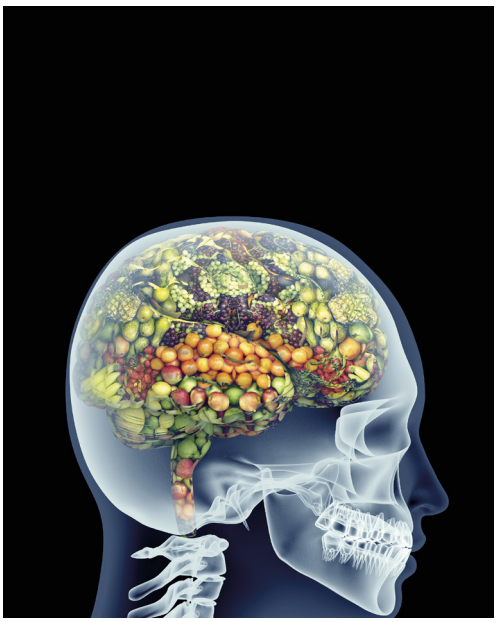
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NEUROENDOCRINE CONTROL OF FEEDING BEHAVIOR

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In both human and rodent proper feeding behavior is essential for survival. Various factors contribute to modulate how dedicated neuro circuits control feeding behavior.
Image: Original drawing by Serge Luquet.

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The hypothalamus plays a crucial role in the regulation of food intake and energy homeostasis. Hypothalamic neuronal circuits thus represent a privileged target for the treatment of eating disorders and metabolic diseases. The present eBook constitutes a unique collection of research articles and reviews that highlight new concepts and recent findings about the neuroendocrine control of feeding behavior.

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EDITORIAL
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Editorial: Neuroendocrine Control of Feeding Behavior

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Editorial on the Research Topic

Neuroendocrine Control of Feeding Behavior

Obesity and metabolic disorders represent major worldwide health threats. Neuroendocrine mechanisms, which play a pivotal role in the integration of hunger and satiety signals, and the regulation of energy homeostasis, holds the promises of new strategies for treatment of feeding-related diseases. This Research Topic compiles a series of review and research articles that provide a broad view of the current knowledge on the complex neuroendocrine control of feeding behavior and energy expenditure, and highlights new concepts in the field.

The hypothalamus is a key region of the brain regulating food intake and energy balance. Interestingly, deregulation of feeding behavior, causing weight loss or obesity, has been associated with high- or low-grade hypothalamic inflammation, respectively (1, 2). In their review, Le Thuc et al. focus on hypothalamic inflammation, with special emphasis on how chemokines can influence, at the hypothalamic level, the deregulation of energy balance and body weight. Indeed, in addition to being essential mediators of the inflammatory response, chemokines exert important roles at the central level by activating and attracting cells of the immune system, regulating neuronal survival and death, and also modulating the activity of certain neurons (3). Therefore, the chemokinergic system could be responsible for the deregulation of feeding behavior associated with inflammation, in both appetite and weight loss, and the development of obesity.

In relation to obesity, it has been shown that individual differences in neurobiological mechanisms controlling food intake may explain why some individuals are more susceptible to weight gain than others (4). Interestingly, one of these mechanisms is impulsivity, generally considered as the tendency to act rapidly without full consideration of consequences (5). In this respect, Michaud et al. provide a comprehensive review on alterations related to impulsivity in obesity and addiction, considering results from the personality, neurocognitive, brain imaging, and clinical fields. Overall, this review provides an approach to understand the association between obesity and addictive behaviors and, on these bases, suggests therapeutic interventions for prevention and treatment of obesity.

Obesity and diabetes have been also linked to cognitive dysfunction (6). In her review, Small investigates the mechanisms underlying the association between obesity and diabetes, and cognitive impairments and brain dysfunction, which at present are still unknown. Although studies have shown integrity of the dopamine (DA) system in cognitive dysfunction associated with diabetes and obesity, a critical role for DA adaptation in response to diet, adiposity and metabolic dysfunction has been proposed, which may explain the neurocognitive impairment observed in diabetes and obesity. However, the mechanisms underlying the involvement of the DA system in these effects remain to be clarified and will be the focus of future research.

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In their study, Hankir et al. investigated whether alterations in fat appetites after Roux-en-Y gastric bypass (RYGB) associate with variations in brain μ-opioid receptors (MORs). Results from diet-induced obese male rats, undergoing RYGB, show an association between suppression of appetite, at a stage of weight loss after RYGB, and reduction of MORs, suggesting that the reduction in MOR signaling may contribute to sustained weight loss.

In relation to appetite and obesity, Santiago and Hallschmid investigated the effect of intranasal administration of insulin before sleep in healthy young and elderly humans on eating behavior in the subsequent morning. In fact, it has been shown that intranasal administration of insulin in humans reduces food intake, independently of its glucoregulatory action (7). The results of this study show that intranasal insulin administration before nocturnal sleep induces a reduction in breakfast intake in healthy subjects, with no change in energy expenditure, suggesting that, depending on sleep period, insulin may display beneficial metabolic effects and even treat or prevent insulin resistance in the brain.

Glucocorticoid hormones (GCs) are essential in the regulation of glucose and fatty acid metabolism, as well as appetite. Conditions of excess or deficiency of GC levels, such as those encountered in Cushing's syndrome or Addison's disease, respectively, are associated with severe metabolic alterations (8). Moisan and Castanon review the role of corticosteroid-binding globulin (CBG) in the regulation of GC levels. In addition to studies demonstrating the importance of CBG in influencing genetic variability of plasma GC levels, a link between CBG levels and body composition/insulin levels has also been indicated (9). Overall, recent studies have proposed a role for CBG in metabolic disorders associated with impairment of GC levels.

Ghrelin is a 28-amino acid acylated peptide produced mainly in the stomach, which has the ability to stimulate growth hormone secretion; in addition, ghrelin stimulates feeding, adiposity, and weight-gain (10). Different mechanisms are involved in the regulation of ghrelin production and signaling. It has been recently demonstrated that circulating ghrelin is in part protected from degradation by binding to immunoglobulins (Ig) (11). The review by Fetissov et al. summarizes the results on acylated ghrelin (AG) and des-acyl ghrelin (DAG) reactive Ig in conditions of altered appetite and energy balance. Overall, the authors suggest that Ig display a role in modulating the biological activities of ghrelin, including those in conditions of altered energy balance, and propose the existence of a functional link between Ig and ghrelin.

In addition to AG, the ghrelin gene-derived peptides include DAG, the most abundant form in the plasma, which binds to a yet unknown receptors and is devoid of endocrine activities, and obestatin, which displays metabolic effects but whose functions and binding characteristics are still to be fully determined (10, 12). Due to the absence of reliable assays to measure all three peptides, in their study, Hassouna et al. developed selective immunoassays combined with a highly sensitive targeted mass spectrometry method to measure and characterize the ratios of the different preproghrelin-derived peptides in mice. To validate their analyses, a preproghrelin deficient mice, that does not produce any of the peptides, was used as negative control. The results show that both forms of ghrelin and obestatin can be detected in the gastrointestinal tract of mice. Interestingly, in this organ, obestatin was found to be far less abundant than AG and DAG, likely because of reduced processing rate of preproghrelin into obestatin, or degradation of the peptide itself. It remains to be established if the main source of obestatin or its processing is outside of the gastrointestinal tract.

The hypothalamus-brainstem circuit defines a fundamental neural substrate that integrates circulating signals of hunger and satiety, nutrient, and hormones to promote adaptive behavioral and metabolic response to changes in energy demands. In order to access neural circuits these signals have to cross the blood brain barrier (BBB) (13, 14). Haddad-Tóvolli et al. provide an extensive review covering the major advances in the understanding of the BBB structure with a specific focus on hypothalamic areas. They discuss how structural adaptation of the BBB and neighboring cells such as endothelial cells, astrocytes, pericytes, tanycyte, and microglia orchestrate the regulated passage of signals from blood to brain. They also call our attention on how nutrient overload might damage the integrity and functionality of the BBB, and in turn affect neural processing of peripheral signals and appropriate control of energy homeostasis.

In the same line several contributions draft important observations regarding brain nutrient sensing. Indeed, in addition to providing energy to brain cells through their catabolism, nutrients can also be detected directly and can act as hormonal-like signals to regulate neuron and glial cell activity (15–18). Nutrient overload during consumption of energy-rich diet (fat and sugar) or in the context of obesity is associated with brain inflammatory response (19). However, it is unclear whether nutrient can directly target brain cells to create adaptive responses. Belegri et al. compared the consequence of free choice access to high-fat high-sugar diet (fcHFHS) and brain-specific infusion of lipid emulsion on endoplasmic reticulum (ER) stress and unfolded protein response (UPR) in the hypothalamus. They observed that short term exposure to a fcHFHS diet, followed by food restriction induces hypothalamic ER stress in rats, a response that was achieved comparably by direct lipid—but not glucose infusion in the brain, suggesting a direct contribution of brain lipid-sensing in that processes. This work, together with other advances in the field over the last decade, has promoted the concept that lipids entering the brain can affect neural activity and regulate, to the same extend as hormones, neural output. Bruce et al. provide an expert overview on lipid processing in the brain and cover several aspects of the mechanism by which neuron lipid sensing can result in the control of energy homeostasis. They specifically draw our attention on physiological processes by which hypothalamic neurons can detect circulating fatty acids and orchestrate the synthesis and release of triglyceride (TG) rich particles by the liver through the modulation of the autonomic nervous system outflow.

Indeed, circulating TGs represent a major source of lipid-substrate for metabolically active tissues such as heart and muscle. TGs typically increase after a meal and are packaged as chylomicrons released by the digestive tract (20). TGs also are part of liver-born lipoproteins including very-low or low

density lipoproteins (VLDL, LDL). Lipoprotein lipase (Lpl) is the rate limiting enzyme for the catabolism of TGs into free fatty acids. Lpl is actively expressed in the brain, suggesting a role for brain TG sensing (21). Laperrousaz et al. elegantly show that hypothalamic Lpl is an important mediator of brain adaptive response to cold and thermogenesis. Using hypothalamic-specific invalidation approaches they demonstrate that mice lacking LPL in the hypothalamic region show increase energy expenditure and conserved body temperature. This work points out that Lpl-mediated TG-sensing in lipid-sensing neurons contribute to central regulation of thermogenesis.

Aside of lipid-sensing neurons, the hypothalamus also contains glucose-activated and glucose-inhibited neurons. The ability of these neurons to respond to glucose is an important mechanism in hypothalamic control of energy homeostasis (22). Among the different mechanisms involved in glucose sensing, Kohno et al. call our attention on sweet taste receptor-mediated glucose sensing. Sweet taste receptors are composed of heterodimers of taste type1 receptor2 (T1R2) and taste type1 receptor3 (T1R3). These receptors are widely distributed in various organs including the hypothalamus. The authors used calcium imaging to probe sweet-taste receptor signaling in arcuate neurons. They demonstrate that artificial sweeteners such as sucralose can trigger calcium response mostly in non-POMC neurons. They point to an important mechanism by which artificial sweeteners, that are widely abundant in modern food environment, may potentially alter brain-nutrient sensing and energy homeostasis.

Finally, among nutrients, amino acids (AA) are also known to signal in neural substrate regulating energy homeostasis (17). Nutritional protein input together with AA quality have great influence on metabolism and behavior. Several redundant mechanisms have evolved to insure proper balance in AA quality and quantity. Heeley and Blouet review the literature pertaining to brain AA sensing mechanisms and neural coupling to adaptive behavior. Notably, the authors highlight how bidirectional changes in essential AA availability are detected through mTOR and general control non-derepressible 2 (GCN2) pathway to adapt feeding behavior and tropism.

Several elaborated strategies are in place to optimize food consumption. Food intake responds to energy demands and metabolic needs but also to reward and emotional inputs that are processed in several brain structures integrating cognitive inputs. Competing signals such as anxiety and palatable food reward are often at play in adapted strategies and decision making behavior (23, 24). In this context, Lockie et al. explored reward-driven feeding behavior in mice in an anxiogenic context. They show that food-deprivation or ghrelin injection used as a proxy of hunger signal increase food-reward seeking and consumption in anxiogenic environment while glucose injection or *ad libitum* feeding reduce it. This works highlight how metabolic signal/nutrient can influence the assessment of safety in food-reward seeking in a risky environment. Reward encoding depends on dopamine release in the mesolimbic system (MCL) (24). The gut-brain axis has emerged as one key pillar of energy homeostasis (25). The role of the gut microbiota has particularly drawn much attention in the recent years as potentially holding important keys in whole-body homeostasis (26, 27). While microbiota manipulation can be achieved through prebiotic supplementation and has readily impact on body weight control, it remains unclear whether reward-driven components can be manipulated through digestive fibers consumption. Delbès et al. examined this aspect and show that prebiotic supplementation affects various components of food reward seeking behavior, gut microbiota ecosystem and molecular adaptation in both hypothalamic and mesolimbic structures. They found that energy-rich diet and probiotic supplementation can exert synergistic action on food reward seeking behavior and brain expression of neuropeptides involved in the regulation of body weight homeostasis. Both the nature of the diet (regular chow or HFHS) as well as the timing at which prebiotic supplementation is introduced over the course of obesogenic diet exposure greatly influence the molecular and behavioral changes underlying reward-driven behavior.

While overfeeding represents one common hallmark of obesity in modern food environment, anorexia nervosa lies at the opposite site of the spectrum and represents a devastating eating disorder whose mechanism is still largely undefined, partly due to the lack of animal model for anorexia (28). Activity-based anorexia (ABA) is a model of anorexia-like body weight loss and decreased feeding in which animals are subjected to running wheel while giving restricted-time scheduled access to food. Using that model Scharner et al. demonstrate that while female rats undergoing ABA protocol do not show alteration of short-term meal ultrastructure, brain c-fos analysis (as a proxy for neuronal activation) reveals important differences between *ad libidum* and ABA animals characterized by increased c-fos signal in brain structures controlling energy homeostasis such as the arcuate nucleus, supraoptic nucleus, locus coeruleus (LC) and nucleus of the solitary tract. This work provides an important insight into one of the rare models of body weight loss although, as pointed by the authors, this model does not fully recapitulate human anorexia as animals do not voluntarily reduce nutrient intake.

Comparative studies conducted in distant species can reveal fundamental regulatory mechanisms that have exerted strong evolutionary pressure. Teleost fish, that diverged from the mammalian lineage about 450 MYA, represent very suitable models in which to identify conserved neuroendocrine systems involved in the control of food intake and energy expenditure (29). Delgado et al. review the literature concerning the action of various neuropeptides including proopiomelanocortin (POMC), neuropeptide Y (NPY), agouti-related peptide (AgRP), cocaine- and amphetamine-regulated transcript (CART), orexin, cholecystokinin (CCK) and melanin-concentrating hormone (MCH), and various hormones e.g., insulin, leptin, ghrelin, and glucagon-like peptide 1 (GLP-1). They also discuss their implication in the hypothalamic integration of metabolic information that elicits a coordinated feeding response in fish. In spite of discrete species variations, most of these regulatory mechanisms have been highly conserved from fish to mammals.

In a sister review, Volkoff reminds us that about 30 neuropeptides are potentially involved in the regulation of feeding in fish. In addition to those aforementioned,

thyrotropin-releasing hormone (TRH), orexin, galanin, apelin, and secretoneurin exert orexigenic effects whereas gonadotropin-releasing hormone 2 (GnRH2), prolactin-releasing peptide (PrRP), corticotropin-releasing factor (CRF) and its paralogs urotensin I and urocortin 3, arginine vasotocin (AVT), pituitary adenylated cyclase-activating polypeptide (PACAP), the octadecaneuropeptide (ODN), peptide YY, amylin, RFamide-related peptide-3 (RFRP-3), and nesfatin-1 act as satiety factors.

NPY is one of the most potent orexigenic peptides in the brain of mammals (30). The sequence of frog NPY is almost identical to that of the human peptide (31) and, in frog tadpoles as in rodents, intracerebroventricular (icv) injection of NPY stimulates feeding behavior (32). Matsuda et al. have investigated the downstream mechanisms through which NPY exerts its orexigenic activity in bullfrog larvae. They show that the stimulatory effect of NPY on food intake is abolished by co-administration of the selective orexin receptor antagonist SB334867. These data indicate that, in premetamorphic larvae, the orexigenic effect of NPY is mediated via the orexin/orexin receptor system.

Owing to the economic importance of poultry production, the neuroendocrine control of feeding behavior has been extensively studied in birds, notably in neonatal chicks and young chickens (33, 34). Tachibana and Tsutsui summarize the effects of various hormones and neuropeptides on feeding in birds, and highlight a few differences with what is known in mammals. For instance, ghrelin which is a potent orexigenic peptide in mammals (35) inhibits food intake in chickens (36). Reciprocally, PrPR which exerts an anorexigenic effect in mammals (37) stimulates feeding behavior in neonatal chicks (38).

Gonadotropin-inhibitory hormone (GnIH; also called RFRP3) is a member of the RFamide family of neuropeptides. The inhibitory effect of GnIH on the hypothalamo-pituitary-gonadal axis has been initially discovered in birds (39) and subsequently confirmed in fish and in mammals (40). Since energy homeostasis and reproduction are intimately correlated (41), Tsutsui and Ubuka review the evidence that GnIH exerts a coordinate control on feeding and reproductive behaviors in vertebrates.

26RFa/QRFP, another member of the RFamide family of neuropeptides, is the natural ligand of GPR103 now renamed QRFPR (42). Functional characterization of 26RFa/QFRP has revealed that *icv* injection of the peptide stimulates feeding behavior (43, 44). 26RFa/QRFP also acts on pancreatic β-cells to inhibit basal and glucose-induced insulin secretion (45, 46). Chartrel et al. review the current knowledge on the involvement of 26RFa/QRFP in the regulation of food intake and glucose homeostasis.

In a sister paper, Gesmundo et al. describe the involvement of neuroendocrine signals, including melatonin, galanin, and 26RFa/QRFP, in the control of insulin secretion. Their report highlights the key role of neurohormones in the complex regulation of β-cell activity. Together with other players, these neuroendocrine factors and their receptors represent potential therapeutic targets for the treatment of type 1 and type 2 diabetes, and metabolic disorders.

It is firmly established that the endocannabinoid system is implicated in the control of food intake and energy homeostasis (47, 48) but the underlying mechanisms have long remained unknown. Koch reviews the current knowledge on cannabinoid receptor type 1 (CB1) signaling in the regulation of feeding behavior.

Secretin, glucagon, glucagon-like peptides (GLP1 and 2), growth hormone-releasing hormone, vasoactive intestinal peptide (VIP) and PACAP belong to the same family of regulatory peptides also called the secretin family (49). Sekar et al. review the abundant literature regarding the actions of these peptide hormones and neuropeptides in the central control of feeding behavior. They point out the therapeutic potential of selective and stable analogs of these peptides, notably GLP-1 receptor agonists for the treatment of obesity and metabolic disorders.

The ventromedial nucleus (VMN) of the hypothalamus, which plays a prominent role in the regulation of food intake and energy expenditure, actively expresses the PACAP-selective receptor PAC1R (50). Consistent with this observation, microinjection of PACAP into the VMN strongly reduces food intake (51). Hurley et al. have developed a novel binge-eating model that allows to distinguish homeostatic feeding drive (hunger) from hedonic feeding drive (palatability). Their data show that injection of PACAP into the VMN decreases homeostatic feeding while injection of PACAP into the nucleus accumbens reduces hedonic feeding. These two distinct mechanisms likely contribute to the global anorexigenic effect of PACAP (52).

The central control of energy balance relies not only on neurons but also on glial cells, endothelial cells, and ependymocytes/tanycytes (53–55). Freire-Regatillo et al. review the involvement of non-neuronal cells in the transport and metabolism of hormones and nutrients participating to the neuroendocrine control of appetite and energy expenditure.

The term "endozepines" designates a family of regulatory peptides including diazepam-binging inhibitor/acyl-CoA-binding protein (DBI/ACBP) and its processing fragments, the triakontatetraneuropeptide TTN and the octadecaneuropeptide ODN, that are produced by astroglial cells and act as endogenous ligands of benzodiazepine receptors (56). Intracerebroventricular (icv) administration of ODN substantially reduces food consumption (57, 58). At the hypothalamic level, the anorexigenic effect of ODN can be ascribed to stimulation of POMC mRNA and inhibition of NPY mRNA expression (59). Guillebaud et al. here show that the DBI gene is expressed in tanycytes in the rat brainstem. Icv injection of ODN into the 4th ventricle causes a marked reduction of food intake and, in anesthetized animals, inhibits the swallowing reflex. These observations indicate that ODN acts, as an anorexigenic factor, not only within the hypothalamus, but also at the brainstem level to modify the excitability of neuronal networks implicated in feeding behavior.

DBI/ACBP belongs to the acyl-CoA-binding domain-containing protein (ACBD) family that encompasses multiple members including ACBD7 (60). Expressed in the arcuate nucleus, ACBD7 is the precursor of the non-adecaneuropeptide

NDN which, like ODN, is a potent anorexigenic neuropeptide (61). Based on these observations, Lanfray and Richard describe the neurochemical mechanisms regulating the activity of ACBD7 neurons, and the downstream neuronal circuits involved in the anorexigenic effect of ODN.

There is now evidence that microRNAs (mRNAs) are involved in the central regulation of energy balance. In particular, the miRNA-processing enzyme DICER plays a pivotal role in the development, activity and survival of POMC neurons (62). Derghal et al. review the roles of miRNAs in the regulation of the melanocortin system and focus on the involvement of miRNAs in the control of POMC neurons by leptin.

The review articles and original research papers gathered in the present e-book illustrate the most recent progress in the understanding of the neuroendocrine regulation of feeding behavior and energy homeostasis. It is our hope that this Research Topic will become a major set of references for all researchers involved in this rapidly expanding field.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Hypothalamic Inflammation and Energy Balance Disruptions: Spotlight on Chemokines

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The hypothalamus is a key brain region in the regulation of energy balance as it controls food intake and both energy storage and expenditure through integration of humoral, neural, and nutrient-related signals and cues. Many years of research have focused on the regulation of energy balance by hypothalamic neurons, but the most recent findings suggest that neurons and glial cells, such as microglia and astrocytes, in the hypothalamus actually orchestrate together several metabolic functions. Because glial cells have been described as mediators of inflammatory processes in the brain, the existence of a causal link between hypothalamic inflammation and the deregulations of feeding behavior, leading to involuntary weight loss or obesity for example, has been suggested. Several inflammatory pathways that could impair the hypothalamic control of energy balance have been studied over the years such as, among others, toll-like receptors and canonical cytokines. Yet, less studied so far, chemokines also represent interesting candidates that could link the aforementioned pathways and the activity of hypothalamic neurons. Indeed, chemokines, in addition to their role in attracting immune cells to the inflamed site, have been suggested to be capable of neuromodulation. Thus, they could disrupt cellular activity together with synthesis and/or secretion of multiple neurotransmitters/mediators involved in the maintenance of energy balance. This review discusses the different inflammatory pathways that have been identified so far in the hypothalamus in the context of feeding behavior and body weight control impairments, with a particular focus on chemokines signaling that opens a new avenue in the understanding of the major role played by inflammation in obesity.

Keywords: neuroinflammation, hypothalamus, chemokines, energy balance, metabolic diseases, high-fat diet, obesity, anorexia

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INTRODUCTION

Energy balance is finely regulated *via* a bidirectional communication between the brain and the peripheral organs. One brain area is particularly important in this regulation: the hypothalamus. The hypothalamus shelters, in its different nuclei, several neuronal populations producing peptides that are either orexigenic or anorexigenic. The activity of these neuropeptidergic circuits is, among others, modulated by peripheral signals, of neural or hormonal nature, or by nutrients

themselves (**Figure 1**). Thus, it would make sense that the function of these neuropeptidergic circuits would be impaired in case of feeding behavior deregulation, whether it is a loss of appetite or a food overconsumption. Numerous studies, based either on lesion, pharmacological, or genetic approaches, indeed confirmed this [for review see Ref. (1)]. Interestingly, hypothalamic inflammation has already been linked to energy balance disruptions: high-grade hypothalamic inflammation has been associated to involuntary weight loss and, on the contrary, low-grade hypothalamic inflammation has been associated to obesity (2, 3). Importantly, these feeding behavior deregulations represent major public health issues, especially obesity. Indeed, obesity, which keeps developing since the end of the 20th century, is often associated to potentially deadly comorbidities such as diabetes, cardiovascular diseases, liver diseases, and cancers. Yet, a loss of appetite, consecutive to some inflammatory pathologies such as cancer, can also have severe consequences, as it can impair recovery by inducing a deficit in energy.

Hence, understanding the molecular mechanisms linking hypothalamic inflammation and feeding behavior deregulations could, in the long-term, allow identifying potential therapeutic targets. As previously mentioned, we will focus in this review on hypothalamic inflammation, even though peripheral inflammation is also often associated with energy balance deregulations: in involuntary weight loss, inflammation is rather firstly systemic and a consequence of a primary pathology. In obesity, both systemic and hypothalamic inflammations have been described, and even if this is still debated, recent studies suggest that hypothalamic inflammation precedes systemic inflammation associated to the obese state (4–6).

In this context, in particular when there is systemic inflammation, it is important to understand how the periphery and the brain can communicate, especially regarding inflammatory signals. The most described brain–periphery communication pathways are the neural and the humoral pathways. The neural pathway involves primarily the vagus nerve—which expresses cytokine receptors—and the dorso-vagal complex, whereas the humoral pathway involves circulating cytokines that are overexpressed with inflammation and that can (1) bind to their receptors on the membrane of endothelial cells, (2) get to the brain at the level of the circumventricular organs and of the choroid plexus when the blood–brain barrier (BBB) is incomplete, or

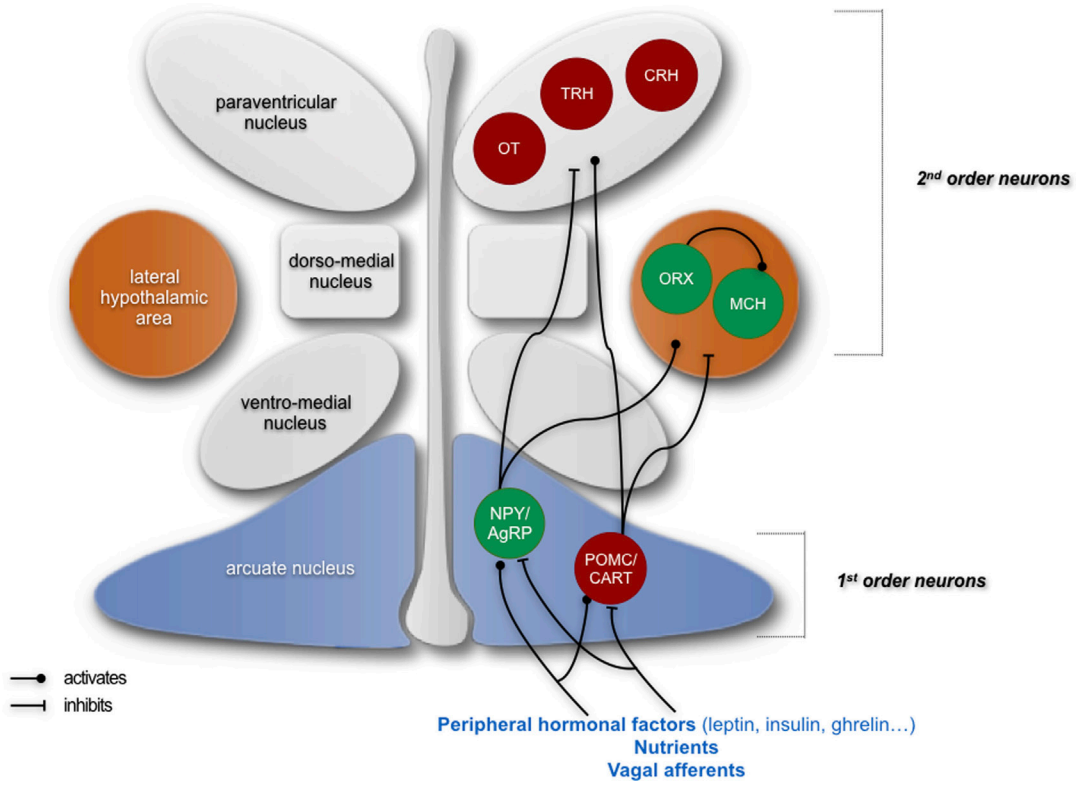


FIGURE 1 | Simplified schematics summarizing the neuropeptidergic hypothalamic networks that are involved in homeostatic regulation of feeding behavior. Signals giving indications about the energy status of an organism are emitted from the periphery and are first integrated by first-order neurons located in the arcuate nucleus, producing either orexigenic neuropeptides (NPY and AgRP) or anorexigenic peptides (POMC and CART). These neurons project to second-order neurons, such as neurons producing the anorexigenic factors oxytocin (OT), thyrotropin-releasing hormone (TRH), and corticotropin-releasing hormone (CRH) in the paraventricular nucleus and neurons producing orexigenic peptides orexin (ORX) and melanin-concentrating hormone (MCH) in the lateral hypothalamus. The integration of peripheral signals by these neuropeptidergic systems participates in the homeostatic regulation of feeding behavior and the maintenance of a suitable weight by ensuring an appropriate food intake as well as appropriate energy intake and expenditure. In green: neurons producing orexigenic peptides; in red: neurons producing anorexigenic peptides. Adapted from Le Thuc and Rovère (7).

(3) reach directly the brain thanks to the increased BBB permeability induced by inflammation (8). However, one must also consider the cellular pathway, which involves the infiltration of immune cells and activation of microglia in the brain parenchyma, and also the microbiota pathway, for example.

Studies that aimed to identify inflammatory mediators allowing bidirectional communication between the periphery and the brain during inflammation mainly focused on the role of peripheral cytokines. Yet, it has been suggested that in the context of disruptions of proper control of food intake and body weight, the central production of inflammatory mediators is more important than the peripheral one, or at least "necessary," in particular in the models of inflammation induced by the bacterial lipopolysaccharide (LPS), where it has been suggested that its effects do not require the signaling from peripheral cytokines (9–11).

Hence, in the context of disruptions of proper energy balance control by hypothalamic circuits, a group of pro-inflammatory mediators might be as relevant as the canonical cytokines, if not more, downstream of the latter: the chemokines. Chemokines are a subgroup of cytokines, small (8–14 kDa) heparin-binding proteins, mainly described for their chemoattractant properties for immune cells to the affected site.

Chemokines and their receptors are suspected to be mediators of the effects of neuroinflammation, from attraction of immune cells to behavioral changes. Beyond participating in the immune response, chemokines and their receptors (the "chemokinergic system") are constitutively expressed in the brain, in a specific manner, both area wise and cell type wise. Interestingly, it is comparable to the neurotransmitters or neuropeptidergic systems (12, 13). Furthermore, the chemokinergic system has been demonstrated to interact with the neurotransmitter and neuropeptidergic systems (14, 15) and some recent studies suggest that the chemokinergic system is able to directly modulate neuronal activity (16–18). Thus, the chemokinergic system could be responsible for the behavioral changes associated to inflammation.

In this review, we discuss how inflammation, with a focus on chemokines, can participate in the deregulation of the hypothalamic control of energy balance and body weight: first, in appetite and weight loss and, second, in the establishment and/or the development of obesity.

INVOLUNTARY WEIGHT LOSS AND INFLAMMATION

Weight loss illustrates an unbalance between energy intake and energy expenditure in favor of expenditure. While both involuntary and voluntary weight losses can be caused by a decrease in food intake or an increase in energy expenditure (increase in basal metabolism, e.g., thermogenesis), only an involuntary weight loss can be explained by an uncontrolled decrease in food intake or by an increase in energy expenditure.

There are multiple possible causes for an involuntary weight loss. This weight loss can be a symptom of a severe primary disease such as cancer, where it is particularly preoccupying when it reaches or exceeds 10% in 1 year. In most cases, one can link an involuntary weight loss to psychic troubles, such as depression, that can induce a sustainable loss of appetite, thus weight loss. Excessive consumption of certain substances (e.g., drugs of abuse and alcohol) can also induce an involuntary weight loss. Yet, a weight loss is only considered "involuntary" when it is not a consequence that can be expected from a specific medical treatment of a known medical condition (19). Moreover, aging is often associated to a loss of appetite which induces, in the long-term, severe malnutrition and, obviously, weight loss. This can also be associated to sarcopenia, a geriatric syndrome characterized by loss of muscle mass and function (19). However, as previously mentioned, an involuntary weight loss can have organic causes such as gastrointestinal diseases [e.g., Crohn's disease, celiac disease (weight loss related to food malabsorption), and digestive ulcer], cardiovascular, endocrine, autoimmune, infectious diseases (e.g., HIV infection, hepatitis, and tuberculosis), neurological diseases (e.g., dementia and Parkinson's disease), or cancer (19, 20). In the case of cancer, weight loss is one of the first symptoms with 50% of patients with cancer reporting weight loss (21). It should be noted that the majority of the abovementioned organic causes are commonly associated with "high-grade"—in other words high-intensity—inflammation. Interestingly, even if benign and time-restricted, numerous pathological states are strongly inflammatory and associated to a reduced appetite or "anorexia." This anorexia is part of a classical defense mechanism of an organism against infection, lesions, etc., that is referred to as "sickness behavior" (22, 23). Sickness behavior involves various behavioral changes that primarily affect mood and energy balance, which develop parallel to infection or to another pathology (22).

Like other strategies adopted by the body to promote healing, anorexia is supposed to be temporary and beneficial. However, in the case of chronic diseases such as cancers, both appetite and weight loss can persist, leading to a further degraded state of the subject (hypoglycemic malaise, amenorrhea in women, decalcification leading to more fragile bones, teeth falling, etc.), comparable, in some aspects only linked to undernutrition, to what is observed with anorexia nervosa (24). Prolonged anorexia, irrespective of its type and its causes, is often a contributory factor to the onset of cachexia. Cachexia is a complex syndrome that cannot be fully reversed by conventional nutritional support and leads to progressive functional impairment, i.e., pathological thinness, associated with deep asthenia and organs dysfunction [decreased muscle strength, fatigue, adipose tissue (AT) dystrophy, etc.] (25, 26). Hence, cachexia can be defined as a complex syndrome in which inflammation leads to early satiety and anorexia, decreased fat mass, and weakness (25, 26). Prolonged loss of appetite may even be fatal. One can then assume that inflammation, here high-grade, could disrupt the proper functioning of the hypothalamic systems that are involved, and thus the regulation of feeding behavior. Hence, considering the serious consequences of unresolved anorexia, it is important to better understand the mechanisms linking inflammation and the cerebral centers that control energy homeostasis, because if the link between high-grade inflammation and loss of appetite and weight is established and extensively recognized, the underlying molecular mechanisms have not yet been fully resolved.

Inflammatory Pathways in the Hypothalamus and Involuntary Weight Loss

In the hypothalamus, some studies have been able to identify some inflammatory pathways, involving different cell types, to be relevant for weight loss.

At the molecular level, as previously mentioned, the literature related to the deregulation of the control of energy balance in the anorexia–cachexia syndrome has focused on the role of pro-inflammatory cytokines, such as interleukin (IL)-1β, IL-6, and TNF-α. It has been shown that pro-inflammatory cytokines, whose production can be induced by infectious agents affecting peripheral organs, notably by LPS—the most commonly used model for modeling disease behavior and anorexia associated with inflammation (27)—participate in the induction of sickness behavior.

Some studies deciphering the kinetic aspects of the effect of central or peripheral cytokine injection suggest that pro-inflammatory mediators exert their effect primarily at the central level versus periphery (28, 29). Numerous studies have focused on IL-1β and have shown that its intracerebroventricular (ICV) injection induces profound behavioral changes in rodents (30). Furthermore, the central role of cytokine signaling at the central level has been confirmed, for example, by the fact that the central injection of the IL-1β receptor antagonist prevents the effects induced by the peripheral injection of this cytokine (31, 32). In addition, some studies suggest that inflammatory inducers such as LPS are not capable of directly inducing sickness behavior and require downstream actors such as cytokines. Indeed, for example, unlike the central injection of LPS, central injection of IL-1 is able to induce sickness behavior in mice whose LPS toll-like receptor (TLR) 4 is non-functional (33).

As mentioned earlier, cytokines, involved in the induction and regulation of sickness behavior at the central level, can be released into the circulation by the immune cells (8) or directly produced by neurons and glial cells in the central nervous system (CNS) (34–39). In the context of systemic diseases, the central effects of these cytokines appear to be independent of the place of their secretion. As previously mentioned, when produced in the periphery, cytokines have the following two main pathways to act on the brain: a neural pathway and a humoral pathway. Most cytokines act in a paracrine manner at the site of infection, suggesting that neural afferents may be the target of pro-inflammatory cytokines. Interestingly, the perineural sheath of the vagus nerve contains immune cells that are capable of producing IL-1, in particular in response to LPS (40). Furthermore, the sensory neurons of the vagus nerve express IL-1 receptors and it has been shown that this cytokine stimulates the sensory activity of the vagus nerve (40). Vagotomy experiments have demonstrated the importance of the vagus nerve in the transmission of information from the periphery to the brain: for example, after injection of LPS or IL-1 at the periphery, vagal afferents are involved in the induction of the sickness behavior and in the neural activation of the brainstem, the hypothalamus, and the limbic structures (40).

Chronic administration of cytokines can reproduce the characteristics of anorexia–cachexia syndrome (38, 41–44), while blocking the signaling of one of them, such as TNF, by the use of neutralizing antibodies, inhibits its development (42, 45, 46). Similarly, administration of an IL-1 receptor antagonist prevents anorexia in cancer animal models (47). Some studies have suggested that endogenous brain IL-1 is a mediator of LPS-induced anorexia by acting on the expression of cytokines in the hypothalamus (9). In addition, it appears that IL-1β can act on ARC POMC neurons (48) and that TNF-α indirectly increases energy expenditure *via* β3 adrenergic signaling in the brown AT (thermogenesis) (49). By different approaches, it has been shown that interfering with mediators of inflammation coincides with a reduction in hypothalamic inflammation and prevents weight loss in animal models of anorexia: for example, the inhibition of the "adenosine monophosphate protein kinase" in the hypothalamus reduces hypothalamic inflammation, which is accompanied by an increase in food intake in the case of cancer-associated anorexia, allowing better overall survival (50). Interestingly, in the context of cancer-associated cachexia, the administration of ghrelin, an appetite-stimulating hormone, increases food intake and is accompanied by a decrease in IL-1β (51).

Focusing on the LPS-induced anorexia model, it is interesting to underline that some studies suggest that this loss of appetite is independent from the vagal afferents and that it only depends on central inflammatory mechanisms, where the central effects of peripheral LPS could be, among others, mediated by some cytokines and/or *via* receptors for LPS expressed by some brain cells (11, 52). Indeed, microglia expresses TLR4, through which LPS has been shown to exert some of its effects (53–56). Furthermore, Hines and colleagues have shown that disrupting TLR4 signaling prevents microglial activation, inhibits the production of cytokines, and prevents the development of the sickness behavior that are induced by peripheral LPS (57). In contrast, a recent study by Reis et al. has shown that if microglia and TLR4 are necessary for LPS to acutely induce inhibitory effects on orexigenic agouti-related peptide (AgRP)/neuropeptide Y (NPY) neurons, conversely, LPS can actually acutely increase the firing activity of anorexigenic pro-opiomelanocortin (POMC) neurons in a microglia/TLR4-independent manner (58). In addition, the same study presents results which further supports that mediators of inflammation such as microglia and TLR4 can, at least acutely, affect basal food intake and hormonal-dependent modulation of feeding behavior. Indeed, the inhibition of microglia alone, *via* ICV injection of minocycline, leads to an increase in food intake, comparable to the one induced by ICV ghrelin (58). Interestingly, the co-injection of ghrelin and minocycline does not synergistically increase food intake. This implies that the inhibition of microglia itself, as it increases food intake, interferes or prevents ghrelin's orexigenic effects (58).

Changes in gene expression of hypothalamic anorexigenic and orexigenic neuropeptides were assessed in mice or rats that received LPS IP injections (59–61). Generally, peripheral LPS appears, initially, to reduce the expression of the orexigenic peptides and, on the contrary, to increase that of the anorexigenic peptides. In later stages, the expression of orexigenic peptides

increases again, probably to promote food intake and compensate for weight loss.

Some authors have used other models to study to understand links between inflammation in the brain and the induction of sickness behavior.

For example, Jang and colleagues sought to determine the mechanisms behind anorexia and weight loss associated with illness *via* administration, in mice, of either bacterial or viral products: LPS and human immunodeficiency virus-1 transactivator protein (Tat), respectively (62). They found in both cases that pro-inflammatory cytokines such as IL-β, IL-6, and TNF-α were upregulated in the hypothalamus. Furthermore, AtT-20 and SH-SY5Y cells treated with either Tat or LPS exhibit increased POMC transcriptional activity. In addition, the injection of Tat or LPS in the hypothalamus of mice induces in both cases a decrease in food intake and in body weight. They identified POMC as a potential mediator of illness-induced anorexia and as a possible downstream target of NF-κB. Indeed, they showed through different approaches that the NF-κB pathway in the melanocortin system plays an important role in illness-induced anorexia and body weight loss: both administration of AgRP, an endogenous melanocortin antagonist and the inhibition of the NF-κB pathway specifically in the POMC neurons ($Ikk\beta^{\Delta Pomc}$ mice), significantly blunted the effects of Tat and LPS on food intake and body weight. Interestingly, the authors also present results suggesting that leptin-induced anorexia is also dependent on the NF-κB pathway (62).

Another study has shown that a pathway involving TLR2 participates in the induction of sickness behavior through a microglia-POMC neurons axis (63). In this publication, the authors show, by ICV injection of Pam3CSK4, a specific synthetic TLR2 agonist that TLR2 central signaling is able to trigger sickness behavior through activation of microglial cells, which express TLR2, and *via* the NF-κB and COX pathways. The authors also show that activation of central TLR2 is able to modify the synaptic architecture in the ARC, especially at the level of the POMC neurons: it reduces the rate of GABAergic contacts on POMC neurons, whereas it increases the vesicular glutamate transporter 2 (vGLUT2) contacts on POMC soma, translating an increased excitatory state, in correlation with an increased microglial occupancy.

In addition, Murray et al. used the peripheral administration of the viral mimetic poly I:C to induce type-I interferons (IFN-I) overexpression in the brain. Using IFN-I receptor 1 (IFNAR1)-deficient mice, the results of the authors present suggest that IFN-I are involved in the induction of sickness behavior, including anorexia, whereas IL-6 participates in sickness behavior but not in anorexia (64).

Furthermore, a local increase in serotonin levels in the hypothalamus has already been linked to anorexia and cachexia (65–67) and a recent study showed that inflammation induced by peripheral treatment by either IL-6 and/or TNF-α is associated with an impairment in local serotonin turnover in the hypothalamus, a decrease in NPY and AgRP gene expressions, and a decrease in food intake in comparison to control conditions (68). Authors were able to identify upstream inflammatory regulators including interferon gamma (IFN-γ), transforming growth factor beta (TGF-β), IL-6, and IKBKG, an enzyme crucial for the activation of the NF-κB pathway. This study thus suggests that peripheral inflammation reaches the hypothalamus where it impairs serotonin turnover, which is associated to a decrease in food intake.

Taken together, numerous studies support the hypothesis that inflammation at the hypothalamic level is able to disrupt the proper function of neuropeptidergic circuits of the hypothalamus and thus to induce an involuntary weight loss.

However, mediators linking inflammation and its consequences at the level of central systems regulating energy homeostasis, and ultimately on weight, have not been determined with certainty, and one may note that the nature of the mediators responsible for the central effects of IL-1β has been especially poorly described. Thus, chemokines can represent interesting candidates to study further understanding of the underlying mechanisms.

Chemokines in the Modulation of Hypothalamic Neuropeptidergic Circuits in Inflammation-Associated Involuntary Weight Loss

So far, very few studies have sought to determine if chemokines have a role in the induction of involuntary weight loss associated with inflammation by impairing the homeostatic regulation of energy balance by hypothalamic neuropeptidergic circuits.

A study published in 1994 by Plata-Salamán and Borkoski aimed to investigate how chemokines could act on the regulation of feeding. Indeed, as previously mentioned, chemokines are produced in multiple types of cells as a response to pathological conditions such as infection, inflammation, injury, and trauma. Among the stimuli that can induce the release of these chemokines, we can list LPS, and also the IL-1β, TNF-α, and IFN, which have been associated to food intake suppression by direct action on the CNS. Thus, the authors aimed to determine if chemokines could be involved, as downstream mediators, in the decrease in food intake induced by inflammatory signals such as LPS. In order to do so, they used, in rats, ICV microinfusion of different chemokines from two different subfamilies: CXC and CC, also known as the α and β subfamilies. This way, they tested the effect of CXC-motif chemokine ligand (CXCL)-1, 4, 7, 8, 10 and CC-motif chemokine ligand (CCL)-2–4, 5. Even though it is at a lesser extent than the cytokine IL-1β, their results indeed identify certain chemokines as capable of acutely decreasing food intake: CXCL4, CXCL8, CXCL10, CCL2, and CCL5. Interestingly, in their model, these chemokines would affect feeding behavior at different time scales: if they all reduced food intake in the 2 h following injection, only CXCL8, CXCL4, and CCL2 were able to reduce food intake over the whole dark phase and only CXCL8 and CXCL4 were able to decrease the total daily food intake (69). This study is particularly important as it sets chemokines as contributors, in the brain, to the effects of infection/inflammation on feeding behavior. Nevertheless, no mechanistic insights were then provided.

As previously acknowledged, very few studies aimed at better characterizing how chemokines could modulate feeding behavior after this study by Plata-Salamán and Borkoski.

Yet, in a recent study from our laboratory (70), we sought to identify and characterize chemokines that could possibly

deregulate the hypothalamic circuits to alter food intake and energy balance in anorexia and weight loss. We thus assessed the gene expression of several pro-inflammatory mediators in the hypothalamus of mice that had received an acute ICV injection of LPS. After confirmation of LPS-induced inflammation in our mice by observing the overexpression of several pro-inflammatory mediators, we then identified ligands of the chemokine receptors CCR (CC-motif chemokine receptor)-2 and 5 as the most overexpressed. Among them, CCL2 (also known as monocyte chemoattractant protein 1) caught our attention as it has been described as particularly important in the context of LPS-induced neuroinflammation and suggested to be able to reduce food intake (69, 71–73). Thus, we focused on the central signaling of CCL2 and its receptor CCR2 and could demonstrate that it is mandatory for both metabolic and behavioral changes induced by LPS (70). Indeed, inhibiting CCR2 signaling by combined ICV injection of a specific antagonist of CCR2, INCB3344, together with LPS, prevents the weight loss that is induced by ICV injection of LPS alone. Similarly, the weight loss induced by ICV injection of LPS is reduced in mice deficient for CCR2 in comparison to control animals. Experiments in metabolic cages demonstrate that central injection of LPS decreases both food intake and locomotor activity, whereas it increases fat oxidation and induces a shift in used energy substrate in favor of lipids versus carbohydrates. These two last points probably illustrate an increased use of the energy stocked in the AT as lipids. Interestingly, these LPS effects highlighted by the metabolic cages experiments were also reduced when interfering with central CCR2 signaling by co-ICV injection of LPS and INCB3344.

We then identified the neurons, which produce melanin-concentrating hormone (MCH), a peptide known to elicit food intake and to decrease energy consumption, as targets for CCL2 (74). After ICV injection of LPS, we observed a sustained decrease in gene expression and protein levels of MCH, which is blunted/prevented by pharmacological (using INCB3344) or genetic inhibition of CCL2 signaling. Interestingly, we found that, similar to LPS, ICV injection of CCL2 promotes neuroinflammation, together with a decrease in both MCH expression and body weight. Immunostaining experiments showed that 70% of the MCH neurons of the lateral hypothalamus (LHA) express CCL2 receptor. These neurons responded to CCL2 by decreasing both electrical activity and MCH release. Thus, it seems that the inhibition of the MCH system by LPS depends primarily of the central signaling of CCR2. Moreover, in experiments of perifusion of hypothalamic explants treated with KCl and CCL2, MCH secretion is totally inhibited, while co-application of KCl and INCB3344 only, leads to enhanced secretion of MCH. This suggests that endogenous CCL2 could participate, in normal conditions, in modulating MCH system and thus energy balance regulation.

Thus, it appears that the central CCL2/CCR2 axis is able to directly act on neurons producing the orexigenic peptide MCH by reducing their activity and both the expression and secretion of the peptide, leading to a reduced food intake and to an increased use of energy stores. Hence, the central CCL2/CCR2 axis, by acting through MCH neurons, appears as a major actor in appetite and weight loss associated with LPS-induced inflammation (**Figures 2** and **3**). This study was the first to demonstrate that a chemokine can play a role, at the central level, in energy balance deregulation, by acting directly on neuropeptidergic systems in the hypothalamus.

OBESITY AND INFLAMMATION

As previously mentioned, interestingly, inflammation is also a characteristic for an opposite disorder of the energy balance disorder of the regulation of food intake and body weight: obesity. Yet, different from the inflammation associated to involuntary weight loss, this inflammation is of low intensity, also referred to as a "low-grade" inflammation (79). Obesity is characterized by an excessive fat mass distributed throughout the body that can be harmful to health [source: World Health Organization

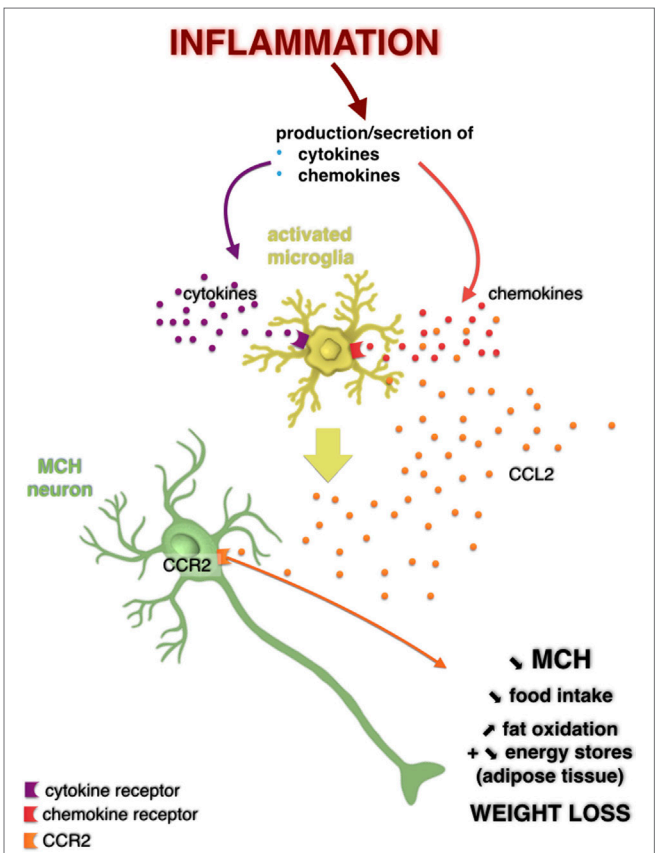


FIGURE 2 | Potential action of the CC-motif chemokine ligand (CCL) 2/CCR2 signaling pathway on melanin-concentrating hormone (MCH) neurons in a weight loss model induced by a central injection of lipopolysaccharide. Hypothalamic inflammation is characterized by overexpression of inflammatory mediators such as cytokines and chemokines. It is possible that these bind to their receptors expressed by glial cells such as microglia, which then activated, can produce even more cytokines and chemokines, including CCL2. However, it is not excluded that CCL2 could act directly on MCH neurons that expressed its receptor in the lateral hypothalamus. This would result in a decreased MCH neuronal activity and in a decreased secretion of this neuropeptide, which is associated to a loss of appetite, an increased fat oxidation, likely reflecting a decrease in energy stores in adipose tissue, and thus a loss of weight. Adapted from Le Thuc and Rovère (7).

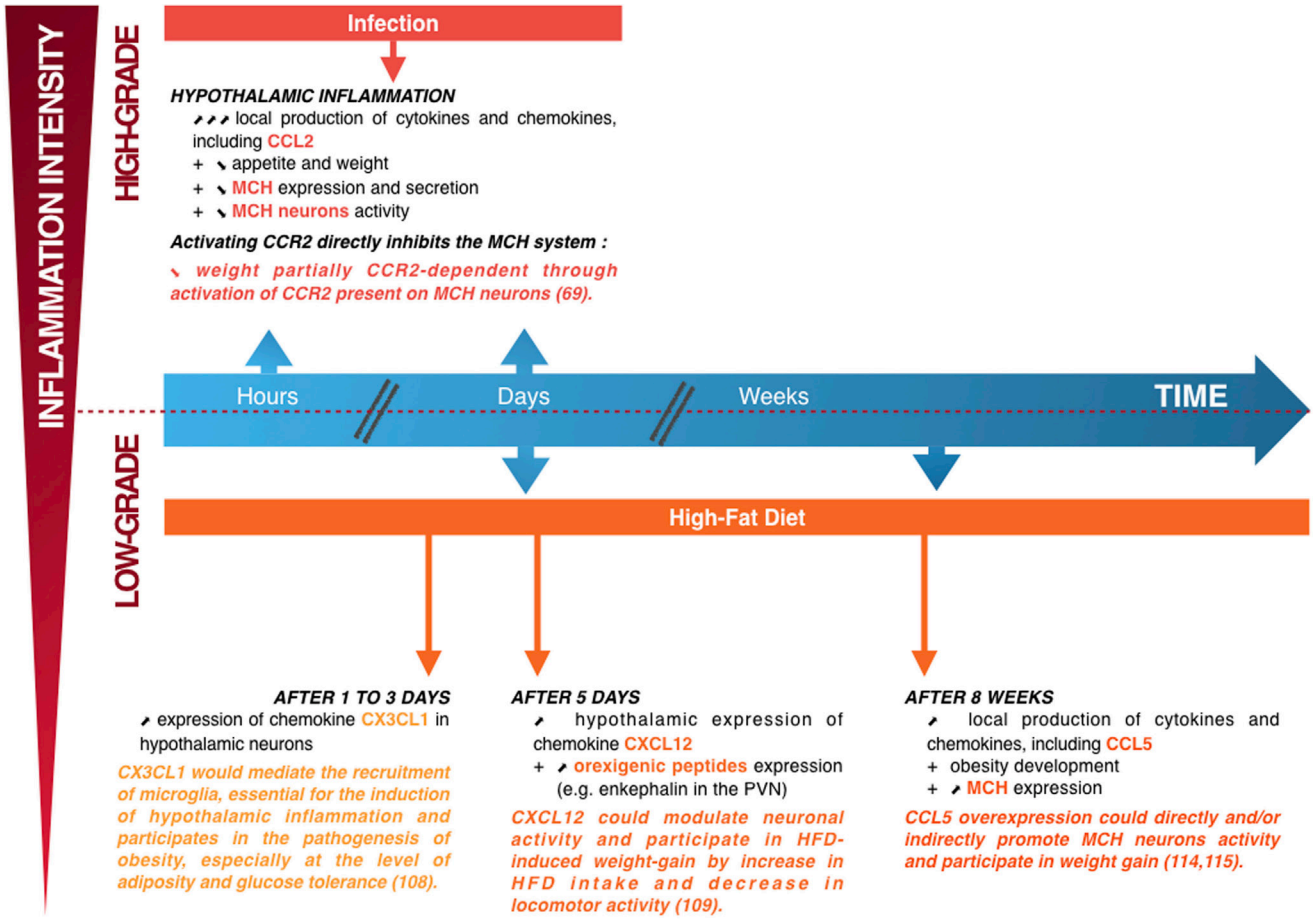


FIGURE 3 | Summary diagram showing the links between hypothalamic inflammation and deregulation of weight. *Upper part*: when lipopolysaccharide induces high-grade hypothalamic inflammation, there is an acute and increase in local production of cytokines and chemokines, including CC-motif chemokine ligand (CCL) 2 (a few hours to a few days) associated with weight loss (a manifestation of the sickness behavior associated with a high-intensity infection). This weight loss is partially dependent on the CCL2/CCR2 signaling pathway, by direct inhibition of the lateral hypothalamus (LHA) neurons producing the orexigenic peptide melanin-concentrating hormone (MCH) (70). *Lower part*: short (1–5 days) or prolonged (several weeks) high-fat diet (HFD) consumption is associated with chronic low-grade hypothalamic inflammation that appears to be linked to the development of obesity, together with an overexpression of orexigenic neuropeptides such as enkephalin in the paraventricular nucleus or MCH in the LHA. The chemokine CX3CL1 appears essential to the induction of hypothalamic inflammation, with an important role in the recruitment of microglia, whereas the chemokines CXC-motif chemokine ligand 12 and CCL5 could modulate neuronal activity and participate in HFD-induced weight loss (75–78). Adapted from Le Thuc and Rovère (7).

(WHO)]. One is considered obese if one's body mass index is greater than or equal to 30 (80, 81). Worldwide, close to 13% of the population were obese in 2014 and the prevalence of obesity nearly doubled between 1980 and 2014 (source: WHO). Obesity is a concern as it represents a risk factor for chronic diseases such as cardiovascular diseases (hypertension, heart disease, and stroke), diabetes, hepatic steatosis, respiratory diseases, musculoskeletal disorders (osteoarthritis, etc.), certain cancers (endometrium, breast, colorectal, etc.), and neurodegenerative disorders (79–81). Furthermore, childhood obesity, which is progressing in an alarming manner, can promote respiratory difficulties, high blood pressure, the emergence of markers of cardiovascular disease, fractures, insulin resistance, and psychological problems. All these aspects in turn increase the risk of adult obesity, premature death, and disability in adulthood (source: WHO).

While the development of obesity may be explained by some genetic aspects or be consecutive to a primary disease and/or its treatment (hormonal and/or psychological factors, drugs, etc.), its most common cause is a change in the population's life-style. This encompasses an increase in sedentariness and hypercaloric diets overconsumption, where the excess in calories intake most often comes from lipids and also carbohydrates (80, 81). As obesity represents a major and growing public health issue, it becomes important to identify and understand its causes and mechanisms. Therefore, understanding the role played by inflammation, both peripheral and central, in the establishment and/or development of obesity and its comorbidities might allow to identify new potential targets in the fight against obesity.

The inflammation associated to obesity exhibits several specificities: first, as mentioned previously, it is low grade and chronic. So far, it has been mostly described in peripheral tissues

(AT, liver, pancreas, etc.). Nevertheless, more recently, it has been shown to also occur in the CNS: a hypercaloric challenge, especially a high-fat diet (HFD), even on the short term, can induce an inflammation in the hypothalamus that is sustained in models of nutritional obesity (82).

Obesity and Peripheral Inflammation

Studies have associated inflammation and obesity for a long time (79). Yet this inflammation is not "typical," as it cannot be associated to the cardinal signs of redness, swelling, heat, and pain. Thus, the inflammation associated to obesity is of different nature. First, it is aseptic: it is caused by the overconsumption of specific nutrients (lipids and carbohydrates); thus, the trigger of this inflammation can be considered to be of metabolic nature. Interestingly, not only the trigger of this inflammation is metabolic, but this inflammation first targets the cells that specialized in metabolism: e.g., adipocytes, hepatocytes, pancreatic β-cells, and myocytes (but also the neurons of the peptidergic systems involved in the regulation of feeding behavior, as detailed further). Hence, this inflammation is often referred to as "metabolic inflammation" or "metainflammation" (79).

The characteristics of the inflammation associated with obesity, in periphery at least, are: (i) being of metabolic nature—it is induced by nutrients and is orchestrated by "metabolic" cells; (ii) being low grade, with moderate and localized overexpression of pro-inflammatory mediators; (iii) creating an impaired milieu where the tissue "composition" in terms of immune cells favors an inflammatory environment in the tissues; and (iv) being sustained in time, without any apparent resolution (79). Furthermore, peripheral metabolic inflammation associated with obesity is deleterious at many levels and, as a consequence of poor eating habits favoring the obese phenotype, inflammation itself promotes tissue dysfunctions, which also contribute to development of obesity, including resistance to insulin and leptin.

Obesity and Hypothalamic Inflammation

In the context of nutritional obesity, inflammatory pathways are not only activated in peripheral tissues but also activated in central areas involved in the control of energy metabolism, especially in the hypothalamus. Several questions then deserve to be asked and answered such as "What are the signals that induce this inflammation?" and "Is inflammation a cause or a consequence of obesity?"

The relationship between nutritional obesity and hypothalamic inflammation was first described by De Souza and his collaborators (83). The authors demonstrated in rats that a 4-month period of HFD feeding activates inflammatory pathways in the mediobasal hypothalamus (MBH), such as JNK and NF-κB, leading to the production of canonical pro-inflammatory cytokines such as IL-1β, TNF-α, and IL-6 and to deficiencies in insulin and leptin signaling. These observations on hypothalamic inflammation associated to nutritional obesity were confirmed in rats, obese mice, and non-human primates (82, 84–92). Interestingly, some studies have shown that the inflammation induced by the consumption of HFD, likely a response to overnutrition, develops much faster in the hypothalamus than in the peripheral tissues. Indeed, inflammation in the AT, for example, is only observable after several weeks or even months, whereas in the hypothalamus, the consumption of a HFD during a much more restricted period of 24–72 h is enough to induce overexpression of pro-inflammatory cytokines and gliosis—which corresponds to an activation and proliferation of glial cells such as microglia and astrocytes, an inflammation hallmark in the brain (82).

Thus, it appears that the inflammation induced by the excess of nutrients in the hypothalamus precedes the establishment of obesity, as well as peripheral inflammation and metabolic disturbances. It is worth mentioning that the gliosis induced by the consumption of a HFD for a short-period of time (2–3 weeks) is actually reversible, whereas it reappears after longer periods of HFD consumption (8 months) (82). This suggests that the initial short-term gliosis is rather a protective mechanism induced to protect against the "injury" induced by the overload of fat, which is in line with the known roles of these cells, and that with time, the consumption of HFD triggers a durable gliosis and inflammation that would then play a major role in the development of the pathophysiology induced by overnutrition. For example, a study by Dalvi et al. further supports that acute gliosis associated with a moderate period of HFD feeding would actually be protective: as long as the inflammation is restricted to glial cells, the organism seems to attempt to limit brain injury by overexpressing in parallel some protective factors such as the heat shock protein 70 and the ciliary neurotrophic factor. After a longer period of HFD feeding, overexpression of inflammatory mediators within the neurons suggests an exhaustion of neuroprotective mechanism in the hypothalamus, leading to a deregulation of the expression of certain neuropeptides that will favor a positive energy balance (93).

What is the signal that triggers hypothalamic inflammation? It has been suggested that it is the excess of nutrients, which are major physiological regulators of hypothalamic neural networks, that induces the establishment of inflammation at the level of the hypothalamus. Some studies attribute the triggering of hypothalamic inflammation to, more especially, saturated fatty acids (SFA), especially long-chain SFA, due to their accumulation in the hypothalamus when consuming HFD, which could, among others, induce inflammatory signaling by activating the TLR4 pathway. It is interesting to note that other fatty acids, such as long-chain polyunsaturated fatty acids, appear, conversely to long-chain SFA, to be beneficial, especially in the diet-induced obesity (DIO) context, as they have anti-inflammatory properties (94–97). In contrast, not only nutrients but also hormones seem to play a role in the regulation of glial cells function. Indeed, as illustrated by a study by Gao et al., showing that monogenic obese mice with deficient leptin signaling [*ob/ob* (leptin deficient) and *db/db* (leptin receptor mutation)] exhibit less microglial activation than wild-type controls both on chow and on HFD (98). The authors further demonstrated that the lack of leptin signaling affects also the microglial function in the hypothalamus as the expression of several inflammatory mediators is reduced (98). This indicates that the signaling of leptin, already described as a pro-inflammatory adipokine (99), could affect microglial activation. Other mechanisms have been proposed as mediators of the hypothalamic inflammation associated with overnutrition such as the endoplasmic reticulum stress (86, 100, 101). Similarly,

oxidative stress could be an initiating factor and participate in the maintenance of the hypothalamic inflammation induced by nutrition. Indeed, at the periphery, oxidative stress, along with the production of reactive oxygen species (ROS), has been shown to precede severe metabolic disturbances and insulin resistance (102). Yet, the brain alone uses a large amount of the oxygen and calories consumed by the body, making it particularly vulnerable to excessive ROS production and oxidative stress. Indeed, mitochondria naturally produce ROS in physiological conditions, especially during the oxidation of nutrients such as glucose or fatty acids (103), and it has been shown that an excessive production of ROS in the hypothalamus occurs in the obese rat. This impairs not only the local detection of glucose but also the associated physiological response, such as peripheral insulin secretion (104). Autophagy is another possible pathway. It is a cellular process allowing the elimination of damaged cytoplasmic elements and organelles in order to maintain internal homeostasis and structural integrity and also plays a key role in cellular responses to metabolic stress. Under conditions of overnutrition, endoplasmic reticulum stress and oxidative stress can induce autophagy (105, 106). Impairing the pathways of autophagy can increase food intake and weight, in association with overactivation of the IKKβ/NF-κB pathway in the hypothalamus, illustrating the connection between autophagic and inflammatory pathways in the hypothalamus.

Over the recent years, the importance of the role played in hypothalamic inflammation in the onset of metabolic disorders in obesity was indeed underlined and the potential of counteracting inflammation in the hypothalamus as a strategy in the fight against obesity was highlighted.

For example, a recent study by Douglass and colleagues demonstrates that astrocytes in the MBH of HFD-fed mice mediate hypothalamic inflammation together with DIO *via* their own inflammatory signaling (107). Indeed, the authors showed that the inducible and specific deletion of IKKβ in astrocytes, which should blunt astrocytic inflammatory capacity, reduces the mice susceptibility to DIO (reduced weight gain and fat mass associated with a decrease in food intake and an increase in energy expenditure), improves glucose tolerance and insulin sensitivity (according to glucose and insulin tolerance tests), and finally, reduces HFD-induced astrocytosis in the MBH.

Furthermore, it has been shown that subcutaneous application of liraglutide or canagliflozin (an inhibitor of the sodium-glucose cotransporter 2) in obese and insulin-resistant rodents is able to disrupt the activation of microglial cells in the hypothalamus, which is associated with an improvement of insulin and glucose homeostasis. In addition, when the ICV injection of IL-4 in HFD-fed rats increases further HFD-induced inflammation in the hypothalamus and causes excessive weight gain, the ICV injection of an IKKβ/NF-κB blocker allows, on the contrary, to prevent hypothalamic inflammation, which is associated with both a decrease in body weight and fat mass and improvements in glucose metabolism and general energy homeostasis in DIO animals (86, 108–111).

Another study by André et al. shows that overnutrition will induce, in addition to an increase in body weight and in adiposity, an increase in the number of microglial cells in the ARC. The authors show the inhibition of microglia expansion in the ARC, achieved by the central delivery of an antimitotic drug, allows to limit food intake and the increase in body weight and in adiposity and also to restore leptin sensitivity. This is associated with a "predictable" inhibition of the upregulation of inflammatory pathways in the ARC that is normally associated with overnutrition, but also in periphery (112).

Taken together, studies studying the hypothalamic inflammation associated with obesity support that this inflammation plays a key role in the metabolic dysfunctions associated with obesity and represents a very interesting target.

Research aiming to understand how the central regulation of energy balance is altered in the context of overnutrition has mainly focused on neurons so far. Yet, as previously mentioned, since hypothalamic inflammation, especially its gliosis feature, has been associated to overnutrition, a strong interest in the role of glial cells (mainly microglia and astrocytes) emerged more recently. Interestingly, these glial cells, once activated, are capable of overproducing, locally, pro-inflammatory mediators such as cytokines and chemokines that can, in turn, affect the neuropeptidergic systems of the hypothalamus, potentially participating in the onset of obesity.

Interestingly, chemokines and their receptors have been demonstrated to be expressed by glial cells, and also by neurons. Especially, chemokine receptors appear to be more expressed by neurons than cytokine receptors in some nuclei of the hypothalamus, allowing to ask the following question: "Could not chemokines act as one of the latest inflammatory mediator that would link inflammation to the disruption of the proper functioning of the neuropeptidergic systems involved in the regulation of the energy balance to promote weight gain and thus the development of obesity?"

Chemokines in the Modulation of Hypothalamic Neuropeptidergic Circuits in DIO

As it was the case in the context of hypothalamic inflammation and involuntary weight loss, only few studies have aimed to determine and understand if and how chemokines are able to alter the function of hypothalamic neuropeptidergic circuits and thus participate in the development and/or the maintenance of obesity.

A study by Morari and colleagues has identified fractalkine (or CX3CL1) as involved in the early activation of hypothalamic inflammation in a murine model of DIO, likely through recruitment of microglial cells which express CX3CR1, the receptor for CX3CL1 (75). In this study, the authors show that, early after HFD introduction to the mice (1–3 days), CX3CL1 is induced in hypothalamic neurons of obesity-prone mice, unlike what was observed in obesity-resistant mice. Interestingly, the inhibition of CX3CL1 in the MBH by an approach of small interfering RNA allows a reduction in inflammation, glucose intolerance, and diet-induced adiposity (no significant difference in body weight was observed though). It suggests that CX3CL1, mediating early recruitment of microglia induced by HFD and thus participating in the induction of hypothalamic inflammatory response, participates in the pathogenesis of obesity as it impairs glucose tolerance and adiposity (**Figure 3**).

In addition, one recent study highlights the role of the axis CXCL12/CXC-motif chemokine receptor 4 (CXCR4) in the paraventricular nucleus (PVN) of the hypothalamus in mediating both neuronal and behavioral effects of the consumption of a HFD in rats (76). In more details, the authors show that a 5-day consumption of a HFD induces an overexpression of CXCL12 and its receptors CXCR4 and CXCR7 both in the PVN and in the perifornical lateral hypothalamus (PFLH). The authors also show that HFD is able to induce an overexpression of orexigenic neuropeptides enkephalin and galanin in the PVN and orexin (ORX) and MCH in the PFLH. Moreover, HFD is associated with an increase in the number of $CXCR4^+$ cells in the PVN. Conversely, in the arcuate nucleus, the levels of CXCL12 and CXCR4 were too low to be detected. The ICV injection of CXCL12, next to the hypothalamus, is able to recapitulate the effects of HFD consumption: (1) it reduces novelty-induced locomotor activity, as a 5-day HFD feeding period does; (2) it is associated to an increase in gene expression of enkephalin in the PVN; and (3) it induces an acute increase in calorie intake (by overconsumption of the HFD only and no consumption of chow diet, as rats had access to both diets). This last point interestingly suggests that CXCL12 could participate in the control ingestive behavior, especially since some different studies also previously demonstrated that CXCL12 could affect other systems such as the MCH and vasopressin in the hypothalamus or dopamine in the substantia nigra (18, 113, 114). Taken together, these results suggest that CXCL12 could modulate the activity of orexigenic peptide-producing neurons, especially the encephalin neurons in the PVN. This would favor HFD intake and decrease locomotor activity and, thus, participate in HFD-induced weight gain (**Figure 3**).

The chemokine CCL5, also known as RANTES for Regulated on Activation, Normal T Cell Expressed and Secreted, has already been linked to obesity-associated inflammation in periphery. Indeed, obesity is associated with an increase in CCL5 secretion and gene expression in AT in obese human and mice. There, CCL5 is suspected to mediate the increase in the local accumulation of T cells and macrophages, which is involved in the complex genesis of chronic inflammation (115, 116). In addition, our joint study with Dr. Karine Clément's team proposed CCL5 as biomarker of weight evolution in patients undergoing bariatric bypass surgery Roux-en-Y as its levels were nearly eightfold higher in the serum of obese patients than in the one of control patients. CCL5 seems to be the only chemokine of which serum levels appear to be correlated with caloric intake in patients undergoing bariatric bypass surgery Roux-en-Y: they rapidly decrease after surgery, as caloric intake decreases and, later on, when patient's caloric intake and body weight start increasing again, they also increase again (77). This justified further interest in the potential role of CCL5 as a modulator of the activity of hypothalamic neurons regulating food intake and which could possibly promote the establishment and/or development of obesity. Thus, our group characterized its expression profile, both at the peripheral and hypothalamic levels in a model of nutritional obesity in mice. In this model, we found both peripheral and central inflammations, as evidenced by the overexpression of canonical pro-inflammatory mediators in the serum and hypothalamus. CCL5 was also found to be overexpressed in the periphery and in the hypothalamus of obese animals (78). An ICV injection of CCL5 on the expression of orexigenic peptides MCH and ORX seems able to increase the expression of both ORX and MCH, transiently for ORX and in a more stable and persistent manner for MCH. Furthermore, it would appear that CCL5 is capable of depolarizing MCH neurons, thus facilitating their activation (78). These results indeed suggest that CCL5 is acutely able to modify the activity of the hypothalamic orexigenic MCH system. Taken together, these results suggest that the overexpression of CCL5 would promote the overactivation of hypothalamic MCH neurons and thus participate in weight gain (**Figure 3**). Whether CCL5 is a cause or a consequence of obesity, or if it could effectively disrupt the proper function of the neuropeptidergic circuits of the hypothalamus, which regulate energy balance and promote the establishment of obesity, is nevertheless still matter of investigation.

CONCLUSION

As of today, a substantial amount of publications supports that hypothalamic inflammation mediates disruptions in the hypothalamic control of energy homeostasis, especially regarding body weight regulation. If some pathways and cellular actors have been identified, mechanisms are still ill-described and poorly understood.

Chemokines have long been considered as essential mediators of the inflammatory response, but more particularly because of their ability to activate and attract immune cells to the affected site. However, at the central level, the literature has also attributed to chemokines and their receptors, beyond their role in the attraction of leukocytes in the cerebral parenchyma, important roles in neuronal survival as in neurotoxicity, cerebral development, communication between immune and glial cells, and also communication between neurons and glial cells (since all these cell types have been shown to be able to express the actors of the chemokinergic system) and also in neuromodulation (15, 18).

Several studies now support the hypothesis that chemokines are actually able to modulate the activity of certain neurons. We and others were able to demonstrate that their overexpression is able to deregulate the neuropeptidergic systems of the hypothalamus, which participate in the regulation of the energy balance and participate in the development of deregulations of the latter, whether it is an excessive weight loss or gain (**Figure 3**). Moreover, this does not, in any way, exclude that, under physiological conditions, the chemokines are actors of this regulation. These studies on chemokine central signaling now identify chemokines as novel potential therapeutic targets against deregulations of the energy balance.

ETHICS STATEMENT

The protocols were carried out in accordance with French standard ethical guidelines for laboratory animals and with approval of the Animal Care Committee (Nice-French Riviera, project agreements no. 04042.01 and 04464.01).

AUTHOR CONTRIBUTIONS

OLT conceived and wrote the manuscript. OLT also made the figures. OLT and KS realized the bibliography researches. CC, JLN, NB, and CR critically appraised and revised the manuscript and figures. All authors listed made substantial, direct, and intellectual contribution to the work and gave permission for this manuscript to be published.

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REVIEW
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Overlapping Neural Endophenotypes in Addiction and Obesity

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Impulsivity refers to a tendency to act rapidly without full consideration of consequences. The trait is thought to result from the interaction between high arousal responses to potential rewards and poor self-control. Studies have suggested that impulsivity confers vulnerability to both addiction and obesity. However, results in this area are unclear, perhaps due to the high phenotypic complexity of addictions and obesity. Focusing on impulsivity, the aim of this review is to tackle the putative overlaps between addiction and obesity in four domains: (1) personality research, (2) neurocognitive tasks, (3) brain imaging, and (4) clinical evidence. We suggest that three impulsivity-related domains are particularly relevant for our understanding of similarities between addiction and obesity: lower self-control (high Disinhibition/low Conscientiousness), reward sensitivity (high Extraversion/Positive Emotionality), and negative affect (high Neuroticism/Negative Emotionality). Neurocognitive studies have shown that obesity and addiction are both associated with increased impulsive decision-making and attention bias in response to drug or food cues, respectively. Mirroring this, obesity and different forms of addiction seem to exhibit similar alterations in functional MRI brain activity in response to reward processing and during self-control tasks. Overall, our review provides an integrative approach to understand those facets of obesity that present similarities to addictive behaviors. In addition, we suggest that therapeutic interventions targeting inhibitory control may represent a promising approach for the prevention and/or treatment of obesity.

Keywords: obesity, addiction, impulsivity, brain, personality and neurocognitive characteristics

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INTRODUCTION

Obesity and addiction are complex and heterogeneous conditions at the intersection of biology and mental health. A bulk of scientific literature has highlighted the importance of neurobiological and neuropsychological factors in the pathophysiology of obesity (**Figure 1**) (1, 2). More importantly, growing evidence suggests that obesity shares common mechanisms with addiction in terms of neurobiological systems that underlie reward and self-regulation processes (3–5). The goal of this review is to critically assess the putative overlaps between addiction and obesity in four domains: (1) personality research, (2) neurocognitive task, (3) brain imaging, and (4) clinical evidence.

BRAIN MECHANISMS OF APPETITE CONTROL AND UNDER CONTROL

Three interconnected brain systems control food intake and eating behavior: (1) the hypothalamus, which responds to internal energy-balance signals, (2) the limbic system [amygdala/hippocampus, insula, orbitofrontal cortex (OFC), and striatum], which is involved in learning and memory and

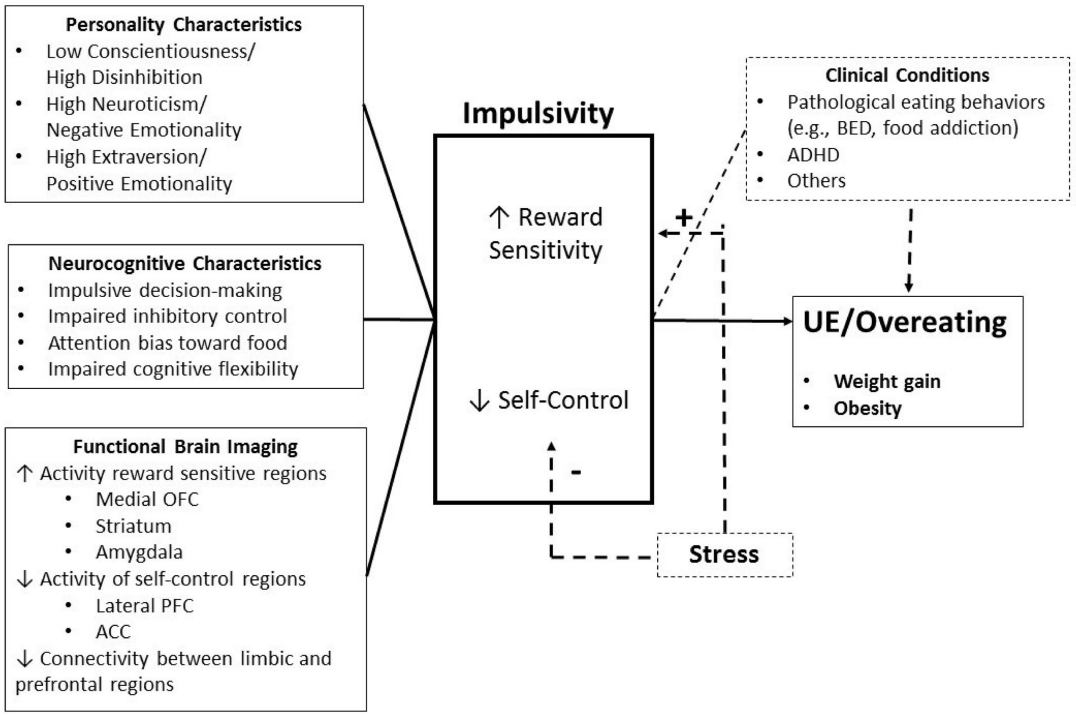


FIGURE 1 | Brain endophenotype of obesity vulnerability. Personality, cognitive, and functional brain imaging characteristics that increase obesity vulnerability. Uncontrolled eating (UE) results from an interaction of elevated reward sensitivity and poor self-control. OFC, orbitofrontal cortex; PFC, prefrontal cortex; ACC, anterior cingulate cortex; BED, binge-eating disorder; ADHD, attention deficit/hyperactivity disorder; BMI, body mass index.

encodes the value or incentive salience of foods, and (3) the cortical (mostly prefrontal) cognitive control system, which enables behavioral self-regulation (6, 7). The normal function of these systems maintains energy homeostasis, enables learning about the nutrient content of foods, and promotes motivation to seek and consume foods as appropriate.

However, individual differences in neurobiological mechanisms involved in the control of food choices and food intake likely explain why some individuals are more susceptible to weight gain than others (8). Indeed, obese individuals may have neurocognitive characteristics that predispose them to overeating upon exposure to favorable environmental or endogenous conditions. One such characteristic is impulsivity. Although many definitions exist (9–14), impulsivity is generally considered as the tendency to act rapidly without full consideration of consequences (15). Sharma et al. (16) recently conducted a meta-analytic principal-components analysis and proposed that impulsivity is a multidimensional construct that includes various distinct psychological components such as disinhibition, neuroticism, extraversion, sensation seeking, inattention, impulsive decision-making, insufficient inhibitory control, and lack of cognitive flexibility (16–19).

Impulsivity is a key component of several neuropsychiatric disorders such as attention deficit/hyperactivity disorder (ADHD), mania, and personality disorders (20, 21). Numerous studies have reported that impulsivity, a personality trait generally observed in individuals with addiction (22–26), may also be associated with high-calorie dietary choices, undercontrolled eating, and the development of obesity (27–31). For instance, individuals characterized by frequent disinhibited behavior and elevated response to potential rewards may be more vulnerable to develop unhealthy weight gain when exposed to the so-called "obesogenic" food-abundant environment (8, 28, 32). Neurobehavioral processes that lead to impulsivity result from the interaction of high arousal response to potential rewards (i.e., reward sensitivity) and poor self-control (i.e., rash spontaneous impulsivity) (14, 28). The reward system is generally thought to encompass projection sites of mesolimbic dopamine neurons, while self-control is dependent on the prefrontal cortex (PFC), especially the lateral PFC, and dorsal anterior cingulate cortex (ACC). Individual differences in impulsivity might constitute a common denominator across obesity and drug addiction. In this regard, several studies have suggested the existence of similarities between addiction and obesity in reward processing (4, 5, 33, 34). In fact, addictive drugs are thought to be addictive by virtue of their actions on neural systems that primarily control appetitive responses to natural rewards such as food (4, 34–36). Dopamine circuitry plays an important role in encoding the reinforcing values of addictive substances (37, 38).

Considering that some neurobehavioral characteristics that confer vulnerability to addiction may also represent risk factors for obesity, this review is aimed at tackling the following question: is the impulsive and poor self-control phenotype identified in drug addiction also present in obesity? The next sections review the evidence in terms of personality, neurocognitive tasks, neuroimaging, and clinical evidence.

PERSONALITY CHARACTERISTICS

Personality traits reflect tendencies for cognitive, emotional, and behavioral responses to events and environments. Traits that capture impulsive tendencies have been associated with unhealthy weight gain and addiction (39). A recent meta-analytic principal component analysis of personality questionnaires identified three distinct impulsivity subdomains (16): (1) Disinhibition versus Constraint/Conscientiousness, (2) Neuroticism/Negative Emotionality, and (3) Extraversion/Positive Emotionality. These dimensions map well to the "Big Five" personality framework (40), the UPPS (Urgency, Perseverance, Premeditation, Sensation Seeking) scale (19), and many other impulsivity conceptualizations (9, 11). Therefore, we use this three-factor decomposition of impulsivity (16) as a base framework to organize evidence that personality-measured impulsivity is associated with addiction and obesity (**Table 1**).

High Disinhibition and Low Constraint/ Conscientiousness

The Disinhibition versus Constraint/Conscientiousness factor is comprised of two subfactors associated with behavioral dyscontrol: lack of planning, leading to an inability to refrain from hasty actions, and a lack or perseverance, leading to an inability to maintain self-control in the face of adversity (16). This factor relates to the following measures from commonly used personality scales: lack of perseverance and lack of premeditation from the UPPS, low Conscientiousness from the NEO-Personality Inventory-Revised NEO-PI-R, and motor impulsivity and non-planning impulsivity from the Barratt Impulsiveness Scale (BIS) (16).

Low scores on Conscientiousness have been related to various addictive behaviors (41) including illegal substance abuse (42–44), gambling problems (45), smoking (46–48), and alcohol use (49, 50). Furthermore, lower Conscientiousness increases the risk of relapse after treatment (51). Lack of planning or premeditation assessed using the UPPS scale is also an independent predictor of addiction (52). Thus, the high Disinhibition and low Conscientiousness domain of impulsivity is consistently associated with a higher risk of addiction, supporting the importance of self-control in resisting drug abuse.

Similarly, obesity has consistently been associated with a reduced level of Conscientiousness (28, 53) as measured by the NEO-PI, an association confirmed in a large meta-analysis involving close to 50,000 individuals (54). In a large heterogeneous sample using the BIS, Meule and Blechert (31) found that higher attentional and motor impulsivities were predictive of higher body mass index (BMI) after statistical adjustment for age and sex. However, the effect was small, and non-planning impulsivity was not significantly associated with BMI (31). Finally, studies using the UPPS have also found an association between BMI and lack of perseverance, which is the inability to persist with challenging tasks (55, 56). Furthermore, higher levels of habitual disinhibition, as measured by the Three-Factor Eating Questionnaire, have been associated with body weight gain over time (57). Disinhibition here refers to a tendency to overeat upon exposure to palatable foods or stressful situations, a trait related to consciousness and self-control. In light of these studies, obesity seems to be associated with high Disinhibition and low Conscientiousness. These traits may increase the tendency of an individual to overeat in certain situations and may complicate the maintenance of behaviors associated with body weight reduction in obese individuals (58).

Neuroticism/Negative Emotionality

The factor Neuroticism/Negative Emotionality reflects a tendency to act rashly in response to negative emotions and to experience cravings when in negative mood states (16). It is reflected in neuroticism in the NEO-PI-R, negative urgency in the UPPS, and attentional impulsivity in the BIS (16).

Neuroticism (NEO-PI-R) has been related to various addiction syndromes, including substance abuse (42–44), problem gambling (45), smoking (46–48), and alcohol use (49, 50), and also with increased risk of relapse after treatment (51). Other studies have also reported an association between negative urgency (UPPS) and substance addiction (59–62). In sum, individuals with addictive behavior may engage in drug use as a way of coping with stress and negative emotion.

The relationship between obesity and neuroticism is less evident. While previous reviews have reported a link between the two (28, 53), a recent meta-analysis found no association (54). A possibility for this lack of significant relationship is that body weight is specifically linked only to some facets of negative emotionality. For example, it has been consistently shown that only the impulsiveness subfactor ("N5:Impulsiveness") of the NEO-PI-R correlates with adiposity (39, 63). Findings from the UPPS

TABLE 1 | Summary of the main associations between addiction or obesity and impulsivity measurements.

	Addiction	Obesity
Personality characteristics		
High Disinhibition and low Constraint or Conscientiousness	+	+
High Neuroticism/Negative Emotionality	+	+, NS
High Extraversion/Positive Emotionality	+	+, NS
Neurocognitive characteristics		
Impulsive decision-making	+	+
Impaired inhibition	+, NS	+
Inattention	+	+, NS
Impaired set-shifting	+, NS	+
Functional brain imaging		
Reward/motivation system		
Medial OFC/VMPFC	↑ Activity	↑ Activity
Striatum	↑ Activity	↑ Activity
Amygdala	↑ Activity	↑ Activity
Self-regulation system		
Lateral PFC	↓ Activity	↓ Activity ↓ OFC–PCF connectivity
ACC	↓ Activity ↓ ACC–striatum connectivity	↓ Activity

+, positive associations; NS, association not significant; ↑, increase; ↓, decrease; OFC, orbitofrontal cortex; VMPFC, ventromedial prefrontal cortex; PFC, prefrontal cortex; ACC, anterior cingulate cortex.

support this notion, as negative urgency, a tendency to experience strong impulses during negative affect, has been linked to greater BMI (55, 56). Other factors that could obscure the link between obesity and Neuroticism/Negative Emotionality include the fact that the association may be present only in women and that neuroticism may also predispose to underweight, *via* a link to eating disorders (64). This could obscure a linear relationship between obesity and neuroticism in population studies. Finally, the link between neuroticism and obesity could be driven by two questions in the Neuroticism scale of the NEO PI-R that specifically target uncontrolled eating (UE) behavior (65, 66).

In summary, the association between the Neuroticism/Negative Emotionality domain and obesity is somewhat less consistent than that with Conscientiousness and Disinhibition. Nonetheless, this personality trait may predispose an individual to overeating in conditions of emotional distress (67), which may lead to adiposity in the long term.

Extraversion/Positive Emotionality

The Extraversion/Positive Emotionality factor refers to sensation seeking and sensitivity to appetitive or rewarding cues (16). Individuals with high Extraversion/Positive Emotionality are sensitive to positive environmental stimuli and more likely to engage in impulsive or reward-seeking behaviors when they experience positive emotions. They are said to seek novel and exciting experiences. Extraversion/Positive Emotionality correlates with the Extraversion domain in the Five-Factor Model of personality and with Sensation Seeking of the UPPS (16). The Sensitivity to Reward portion of the Sensitivity to Punishment and Sensitivity to Reward Questionnaire (SPSR) is a self-report questionnaire that also assesses this dimension (28, 68).

Numerous studies suggest that reward-driven impulsivity represents a risk factor for both drug addiction and overeating by enhancing the motivation to obtain drugs or palatable foods (69, 70). Higher scores in Extraversion have been related to drug addiction (47). A related trait, positive urgency, the tendency to act rapidly in response to positive emotions, was also correlated to substance addiction (59–62). In addition, Sensation Seeking is commonly associated with substance-use disorders and alcohol problems (62). In sum, the literature is consistent in associating the Extraversion/Positive Emotionality domain of impulsivity to addictive disorders.

Some studies have proposed that high BMI is associated with increased levels of Extraversion (28, 53). Higher scores in Extraversion also seem to predict prospective weight gain (after 2 years) (71). However, contradictory findings do exist, with a meta-analysis (54) failing to show a consistent relationship between obesity and Extraversion in longitudinal studies. However, Davis et al. (72) found that reward sensitivity, as assessed by the SPSR, was associated with maladaptive eating behaviors such as preference for high-calorie foods and overeating (72). They suggested that some individuals may have greater reactivity to food cues and that weight management, in these individuals, may represent a continuous struggle in the modern obesity-promoting food environment. Using the SPSR, this group also demonstrated an inverted U-shaped relationship between reward sensitivity and BMI in a sample of subjects covering a large spectrum of adiposity values, suggesting that lean and severely obese subjects were less sensitive to reward than overweight and obese subjects (73). By using the Behavioral Activation Scale, other groups have also provided evidence of a quadratic relationship between BMI and reward sensitivity (74, 75). To explain this curvilinear relationship, Davis and Fox (73) proposed that both hyper- and hyposensitivity to reward could predispose to obesity. The possibility of an inverted U-shape relationship between BMI and Extraversion suggests that differences in the range of sampled BMI across studies might account for the discrepancies in the literature. In addition to this, gender might modulate the correlation between Extraversion and BMI. For women, lower scores in Extraversion seem to relate to higher adiposity (76, 77), while the opposite has been reported in males (76, 78).

Overall, although contradictory findings do exist, the current evidence points in the direction of similar impulsivity profiles in obesity and addictive disorders. Specifically, these two disorders seem to share lower cognitive control (high Disinhibition/low Conscientiousness), and a tendency toward making impulsive decisions in response to positive (high Extraversion/Positive Emotionality) and negative (high Neuroticism/Negative Emotionality) mood states. **Figure 2** displays a comprehensive overview of personality differences in obesity and addiction as derived from Ref. (39, 42, 79). This shows that while, on a broad level, obesity seems to be similar to addictive behaviors, there are also differences at the finer level of personality subscales.

NEUROCOGNITIVE TASKS

Laboratory-based neurocognitive tasks can be used to measure inhibitory control or self-regulation. Commonly used examples are the delay discounting task, the stop-signal task (SST), the Go/No-Go task, the Stroop task, and the Wisconsin card sorting task (WCST) (80). These neurocognitive tests assess various dissociable dimensions of impulsivity, including impulsive choice, impulsive responding, and inattention (15, 81). Sharma et al. (16) also performed a meta-analytic principal-components factor analysis of the most commonly used behavioral task measures of impulsivity and they identified four major domains: (1) impulsive decision-making, (2) inattention, (3) inhibition, and (4) shifting. The next sections describe how these four domains of impulsivity are associated with addiction and obesity (**Table 1**).

Impulsive Decision-Making

Impulsive decision-making (or impulsive choice) refers to a tendency not to delay gratification and to prefer immediately available rewards (16). It is typically tested with the delay discounting task, in which participants must choose between an immediate, smaller monetary sum and a larger, delayed amount (82). A steeper delay discounting rate is associated with a greater preference for immediate rewards, which reflects impulsive decision-making.

Kirby and Petry (83) have demonstrated using a questionnaire version of this task that substance-addicted individuals have higher discounting rates for delayed rewards than controls. Two meta-analyses also provided strong evidence that steeper impulsive discounting rate is associated with the severity and the frequency of addictive behaviors (84, 85). The magnitude of the

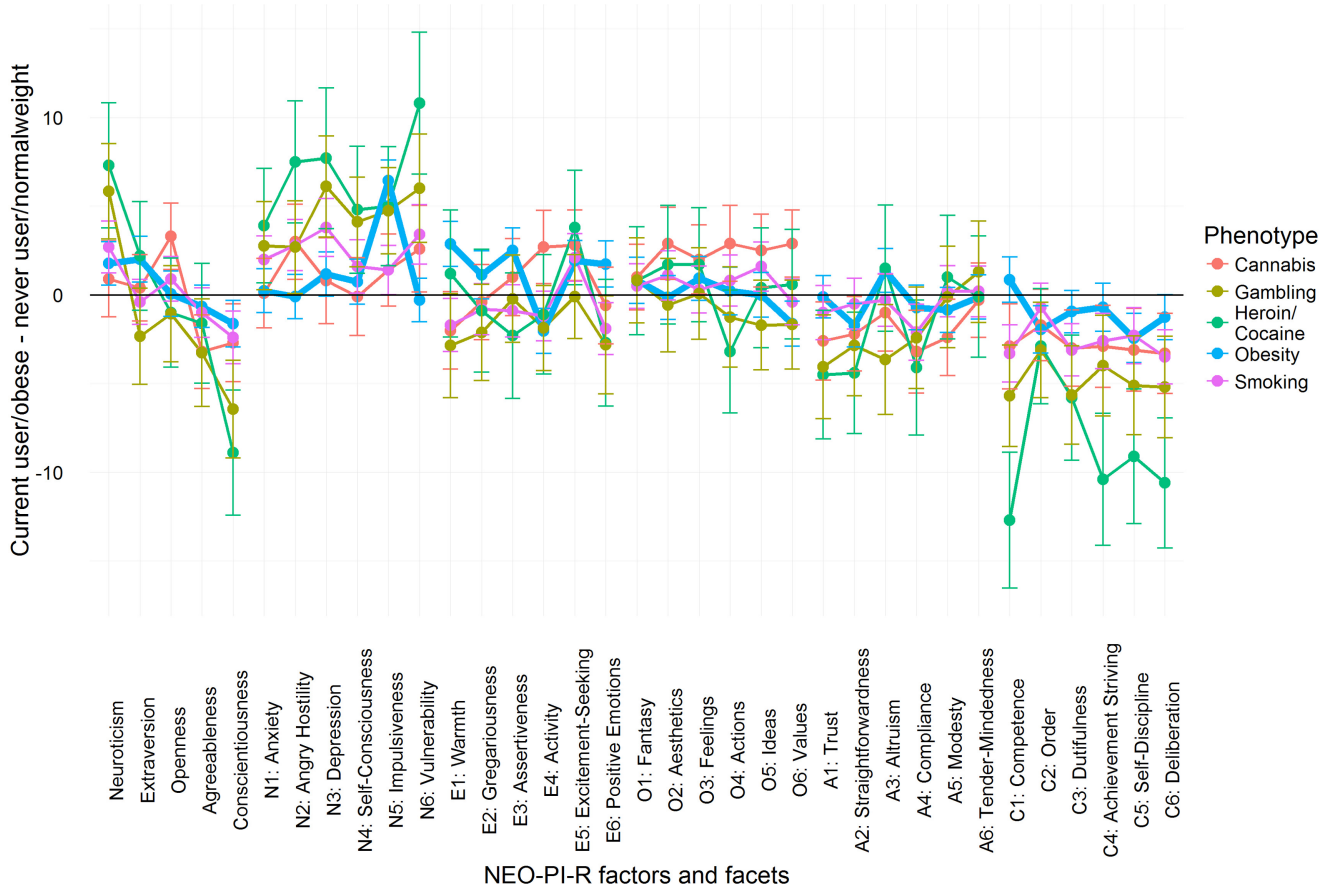


FIGURE 2 | Personality profiles of obesity and addictive phenotypes according to NEO-personality inventory revised. We present the difference in T-score units between obese minus normal weight group and addiction phenotype group minus control group. On a broad factor level, all phenotypes share higher Neuroticism (high Negative Emotionality) and lower Agreeableness and Conscientiousness (high Disinhibition). However, on a finer facet level, the profiles become less similar. For instance, obesity sets apart from other addictions only peaking at one facet of Neuroticism, and not on all facets of Conscientiousness. Therefore, while there are broad similarities, obesity and addictive phenotypes are not fully similar to each other. Mean scores were obtained from these papers (39, 42, 79).

association was similar between various types of addictive problems (alcohol, gambling, tobacco, cannabis, opiates, and stimulants) (85). The same group also reported a similar relationship in obesity: although results vary, their meta-analysis concluded that obesity is associated with steeper delay discounting of future monetary and food rewards (86). Interestingly, Weygandt et al. (87) recently found that less functional MRI (fMRI) activation of inhibitory-control areas during a delay discounting task is associated with poor weight loss maintenance in the long term. More specifically, obese subjects seem to have greater delay discounting for food compared to other type of rewards. Similarly, substance-addicted subjects have greater delay discounting for drugs compared to other type of rewards (28, 85, 86). Impulsive decision-making in addiction and obesity may explain why some individuals engage in maladaptive behaviors that are immediately rewarding but detrimental in the long run.

Another perspective in impulsive decision-making revolves around the concept of risk sensitivity. Risk sensitivity refers to the individual degree of attraction or aversion to uncertain outcomes (88). A moderate risk-seeking behavior may confer advantages in the discovery of new environments and resources and might lead to experiencing exciting adventures. However, an excessive attraction toward risk may also be associated with adverse consequences and might have a role in the development of drug addiction. In recent years, the concept of risk sensitivity has been used to describe impulsive behavior in addiction and obesity (89, 90). Both addiction and obesity might involve to some extent an approach tendency toward short-term pleasure despite the risk of long-term negative consequences (89, 91). Several studies have suggested the existence of addiction-related alterations in risky choices. For example, compared with healthy controls, participants who binge drink exhibited increased risk-seeking when anticipating large unlikely monetary losses (92). Risky decision-making and higher delay discounting also appear to hamper the maintenance of abstinence following treatment (93).

Relatively few studies have directly examined risk-taking similarities or differences between addiction and obesity to date. One study found that obese individuals with and without binge-eating disorder (BED) made as many risky choices in a monetary task as drug addicts (94).

Inhibition

The inhibition domain refers to the ability to suppress prepotent motor responses (16). Tasks that test inhibition include the Go/No-Go and the SST (80, 82). In the Go/No-Go task, individuals are asked to answer as quickly as possible when a repeated visual stimulus appears (Go signal) but to inhibit their response when a rare stop signal appears (No-Go signal). In the SST task, the stop signal is presented after the Go signal to measure the ability of an individual to stop an already initiated response (95).

Considerable evidence links drug addiction to impaired inhibitory control (96–98). A meta-analysis of 97 studies using the SST or Go/No-Go tasks reported that impaired inhibitory control is generally observed in subjects with heavy substance-use disorders and pathological gambling (99). However, there was lack of evidence for inhibitory deficit in subjects diagnosed with cannabis, opioid, or Internet addiction (99).

Similarly, obesity has been linked to poor inhibitory control. A comprehensive literature review found that obese and overweight individuals have lower inhibitory-control performance in food-specific versions of the SST (100). The authors proposed that the SST may be a good marker to identify individuals at high risk of weight gain or less responsive to weight loss interventions (100). Poor inhibitory control is also associated with higher prospective weight gain (101, 102) and food intake (103). Furthermore, a recent meta-analysis confirmed that obese adults display inhibitory-control deficits compared to lean controls (104). Similar findings have been reported in children and adolescents (104–108). However, Loeber et al. (109) found no significant differences between lean and obese participants in performance during a food-related Go/No-Go task. Furthermore, others did not find an effect of BMI *per se* on SST performance in response to food, but rather a complex interaction between BMI and impulsivity (110).

Furthermore, Voon et al. (111) used a serial reaction time task adapted from rodent experiments to assess a somewhat different form of motor impulsivity: waiting impulsivity or premature responding. They found that premature responses were significantly higher in addicted individuals (alcohol, smoking, and drugs) but not in obese or BED subjects. Thus, certain forms of motor impulsivity seen in addiction are not present in obesity.

Inattention

The third impulsivity domain considered here refers to the ability to focus attention on specific activities while suppressing the response to distracting stimuli (16). The Stroop task is typically used to measure the inattention domain of impulsivity (16). This task requires participants to identify (usually verbally) the color of a written color word, without reading the word itself. When the word is printed in a color that is incongruent with the word (for example, the word blue printed in green), there is a conflict between word reading and color naming. PFC has been implicated in the performance of the Stroop task (112).

A refinement of this task, the "addiction-Stroop," in which the distractor stimuli represent the addictive substance of interest, has also been used to assess altered attentional processes associated with addictive behaviors (113). Indeed, there is considerable evidence that individuals with addiction have an attentional bias toward drug-related cues, which may play an important role in drug craving, consumption, and relapse (114). Similarly, some studies have reported that obese individuals may have attentional biases toward food-related cues, which may increase food consumption and weight gain over time (115). Hall et al. (116) found that elevated levels of inattention were predictors of high-calorie snack consumption. Furthermore, a recent study demonstrated that obese individuals are characterized by lower scores on the traditional Stroop task (117). Even though some reviews reported inconsistent associations between attentional bias for food-related cues and obesity (28, 115, 118, 119), we previously concluded in a comprehensive review that the Stroop task seems to be one of the most consistent cognitive control tasks demonstrating replicated associations with obesity and weight-related eating behaviors (28).

Shifting

Behavioral flexibility, or the ability to switch attentional or task set in response to changing rules, has also been linked to impulsivity (16). It is typically evaluated with the WCST (16). During this task, participants are asked to match a response card to one of four category cards based on specific rules (e.g., color, shape, or number) (120). The rules change over time and subjects need to modify their response accordingly. A tendency to fail to switch is called perseveration, and it may reflect a form of impulsivity. Poor cognitive flexibility has been associated with compulsive behaviors (121, 122).

A recent review by Morris and Voon (122) argued that the links between cognitive flexibility assessed using the WCST and addiction are inconsistent. Indeed, some studies reported impaired cognitive flexibility in substance-addicted (123) and non-substance-addicted (gambling, bulimia) individuals (124). However, others found no significant association between performance on the WCST and addiction (125–127). With respect to obesity, a recent study reported impaired performance on the WCST in obese individuals compared to individuals with other eating disorders (128). In addition, a meta-analysis (121) and systematic review (118) both reported impaired WCST performance in obese individuals compared to controls. However, overweight rather than obese individuals were not characterized by set-shifting impairment (121).

Overall, current evidence from neurocognitive tasks is that obese and addicted individuals are both generally characterized by higher impulsive decision-making and attentional bias in response to drug or food cues. In addition, obesity is usually associated with altered cognitive flexibility (set-shifting) assessed with the WCST and poor inhibitory control assessed with the SST.

NEUROIMAGING

Neuroimaging has been used to investigate functional and anatomical neural correlates of the vulnerability to drug abuse and overeating. Vulnerability to addiction can be considered as resulting from the interaction of increased incentive response to drug cues, propensity for habit formation, poor self-control, and

heightened negative emotionality (129, 130). These processes are related to different but interconnected brain systems: (1) the mesolimbic dopamine system, implicated in reward, motivation, and habit formation, which includes the ventral tegmental area, ventral striatum, anterior insula, OFC, amygdala, and hippocampus and (2) cognitive control circuits, implicated in self-regulation, including middle and inferior lateral PFC, ACC, and insula (131). Previous neuroimaging studies have shed light on the role of the mesolimbic system in the pathophysiology of addiction (132–139). Participants with addiction seem to exhibit increased fMRI activation in ventral striatum, amygdala, and medial regions of OFC in response to drug cues (133). In general, these results are consistent with the observation that participants with drug addictions exhibit a heightened attentional or motivational focus toward drug-related stimuli (130).

With regards to cognitive control circuits, adolescents who initiate substance use seem to exhibit reduced blood oxygen level dependent (BOLD) activity in the dorsolateral prefrontal cortex (DLPFC), putamen, and inferior parietal cortex during a Go/No-Go task, suggesting that baseline dysfunction in these areas could predict the initiation of drug use (140, 141). In this vein, theoretical work has highlighted the key role of PFC areas in the endophenotype of addiction vulnerability (112). For instance, participants with addiction seem to exhibit prefrontal dysfunction, implicating the dorsal PFC (dACC and DLPFC) involved in self-control, the ventromedial prefrontal cortex (VMPFC) involved in emotional regulation and salience attribution, as well as the ventrolateral prefrontal cortex and lateral OFC involved in inhibitory or automatic responses (112). It has been proposed that the PFC is involved in addictive behaviors through its capacity to regulate subcortical regions implicated in reward processes (112, 142). For example, the strength of the connectivity between dACC and striatum has been negatively associated with the severity of nicotine addiction (143). PFC dysfunction might be implicated in an endophenotype named *impaired response inhibition and salience attribution* (112). This endophenotype both increases sensitivity to drug cues and reduces the capacity to inhibit maladaptive behaviors (144). Consistent with these findings, drug craving seems to involve the amygdala, ACC, OFC, and DLPFC (145), suggesting the involvement of both reward-related and inhibitory-control resources.

Numerous brain imaging studies also support the notion that vulnerability to weight gain and overeating may result from the interaction between elevated food reward sensitivity (incentive salience of the cue) and poor inhibitory control. In response to visual food stimuli, participants with obesity exhibit increased activation in the dorsomedial PFC, the ventral striatum, the parahippocampal gyrus, the precentral gyrus, the superior/inferior frontal gyrus (IFG), and the ACC relative to lean subjects (119–121). These brain regions are thought to encode reward responses, incentive salience, motor coordination, and memory. Longitudinal study designs have shown that increased BOLD activity in reward-related areas (i.e., ventral striatum and OFC) predicts weight gain, suggesting a link between heightened reward responsivity and the development of obesity (146, 147). With regards to inhibitory-control circuits, participants with obesity seem to show consistent blunted activity in the DLPFC and insula in response to visual food cues (148), suggesting a reduced engagement of neural resources associated with inhibition, executive control, and interoceptive awareness. Of note, longitudinal studies have reported that increased activation in the DLPFC in response to high-calorie food images is associated with successful voluntary weight loss (149, 150). An interesting possibility is that self-control processes in the DLPFC may downregulate the activity of the VMPFC and thus, modulate eating choices (151). Supporting this model, stronger functional coupling between the DLPFC and the VMPFC has been associated with successful dietary weight loss (102) and healthier dietary decisions (151). Furthermore, other fMRI studies have reported that the regulation of food craving was associated with increased activity in the DLPFC, IFG, and dorsal ACC (152–154).

A few neuroimaging studies in obesity have specifically addressed cognitive control processes by using cued inhibitory-control paradigms. Here, fMRI studies have found negative associations between brain activation in executive-control regions (lateral PFC) and BMI (155–157). Longitudinal studies have reported that activity in the DLPFC during cognitive control tasks seems to predict successful weight loss after treatment (87, 102). Conversely, impairment of cognitive control over appetitive regions may (1) decrease the acquisition of behaviors leading to successful weight loss and (2) enhance the motivation to consume palatable foods, even in the absence of energy requirements (6, 158).

Together, the aforementioned studies suggest that participants with obesity and patients with addictions present similar functional alterations in frontal regions and in mesocorticolimbic circuits. However, to date few neuroimaging studies have directly compared the impact of obesity and various types of addictions on brain activation. This last point is especially relevant, since food and drug cues seem to activate similar brain regions involved in reward processes, such as striatum, amygdala, OFC, and insula (135). A previous meta-analysis observed that participants with obesity and subjects with different forms of substance addiction exhibited similar heightened BOLD activity in the amygdala and ventral striatum in response to the relevant cues (food in obesity and drugs in addiction) (159).

Overall, current fMRI studies provide evidence for the existence of shared neural mechanisms associated with obesity and different forms of addiction. Poor inhibitory control in combination with increased reward sensitivity and attention to cues (foods or drugs) may be relevant for both obesity and addictive disorders.

CLINICAL EVIDENCE

Binge-Eating Disorder

Binge-eating disorder (BED) is an eating disorder characterized by recurrent episodes of consumption of larger than normal amounts of food in short periods of time (160). These binges are associated with a sense of loss of control and subsequent distress and culpability. Many studies report that individuals with BED display increased impulsivity, altered reward sensitivity, and

altered attentional and memory biases to food-related stimuli (161, 162). For example, individuals with BED have steeper delay discounting of rewards (163) and lower activation in the PFC regions during inhibitory-control tasks (164, 165), suggesting that impulsivity may be importantly related to BED. BED presents phenotypic similarities with substance-use disorders (166). Indeed, substance-use disorders and BED are both characterized by loss of control over consumption, and chronic overconsumption despite negative consequences (167).

The observation that BED shares behavioral and neural underpinnings with substance-use disorders has led to the use of the expression "food addiction," specifically with respect to individuals who meet BED diagnostic criteria, but also more generally as an explanation for obesity. The model hypothesizes that hyper-palatable foods may lead to an addictive response in vulnerable and high-risk individuals (168, 169). Individual variations in "food addiction" can be operationalized by means of scales such as the Yale Food Addiction Scale (YFAS) (166, 170, 171) or the YFAS 2.0 (a revised version adapted for the DSM-5 criteria for substance-related and addictive disorders) (172). However, the model of "food addiction" in humans remains controversial (173–177). The main criticism is that the model is based mostly on animal studies and that the type and quantity of food that characterize "food addiction" are imprecise (173, 174, 177). Furthermore, animals rarely exhibit addition-like behaviors toward sugar; these behaviors only occur when access to sugar is intermittent, and not because of some neurochemical effect of sugar (177). This failure in characterizing what constitutes an addictive agent in foods has led to some theorists to advocate in favor of referring to the phenomenon as "eating addiction" instead (178). We have proposed the term "UE" (65). In addition, even though "food addiction" scores are positively correlated with several measures of adiposity (179), not all individuals with obesity or BED exhibit "food addiction," and conversely, some individuals displaying "food addiction" are not obese (174, 180). Davis (171) suggests that "food addiction" constitutes the last stage of an overeating spectrum (65) and may represent an extreme subtype of BED. In a similar vein, BED has been strongly associated with obesity; however, BED can also occur in individuals with a wide spectrum of body weight (181). As suggested by previous studies, obese individuals with BED seem to represent a specific and possibly rare subtype of obesity (166, 182). Nonetheless, while the lines between BED, "food addiction," and obesity are ill-defined, these conditions seem to share common characteristics including impulsivity and reward dysfunction.

Attention Deficit/Hyperactivity Disorder

Attention deficit/hyperactivity disorder is a neurodevelopmental disorder characterized by inattention, hyperactivity, and impulsivity (160). Neuroimaging studies have suggested a link between ADHD and dysfunction in frontostriatal circuits. For instance, anatomical studies have observed that participants with ADHD exhibit cortical thinning in the PFC, associated with inhibitory-control deficits (183, 184). A frequent comorbidity of ADHD is substance-use disorders (185–187). For example, a longitudinal study found that children and adolescents with ADHD are at higher risk of substance-use disorders and tobacco smoking after a 10-year follow-up period (188).

There is also growing evidence of a link between ADHD and obesity. However, this relationship remains controversial (189, 190). A recent meta-analytic report found a significant association between obesity and ADHD in both children and adults after controlling for possible confounding factors (e.g., gender, study design, country, and study quality) (190). Conversely, another recent meta-analysis reported that the strength of the association between ADHD and obesity was weak. Nevertheless, the effect size increases with age suggesting that the association is stronger in adults than children (189). Two longitudinal studies found that individuals with ADHD are at higher risk of obesity than controls (191, 192). A recent systematic review found that the strength of the association between ADHD and disordered-eating behavior was moderate (193). Furthermore, genetic correlations were found between ADHD, BMI, and smoking (194). To explain the link between ADHD and obesity, researchers have hypothesized that these two disorders exhibit common neurocognitive features, such as impulsivity and inattention (195). Davis et al. (196) also suggested that individuals with ADHD may be more inattentive to their internal signals of hunger and satiety, which may lead to subsequent overeating. Interestingly, the pharmacological treatment of ADHD with dopaminomimetics may facilitate weight control by modulating satiety signals and eating behaviors (197). Overall, ADHD appears to be associated with both addiction and obesity and with the neural endophenotypes that predispose to both, namely, self-control deficits and impulsivity.

Stress or Emotion Dysregulation

Stress is a ubiquitous risk factor across several psychiatric disorders, and it has important implications for our current understanding of addiction and obesity (198, 199). Studies have shown associations between stress and drug craving (200, 201). Chronic exposure to life stressors also predisposes to the transition from casual drug use to substance abuse (202), and it seems to increase the risk of relapse among abstinent users (202). Stress is one of the central elements of the model of addiction proposed by Koob and Le Moal (203). According to this framework, addiction can be conceived as a continuous process of hedonic and homeostasis dysregulation (204). The *spiraling distress* cycle describes how continued drug use along with failures in self-regulation can cause chronic dysregulation of the reward system. As the drug use escalates, patients reach a pathological state that is characterized by increased negative affect and distress, which are particularly pronounced after drug withdrawal. The model hypothesizes that this aversive emotional state constitutes a powerful motivator for drug-seeking, since patients at severe stages of drug addiction will consume drugs to find relief from distress (203).

With regards to obesity, mounting evidence suggests that stress can modify eating patterns (198, 205). Negative mood states or chronic stress increase subjective appetite or food cravings, selective attention toward food, and individual preferences for high-calorie snacks (e.g., sweets and chocolate) (206–209). Increments in food seeking and food consumption during emotionally demanding situations might relate to the fact that

eating a so-called "comfort food" promotes improvements in negative affect (210, 211), in line with the model of Koob and Le Moal. The relationship between stress and food intake, however, presents remarkable interindividual variations. Indeed, stress can be associated with both augmented and diminished appetite (205), with around 30% of the population experiencing increases in appetite, 48% appetite suppression, and the rest no change (212). Studies have suggested that obesity constitutes a crucial predictor of increases in food intake during stress. For instance, while work stress has been associated with weight gain in male participants with elevated BMI, the same psychological stressor leads to weight loss in lean participants (213). Finally, individuals with obesity seem to suffer a higher numbers of adverse life events and chronic stressors compared to lean individuals (198).

Stress acts on brain areas involved in both sides of appetite regulation: the reward/motivation system and the inhibitory-control pathways. For example, Tryon et al. (214) found that in response to high-calorie food pictures, women characterized by higher chronic stress have increased activation in brain regions involved in reward and motivation as well as reduced activation in prefrontal regions. These women also demonstrated greater consumption of high-calorie foods after the scanning session. In a similar vein, Maier et al. (215) compared the neural responses between participants assigned to a laboratory stressor versus those assigned to a neutral condition during a food choice task. Subjects assigned to the stressor put greater value on the taste of the food items presented. Paralleling this, bilateral amygdala and right nucleus accumbens reflected the relative taste value of chosen options more strongly in stressed compared to control participants. The authors interpreted these findings as suggesting that acute stress may increase the rewarding attributes of food stimuli (215). Furthermore, Jastreboff et al. (216) observed that obese individuals exhibit increased activation in striatal, insular, and hypothalamic regions in response to stress and favorite-palatable food cues compared to lean individuals. These increased corticolimbic-striatal activations in response to food cues and stress were also positively associated with food craving ratings, suggesting that some individuals may be at higher risk to consume high-calorie foods during stressful periods (216). On the basis of the theoretical model proposed by Sinha and Jastreboff (198), highly palatable food cues in combination with chronic stress exposure could modulate emotions, metabolic responses (e.g., glucose and energy-balance hormones), and stress-responsive hormones (e.g., adrenocorticotrophin cortisol) that influence brain regions involved in reward, motivation, self-control, and decision-making. Thus, stress sensitivity likely interacts with reward systems to promote either drug use or overeating (or both) in vulnerable individuals (217).

CONCLUSION

Evidence of Non-Overlap

Despite the similarities exposed here there is also evidence that obesity and other addictive behaviors differ and may only overlap partially (218). While some studies have observed higher rates of addictive disorders in obese populations (219, 220), others have reported a lack of significant relationships between addiction and obesity (221–224). Methodological aspects (224) as well as the remarkable intrinsic complexity and heterogeneity associated with obesity and addiction (225) might help to explain the discrepancies observed between studies. Multiple factors (e.g., impulsivity and depressive symptoms) might interact with obesity/eating behavior in complex ways that are difficult to account for in studies with relatively small sample sizes. These factors may explain conflicting studies in the literature. Furthermore, an interesting possibility is that some subtypes of obesity might be at higher risk for developing addictive behavior (33). For instance, some post-bariatric surgery patients seem to exhibit increased rates of addictive problems (226–228). This phenomenon is commonly referred to as "cross addiction" or "addiction transfer."

Limitations of the present review should be acknowledged. Obesity results from a chronic positive imbalance between energy intake and energy expenditure. Almost all studies in obesity and impulsivity presented here describe obese participants in terms of the BMI (kg/m^2). While the BMI is an indicator of total adiposity, an important disadvantage is that it might not necessarily be associated with addictive-like eating patterns. In this vein, it is thus crucial to include a description of the participants in terms of their eating behavior or their UE patterns. Furthermore, clinical conditions that often present in comorbidity with obesity, such as BED or ADHD are not systematically evaluated and excluded in all the studies included in the present review. This point constitutes an important limitation that might obscure or inflate the overlap between addiction and obesity.

Concluding Sentences

Addiction and obesity are health problems with high phenotypic complexity. Growing evidence from personality, cognitive neuroscience, and brain imaging studies suggest that the combination of reduced cognitive control and, to a lesser extent, increased reward sensitivity is a risk factor for the development and maintenance of both syndromes. This is especially true in the domain of cognitive control (**Figure 2**) as measured by the Conscientiousness versus Disinhibition factors on personality questionnaires, by cognitive tasks of executive function, or by diminished recruitment of areas associated with cognitive control, such as the lateral PFC, in fMRI studies. Individuals characterized by high food drive and high cognitive control might better control their body weight in an environment rich in palatable foods.

The present review provides a comprehensive view of impulsivity-related alterations in obesity and addiction, covering results from the personality, neurocognitive, neuroimaging, and clinical fields. The conclusions of the review have the potential to inform clinical approaches aimed at the prevention or treatment of obesity. Diminished self-control is a predictor of poorer treatment outcomes in substance abuse disorders (51) and might also be one in obesity treatment. The findings of the present review might, as such, be of interest to cognitive behavioral therapists aiming to foster impulse control strategies in participants with obesity. Specific inhibitory-control interventions may also

represent a promising approach for the prevention of obesity in individuals with poor self-control and high reward sensitivity.

AUTHOR CONTRIBUTIONS

AM: design and conception of the manuscript; wrote the manuscript; and gave final approval. UV and IG: wrote and critically revised the manuscript; gave final approval. AD: design and conception of the manuscript; wrote and critically revised the manuscript; study supervision and responsible for funding; and gave final approval.

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Dopamine Adaptations as a Common Pathway for Neurocognitive Impairment in Diabetes and Obesity: A Neuropsychological Perspective

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Evidence accumulates linking obesity and diabetes with cognitive dysfunction. At present the mechanism(s) underlying these associations and the relative contribution of diet, adiposity, and metabolic dysfunction are unknown. In this perspective key gaps in knowledge are outlined and an initial sketch of a neuropsychological profile is developed that points toward a critical role for dopamine (DA) adaptations in neurocognitive impairment secondary to diabetes and obesity. The precise mechanisms by which diet, metabolic dysfunction, and adiposity influence the DA system to impact cognition remains unclear and is an important direction for future research.

Keywords: dementia, diabetes, obesity, dopamine, cognition, executive function, memory, associative learning

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NEUROCOGNITIVE IMPAIRMENTS IN TYPE 2 DIABETES (T2D)

T2D is associated with cognitive decline, brain dysfunction, and dementia (Biessels et al., 2014; Koekkoek et al., 2015; Stoeckel et al., 2016). One recent study estimated that the combined overall relative risk for dementia is 73% higher in people with, compared to without T2D, indicating that between 1 in 10 and 1 in 15 incidences of dementia may be attributable to T2D (Biessels et al., 2014). Although glucose intolerance is diagnostic of T2D, a recent systematic review of 86 papers examining T2D and cognition only reported a weak association between glycaemia, and cognition (Geijselaers et al., 2015) and there is even less evidence for an association with other measures of peripheral glucose regulation and cognitive function (e.g., insulin concentration, insulin action, insulin resistance) (Geijselaers et al., 2015). Thus, although T2D is by definition associated with altered glucose metabolism, it is not clear that altered glucose metabolism contributes to cognitive change. The mechanism behind the link between cognitive dysfunction and T2D is therefore not clear.

NEUROCOGNITIVE DEFICITS MAY ARISE FROM CHRONIC CONDITIONS ASSOCIATED WITH T2D

The majority of human studies linking T2D to cognitive decline are performed in older individuals with long-standing diagnoses of diabetes (Stoeckel et al., 2016). This poses a problem for interpreting the pathophysiology of the link between T2D and cognition because individuals with chronic T2D exhibit a number of pathologies associated with cognitive decline such as damage

to the blood brain barrier (BBB), neuroinflammation (Banks et al., 2012; Steculorum et al., 2014), cerebral atrophy, and small vessel disease (Biessels and Reijmer, 2014; Akrivos et al., 2015; Ramos-Rodriguez et al., 2016; Stranahan et al., 2016). The co-occurrence of these pathologies that are secondary to diabetes has led to controversy over whether it is T2D (Biessels and Reagan, 2015) or complications arising from T2D that leads to cognitive decline (De Felice and Ferreira, 2014). To rule-out confounds associated with the secondary complications of T2D it will be informative to study cognition in populations free from other chronic conditions and in populations prior to the onset of T2D. For example, it would be informative to characterize neurocognition in youth before and after the onset of prediabetes, since this population will be free from other chronic conditions that could influence cognitive function.

IT IS UNKNOWN IF NEUROCOGNITIVE DEFICITS ARE ASSOCIATED WITH T2D OR ADIPOSITY OR BOTH

Perhaps the most important limitation of the current literature is the failure to disentangle effects of metabolic dysfunction on cognition from those of adiposity and diet. Obesity has been associated with altered brain structure and function in animal models and in metabolically and neurologically healthy adults and children (Elias et al., 2003; Reinert et al., 2013; Hsu and Kanoski, 2014; Yau et al., 2014; Bocarsly et al., 2015), while diets high in saturated fat and cholesterol are correlated with compromised cognitive flexibility and processing speed in pre-pubertal children after adjusting for age, sex, socioeconomic status, IQ, VO_{2max}, and BMI (Khan et al., 2015b). Consumption of a high fat diet (HFD) can also negatively impact brain and brain function well-before obesity onset. For example, in animal models hypothalamic insulin resistance is observed following acute exposure to HFD before changes in adiposity occur (Clegg et al., 2011) and impaired performance on hippocampal-dependent tasks is observed after only 72-h access to a HFD when animals have actually lost weight, presumably due to neophobia (Kanoski and Davidson, 2010). These findings suggest that obesity can impact cognition independently from metabolic disease and that diet can impact metabolic function and cognition independently of obesity.

To date, studies have rarely taken obesity and diet into account when examining the relationship between T2D and cognition. For example, patients with T2D exhibit reduced activity in the default mode network (Musen et al., 2012), which has been associated with a wide range of neurological conditions and cognitive impairments (Browndyke et al., 2017; Contreras et al., 2017; Jockwitz et al., 2017; von Rhein et al., 2017) but BMI, which was higher in T2D, was not accounted for. Similarly, the putative confound of glucose intolerance is often not considered when examining the relationship between obesity and cognition. For example, a prospective study examining the impact of obesity on cognition excluded participants for many medical conditions likely to influence cognition, including stroke, dementia, myocardial infarction, and atrial fibrillation but NOT diabetes (Gunstad et al., 2010). They did however, include "glucose intolerance" in their mixed model regression analyses and found that this variable was related to cognitive impairments that also correlated with their adiposity measures (waist-hip ratio). In another study deficits in executive function and declarative memory were observed in 38 middle-aged adults with insulin resistance but without T2D compared to 54 age, gender, education but NOT BMI matched controls. Since the insulin resistance group had significantly higher BMI these deficits may be equally attributable to BMI (Bruehl et al., 2010).

Failure to account for confounds between diet, obesity, and metabolic dysfunction also pervade the animal literature. Rats prone to develop diabetes upon HFD are often used as a model of T2D (Levin and Routh, 1996). These models have been associated with deficits on the water maze (Li et al., 2002; Stranahan et al., 2008b), object recognition test (Stranahan et al., 2008a), contextual cue conditioning (Grillo et al., 2011), and discrimination and reversal learning (Kanoski et al., 2007, 2010). HFD has also been shown to increase inflammatory cytokines and impair neuroplasticity and learning and memory in the hippocampus (Erion et al., 2014). The extent to which adiposity or insulin resistance contributed to these observations is not known. However, impaired cognition is also observed with the streptozotocin (STX)-induced diabetic model, which impairs insulin production without increasing adiposity or requiring a high fat diet, indicating that metabolic dysfunction alone is sufficient to impair cognitive function (Stranahan et al., 2008a).

Importantly, when more than one variable is measured interactions between adiposity, diet, and impaired glucose tolerance are revealed. In obese humans without T2D, insulin sensitivity mediates the relationship between working memory-related activation in the right parietal cortex and BMI (Gonzales et al., 2010), while brain insulin action is selectively impaired in the prefrontal cortex in overweight and obese, but not diabetic adults compared to their lean counterparts (Kullmann et al., 2015), highlighting interactions between adiposity and glucose tolerance on brain function.

In summary, the relative contribution of diet, impaired glucose tolerance, and adiposity to neurocognitive impairment is largely unexplored and unknown.

CHARACTERIZATION OF GLYCEMIA

Another factor clouding the association between T2D and cognitive impairment is the use of a variety of methods to characterize glycemia, each of which reflect different, and sometimes independent, aspects of glucose metabolism (Geijselaers et al., 2015). Insulin sensitivity can be measured using a variety of techniques. HbA_{1c}, which reflects the mean glucose concentration over a period of 8–12 weeks is the most frequently used measure. Fasting blood glucose concentrations are also frequently measured, which reflect nocturnal hepatic gluconeogenesis that is influenced by hepatic insulin sensitivity, but a recent review found that studies often fail to report whether measurements are taken at the same time of day (Geijselaers et al., 2015). Other measures include post-prandial

glucose concentrations, reflecting insulin secretory responses and HOMA-IR to measure insulin resistance. HbA_{1c} shows the strongest association to insulin resistance, followed by post-prandial measures. Fasting glucose, by contrast, seems to be unrelated to cognitive performance (Geijselaers et al., 2015). Interestingly, one study found that insulin resistance was related to declarative memory whereas HbA_{1c} was associated with executive dysfunction (Bruehl et al., 2010), hinting at the possibility that the different measures are associated with distinct pathophysiological effects on the brain and highlighting the need for more comprehensive measures of glucose metabolism.

NEUROCOGNITIVE IMPAIRMENTS MAY BE RELATED TO CENTRAL RATHER THAN (OR IN ADDITION TO) PERIPHERAL IMPAIRMENTS IN GLUCOSE TOLERANCE

Insulin receptors are widely distributed in the brain, with the highest concentrations in the olfactory bulb, hypothalamus, cerebral cortex, cerebellum, and hippocampus (Havrankova et al., 1978; van Houten et al., 1979). Brain-specific deletion of the insulin receptor in mice results in glycogen synthase kinase 2 beta activation resulting in hyperphosphorylation of tau protein, a hallmark of early Alzheimer's Disease (AD) (Schubert et al., 2004). There is also evidence from animal studies that disrupted central insulin and insulin-like growth factor-1 (IGF-1) signaling may lead to disrupted neurotransmitter (e.g., dopamine) and astroglial cell function, brain endothelial cell function involved in formation and regulation of BBB, mitochondrial metabolism and oxidative stress, clearance of Aβ and/or amyloid fibrils, cholesterol synthesis in the brain (important for myelination and membrane function), glucose and lipid metabolism in select regions of the brain, and regulation of central energy balance, which could relate to both metabolic and neurocognitive dysfunction (Brüning et al., 2000; Convit et al., 2003; Schubert et al., 2004; Suzuki et al., 2010; Kleinridders et al., 2014; Stouffer et al., 2015). While these data suggest a likely role for central insulin resistance in impaired neurocognitive function, it is important to note that central insulin resistance has a complicated relationship with peripheral glycemic control (Ketterer et al., 2011). Central insulin resistance is thought to result from a combination of impaired insulin receptor signaling and decreases in the transport of insulin across the BBB (Banks et al., 2012), which can occur secondary to peripheral glucose intolerance (Niswender et al., 2003). Conversely, central insulin signaling contributes to peripheral glucose regulation (Brüning et al., 2000; Heni et al., 2014) to create a dynamic brain-gut axis regulating glucose metabolism. Importantly, however, central insulin resistance can occur independently from peripheral impairments in glucose tolerance. Post-mortem studies of brain tissue from patients with AD but not T2D, reveal disrupted brain insulin signaling (De Felice and Ferreira, 2014; Yarchoan and Arnold, 2014). Accordingly, treatment with intranasal insulin, which results in direct insulin transport from the nasal cavity to the CNS via intraneuronal and extraneuronal pathways (Reger and Craft, 2006), improves cognition in patients with (Reger et al., 2008; Craft et al., 2012) and without (Hallschmid et al., 2007, 2008) dementia. These findings underscore the importance of concurrent measures of peripheral and central insulin resistance.

One promising avenue for future research is in using intranasal insulin in combination with neuroimaging methodologies and neuropsychological testing to assess the role of central insulin resistance in neurocognition (Tschritter et al., 2006; Ketterer et al., 2011; Grichisch et al., 2012; Kullmann et al., 2013, 2015; Heni et al., 2014, 2016). For example, intranasal insulin decreases the blood oxygen dependent (BOLD) response in the hypothalamus and PFC increases BOLD response in the striatum (Schilling et al., 2014) and insular cortex (Heni et al., 2012) and increases brain energy levels (Jauch-Chara et al., 2012). Critically, these effects are blunted in obesity (Tschritter et al., 2006) with evidence that hypothalamic insulin resistance is driven by visceral fat and frontal insulin resistance by peripheral insulin sensitivity (Kullmann et al., 2015). Collectively, these findings indicate a complex relationship between peripheral glucose control and central insulin resistance and they raise the possibility that central insulin resistance contributes to cognitive impairment in concert with, or independently from peripheral impaired glucose tolerance.

NEUROCOGNITIVE DEFICITS MAY BE DOMAIN-SPECIFIC AND DIFFERENTIALLY INFLUENCED BY DIET, ADIPOSITY, AND METABOLIC DYSFUNCTION

Although obesity and T2D are occasionally associated with global measures of brain atrophy (Enzinger et al., 2005; Gunstad et al., 2010; Raji et al., 2010; Brooks et al., 2013) and cognitive decline (Liang et al., 2014), many studies suggest that executive function is the domain most affected in both adults (Gunstad et al., 2007; Sabia et al., 2009; Fitzpatrick et al., 2013) (Volkow et al., 2009) and children (Convit et al., 2003; Reinert et al., 2013; Liang et al., 2014). For example, negative correlations are observed between BMI and performance on tasks of executive function but not episodic verbal memory (Volkow et al., 2009) with BMI negatively, and executive performance positively, correlated with baseline prefrontal glucose metabolism. Similarly, a recent meta-analysis of 21 studies concluded that obesity is associated with impairments in decision-making, planning and problem solving with less evidence for associations with verbal fluency and learning and memory (Fitzpatrick et al., 2013). Correspondingly, structural changes (Enzinger et al., 2005; Pannacciulli et al., 2006; Raji et al., 2010; Fotuhi et al., 2012; Bocarsly et al., 2015) and reduced brain connectivity (Musen et al., 2012) are observed in the parietal and prefrontal cortex (PFC), which are critical for executive function.

There is also strong evidence from animal work that HFD produces hippocampal insulin resistance (Biessels and Reagan, 2015) and damage (Hsu and Kanoski, 2014), resulting in impaired hippocampal-dependent cognitive functions (Kanoski and Davidson, 2011). Likewise, hippocampal atrophy is observed in obese humans (Raji et al., 2010) and altered hippocampal

white matter connectivity is found in T2D (van Bussel et al., 2016). However, there are inconsistent findings with respect to alterations in hippocampal-dependent episodic memory tasks (Fitzpatrick et al., 2013). For example, significant deficits in working memory and in reinforcement learning are observed in the absence of episodic learning and memory impairment, in obese vs. healthy weight adults that are matched for age, gender, education, and IQ (Coppin et al., 2014). In contrast, impaired episodic memory and decreased hippocampal volume is observed as a function of glucose tolerance (Convit et al., 2003) and intranasal insulin increases the functional connectivity between the hippocampus and PFC in people with T2D (Zhang et al., 2015). Other studies report hippocampal-dependent impairments as a function of saturated fat intake (Francis and Stevenson, 2011) and central, but not whole body adiposity (Khan et al., 2015a). Collectively these data suggest that episodic memory may be affected by diet and metabolic dysfunction while being unrelated to BMI and whole body obesity.

This emerging neuropsychological profile provides an important insight into the pathophysiological mechanism that gives rise to neurocognitive impairment in obesity and T2D. Brain functions associated with diabetes and obesity tend to rely upon DA signaling (**Figure 1**). For example, the dopaminergic fronto-striatal loop plays a well-known role in working memory, cognitive flexibility, reinforcement learning, and incentive motivation (Frank and Fossella, 2011). It is also critical for response inhibition, the failure of which is associated with addictive like behaviors including overeating (Lokken et al., 2009; Maayan et al., 2011; Lee et al., 2013; Guo et al., 2014; Zhao et al., 2016). Finally, a role for DA in memory via a projection from the ventral tegmental area to the hippocampus has been described (Shohamy and Adcock, 2010). As reviewed above, these DA-dependent cognitive processes are altered in obesity/T2D, raising the possibility that a common pathway by which diet, adiposity and metabolic dysfunction might coalesce to impact cognition is by producing alterations in the

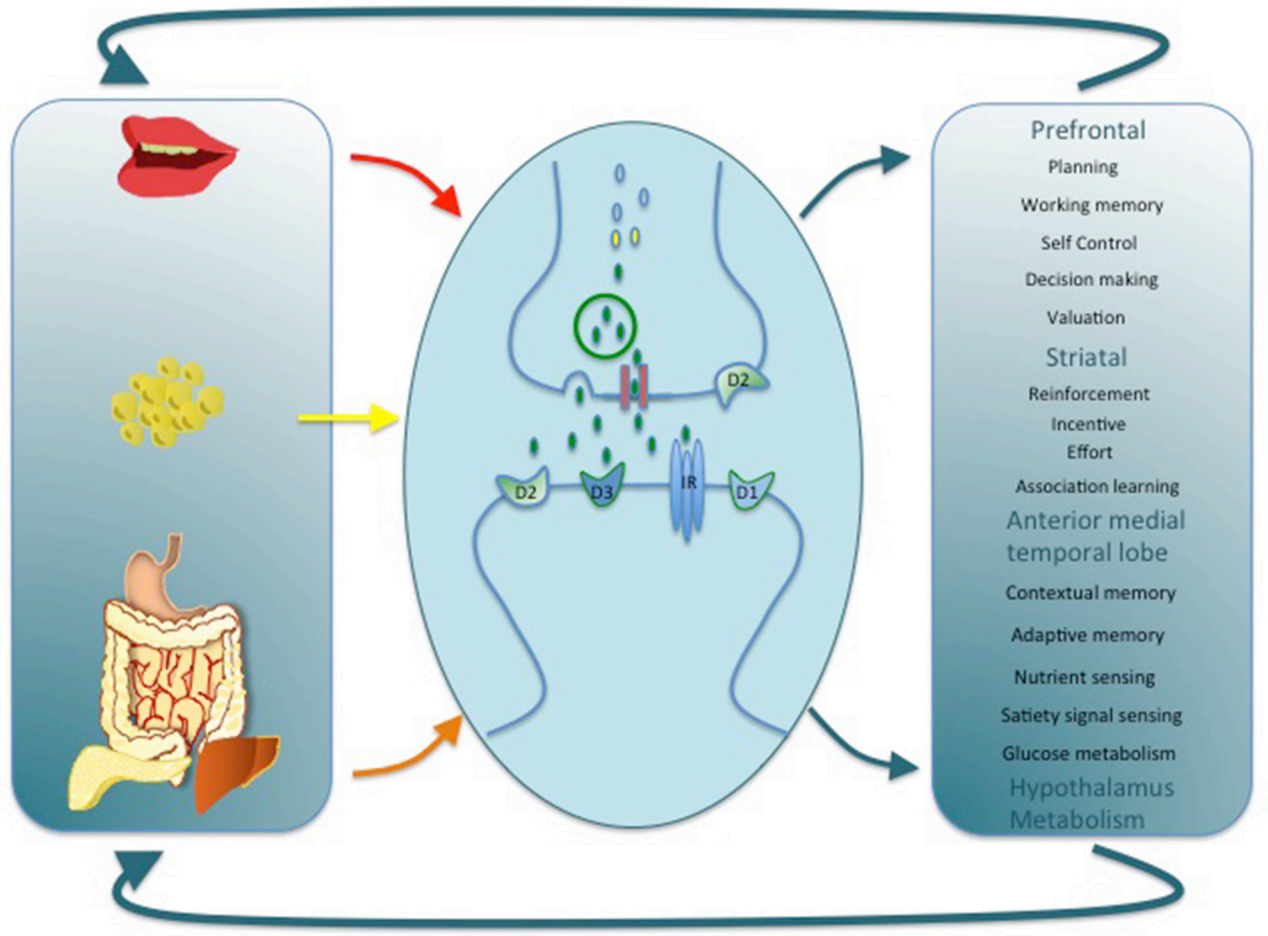


FIGURE 1 | This cartoon depicts alterations in DA signaling as a common link by which diet, adiposity, and metabolic dysfunction might impact cognition, motivation, and energy balance. A variety of mechanisms at the cellular and molecular level could support this association by regulating pre and post-synaptic DA receptor expression, DA synthesis, release, and reuptake. Alterations at any level may in turn have a wide impact on brain function and provide a parsimonious explanation for a number of dysfunctions associated with obesity and T2D.

DA system (**Figure 1**). Interestingly, although DA adaptations are considered integral in the development of compulsive behaviors and alterations in reward sensitivity they are not typically considered as a potential mechanism behind other neurocognitive complications in T2D and obesity.

There are consistent findings in the animal literature that HFD, and adiposity alter DA signaling at the cellular, and molecular levels (Anderzhanova et al., 2007; Johnson and Kenny, 2010; van de Giessen et al., 2012, 2013; Sharma and Fulton, 2013; Tellez et al., 2013; Cansell et al., 2014; Adams et al., 2015; Woods et al., 2016), as well as mounting evidence for altered DA signaling in human obesity, especially reflected in changes in DA receptor density, (Wang et al., 2001, 2011; Dunn et al., 2010; Steele et al., 2010; Eisenstein et al., 2013, 2015; Guo et al., 2014; Cosgrove et al., 2015; Horstmann et al., 2015; Karlsson et al., 2015; Caravaggio et al., 2015b; Dang et al., 2016; Gaiser et al., 2016). Additionally, there is evidence that central and peripheral insulin resistance might impact DA function. Central insulin signaling, acting through the downstream modulator Akt, is a potent modulator of DA transporter (DAT) activity, which fine-tunes DA signaling at the synapse (Kleinridders et al., 2015), demonstrating a pathway by which central IR could influence the DA system. Insulin administration also suppresses DA release by clearing DA from the synapse and concomitantly reducing the rewarding properties of food (Figlewicz and Sipols, 2010). Likewise, peripheral insulin sensitivity is associated with reduced endogenous DA levels (Murzi et al., 1996; Caravaggio et al., 2015a) and peripheral glycemia with PFC-striatal-hippocampal functional connectivity (Page et al., 2013). Thus, diet, adiposity, and insulin resistance could each impact DA signaling with the potential for additive effects and interactions. Future work aiming to disambiguate the unique and interacting effects will therefore be an important step toward understanding neurocognitive impairment in T2D and obesity.

SUMMARY

In summary, a consensus is emerging that obesity and diabetes are accompanied by cognitive impairments and brain dysfunction and that at least some of these effects are secondary to their onset. Multiple mechanisms have been proposed to underlie these associations but at present it is unclear which mechanism, or mechanisms, are critical. Also unclear is whether diet, obesity and metabolic dysfunction have distinct and/or converging pathways to neurocognitive impairment. However, work emerges to suggest that all three factors may influence DA signaling, which is provocative since the cognitive impairments that characterize diabetes and obesity uniformly rely upon the integrity of the DA system. It is therefore proposed that adaptations in DA signaling secondary to diet, adiposity and metabolic dysfunction underlie much of the neurocognitive impairment observed in diabetes and obesity.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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Suppressed Fat Appetite after Roux-en-Y Gastric Bypass Surgery Associates with Reduced Brain μ-opioid Receptor Availability in Diet-Induced Obese Male Rats

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Brain μ-opioid receptors (MORs) stimulate high-fat (HF) feeding and have been implicated in the distinct long term outcomes on body weight of bariatric surgery and dieting. Whether alterations in fat appetite specifically following these disparate weight loss interventions relate to changes in brain MOR signaling is unknown. To address this issue, diet-induced obese male rats underwent either Roux-en-Y gastric bypass (RYGB) or sham surgeries. Postoperatively, animals were placed on a two-choice diet consisting of low-fat (LF) and HF food and sham-operated rats were further split into *ad libitum* fed (Sham-LF/HF) and body weight-matched (Sham-BWM) to RYGB groups. An additional set of sham-operated rats always only on a LF diet (Sham-LF) served as lean controls, making four experimental groups in total. Corresponding to a stage of weight loss maintenance for RYGB rats, two-bottle fat preference tests in conjunction with small-animal positron emission tomography (PET) imaging studies with the selective MOR radioligand [^{11}C]carfentanil were performed. Brains were subsequently collected and MOR protein levels in the hypothalamus, striatum, prefrontal cortex and orbitofrontal cortex were analyzed by Western Blot. We found that only the RYGB group presented with intervention-specific changes: having markedly suppressed intake and preference for high concentration fat emulsions, a widespread reduction in [^{11}C]carfentanil binding potential (reflecting MOR availability) in various brain regions, and a downregulation of striatal and prefrontal MOR protein levels compared to the remaining groups. These findings suggest that the suppressed fat appetite caused by RYGB surgery is due to reduced brain MOR signaling, which may contribute to sustained weight loss unlike the case for dieting.

Keywords: bariatric surgery, caloric-restriction, fat appetite, Brain μ-opioid receptors, positron emission tomography imaging

INTRODUCTION

The current obesity pandemic is primarily a consequence of the sudden widespread ease of access to palatable, energy-dense foods (Swinburn et al., 2011). Overeating in the face of plenty results from a complex interaction of emotional, cognitive and hedonic pressures which outweigh homeostatic processes in place to maintain stable body weight (Alsiö et al., 2012). A rich heritage of pharmacological studies has provided a picture of how opioidergic circuits interface with the brain systems that orchestrate feeding behavior (Bodnar, 2013). In rodents, agonists of the μ-opioid receptor (MOR) in particular have consistently been demonstrated to stimulate food intake when administered into various hypothalamic nuclei (Stanley et al., 1988), the central nucleus of the amygdala (Gosnell, 1988), ventromedial prefrontal and orbitofrontal cortices (Mena et al., 2011), and especially the striatum (Bakshi and Kelley, 1993). Here, acute administration of a selective MOR agonist into the nucleus accumbens potently stimulates, whereas a MOR antagonist inhibits fat intake in sated and fasted rats, respectively (Zhang et al., 1998). Moreover, intracerebral administration of antisense oligonucleotides against the MOR inhibits food intake and lowers body weight (Leventhal et al., 1996) while chronic administration of an irreversible MOR antagonist into the nucleus accumbens inhibits high-fat (HF) diet intake and prevents weight gain in rats (Lenard et al., 2010). These findings provide a rationale for the evaluation of MOR antagonists in weight loss studies performed on obese patients (Greenway et al., 2010; Ziauddeen et al., 2013).

Despite being by far the most efficacious treatment for obesity, little is known about how bariatric surgery affects the brain control of food intake to achieve sustained weight loss (Manning et al., 2015). Recently, a human positron emission tomography (PET) imaging study with the selective MOR radioligand [^{11}C]carfentanil revealed widespread changes in brain MOR availability in obese subjects compared to lean, which were completely normalized after bariatric surgery (Karlsson et al., 2016). While a separate study reported modest changes in brain MOR availability in obese subjects after dieting (Burghardt et al., 2015), a direct comparison between these differing means of weight loss under standardized conditions has not been performed. This forms an important basis for gaining a better understanding of the central molecular underpinnings of successful long-term weight reduction unique to bariatric surgery. Additionally, it remains unclear whether alterations in brain MOR signaling following Roux-en-Y gastric bypass (RYGB), the most frequently employed bariatric surgical procedure, relates to the documented postoperative suppression in fat appetite in human (Kenler et al., 1990; Olbers et al., 2006; Thomas and Marcus, 2008) and animal (Zheng et al., 2009; le Roux et al., 2011; Shin et al., 2011b; Hao et al., 2016) studies. We therefore performed detailed fat intake and preference measurements in conjunction with *in vivo* small-animal [^{11}C]carfentanil PET imaging studies in lean and diet-induced obese rats in comparison with animals that experienced identical weight loss from RYGB or chronic caloric-restriction. We then analyzed MOR protein expression in various brain regions by Western Blot.

MATERIALS AND METHODS

Animals

Sixteen male Wistar rats (RjHan:WI, outbred, Janvier, Le Genest-Saint-Isle, France) were used for our studies. Food and water were provided *ad libitum* unless otherwise stated. Animals were initially group housed and maintained on a 12-h light/dark cycle (lights on at 07:00 h) in facilities with an ambient temperature of 21–23°C and 40–60% humidity. All experiments were approved by the Institutional Animal Care and Use Committee at the Universität Leipzig with permission of the local government of Saxony (Regional Administrative Authority Leipzig, TVV 63/13, Germany). When indicated, diet induced obesity (DIO) was induced in 9 week old rats ($n = 12$) initially weighing approximately 350 g by feeding them for 5 weeks with a HF diet, which provides 58% of total energy as fat, 25.5% as carbohydrate, and 16.5% as protein (EF D12331, Ssniff GmbH, Soest, Germany). A separate group of rats always maintained on standard laboratory chow with 9% kcal derived from fat (RM1 diet; Ssniff GmbH, DE-59494, Germany) served as lean controls ($n = 4$).

Abdominal Surgeries and Postoperative Care

All abdominal surgical procedures were performed after an overnight fast by a bariatric surgeon according to a previously established protocol (Hankir et al., 2015; Seyfried et al., 2016). Briefly, animals were anesthetized with 5% isoflurane in 2 l/min oxygen and then maintained on 1.8–2.3% isoflurane in 0.5 l/min oxygen. Following induction of anesthesia, the abdominal wall was opened by a midline incision. For the RYGB procedure ($n = 4$), the jejunum was transected 15 cm aboral to the pylorus to create a 10 cm biliopancreatic limb. The proximal end was anastomosed to the ileum approximately 25 cm oral from the cecum, creating the common channel. The stomach was then transected 3 mm aboral to the gastroesophageal junction. At the proximal end, the gastric pouch (2–3% original stomach size) was anastomosed in an end-to-side fashion to the distal end of the small bowel forming the alimentary limb. At the distal end, the gastric remnant was closed using continues sutures. For the sham procedure ($n = 12$), the small bowel and the gastroesophageal junction were mobilized, and a 1 cm long gastrostomy was performed on the anterior wall of the stomach with subsequent closure. For postoperative analgesia, carprofen (5 mg/kg i.p.) was administered intraoperatively and then daily for 1 week. Postoperatively, all animals were singly-housed.

Postoperative Diet Regimens

Standard laboratory chow with 9% kcal derived from fat (RM1 diet; Ssniff GmbH, DE-59494, Germany) mixed with water (wet diet) was given to all animals for 48 h following surgeries. Afterwards, the lean Sham-LF control group was maintained on standard laboratory chow, whilst the remaining DIO animals were postoperatively given a two-choice diet of standard laboratory chow and HF diet with 58% kcal derived from fat (EF D12331, Ssniff GmbH, DE-59494, Germany). DIO sham-operated animals were then randomly allocated into an *ad*

libitum fed group (Sham-LF/HF group; $n = 4$) or a chronically food-restricted group body weight-matched to RYGB rats (Sham-BWM group; $n = 4$). To achieve this, animals from the Sham-BWM group were given approximately the same amount of HF diet as that consumed daily by the RYGB group in the early light phase, but the amount of LF diet was adjusted accordingly. Body weight and food intake were recorded daily for 12 weeks. This time-point after surgery corresponds to a stage of weight loss maintenance in our RYGB model (Hankir et al., 2015; Seyfried et al., 2016).

Behavioral Protocol for Oral Fat Intake

During weeks 12–14 postoperatively, rats underwent 18-h two-bottle preference tests in which consumption of 5% concentration of an IntraLipid® fat emulsion (Fresenius Kabi, UK) was assessed. This concentration of fat emulsion was chosen based on previous observations (le Roux et al., 2011; and own unpublished data). After habituation with two water bottles for 72-h, *ad libitum* fed animals lacking side preferences had their food withdrawn and were then presented with two other bottles: one that contained 150 ml of water and the other 150 ml of 5% IntraLipid® solution on randomized sessions. Volumes ingested over 18 h were measured using an automated feeding-drinking monitoring system (TSE Systems GmbH, Bad Homburg, Germany). The preference ratio for fat emulsion was calculated as the ratio (%) between the volume ingested of fat emulsion and the total volume of fluid ingested.

Synthesis of [^{11}C]carfentanil

[^{11}C]carfentanil was prepared according to a previously reported procedure (Dannals et al., 1985) applying the "loop method" (Wilson-Pérez et al., 2013). Specific activity as determined by high-performance liquid chromatography was $5.8 \cdot 10^5$ MBq/nmol.

Small-Animal PET Imaging Protocol with the Radioligand [^{11}C]carfentanil

During weeks 15–17 postoperatively, animals underwent PET scanning under *ad libitum* fed conditions (just prior to feeding for the Sham-BWM group) using a dedicated small-animal PET/MR system (nanoScan®, Mediso Medical Imaging Systems, Budapest, Hungary) as previously described (Nagy et al., 2013). All PET scans were performed in the morning hours between 8 a.m. and 10 a.m. Rats were anaesthetized with 1.8% isoflurane in 0.5 l/min 60% oxygen/40% air mixture (Gas Blender 100 Series, MCQ instruments, Rome, Italy) before receiving a bolus intravenous injection of 41.6 ± 6.4 MBq [^{11}C]carfentanil via the lateral tail vein. Simultaneous to tracer injection, a dynamic 35 min PET scan was initiated, during which animals were maintained at 37°C with a thermal bed system under isoflurane anesthesia. As animals' heads were too large to fit into the MR coil for subsequent MR imaging, for anatomical orientation a second static 15 min PET scan was performed immediately afterwards following intravenous bolus injection of approximately15 MBq [^{18}F]- fluordeoxyglucose (FDG). The list-mode data for the [^{11}C]carfentanil scan were rebinned into 28 frames (12 × 10, 6 × 30 s, 5 × 1 and 5 × 5 min) and iteratively reconstructed with 3D-ordered subset expectation maximization (OSEM), 4 iterations and 6 subsets using an energy window of 400–600 keV, coincidence mode of 1–5 and ring difference of 81. Cerebellum was selected as a reference region as a previous study performed on rats revealed minimal uptake of [^{11}C]carfentanil in this brain region following intravenous administration (Quelch et al., 2014). [^{11}C]carfentanil binding potential (BP_{ND}) was calculated using the simplified reference tissue model (Lammertsma and Hume, 1996) for hypothalamus, thalamus, amygdala, striatum, cingulate cortex, insular cortex, prefrontal cortex and orbitofrontal cortex, again using the cerebellum as a reference region. This was performed after automatic co-registration of the [^{18}F]-FDG PET images with a standard [^{18}F]-FDG rat brain atlas using PMOD (v.3.6, PMOD Technology, Zurich, Switzerland) software. The final resolution of rendered PET images for analysis was 0.2 mm^3.

Western Blot

At postoperative week 18, *ad libitum* fed rats were sacrificed and brains rapidly removed. Hypothalamus, striatum, prefrontal cortex and orbitofrontal cortex were carefully dissected and frozen immediately in liquid nitrogen. Protein was extracted from tissue (50 mg) in radioimmunoprecipitation assay (RIPA) buffer (40 μl/mg) after homogenization and incubation at 4°C for 1 h. Lysate was centrifuged for 15 min and supernatant collected. Sample (10 μg protein determined using a bicinchoninic acid (BCA) protein assay kit (ThermoFisher Scientific, Darmstadt, Germany)) was loaded into polyacrylamide gel, resolved by electrophoresis (125 V for 2 h) and transferred onto a nitrocellulose membrane using a semi-dry method (75 mA for 1 h). Membranes were blocked with 3% bovine serum albumin (BSA) for 30 min and after washing were incubated overnight at 4°C with rabbit anti-rat MOR monoclonal primary antibody (AbCam, Cambridge, UK) diluted 1/1000 in 3 % BSA. Membranes were then incubated for 1 h at room temperate with goat anti-rabbit IgG secondary antibody conjugated to horseradish peroxidase (Cell Signaling Technology, Leiden, Netherlands) diluted 1/3000 in 3% BSA. Bands were visualized using an enhanced chemiluminescence (ECL) kit (Biozym Scientific GmbH, Oldendorf, Germany) and quantified using the rolling method with GeneSnap software (v7.12, SynGene, Cambridge, UK). As a loading control, membranes were stripped with 0.2 N NaOH and β-actin was measured using rabbit anti-rat β-actin monoclonal primary antibody (Sigma Aldrich Chemie GmbH, Taufkirchen, Germany) diluted 1/500 in 3% BSA and then the same secondary antibody as for the MOR.

Statistics

Data sets were analyzed for statistical significance using GraphPad Prism software (v.7.02, GraphPad Software Inc., La Jolla, CA). One-way and two-way ANOVAs corrected for multiple comparisons with Tukey's *post-hoc* tests were used as required.

RESULTS

RYGB Surgery Reduces Body Weight, HF Diet Intake, and Preference in Diet-Induced Obese Male Rats

To confirm that our RYGB rat model recapitulates the human phenotype observed after RYGB, body weight, food intake and food preference were regularly measured postoperatively compared to obese and lean control groups. With respect to body weight (**Figure 1A**); preoperative values were similar between Sham-LF/HF (418 ± 18.4 g), RYGB (451 ± 9.4 g), and Sham-BWM (452 ± 4.4 g) groups but was significantly less ($p < 0.01$) for the Sham-LF group (351 ± 3.4 g). From postoperative week 2, body weights started to significantly diverge ($p < 0.0001$) between Sham-LF/HF and post-DIO weight loss groups (RYGB and Sham-BWM) which continued for the duration of the 12 week recording period. Body weights of Sham-LF rats steadily increased during this period so that they were similar to post-DIO weight loss groups by week 12, but significantly less ($p < 0.0001$) than Sham-LF/HF rats.

Concerning total food consumption (**Figure 1B**), cumulative energy intake over the 12 week recording period was considerably lower ($p < 0.01$) for RYGB rats (1428 ± 71.5 kcal) compared to Sham-LF/HF rats (1760 ± 39.7 kcal), but higher ($p < 0.01$) compared to Sham-BWM rats (1074 ± 8.8 kcal). The similar body weights despite different food intakes between RYGB and Sham-BWM rats has previously been attributed to differences in energy expenditure (Hankir et al., 2015). When focusing on HF diet intake alone in groups given access (restricted or free) to it (**Figure 1B**), consumption was markedly less ($p < 0.01$) for RYGB (590 ± 105.5 kcal) and Sham-BWM rats (535 ± 3.5 kcal) compared to Sham-LF/HF rats (996 ± 59.3 kcal). The amount of HF diet given to Sham-BWM rats was ensured to be similar ($p = 0.85$) to that consumed by RYGB rats. Consequently, to cause and sustain weight loss, Sham-BWM rats were given significantly less ($p < 0.05$) LF diet (539 ± 5.3 kcal) than that consumed by RYGB rats (839 ± 75.2 kcal).

With respect to dietary preference in groups with *ad libitum* access to a choice diet (**Figure 1C**), RYGB rats showed an overall reduced preference for HF diet relative to Sham-LF/HF rats over the 12 week recording period [main effect of time $F_{(11, 72)} = 2.24$; $p = 0.028$, main effect of treatment $F_{(1, 72)} = 50.9$; $p < 0.0001$, and interaction $F_{(11, 72)} = 2.40$; $p = 0.013$]. This difference started at postoperative week 8 (64.6 ± 8.4% for Sham-LF/HF rats vs. 33.3 ± 7.7% for RYGB rats; $p < 0.05$) and was maintained until postoperative week 12 (77.1 ± 7.1% for Sham-LF/HF rats vs. 50.0 ± 7.9% for RYGB rats; $p < 0.05$).

RYGB Surgery Suppresses Appetite for High Concentration Fat Emulsion

In order to study the effects of weight loss from RYGB and chronic caloric-restriction on fat appetite in more detail, we then performed two-bottle fat preference tests with a high concentration (5%) IntraLipid® emulsion on RYGB and Sham-BWM groups compared to obese (Sham-LF/HF) and lean (Sham-LF) control groups. The RYGB group presented with a strikingly lower ($p < 0.01$) intake of 5% IntraLipid® relative to the remaining groups (**Figure 2A**). Intake of 5% Intralipid® over 18 h was 40 ± 4.6 ml for RYGB, 96 ± 1.5 ml for Sham-LF/HF, 99 ± 6.2 ml for Sham-BWM and 80 ± 9.7 ml for Sham-LF rats. When calculated as preference over water (**Figure 2B**), the difference between the RYGB group and the remaining groups became more apparent ($p < 0.0001$). Preferences for high concentration fat emulsion were 80 ± 2.8% for RYGB, 98 ± 0.5% for Sham-LF/HF, 99 ± 0.3% for Sham-BWM, and 97 ± 0.7% for Sham-LF rats.

RYGB Surgery Reduces Brain MOR Availability

Having found differential effects of weight loss from RYGB surgery and chronic caloric-restriction on fat appetite, we then sought to determine the potential role of brain MORs for this difference in feeding behavior. We therefore performed small-animal PET imaging with the MOR radioligand [^{11}C]carfentanil

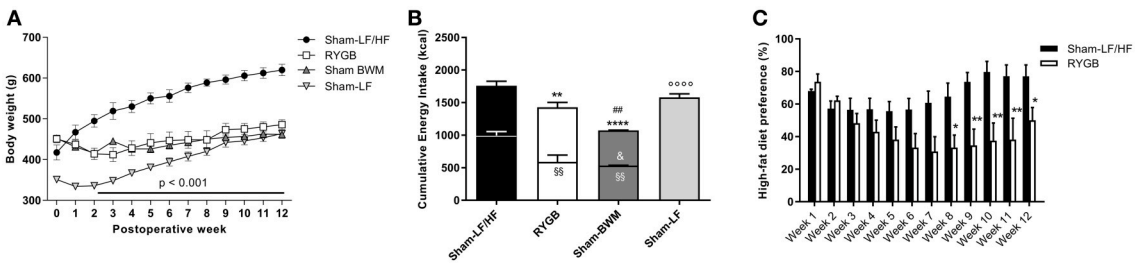


FIGURE 1 | RYGB surgery reduces body weight, food intake and HF diet preference in diet-induced obese male rats. High-fat (HF) diet-induced obese rats were split into 3 groups: Roux-en-Y gastric bypass-operated and then maintained on a choice diet (RYGB), sham-operated and then maintained on a choice diet (Sham-LF/HF) and sham-operated and then body weight-matched to RYGB rats (Sham-BWM). A separate group of never obese sham-operated rats always maintained on a LF diet (Sham-LF) was also added as lean controls. **(A)** Shows weekly body weights and **(B)** total cumulative energy intake over 12 weeks. In **(A)**, horizontal bar denotes significance between the Sham-LF/HF group and the remaining groups. In **(B)**, lower bar segments represent energy in kcal consumed from HF food and upper bar segments represent energy in kcal consumed from LF food. **(C)** Weekly HF diet preference was calculated by dividing the amount of HF diet consumed with the total amount of diet (HF and LF) consumed and expressed as percentage (%). Data are presented as mean ± SEM. $n = 4$ per treatment group. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{****}p < 0.0001$ vs. Sham-LF/HF, $^{\#\#}p < 0.05$ vs. RYGB and $^{\circ\circ\circ\circ}p < 0.0001$ vs. Sham-LF for total energy intake; $^{\S\S}p < 0.01$ vs. Sham-LF/HF for HF food intake; $^{\&}p < 0.05$ vs. RYGB for LF food intake.

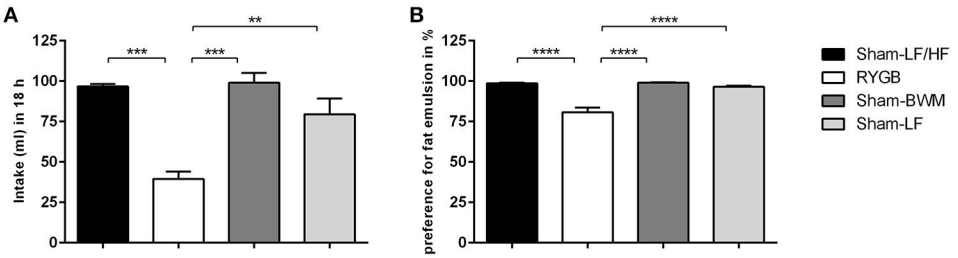


FIGURE 2 | RYGB surgery suppresses intake and preference for high concentration lipid emulsions in diet-induced obese male rats. Two-bottle fat preference tests were performed on Sham-LF/HF, RYGB, Sham-BWM and Sham-LF rats during postoperative weeks 12–14. Animals were presented with two pre-measured bottles: one that contained water and the other HF emulsion containing 5% Intralipid®. **(A)** Volumes ingested were measured over the course of 18 h and **(B)** fat preference was calculated by dividing the volume of 5% IntraLipid® consumed by that of the total volume of liquid (water and 5% Intralipid®) consumed in the session and expressed as percentage (%). Data are presented as mean ± SEM. $n = 4$ per treatment group. $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

which provides a measure of brain MOR availability *in vivo* (Burghardt et al., 2015; Karlsson et al., 2016). On the morning of scans, Sham-LF/HF rats were significantly heavier ($p < 0.0001$) compared to the remaining groups. Sham-LF/HF rats weighed 653 ± 3.3 g, RYGB rats weighed 508 ± 17.3 g, Sham-BWM rats weighed 496 ± 14.1 g and Sham-LF rats weighed 501 ± 18.5 g. After scanning, inspection of PET images revealed a widespread reduction in [^{11}C]carfentanil uptake in the brains of RYGB rats compared to the remaining groups (**Figure 3A**). PET data analysis for all subcortical (thalamus, hypothalamus, striatum, and amygdala) and cortical (prefrontal cortex, orbitofrontal cortex, cingulate cortex, and insular cortex) regions analyzed are shown in **Figures 3B,C**, respectively. In subcortical regions, [^{11}C]carfentanil binding potential (BP_{ND}) was significantly lower in RYGB rats relative to Sham-LF rats in the hypothalamus ($p < 0.05$), and amygdala ($p < 0.01$) and tended to be lower in the striatum ($p = 0.06$). [^{11}C]carfentanil BP_{ND} was also significantly lower in RYGB animals relative to Sham-BWM animals in the striatum ($p < 0.05$). In cortical regions, [^{11}C]carfentanil BP_{ND} was only significantly lower in RYGB rats relative to Sham-LF/HF rats in the insular cortex ($p < 0.05$).

RYGB Surgery Reduces Striatal and Prefrontal MOR Protein Expression

Considering the PET data showing widespread reductions in brain MOR availability after RYGB, we next measured MOR protein expression in brain regions involved in the opioidergic control of feeding including the hypothalamus (Stanley et al., 1988), striatum (Bakshi and Kelley, 1993; Zhang et al., 1998; Lenard et al., 2010), orbitofrontal cortex (Mena et al., 2011), and prefrontal cortex (Mena et al., 2011) in our experimental groups by Western Blot. Immunoblots revealed a distinct 47 kDa band corresponding to the molecular weight of the MOR (**Figure 4**). Analysis of band intensity normalized to β-actin revealed lower values for the RYGB group relative to the remaining groups ($p < 0.05$) in the striatum (**Figure 4B**), and for the RYGB group relative to Sham-LF/HF ($p < 0.05$) and Sham-LF ($p < 0.05$) groups in the prefrontal cortex (**Figure 4C**).

DISCUSSION

Brain MORs are in prime position to be a molecular target of RYGB due to their prominent role in regulating hedonic feeding (Bodnar, 2013). In accordance with previous human (Kenler et al., 1990; Olbers et al., 2006; Thomas and Marcus, 2008) and rodent (Zheng et al., 2009; le Roux et al., 2011; Shin et al., 2011b; Hao et al., 2016) studies, we demonstrated that RYGB reduces HF intake and preference using an established rat model (Hankir et al., 2015; Seyfried et al., 2016). We found that this was associated with widespread reductions in brain MOR availability, particularly in the striatum. Furthermore, levels of MOR protein in the striatum as well as in the prefrontal cortex were downregulated after RYGB, which is consistent with the known stimulatory effects of MOR agonism in these areas on fat intake (Bakshi and Kelley, 1993; Mena et al., 2011). In contrast, no changes in feeding behavior or brain MOR signaling were observed in chronically food restricted Sham-BWM animals. Reduced brain MOR signaling, therefore, may underlie the postoperative suppression in HF food intake, contributing to sustained weight loss caused by RYGB, unlike the case for chronic food restriction.

Previous studies performed on rats support a key role for nucleus accumbens MORs in the "liking" of palatable foods (Richard et al., 2013). In this context, and in line with the findings from the present study of reduced striatal MOR levels after RYGB, patients and rats demonstrate measures of diminished HF food liking postoperatively (Shin et al., 2011b; Ochner et al., 2012). However, patients and rats also show the same response following chronic caloric-restriction (Epstein et al., 1989; Shin et al., 2011a). Considering that we did not find alterations in striatal MORs in Sham-BWM rats, it appears that changes in taste reactivity following weight loss caused by chronic caloric-restriction may be dissociated from striatal MOR signaling.

There is considerable overlap between the effects of RYGB and reduced brain MOR signaling on brain function and behavior. For instance, in human fMRI studies both RYGB and MOR antagonist treatments decrease striatal blood oxygenation level dependent signal when subjects are presented images of HF

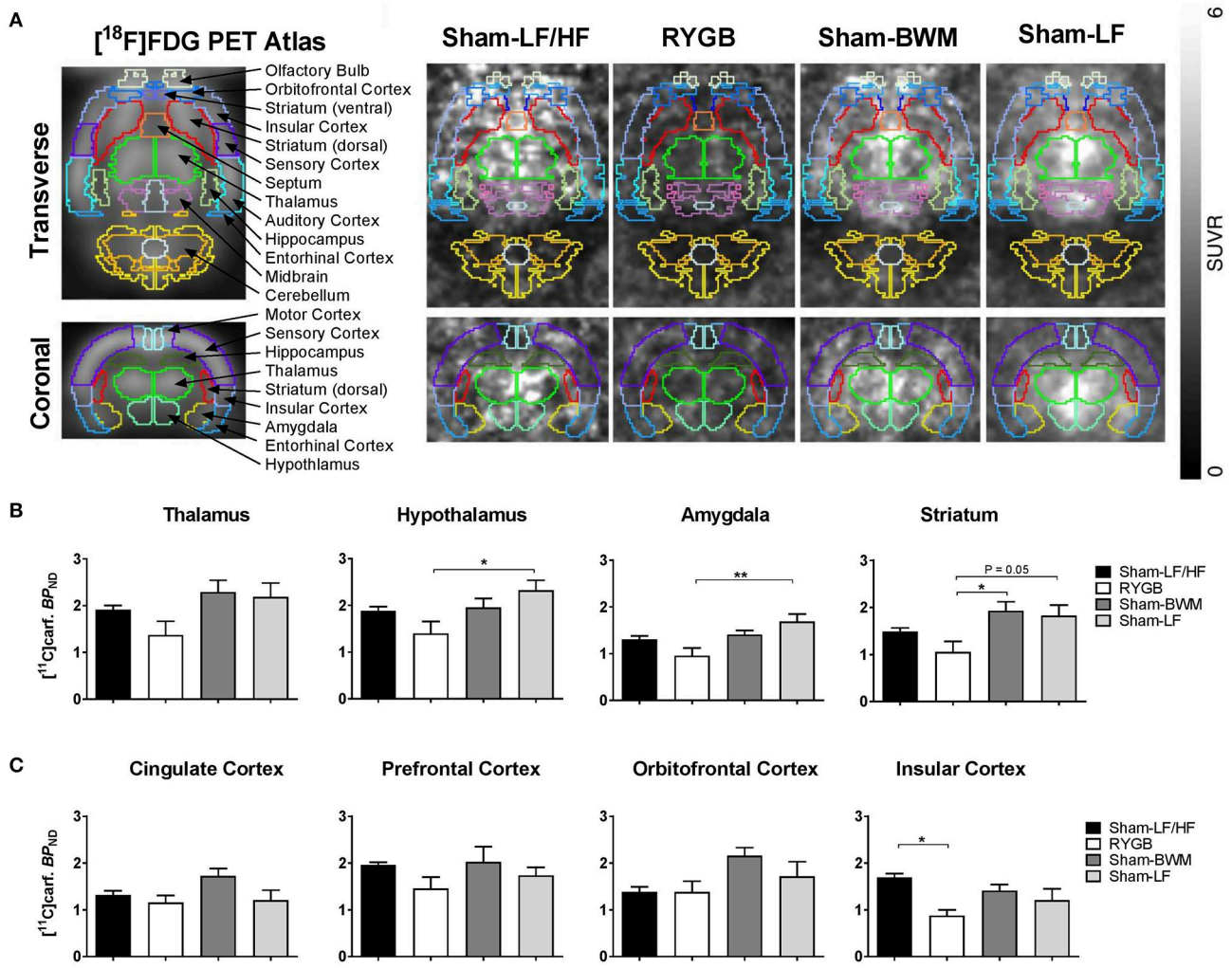


FIGURE 3 | Reduced brain MOR availability at a stage of weight loss maintenance after RYGB in diet-induced obese male rats. During postoperative weeks 15–17, *ad libitum* fed Sham-LF/HF, RYGB, Sham-BWM and Sham-LF rats underwent small-animal PET imaging. Animals were anesthetized with isoflurane and received the selective μ-opioid receptor (MOR) radioligand [^{11}C]carfentanil intravenously at the onset of PET scanning for 35 min. **(A)** (Left) standard [^{18}F]-FDG rat brain PET atlas used to align PET images with brain masks superimposed and labeled. (Right) representative PET images of Sham-LF/HF, RYGB, Sham-BWM and Sham-LF rat brains depicted as a standardized uptake value (SUV) ratio with cerebellum used as reference region based on frames 20–35 min. [^{11}C]Carfentanil binding potential ([^{11}C]Carf. BP_{ND}) was calculated for individual **(B)** subcortical and **(C)** cortical regions of interest. Data are presented as mean ± SEM. $n = 4$ per treatment group. $^{*}p < 0.05$, $^{**}p < 0.01$.

foods (Cambridge et al., 2013; Scholtz et al., 2014) and in rat macronutrient studies, both RYGB and MOR antagonist treatments suppress fat intake when carbohydrate, fat and protein diets are presented simultaneously (Marks-Kaufman and Kanarek, 1990; Wilson-Pérez et al., 2013). In addition, patients who take opioids before RYGB tend to use higher amounts after surgery (Raebel et al., 2013) and a recent investigation revealed that male RYGB rats have markedly higher morphine self-administration rates on a fixed ratio schedule of reinforcement compared to obese, chronically caloric-restricted and lean counterparts (Biegler et al., 2016). These latter findings strongly support those of the present study of reduced brain MOR signaling after RYGB, which may predispose to a compensatory increase in opioid intake as is typically observed with brain receptor downregulation/desensitization during drug tolerance.

The reduced brain MOR availability following RYGB is body weight independent and likely a consequence of altered gut-brain communication. Circulating levels of the orexigenic stomach hormone ghrelin have been previously reported to be decreased in patients (Cummings et al., 2002) and rodents (Shin et al., 2010) after RYGB. Considering that systemic administration of ghrelin increases brain MOR mRNA expression in rats (Skibicka et al., 2012), it is possible that the RYGB-mediated lowering in plasma ghrelin contributes to reduced brain MOR levels postoperatively. Profound changes in gut microbiota also take place after RYGB

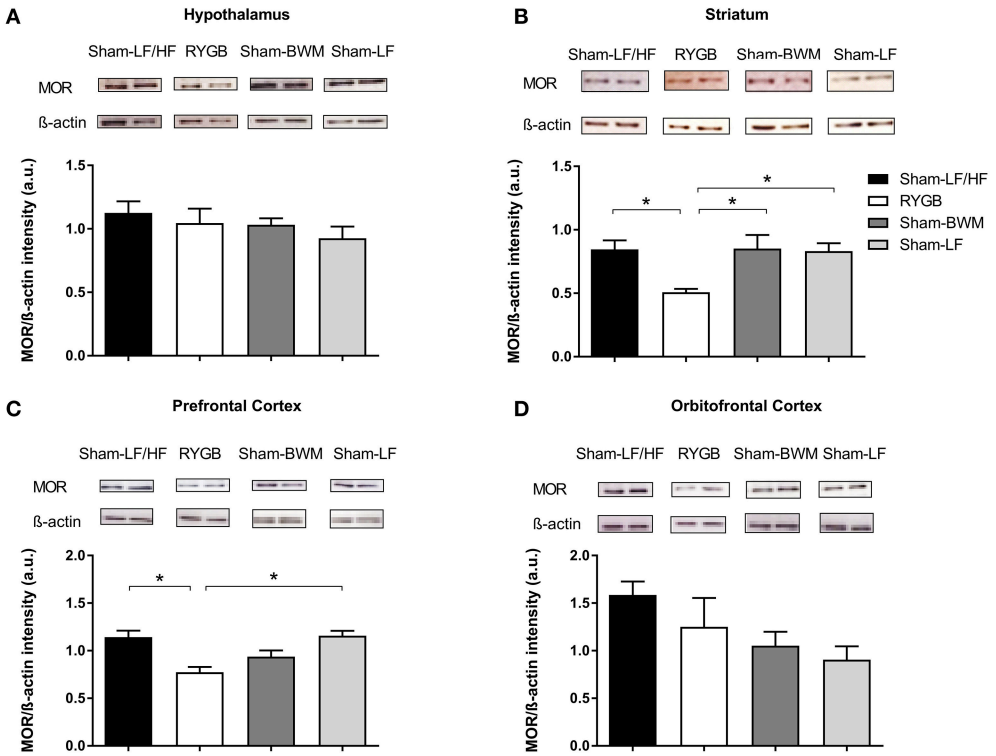


FIGURE 4 | Downregulation of striatal and prefrontal MOR protein levels at a stage of weight loss maintenance after RYGB in diet-induced obese male rats. At postoperative week 18, *ad libitum* fed Sham-LF/HF, RYGB, Sham-BWM, and Sham-LF rats were sacrificed and brains were collected for Western Blot analysis. Representative blots for MOR and β-actin in **(A)** hypothalamus, **(B)** striatum, **(C)** prefrontal cortex, and **(D)** orbitofrontal cortex for each group are presented. In **(A–D)**, MOR protein expression was quantified by normalizing band signal intensity to that of β-actin. Data are presented as mean ± SEM. $n = 3/4$ per treatment group. $^*p < 0.05$.

in humans and rodents (Furet et al., 2010; Liou et al., 2013; Shao et al., 2016). Interestingly, secreted proteins from gut *E. coli* have been found to influence brain opioidergic feeding circuits (Breton et al., 2016) and may too underlie altered brain MOR signaling after RYGB. Future mechanistic studies can address these issues in more detail.

It was recently reported in human [^{11}C]carfentanil PET imaging studies that dieting exerts modest effects (Burghardt et al., 2015) whereas bariatric surgery causes a widespread increase in brain MOR availability in obese subjects (Karlsson et al., 2016). With respect to the study of Burghardt et al., [^{11}C]carfentanil BP_{ND} in various brain regions was not significantly different between fasted obese individuals before (BMI 38) and after (BMI 32) weight loss caused by 4 months of dieting. These findings are comparable with those made in the present study with the Sham-LF/HF and Sham-BWM animals. With respect to the study of Karlsson et al. [^{11}C]carfentanil BP_{ND} in thalamus, ventral striatum, dorsal striatum, amygdala, insular cortex, and orbitofrontal cortex in mildly fasted obese individuals was markedly *increased* 6 months after bariatric surgery (BMI 31) relative to the preoperative state (BMI 40). In contrast, here we report a *reduction* in MOR availability in these brain regions postoperatively. This finding was robust, attaining statistical significance despite the low sample size. A possible explanation for the discrepant findings between the present study and the study of Karlsson et al. is that the timing of our scans corresponded to a late postoperative stage of weight loss maintenance after RYGB, whereas the subjects in the study of Karlsson et al. were scanned at an early stage of active weight loss. We also exclusively report findings from male rats after RYGB surgery, whereas Karlsson et al. performed PET analysis on a mixed population of female patients whom underwent two different bariatric procedures (sleeve gastrectomy and RYGB). Indeed the female sex hormones estrogen and progesterone both have been shown to influence brain MOR expression (Hammer et al., 1994).

A limitation of radioligand PET studies in general is that binding potentials do not provide a strict measure of receptor density. In the case for [^{11}C]carfentanil, binding to the MOR can be reduced as a result of competitive displacement by endogenous opioids (Burghardt et al., 2015). However, we found that in the striatum and prefrontal cortex at least, RYGB downregulates MOR protein expression which may underlie the reduced [^{11}C]carfentanil binding in these regions. Future cerebral microdialysis studies can reveal whether RYGB influences brain opioid release in rats (Difeliceantonio et al., 2012).

In summary, we report that suppressed fat appetite at a stage of weight loss maintenance after RYGB in male rats is associated with reduced brain MOR availability. Bariatric surgery may uniquely target various anatomically and molecularly discrete brain feeding circuits (Haahr et al., 2015) at early and late time points postoperatively (Mumphrey et al., 2016). Thus, treatments which can mimic the dynamic neurochemical signature of RYGB might effectively improve feeding behavior in the long-term, causing and sustaining significant weight loss in obese individuals.

AUTHOR CONTRIBUTIONS

Conceptualization, MKH and WF; investigation, MP, MK, WD, MHK, FS, UK, KS; formal analysis and visualization, GB, PB, SH, OS; writing original draft, MKH and WF; writing review and editing, MHK and WF; funding acquisition and project administration, WF; resources, UK and WF; project design and supervision, WF.

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Central Nervous Insulin Administration before Nocturnal Sleep Decreases Breakfast Intake in Healthy Young and Elderly Subjects

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Peripheral insulin acts on the brain to regulate metabolic functions, in particular decreasing food intake and body weight. This concept has been supported by studies in humans relying on the intranasal route of administration, a method that permits the direct permeation of insulin into the CNS without substantial absorption into the blood stream. We investigated if intranasal insulin administration before nocturnal sleep, a period of reduced metabolic activity and largely absent external stimulation, affects food intake and energy turnover on the subsequent morning. Healthy participants who were either young (16 men and 16 women; mean age ± SEM, 23.68 ± 0.40 years, mean BMI ± SEM, 22.83 ± 0.33 kg/m^2) or elderly (10 men, 9 women; 70.79 ± 0.81 years, 25.27 ± 0.60 kg/m^2) were intranasally administered intranasal insulin (160 IU) or placebo before a night of regular sleep that was polysomnographically recorded. Blood was repeatedly sampled for the determination of circulating glucose, insulin, leptin and total ghrelin. In the morning, energy expenditure was assessed via indirect calorimetry and subjects were offered a large standardized breakfast buffet from which they could eat *ad libitum*. Insulin compared to placebo reduced breakfast size by around 110 kcal (1,054.43 ± 50.91 vs. 1,162.36 ± 64.69 kcal, $p = 0.0095$), in particular decreasing carbohydrate intake (502.70 ± 25.97 vs. 589.82 ± 35.03 kcal, $p = 0.0080$). This effect was not dependent on sex or age (all $p > 0.11$). Sleep architecture, blood glucose and hormonal parameters as well as energy expenditure were not or only marginally affected. Results show that intranasal insulin administered to healthy young and elderly humans before sleep exerts a delayed inhibitory effect on energy intake that is not compensated for by changes in energy expenditure. While the exact underlying mechanisms cannot be derived from our data, findings indicate a long-lasting catabolic effect of central nervous insulin delivery that extends across sleep and might be of particular relevance for potential therapeutic applications.

Keywords: intranasal insulin, food intake behavior, sleep, aging, sex distribution

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INTRODUCTION

Eating behavior is tightly regulated by central nervous circuitries that receive hormonal feedback on body fat stores and nutritional status from the body periphery (Morton et al., 2014). In addition to the adipocyte-derived hormone leptin, insulin is one of the major peripheral signals that contribute to the central nervous control of ingestive behavior. Both leptin and insulin circulate in direct proportion to the size of body fat stores and reach the CNS via active, saturable transport mechanisms across the blood-brain barrier (Baura et al., 1993; Schwartz et al., 1996; William and Banks, 2001). Studies in animals (Woods et al., 1979; McGowan et al., 1992; Air et al., 2002) and humans (Benedict et al., 2008; Hallschmid et al., 2012) have conclusively shown that insulin administered directly to the brain reduces food intake, independent of its peripheral glucoregulatory action. In humans, the inhibitory effect of central nervous insulin on food intake has been mainly investigated by means of the intranasal route of peptide administration (Hallschmid et al., 2004a, 2012; Benedict et al., 2008), a non-invasive method of substance delivery to the brain that largely bypasses the blood-brain barrier (Born et al., 2002; Dhuria et al., 2010). Intranasal administration of 160 IU insulin to healthy, fasted young subjects reduced calorie intake in male, but not female participants (Benedict et al., 2008). Accordingly, intranasal insulin treatment (4 × 40 IU/day) for 8 weeks resulted in loss of body weight and body fat in men but not in women (Hallschmid et al., 2004a). Still, young women who intranasally received 160 IU insulin after a standardized lunch displayed an enhancement of post-prandial satiety and a reduction in the intake of palatable snacks (Hallschmid et al., 2012). Ample evidence for a distinct effect of insulin on food intake-regulatory networks has also been found in related neuroimaging studies (see Heni et al., 2015; Kullmann et al., 2016 for reviews).

Sleep has turned out to be an important factor in the maintenance of energy homeostasis and the regulation of food intake (St-Onge et al., 2016). Habitually short sleep duration is associated with increased body weight (Magee and Hale, 2012; Vgontzas et al., 2014) and a more pronounced risk of impairments in glucose homeostasis (Gangwisch et al., 2007; Cappuccio et al., 2010). Fittingly, individuals exposed to acute sleep deprivation tend to consume more food on the subsequent day (Brondel et al., 2010), to reduce their physical activity (Schmid et al., 2009) and to display a deterioration in glucoregulation (Schmid et al., 2011). It has been proposed that increased energy expenditure due to sleep loss is overcompensated by an exaggerated increase in energy intake, resulting, on the long run, in a higher risk of obesity and related metabolic impairments (Penev, 2012; Schmid et al., 2015). We have previously shown that insulin applied to the CNS before nocturnal sleep increases growth hormone concentrations during early sleep and impacts memory function on the subsequent day (Feld et al., 2016), indicating that central nervous insulin signaling is relevant for sleep-associated neuroendocrine regulation. In the present study, we investigated the effect of intranasal insulin administered before sleep on eating behavior on the subsequent morning. We assumed that the acute enhancement of brain insulin signaling during sleep, i.e., a period of reduced metabolic activity and largely absent external input, exerts a delayed but discernible attenuating effect on breakfast intake, i.e., calorie consumption immediately following the sleep period. This hypothesis was tested in a group of healthy young men and women, thereby enabling the detection of potential sex differences. Considering reports that food-cue elicited changes in brain activity in response to a meal decrease with advancing age (Cheah et al., 2013), we moreover included a group of healthy elderly participants in order to investigate if age is a relevant modulatory factor in this context.

METHODS AND MATERIALS

Participants

Thirty-two healthy young subjects (16 men and 16 women, mean age ± SEM, 23.68 ± 0.40 years) and 19 elderly participants (10 men and 9 women, 70.79 ± 0.81 years) were recruited from the community for this study. All young subjects were normal-weight (BMI, 22.83 ± 0.33 kg/m^2, $p = 0.49$ for men vs. women) while the elderly participants were normal- or mildly overweight (25.27 ± 0.60 kg/m^2, $p = 0.70$ for men vs. women; $p < 0.001$ for young vs. elderly participants). All subjects were non-smokers. The women in the young group were taking oral contraceptives (estrogen dominant, single-phase; Valette, Jenapharm, Jena, Germany), but were otherwise free of medication, as were the men. Clinical examination excluded previous illness prior to inclusion in the study. In order to restrict the burden of experimental participation for the elderly subjects, some assessments (in particular energy expenditure and continuous heart rate monitoring) were omitted and less blood parameters were determined in this group. Written informed consent was obtained from all subjects and the study conformed to the Declaration of Helsinki and was approved by the local ethics committee.

Study Design and Procedure

The experiments were conducted according to a placebo-controlled, double-blind, within-subject crossover design. All participants took part in two experimental sessions which were identical except for the intranasal administration of insulin (Actrapid®, Novo Nordisk, Bagsværd, Denmark) or placebo (vehicle). Sessions were performed in a balanced order, i.e., half of the sample received first placebo and then the active agent, with the reversed order for the other half of the sample. In addition, participants spent an adaption night in the sleep lab (i.e., including the placement of electrodes for polysomnographic recordings), with at least a 24-h delay between adaptation and the first experiment. Experimental sessions were scheduled to be apart as close to 28 days as possible, ensuring that the young women were tested during the same phase of contraceptive intake in both sessions.

Subjects were instructed not to take naps and not to engage in intense physical activities on experimental days. They were told to abstain from caffeine and to follow their usual dinner routines around 1800–1900 h. Participants arrived at the sleep lab at 2000 h. Adherence to the instructions for the experimental day

was confirmed and an intravenous catheter was placed in a vein of the dominant arm. At 2120 h, participants underwent a memory test battery (see Feld et al., 2016, for details and respective results in the group of young subjects) before receiving intranasal insulin or placebo via sixteen 0.1-ml puffs (8 per nostril) in 1-min intervals, amounting to a total dose of 1.6 ml insulin (160 IU) or placebo at 2220 h. This dose was chosen in order to enable comparisons with previous studies on the role of central nervous insulin in the acute regulation of food intake (Benedict et al., 2008; Hallschmid et al., 2012). Subjects went to bed at 2300 h for 8 h of polysomnographically recorded sleep, resulting in an overnight fast of at least 12 h in all subjects. Subjects were awakened at approximately 0700 h; care was taken not to wake participants up from rapid eye movement (REM) sleep or slow wave sleep. In the young subjects, heart rate was recorded throughout the night and energy expenditure was assessed at 0710 h in the morning. At 0815 h, breakfast was offered to all subjects. Throughout the session, blood was repeatedly sampled, without disturbing the participants, from an adjacent room with a thin plastic tube attached to the catheter for the determination of relevant parameters. Venous patency was maintained with a NaCl 0.9% drip.

Assessment of Breakfast Intake

Participants were offered a standardized test buffet of approximately 4,550 kcal at 0815 h and were allowed to eat ad-libitum and undisturbed for 30 min (see **Table 1** for a list of ingredients). All ingredients were weighed before and after eating to calculate the net amount consumed. Participants were allowed to take any leftovers with them in order to prevent them from overconsumption. They were told that this breakfast was scheduled to fill the gap between cognitive tests and provide them with the possibility to follow their usual breakfast routine, so that the experimental nature of this buffet remained undisclosed. One male and three female participants of the young group and one male and two female participants of the elderly group abstained from eating breakfast and were excluded from food intake analyses. All participants rated their hunger, thirst and tiredness on visual analog scales (VAS) before breakfast.

Energy Expenditure

In the participants of the young group, energy expenditure was measured at 0710 h via indirect calorimetry using a ventilated-hood system (Deltatrac II, MBM-200 Metabolic Monitor; Datex-Engström Deutschland, Achim, Germany). Before each use, the device was calibrated with Quick Cale calibration gas to 5% CO_2 and 95% O_2. Due to technical failures, assessments were not possible in two subjects.

Blood Parameters

Blood samples for the determination of blood glucose levels and circulating concentrations of hormones were obtained before intranasal insulin administration and repeatedly throughout the night. Blood glucose was determined immediately after each blood draw (HemoCue Glucose 201 Analyzer, HemoCue AB, Ångelholm, Schweden). The remaining samples were centrifuged and serum and plasma were frozen at −80°C for later analyses.

TABLE 1 | Composition of the test breakfast buffet.

Food	Weight (g)	Energy (kcal)	Carbohydrate (g)	Fat (g)	Protein (g)
NEUTRAL					
Whole milk	750	491	36	26.3	24.8
Buns	240	275	122.4	3.4	6.3
Whole wheat bread	165	360	71	2.3	12
White bread	30	72	14.6	0.4	2.2
Butter	120	928	0.7	99.8	0.8
SWEET					
Orange juice	400	173	36	1	4
Strawberry milk	200	167	18.2	6.8	7.4
Apple	195	104	22.2	1.2	0.6
Orange	180	72	15	0.4	1.8
Banana	179	168	38.3	0.4	2
Pear	140	78	17.4	0.4	0.7
Fruit curd	125	140	19.3	3.3	7.7
Vanilla pudding	125	134	20.8	3.8	3.5
Tangerine	80	35	8.2	0	0.5
Strawberry jam	50	147	35.8	0.1	0.1
Hazelnut spread	40	218	21.6	12.8	2.8
Honey	40	123	30	0	0.1
Sugar	24	98	24	0	0
HEARTY					
Sliced cheese	100	374	0.1	29.2	25.5
Poultry sausage	40	74	0.1	4.3	8.3
Cream cheese (herbs)	40	124	1	11.6	3.2
Cervelat sausage	34	120	0.1	10.2	6.1
Cream cheese	33	87	0.6	7.8	3
Total	3,330	4,562	553.4	225.5	123.4

All values are rounded to the closest decimal.

Insulin concentrations were determined in young and elderly participants (Insulin ELISA Kit, Dako, Glostrup, Denmark). In the young participants, plasma concentrations of total ghrelin (RIA; Linco Research, St. Charles, MO; sensitivity 93 pg/ml, intra-assay and inter-assay CV, 10 and 17.8%) and serum concentrations of leptin (RIA; Linco Research, St. Charles, MO; sensitivity, 0.5 ng/ml, intra-assay and inter-assay CV, 8.3% and 6.2%) were measured at time-points of relevance throughout the night.

Polysomnography and Heart Rate

EEG was recorded continuously from electrodes (Ag-AgCl) placed at C3 and C4 according to the 10-20 System and referenced to two coupled electrodes attached at the mastoids. EEG signals were filtered between 0.16 and 35 Hz and sampled at a rate of 200 Hz using an EEG amplifier system (BrainAmp DC, BrainProducts GmbH, Munich, Germany). Additionally, eye movements and muscle tone were recorded by electrodes placed diagonally above the left and below the right eye and electrodes attached to the chin, respectively. Sleep EEG scoring was carried out independently by two experienced technicians

who were blind to the assigned treatment. Sleep architecture was determined according to standard polysomnographic criteria using EEG recordings from C3 and C4, diagonal EOG and chin EMG (Rechtschaffen and Kales, 1968). For each night, total sleep time, i.e., the time between the first detection of transition from sleep stages 1 to 2 and lights on, was used to calculate relative time spent in the different sleep stages, i.e., wake, REM sleep and NonREM sleep stages 1–4. Heart rate was recorded by electrocardiography (Actiheart, CamNtech, Boerne TX USA) in the young subjects during sleep until shortly after awakening. In the elderly subjects, it was monitored before and after sleep.

Statistical Analyses

R 3.3.1 (R Core Team, 2016) was used for statistical analyses. We used *lme4* (Bates et al., 2015) to build linear mixed-effects models to compensate for missing blood values in some cases (less than four per experiment). Main effects were tested for significance using likelihood-ratio tests with Satterthwaite approximations to degrees of freedom. For the indirect calorimetry data, we used condition (placebo or insulin) and sex as fixed effects and random intercepts for subjects. For breakfast consumption, we built an initial model using condition, sex, age group (young, elderly), macronutrient (carbohydrate, fat, protein) and interactions between condition, sex and macronutrient, condition and sex, condition and age and sex and macronutrient as fixed effects, random slopes for macronutrient and random intercepts for subjects. The inclusion of a random slope takes into account individual food preferences of each subject, thus optimally capturing between-subject differences. To evaluate intake by food type (hearty, neutral, sweet), we replaced macronutrient with food type in the previous model. For the VAS we built a model with fixed effects for treatment, sex, age group and random intercepts for subjects. We used *lsmeans* (Lenth, 2016) to run *post-hoc* comparisons with multivariate *t* adjustment. Levene's test for homogeneity of variance was used to test for equality of variances in our factors of interest, with only sex showing a deviation ($p < 0.01$; $p \geq 0.12$ for age and treatment). For analyses of blood parameters, the fixed factors sex, condition and time were used and areas under the curve (AUC) according to the trapezoidal rule were calculated. Results are presented as means ± SEM. A $p < 0.05$ was considered significant.

RESULTS

Breakfast Intake and Hunger Ratings

Intranasal insulin administration before sleep reduced breakfast intake by 110 kcal or around 9% (**Table 2** and **Figure 1A** and **Supplementary Figure 1**). This hypophagic effect of insulin primarily concerned carbohydrate intake, whereas consumption of fat and protein was not affected [$F_{(2, 170)} = 3.23$, $p = 0.042$ for treatment × macronutrient; **Table 2**]. The insulin effect was modified neither by sex nor age (all $p > 0.11$ for interaction with treatment; **Figure 1B**) and did not specifically affect individual food types [hearty, neutral, sweet; $F_{(2, 233)} = 0.71$, $p = 0.49$ for treatment × food type]. Independent of insulin treatment, men consumed more energy than women [1,276.23 ± 86.76 vs. 913.23 ± 50.64 kcal, $F_{(1, 43)} = 17.34$, $p = 0.0001$; **Figures 1C–F**], in

TABLE 2 | Consumption from the breakfast buffet.

	All subjects			Men			Women			Young subjects			Elderly subjects		
	Insulin	Placebo	*p*	Insulin	Placebo	*p*	Insulin	Placebo	*p*	Insulin	Placebo	*p*	Insulin	Placebo	*p*
Overall consumption	1,054.43 ± 50.91	1,162.36 ± 64.69	0.0095	1,188.79 ± 72.29	1,363.66 ± 97.42	0.0146	893.20 ± 54.30	932.30 ± 47.87	0.0143	1,032.90 ± 60.44	1,128.64 ± 70.78	0.0285	1,092.12 ± 96.11	1,217.89 ± 127.38	0.0210
MACRO-NUTRIENTS															
Carbohydrate	502.70 ± 25.97	589.82 ± 35.03	0.0079	543.82 ± 39.02	678.63 ± 55.67	0.0075	453.36 ± 30.66	488.32 ± 27.31	0.0073	531.03 ± 28.66	587.09 ± 36.26	0.001	453.12 ± 50.91	594.32 ± 72.64	0.0090
Fat	413.85 ± 28.95	430.65 ± 30.92	0.9866	485.51 ± 41.06	516.50 ± 46.48	0.9856	327.84 ± 32.46	332.54 ± 27.67	0.9853	368.75 ± 30.92	404.93 ± 33.51	0.9922	492.76 ± 55.63	473.02 ± 60.48	0.9916
Protein	137.89 ± 8.70	141.89 ± 9.15	1	159.46 ± 13.25	168.54 ± 13.29	1	112.00 ± 7.94	111.43 ± 8.68	1	133.12 ± 10.39	136.62 ± 11.28	1	146.23 ± 16.23	150.55 ± 15.74	1

Results are means ± SEM kcal, rounded to the nearest decimal. P-values are derived from least-squares means with multivariate t adjustment.

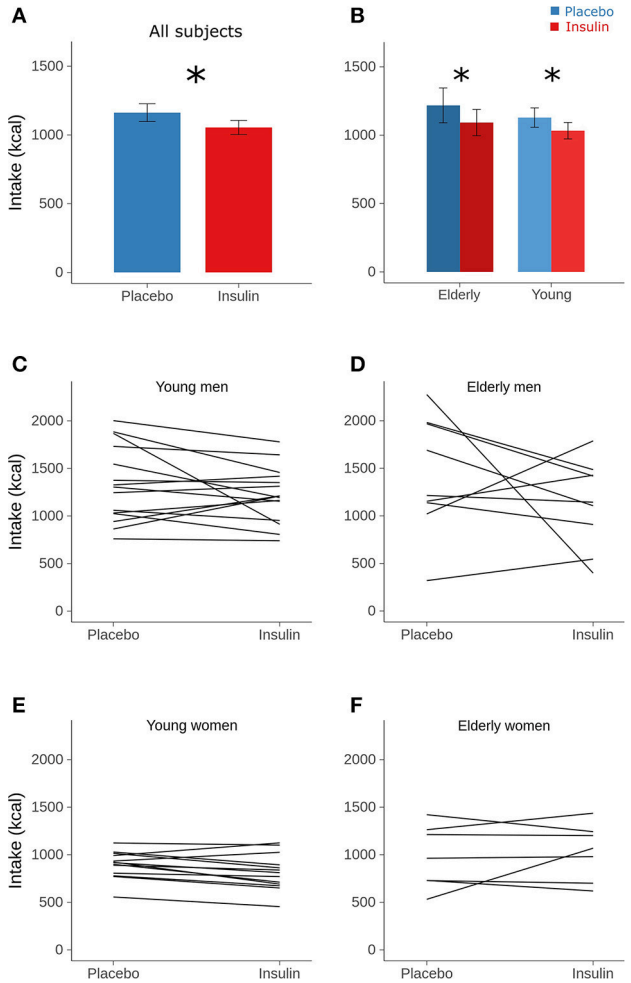


FIGURE 1 | Calorie intake. Mean (± SEM) calorie intake from the breakfast buffet assessed in the morning after intranasal administration of placebo (vehicle; blue bars) and insulin (160 IU; red bars) at 2220 h of the preceding evening in **(A)** all subjects and **(B)** according to age groups. **(C–F)** Individual calorie intake from the breakfast buffet in the placebo (left) and the insulin condition (right) in respectively, the young and elderly men and women. Individual values of both sessions are connected by lines. Note that omitting the male subjects showing the largest insulin effect yielded *p*-values for the factor condition of 0.015 and 0.079 in the groups of young and, respectively, elderly subjects. $n = 14$ young and 9 elderly men, and 14 young and 7 elderly women; $^*p < 0.05$ for comparisons between conditions (least-square means with multivariate *t* adjustment).

particular fat [501.01 ± 43.50 vs. 330.25 ± 29.72 kcal, $t_{(54)} = 4.33$, $p = 0.0008$; $p > 0.05$ for carbohydrates and protein; $F_{(2, 54)} = 4.13$, $p = 0.02$ for sex × macronutrient]. Men also ate more neutral-taste foods than women [248.74 ± 43.48 vs. 168.80 ± 22.92 kcal, $t_{(146)} = 5.73$, $p < 0.0001$; $p > 0.84$ for hearty and sweet foods]. Calorie intake of young and elderly participants was generally comparable [1,080.77 ± 65.85 vs. 1,156.91 ± 111.48 kcal, $F_{(1, 44)} = 0.64$, $p = 0.43$; $F_{(1, 55)} = 0.69$, $p = 0.41$ for sex × age; **Figures 1C–F**] and also did not differ regarding food types [$F_{(2, 231)} = 0.53$, $p = 0.59$]. Age and macronutrient showed a significant interaction [$F_{(2, 54)} = 4.38$, $p = 0.02$] that, however, did not yield significant age-dependent effects on macronutrient intake (all $p > 0.17$).

Overall, intranasal insulin did not alter hunger, thirst and tiredness as rated before breakfast (all $p > 0.50$), and there was no influence of sex on these values (all $p > 0.33$; **Table 3**). Elderly participants reported significantly lower hunger than their young counterparts [43.75 ± 3.81 vs. 67.56 ± 4.52%, $t_{(122)} = -4.64$, $p = 0.0001$], with no differences in thirst and tiredness (all $p > 0.19$; **Table 3**).

Energy Expenditure

Intranasal insulin administration before sleep did not affect resting energy expenditure measured in the young subjects before breakfast [1,637.96 ± 50.25 vs. 1,656.32 ± 50.87 kcal/day for insulin and placebo, respectively, $F_{(1, 29)} = 0.971$, $p = 0.33$; **Figure 2**] and also did not induce sex-dependent changes [$F_{(1, 28)} = 0.07$, $p = 0.80$]. Across conditions, women displayed significantly lower energy expenditure than men [1,418.63 ± 41.56 vs. 1,847.05 ± 46.24 kcal/day, $F_{(1, 28)} = 49.27$, $p < 0.0001$].

Blood Parameters

In the young participants, a transient peak in plasma insulin concentration emerged 10 min after intranasal insulin administration (111.19 ± 9.38 vs. 56.67 ± 4.79 pmol/L after placebo; $p \leq 0.001$) that was followed by a slight dip in blood glucose values (4.38 ± 0.13 vs. 4.86 ± 0.06 mmol/L; $p \leq 0.01$). Neither of these changes was correlated with the intranasal insulin-induced decrease in breakfast intake in the subsequent morning ($r = -0.15$, $p = 0.54$, and $r = 0.02$, $p = 0.95$, respectively, Pearson's coefficients). During the rest of the night, respective values were comparable between conditions [$F_{(6, 156)} = 0.86$, $p = 0.65$ for treatment × time; see Feld et al., 2016, for detailed results], without any statistical difference to the elderly group ($p = 0.24$ for age). Serum insulin as well as blood glucose concentrations were not affected by insulin administration in the elderly subjects (all $p > 0.58$). Across conditions, blood glucose levels were lower in elderly than young [$F_{(1, 105)} = 14.49$, $p < 0.001$ for age] and in female than male individuals [$F_{(1, 25)} = 10.14$, $p < 0.01$ for sex].

Plasma concentrations of total ghrelin, measured only in the group of young subjects, did not differ between conditions [17,940.26 ± 830.37 vs. 17,903.28 ± 763.61 h × pg/ml for insulin and placebo, respectively; $F_{(1, 108)} = 0.01$, $p = 0.95$; $F_{(1, 909)} = 0.0042$, $p = 0.95$ for time × treatment; **Figures 3A,B**]. They were generally elevated in women compared to men [20,258.09 ± 915.24 vs. 15,429.69 ± 967.39 h×pg/ml, $F_{(1, 29)} = 16.31$, $p = 0.0004$]. Serum leptin concentrations in the young participants also remained unaffected by intranasal insulin [154.10 ± 23.25 vs. 156.47 ± 23.75 h × ng/ml, $F_{(1, 31)} = 0.04$, $p = 0.85$; $F_{(1, 406)} = 0.18$, $p = 0.67$ for time × treatment] and, as expected, were markedly higher in women compared to men [259.78 ± 27.57 vs. 50.69 ± 6.30 h × ng/ml, $F_{(1, 30)} = 67.35$, $p < 0.0001$, **Figures 3C,D**].

Sleep and Heart Rate

Total sleep time for the young participants was 461.59 ± 3.37 min in the insulin and 460.70 ± 4.57 min in the placebo condition (*p*

TABLE 3 | Visual analog scale ratings obtained before breakfast.

	Overall			Sex			Age group		
	Insulin	Placebo	*p*	Female	Male	*p*	Elderly	Young	*p*
Hungry	58.96 ± 3.55	60.10 ± 3.63	1	55.84 ± 5.78	62.86 ± 4.26	0.4382	43.75 ± 3.81	67.56 ± 4.52	0.0001
Thirsty	63.00 ± 2.94	61.33 ± 3.25	0.9311	59.31 ± 4.77	64.72 ± 3.96	0.7564	54.69 ± 3.78	65.95 ± 4.17	0.1974
Tired	32.93 ± 3.17	36.98 ± 3.40	0.9837	41.14 ± 4.71	29.60 ± 4.37	0.3319	31.29 ± 4.61	36.83 ± 4.40	0.9398

Results are means ± SEM %. p values are derived from least-square means contrasts with multivariate t adjustment.

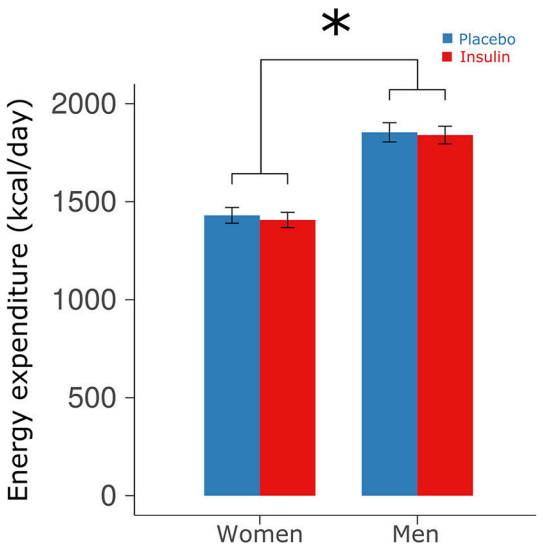


FIGURE 2 | Energy expenditure. Mean ± SEM energy expenditure measured in the young participants via indirect calorimetry in the morning after intranasal administration of placebo (vehicle; blue bars) and insulin (160 IU; red bars) at 2220 h of the preceding evening. Calorimetry was performed in the fasted state at 0710 h before breakfast. *$p < 0.05$ for comparisons between sexes.

> 0.84). Elderly participants spent comparable amounts of time asleep (456.57 ± 11.12 vs. 458.39 ± 5.68 min, $p > 0.89$; $p > 0.57$ for group effect). Intranasal insulin compared to placebo did not alter sleep latency, whole-night sleep architecture and total sleep time (all $p > 0.29$). Heart rate measured in the young subjects throughout the night was unchanged by insulin [58.77 ± 1.59 vs. 58.98 ± 1.48 bpm, $F_{(1, 29,006)} = 0.08$, $p = 0.77$; **Figure 4**]. Independent of treatment, it showed a trend toward increased values in women compared to men [61.19 ± 1.96 vs. 56.63 ± 2.10 bpm; $F_{(1, 30)} = 3.72$, $p = 0.063$]. Heart rate measured in the elderly subjects before and after sleep was not modulated by intranasal insulin (all $p \geq 0.18$).

DISCUSSION

We investigated whether a single dose of intranasal insulin administered to healthy young and elderly subjects before nocturnal sleep attenuates calorie intake from a large, standardized ad-libitum breakfast buffet on the subsequent day. We found that pre-sleep insulin treatment reduced breakfast consumption by around 110 kcal, which is roughly equivalent to a large banana or half a chocolate-caramel bar. This effect emerged against the background of unaltered energy expenditure. Although the number of participants, in particular of elderly subjects, may limit respective conclusions, we did not find indicators that the insulin effect was modified by sex or age. It was neither associated with unwanted side effects on sleep. Our results demonstrate that insulin delivered to the brain via the intranasal route exerts a long-lasting, behaviorally relevant effect on eating behavior. While the exact mediators behind such a delayed hypophagic action of enhanced brain insulin signaling cannot be derived from our data, this finding underlines the efficacy of intranasal insulin in curbing food intake in humans.

We have previously shown that intranasal insulin administered to young male subjects in the fasted state (Benedict et al., 2008) acutely decreases food intake, and reduces the intake of palatable snacks in young female subjects after lunch (Hallschmid et al., 2012). The latter effect concerned post-prandial snacking in the afternoon and likely resulted from effects on reward-processing pathways, which may be assumed to have also played a role in the present experiments, although our study design clearly does not allow for a differentiation between reward- and hunger-related aspects of eating. In the former experiment (Benedict et al., 2008), the hypophagic insulin effect in the young men was observed in the late morning and around 80 min after intranasal delivery of 160 IU insulin, i.e., the same dose as applied in the present experiments. It was found during ad-libitum intake from a breakfast buffet that was comparable in composition and size to the buffet offered in the present study. Insulin administration in the morning led to a macronutrient-unspecific reduction in calorie intake of around 190 kcal in young men, which exceeded the drop in food intake of 110 kcal found in the young male subjects of the present experiments. Both effects developed against the background of well-comparable overall calorie intake (1,350 vs. 1,330 kcal in the respective placebo conditions of the former and, for the young men, the present study). In the former study (Benedict et al., 2008), young female participants did not show an insulin-induced reduction in food intake whereas pre-sleep insulin delivery reduced breakfast size in the young women of the present experiments. There are some indicators that central nervous insulin, with regard to food intake, may be less efficient in female compared to male organisms (Clegg et al., 2003, 2006). However, the women of our previous study (Benedict et al., 2008) ate around 130 kcal less than their female counterparts of the present experiments (769 vs. 895 kcal in the respective placebo conditions), so that a biasing contribution of bottom effects cannot be excluded.

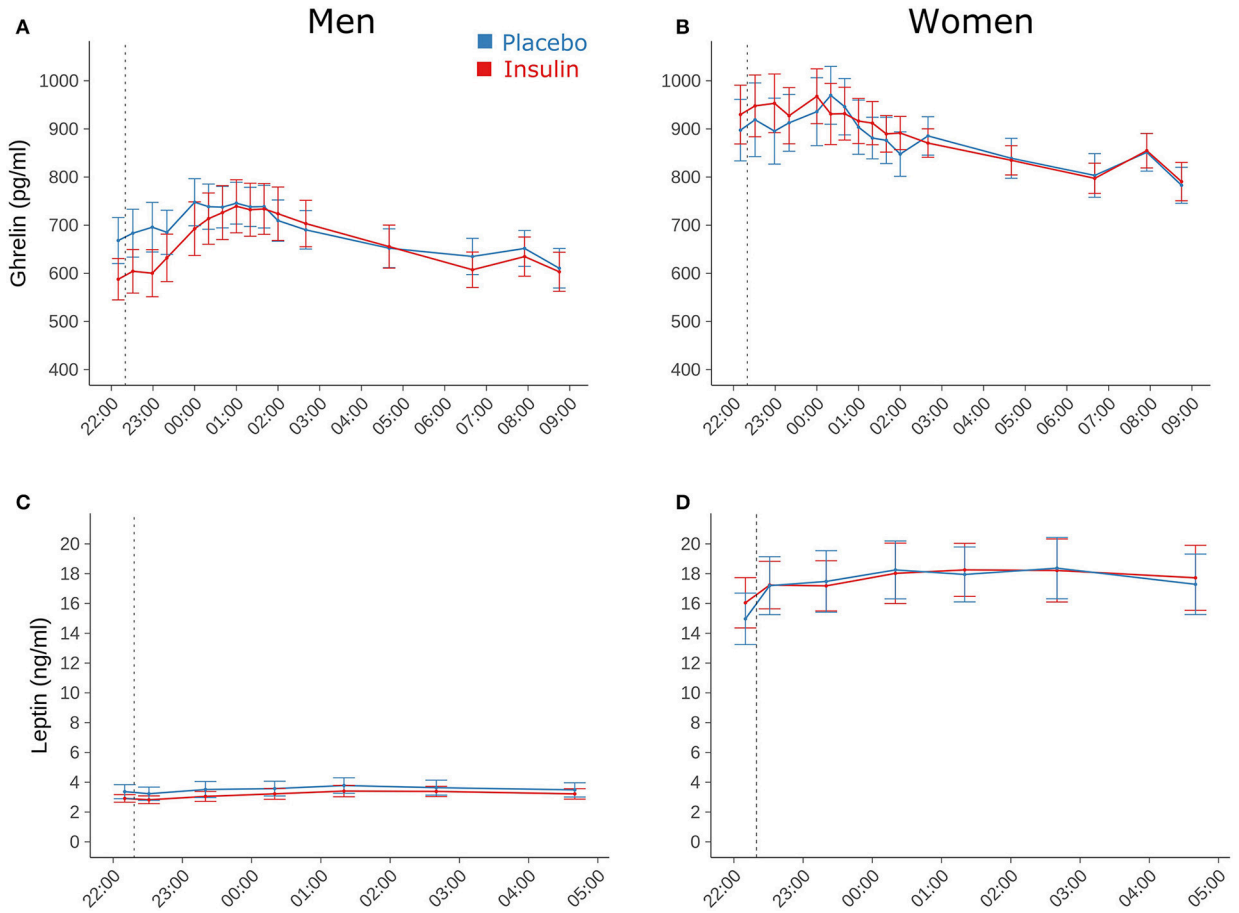


FIGURE 3 | Blood parameters. Mean ± SEM concentrations of **(A,B)** plasma total ghrelin and **(C,D)** serum leptin measured in young male ($n = 16$; left panels) and female participants ($n = 16$; right panels) who were intranasally administered insulin (160 IU; red lines) or placebo (blue lines) at 2220 h (dotted line). Note that leptin concentrations were determined only until 0440 h in the morning.

Basal energy expenditure assessed in the young subgroup before breakfast was comparable between conditions, indicating that the reduction in breakfast intake was not a compensatory effect, but yielded a net decrease in energy intake. This effect was primarily caused by a drop in the consumption of carbohydrates. This finding ties in with experiments in rats indicating that insulin administration to the CNS reduces sugar intake (Figlewicz et al., 2008) and suggests that, in accordance with the major role of peripheral insulin for blood glucose regulation, the anorexigenic effect of the hormone on the brain might focus on carbohydrates. However, the relative paucity of available data in humans (Benedict et al., 2008; Hallschmid et al., 2012; Jauch-Chara et al., 2012) and animals (Woods et al., 1979; McGowan et al., 1992; Air et al., 2002; Clegg et al., 2003, 2006) at the moment does not permit sound conclusions on macronutrient-specific effects of brain insulin on eating behavior (for review see Kullmann et al., 2016; Lee et al., 2016). Animal research has indicated that the adiposity signals insulin and leptin can directly act on the brain reward circuitry to decrease the intake of particularly palatable foods (Figlewicz et al., 2007). Although at a first glance, the insulin-induced reduction in carbohydrate intake would fit with the assumption that intranasal insulin specifically reduces the intake of highly rewarding foods (Hallschmid et al., 2012), sweet items as a food category were not differentially affected here. Considering that hunger ratings were comparable between conditions, it may be concluded that intranasal insulin did not affect hunger motivation, but rather acted via satiating factors that contribute to the termination of a meal. We did not find discernible treatment-induced changes in ghrelin and leptin, two hormones of paramount relevance for food intake control (Morton et al., 2014). A mild insulin-induced decrease in plasma glucose concentration apparently was restricted to the young participants although our study was not designed to detect respective differences between age groups. This dip in blood glucose, which was presumably due to absorption of insulin into the blood stream via the nasal mucosa (Ott et al., 2015), was statistically unrelated to the insulin-induced reduction in breakfast intake. It can be safely excluded to have affected breakfast intake because of its transient nature, and because nocturnal decreases in blood glucose levels rather increase than attenuate food intake in the morning (Schmid et al., 2008).

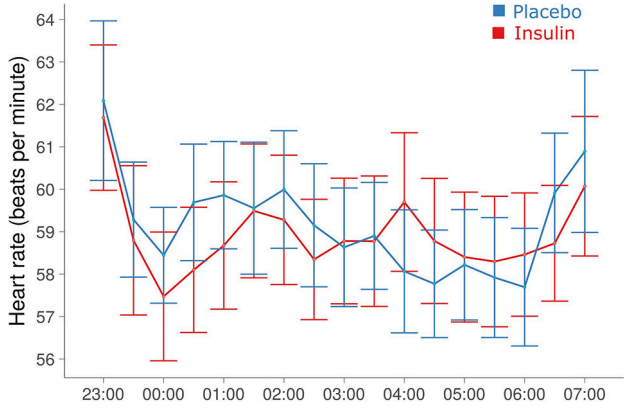


FIGURE 4 | Heart rate during the sleep period. Mean ± SEM heart rate averaged across 30-min blocks from minute-to-minute recordings. No signs of coherent treatment effects were detected (see Results).

The inhibitory impact on ad-libitum food intake exerted by intranasal insulin emerged across 10 h, which is a surprisingly long period of time when compared to respective effects in related studies (Benedict et al., 2008; Hallschmid et al., 2012; Jauch-Chara et al., 2012). Considering that degradation of the insulin molecule in the central nervous compartment can be assumed to be relatively delayed in comparison to its half-life in the circulation of 4–6 min (Duckworth et al., 1998), the enhanced brain insulin signal may in principle be still functionally active after a prolonged period of time. This assumption is supported by the finding that shifts in direct current EEG potentials triggered by intranasal insulin do not yet reach their maximum after around 90 min of recording (Hallschmid et al., 2004b). Alternatively, an indirect mediation of the observed effect might be assumed, but cannot be derived from our data inasmuch as endocrine signals like ghrelin and leptin, but also indicators of sympathovagal balance were unchanged and our study does not warrant a strict differentiation between central and peripheral mediators. Intranasal insulin exerted its anorexigenic effect across an interval of nocturnal sleep which, in itself, has turned out to be a relevant modulator of food intake (Schmid et al., 2015). Total sleep deprivation for one night leads to activity changes in brain functions that favor food intake (Greer et al., 2013), and partial sleep deprivation of 4 h for one night strongly increases breakfast intake in healthy men (Brondel et al., 2010). Since we did not observe differences in total sleep time nor in sleep architecture between conditions, insulin presumably did not exert major effects on sleep. However, sleep might have prolonged the insulin effect by reducing interfering effects of external stimuli, or increases in hunger typically developing in awake subjects across extended periods of time.

The relative reduction in pre-breakfast hunger ratings in the elderly, as compared to young participants, might be related to decreasing responses to food cues (Cheah et al., 2013) and impaired dynamics of satiety-regulating factors (Rolls et al., 1995) that emerge during aging, but was not reflected in differences in actual food intake. Young and elderly participants were also equally responsive to the anorexigenic effect of insulin. However, probably because of the relatively small number of elderly participants the effect appeared to be less robust in this group so that further studies should substantiate this finding. While decreases in central nervous insulin sensitivity have been linked to cognitive deficits and Alzheimer's disease, pathologies associated with advanced age (Freiherr et al., 2013), our results suggest that, at least in healthy individuals, age does not independently affect the role of insulin in central nervous networks that control food intake. Likewise, insulin sensitivity in the body periphery is not independently affected by biological age as long there is no increase in fat mass (Karakelides et al., 2010). Thus, the good health status of our elderly participants, as verified by clinical examination and evidenced by their merely moderately elevated body weight in comparison to our young subjects, might have ensured sufficient potency of the insulin signal. Vice versa, indicators of high cerebral insulin sensitivity have been found to be associated with successful loss of body fat during lifestyle intervention (Tschritter et al., 2012).

CONCLUSION

In sum, we demonstrate that intranasal insulin administration before nocturnal sleep elicits a reduction in breakfast intake in healthy subjects that is not compensated for by changes in energy expenditure. These findings suggest that insulin administered to the brain before sleep may potentiate the satiating effect of food intake in the next morning. The effect observed here was of moderate impact, and potential compensatory changes in eating behavior throughout the rest of the day were not investigated. In previous studies, four daily doses of intranasal insulin, one of them administered before going to bed, induced a reduction in body weight and fat in healthy participants (Hallschmid et al., 2004a), suggesting that on the long run the anorexigenic effect of (pre-sleep) intranasal insulin administration can affect body weight regulation. Thus, central nervous insulin administration regimens focusing on the sleep period may exert beneficial effects on metabolic health and might even help prevent or treat the brain insulin resistance associated with metabolic disorders (Kullmann et al., 2016).

AUTHOR CONTRIBUTIONS

JS conducted the data analyses. MH collected the data and contributed to the analyses. Both authors interpreted the data and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fnins.2017.00054/full#supplementary-material

Supplementary Figure 1 | Boxplots of calorie intake from the breakfast buffet assessed in the morning after intranasal administration of placebo (vehicle; blue bars) and insulin (160 IU; red bars) at 2220 h of the preceding evening (A) according to age groups (elderly subjects, darker shades; young subjects, brighter shades) and **(B)** according to sex.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Emerging Role of Corticosteroid-Binding Globulin in Glucocorticoid-Driven Metabolic Disorders

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Glucocorticoid hormones (GCs) are critical for survival since they ensure the energy supply necessary to the body in an ever challenging environment. GCs are known to act on appetite, glucose metabolism, fatty acid metabolism, and storage. However, to be beneficial to the body, GC levels should be maintained in an optimal window of concentrations. Not surprisingly, conditions of GC excess or deficiency, e.g., Cushing's syndrome or Addison's disease, are associated with severe alterations of energy metabolism. Corticosteroid-binding globulin (CBG), through its high specific affinity for GCs, plays a critical role in regulating plasma GC levels and their access to target cells. Genetic studies in various species including humans have revealed that CBG is the major factor influencing interindividual genetic variability of plasma GC levels, both in basal and stress conditions. Some, but not all, of these genetic studies have also provided data linking CBG levels to body composition and insulin levels. The examination of CBG-deficient mice submitted to hyperlipidic diets unveiled specific roles for CBG in lipid storage and metabolism. An influence of CBG on appetite has not been reported but remains to be more finely analyzed. Finally, only male mice have been examined under high-fat diet, while obesity is affecting women even more than men. Overall, a role of CBG in GC-driven metabolic disorders is emerging in recent studies. Although subtle, the influence of CBG in these diseases could open the way to new therapeutic interventions since CBG is easily accessible in the blood.

Keywords: glucocorticoids, transcortin, obesity, metabolism, lipid storage

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INTRODUCTION

Glucocorticoid hormones (GCs) are adrenal cortex steroids that were named from their role in the regulation of glucose metabolism, mostly through maintaining the reserves of glycogen in the liver. They also play an intricate role in fatty acid metabolism and storage. Finally, they are also important regulators of appetite through complex interactions with orexigenic and anorexigenic neuropeptides and hormones. Excess GC levels, encountered in Cushing's syndrome or GC therapy, lead to central obesity associated with hyperphagia, insulin resistance, and fatty liver development (1).

Under normal physiological conditions, GC secretion (cortisol or corticosterone depending on species) follows a circadian rhythm entrained by light and food intake. These environmental

stimuli trigger the secretion of the hypothalamic peptide corticotrophin-releasing hormone (CRH), which, in turn, stimulates pituitary secretion of adrenocorticotrophic hormone (ACTH) that acts on adrenal glands to induce GC synthesis and release in the blood (2). In plasma, GCs bind with a high affinity but low capacity to transcortin, also called corticosteroid-binding globulin (CBG), and to albumin with a high capacity but low affinity (3, 4). The free fraction of circulating GCs thus constitutes around 3–5% of the total GC pool. Free GCs regulate negatively their own secretion by inhibiting CRH and ACTH release and act on target tissues by binding to specific receptors, the mineralocorticoid and the GC receptors (5). Under stressful conditions, the increase in CRH levels results in increased GC secretion that must be transient to be protective to the body. GCs are essential for survival but not enough or too much GC signaling is deleterious for health (6).

Corticosteroid-binding globulin has been known since 1956 as a plasma protein capable of high-affinity interaction with cortisol (4). It was purified in the early 60s and cloned and characterized since the 90s in many species (7). Recently, CBG has been identified as a major component of GC genetic variability in animals and humans. Consequently, the role of CBG in GC-driven metabolic disorders and obesity gained interest. This mini-review summarizes the recent studies related to the role of CBG in GC-driven metabolic alterations and obesity.

GENETIC STUDIES HIGHLIGHTING THE CENTRAL ROLE OF CBG IN GC VARIABILITY

Large individual differences exist in both basal and stress-induced GC levels. Because this variability is in part of genetic origin, several research groups have used genetic mapping on experimental crosses, i.e., a non-hypothesis-driven approaches, to identify genetic factors contributing to this variability of GC levels (8, 9). The first study to demonstrate the importance of CBG used contrasted pig breeds, the Chinese Meishan vs. the European Large White breed (10). The former shows plasma cortisol concentrations higher than most European pig breeds, as well as a reduced growth rate, low muscle content, and high-fat deposits, all features that may be consequences of their high cortisol levels (11). This genetic mapping study resulted in the detection of a locus containing the gene encoding CBG (called SerpinA6), strongly associated with cortisol levels, in particular stress levels, and explaining 20% of the variance in the F2 population (12). Because CBG was posited as a serious candidate gene of cortisol variability, it was thus investigated further in a follow-up study. New arguments in favor of its involvement were provided by a genetic mapping analysis on the same F2 pig intercross using CBG levels as a trait instead of cortisol. This genetic mapping detected exactly the same genomic locus than when using cortisol levels with higher statistical strength. In addition, CBG mRNA and binding capacity were found different between the parental pig breeds, i.e., higher levels in the Meishan breed (13).

Few years later, a similar genetic mapping approach was used in rodent models. The genome scan detected a highly significant quantitative trait locus associated with poststress corticosterone at the SerpinA6/CBG locus. The cDNA sequence analysis of parental strains revealed a Met276Ile mutation in WKY rat identical to that found in the Biobreeding rat strain that exhibits decreased CBG binding affinity for corticosterone (14). More recently, a series of studies conducted in different pig lines confirmed the genetic linkage or genetic association between SerpinA6/CBG locus and basal cortisol levels in pig (15–18). Finally, in humans, a very significant association between the SERPINA6/SERPINA1 locus and morning plasma cortisol concentrations was reported in a genome-wide association meta-analysis using ~2.5 M SNPs in 12,597 Caucasian subjects, replicated in 2,795 participants (19). Further investigation using part of the cohort showed that common SNPs in this locus were associated with variation in plasma CBG concentrations. These data were replicated in a recent candidate gene study, where SNPs within the SERPINA6 gene were also found associated with morning cortisol and CBG concentrations in a cohort of 1,077 adolescents (20).

In conclusion, CBG appears to be a major component of GC genetic variability in various mammalian species including humans. Given the role of GC in energy metabolism, the association of the locus CBG with body composition and metabolic parameters has also been investigated.

GENETIC STUDIES RELATING CBG AND BODY COMPOSITION

In the first pig study, genetic linkage between the CBG locus and several parameters of body composition was found, namely, backfat weight and thickness, as well as muscle content (13). Interestingly, in this study, plasma CBG levels appeared to be a better predictor of body composition than cortisol levels, as CBG binding capacity showed negative correlation to muscle and positive ones to fat deposits, whereas no significant correlation was found between the same traits and cortisol concentrations. Later, a correlation between the percentage of fat and CBG binding capacity was replicated in a Meishan × Large White F10 intercross (21). However, no genetic linkage to body composition traits was detected in another pig study involving a different breed (Duroc instead of Meishan) (15).

In rodents, many genetic studies have detected adiposity, body weight, insulin levels, or diabetes susceptibility to the locus containing CBG without necessarily testing or even invoking CBG as the causal gene (7).

In humans, a genetic analysis was conducted in a cohort of 44 obese premenopausal women using a genetic marker within the CBG gene (22). No association was found with metabolic or obesity parameters and the CBG genetic marker. However, in patients holding a specific allele of the CBG marker (allele 90), a strong correlation was found between salivary cortisol after dexamethasone suppression test and waist-to-hip ratio, whereas this correlation was not significant for the other patients of the cohort. These results suggest that CBG gene polymorphisms may modulate the influence of the HPA axis on the fat mass distribution in this sample of obese women. In a second study, the same genetic marker in CBG gene was used to genotype a population of 295

men with body mass index (BMI) ranging from 19 to 55 kg/m^2 (23). The frequency of the allele 90 was found markedly increased among men with morbid obesity (BMI > 40) compared to the rest of the population. Furthermore, the CBG polymorphism was found correlated with BMI and waist circumference in the total population. Finally, this CBG gene polymorphism was also used in a cohort of 45 prepubertal obese children. No differences were found for obesity or metabolic parameters between genotypes at the CBG locus, although allele 90 carriers presented increased 24-h free urinary cortisol (24).

Overall, the genetic studies revealed a modest influence of CBG on body composition and obesity. This modest influence of CBG may be explained by the complexity of the regulation of these traits as well as the small size of the populations studied.

DISSECTING METABOLIC ALTERATIONS IN CBG-DEFICIENT MOUSE MODELS

Older studies have reported altered levels of CBG in animal models of obesity. For example, the obese *Zucker* rat presents plasma and adipose tissue CBG levels twice lower than its lean control. Furthermore, in the obese *Zucker* rat, the regulation of CBG by GCs is lost (25, 26). However, in the *Obese* strain chicken model, contrary to the obese *Zucher* rat, CBG is found twice as high as compared with its healthy control (27). The availability of CBG-deficient mice produced by specific knockout of the SerpinA6 gene provided the opportunity to clarify the specific role of CBG in obesity.

A mouse model of CBG deficiency has been first reported in Petersen et al. (28). When submitted to a 6-week-long high-fat diet (HFD: 26 vs. 4% kcal fat for control diet), Cbg KO showed a tendency ($p = 0.08$) for increased body weight compared to WT. However, the weight loss, as well as liver gene expression estimated by gene arrays, was found equivalent between genotypes after 36 h of food deprivation. Using the same mouse model, a recent study examined the metabolic consequences of a 12-week-long very HFD (60 vs. 18% kcal fat for control diet) on body weight, white adipose tissue, and liver (29). Under the very HFD, total body weight gain and food intake were similar in both Cbg KO and WT groups. Interestingly, Cbg KO mice presented a significantly lower subcutaneous white adipose tissue accumulation than WT mice, associated with a slight higher increase in retroperitoneal and epididymal fat, but no differences in mesenteric fat depots. Adipocytes size increased with HFD in both WT and Cbg KO. However, in agreement with the depot-specific fat weights, the increase in adipocytes' size was smaller in the subcutaneous fat from Cbg KO than WT, but bigger in the epididymal fat. Total lipid content in liver of animals under HFD was equivalent between genotypes, but the accumulation of lipids in liver under HFD compared to control diet was found higher in Cbg KO compared to WT. HFD similarly increased serum glucose, insulin, leptin, and cholesterol in both genotypes. Non-esterified fatty acids, triacylglycerols, and urea differed neither between diets nor between genotypes.

In both studies, total corticosterone levels were much lower, whereas free corticosterone levels were higher in Cbg KO compared to WT under both control and HFD in blood sampled in the morning, i.e., at the nadir of secretion in mice. CBG and total corticosterone levels were increased by the 60% HFD in WT, but free corticosterone was not changed as CBG was also increased. Cbg KO displayed a modest increase in total and free corticosterone under HFD. In both studies, overall no differences in liver mRNA expression were detected between genotypes under any of the diets, even for GC target genes, such as phospho-enolpyruvate carboxykinase (PEPCK), tyrosine aminotransferase (TAT), or glucose 6-phosphatase (G6PT), in contradiction with the finding of increased free corticosterone in Cbg KO. In the study by Gulfo et al., a global genotype effect was found for liver 11-beta hydroxysteroid dehydrogenase type 2 (11HSD2), which showed reduced expression in Cbg KO mice and a genotype by diet interaction detected for hexose-6-phosphate dehydrogenase (H6PDH) which expression increased under HFD in Cbg KO contrary to WT. Within fat depots, an opposite pattern of expression was found in epididymal fat, i.e., 11HSD2 was increased in Cbg KO as compared to WT, both under control diet and HFD, while 11HSD2 mRNA was very low in subcutaneous fat in both genotypes regardless of the diet. The authors discuss these results as a within-tissue mechanism to regulate GC excess, based on the finding that 11HSD1 and 11HSD2 strongly regulate local availability of GC. In liver, low 11HSD2 expression would normalize GC signaling by reducing the availability of 11-dehydrocorticosterone that is a substrate of 11HSD1 for regeneration of corticosterone. In epididymal fat, increased 11HSD2 mRNA would inactivate GC, by metabolizing corticosterone in 11-dehydrocorticosterone, as a way to counteract the enlargement of adipocyte area. These interpretations may explain the lack of variation between genotypes in mRNA expression of GC target genes in some tissue, despite higher free GC levels in Cbg KO. However, data collected by our group in Bordeaux provide another line of discussion on these data.

We have also generated a Cbg KO mouse model (30) by first constructing a mouse line floxed for the gene Cbg and then crossing these mice with CMV-Cre mice. In our studies, total and free corticosterone levels were measured not only in the morning (9:00 a.m.) but also in the evening (9:00 p.m.) when the secretion of GC is at its peak (mice are nocturnal animals). In the morning, we found increased free GC levels in Cbg KO as in the study by Gulfo et al. (29) or Petersen et al. (28), but in the evening, this genotype difference was no longer present because the higher total GC levels in WT compensated the increased free GC percentage in Cbg KO. Thus, the lack of genotype effect on mRNA expression in basal conditions is expected since when free GC are high (at night), there are no genotype differences in their levels.

There is a discrepancy between our reports and those of Petersen et al. (28) and Gulfo et al. (29), on the levels of free GC measured after stress, when the rise in GC is important. The latter found either no significant differences in free GCs after stress between WT and Cbg KO or increased levels for Cbg KO, whereas we have shown that Cbg KO show a hypoglucocorticoid response to stress compared to WT (31, 32), including after chronic stress (33). Furthermore, Petersen et al. (28) found a phenotype of hyporesponsiveness to GC after septic shock in the Cbg KO mice.

To explain this paradox, they hypothesized that Cbg KO mice have become insensitive to high levels of free GC, but they did not provide data on the mechanism involved. On their side, Gulfo et al. (29) found higher free GC levels in Cbg KO mice, both under normal and very HFD, associated with lower thymus and higher adrenals weight, thereby pleading in favor of chronically elevated free GC levels. Taken together, the data from the different groups are confusing regarding whether Cbg KO mice are a model of hyper- or hypocorticosteronism. We believe that methodological differences are another point to take in account. We have used isotopic dilution to precisely evaluate free GC, while the other groups have measured free GC directly in the serum samples for levels that are at the limit of ELISA detection. In their studies, free GC levels are lower than expected in WT mice (0.7 and 1% of the total corticosterone, respectively, instead of 7–10% in our studies). This conflicting data will be important to clarify to interpret the data obtained on metabolic alteration in Cbg KO mice.

UNRAVELING THE ROLE OF CBG IN HUMAN METABOLIC DISORDERS AND OBESITY

In humans, plasma CBG was found to be a marker of insulin secretion, being negatively or positively correlated with various markers of obesity (BMI and waist-to-hip ratio) depending on the degree of insulin resistance (34, 35). Indeed, insulin was shown to downregulate CBG levels *in vitro* (36), thereby providing an explanation of low CBG levels in obese hyperinsulinemic subjects and high levels of CBG in diabetic insulin-resistant patients. Plasma levels of CBG were also found to correlate with some markers of the metabolic syndrome (fasting glucose, insulin resistance, and adiponectin) and inflammation (interleukin-6 and C reactive protein) (37–39).

By virtue of its SERPIN structure, CBG is cleaved by the serine protease neutrophil elastase, thereby reducing markedly GC binding and enabling massive local delivery of GCs at the site of inflammation (40). Because elevated levels of elastase have been associated with obesity and insulin resistance (41), a recent study examined the respective levels of high- and low-affinity CBG forms, in a cohort of 100 volunteers. Surprisingly, CBG cleavage was found to be reduced in subjects with central obesity and metabolic syndrome, which was interpreted as the sign of altered CBG protein in obesity that would no longer be sensitive to elastase cleavage. The mechanism of reduced CBG cleavage in obese patients remains to be described (42).

CONCLUSION

From the literature, it is now well established that CBG is a major factor influencing GCs levels and thus GCs action in tissues. The genetic studies provided hints that CBG was associated with increased fat deposits. Few studies have investigated the direct effects of CBG on energy metabolism and obesity. In a model of CBG deficiency in male mice, CBG seems to play a role in lipid metabolism and storage associated with changes in enzymes controlling local GC availability when animals are submitted to a 60% HFD. A potential effect on appetite is not reported, but this function has been studied only grossly. For example, examination of the pattern of eating through the day and the night or nutrient preferences may reveal a role for CBG. In addition, the status of GC activity in Cbg KO mice is contradictory in the literature, precluding clear-cut interpretation of the effect of CBG deficiency on GC-driven metabolism.

Overall, a role of CBG in GC-driven metabolic disorders is emerging but in extreme conditions of very hyperlipidic diets. The role of elastase in obesity and its regulation of CBG binding affinity have opened new avenues of research. Another area not explored yet is the influence of changes in temperature on CBG binding in the context of obesity. The liver being a very metabolic organ with a higher temperature than surrounding tissues, CBG affinity for GCs is probably diminished there. The influence of CBG in metabolic diseases is probably subtle but could be promising as a therapeutic target, given that CBG is easily accessible in the blood.

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All the authors listed have made substantial, direct, and intellectual contribution to the work and approved it for publication.

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Ghrelin-Reactive Immunoglobulins in Conditions of Altered Appetite and Energy Balance

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Part of circulating ghrelin is bound to immunoglobulins (Ig) protecting it from degradation and preserving its functional activity. This review summarizes the data on ghrelin- and desacyl-ghrelin-reactive IgG in conditions of altered appetite and energy balance. Plasma levels and affinity kinetics of such IgG were compared in patients with obesity and anorexia nervosa (AN) and in animal models of obesity including *ob/ob* mice, high-fat diet-induced obese mice, and obese Zucker rats as well as in mice after chronic food restriction and activity-based anorexia and in rats with methotrexate-induced anorexia. We show that plasmatic IgG in both obese humans and animals are characterized by increased affinity for ghrelin. In contrast, patients with AN and anorectic rodents all show lower affinity of ghrelin- and desacyl-ghrelin-reactive IgG, respectively, the changes which were not observed in non-anorectic, chronically starved mice. We also show that affinity of ghrelin-reactive IgG correlate with plasma levels of ghrelin. These data point to common mechanisms underlying modifications of affinity kinetics properties of ghrelin-reactive IgG during chronic alterations of energy balance in humans and rodents and support a functional role of such autoantibodies in ghrelin-mediated regulation of appetite.

Keywords: ghrelin, desacyl-ghrelin, anorexia, obesity, autoantibodies

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INTRODUCTION

Ghrelin is a 28 amino acid acylated enteroendocrine peptide produced mainly in the stomach, which has been isolated and named based on its ability to stimulate growth hormone secretion (1). One of the main properties of ghrelin, independent from growth hormone secretagogue activity, is stimulation of feeding and body weight gain (2–4). Importantly, acylation of the serine 3 residue in the ghrelin's N-terminal by octanoic acid is necessary for binding of the growth hormone secretagogue receptor 1 (GHSR1), designated as ghrelin receptor (5) and biological activity including stimulation of food intake (6–8). However, the active, acylated form of ghrelin is unstable in the circulation and is degraded to desacyl-ghrelin (9, 10). The half-life pharmacokinetics properties of acyl-ghrelin in human plasma are about 10 min (11). The preservation of the acylated ghrelin form in the circulation appears, hence, as a key factor for maintaining its role in regulation of appetite and energy balance. Several mechanisms are involved in the regulation of ghrelin signaling during its production and receptor activation, which can be exploited for development of ghrelin-based therapy of altered appetite (12). Recently, a new regulatory factor of the ghrelin signaling has been identified, which is ghrelin-reactive immunoglobulins (Ig) (13). This review is the first attempt to summarize the data on ghrelin-reactive IgG obtained since their initial identification and the following studies in animal models of altered appetite and energy balance.

METHODS

The review focuses mainly on the data from five papers, all reporting affinity kinetics of ghrelin- and desacyl-ghrelin-reactive IgG. They include a study of patients with hyperphagic obesity and anorexia nervosa (AN) and of *ob/ob* mice by Takagi et al. (13); two studies by François et al. in high-fat diet (HFD)-induced obese mice (14) and in rats with methotrexate (MTX)-induced anorexia (15), a study by Lucas et al. in obese Zucker rats (16) as well as a Legrand et al. study in mice after chronic food-restriction and activity-based anorexia (17). In all mentioned studies, the same protocols of IgG plasma extraction and affinity kinetic assay by the surface plasmon resonance were used as was previously described in details (18). All measurements of ghrelin and desacyl-ghrelin peptides in above mentioned studies were performed using corresponding kits from Mitsubishi Chemical Med Corp. (Tokyo, Japan), selectively detecting acyl- and desacyl-ghrelin with resulting plasma ratios of 1:3 to 1:6 in humans. In the text, we use the terms of ghrelin and acyl-ghrelin as synonyms. For the statistical analysis of ghrelin and ghrelin-reactive IgG properties between different animal models and patients, they are shown as means of folds of changes vs. corresponding controls. In this analysis, we combined two animal models of anorexia including ABA mice and MTX rats and three animal models of obesity including *ob/ob* mice, HFD-induced obese mice, and obese Zucker rats. Group differences shown in **Tables 1**–**4** and **Figure 1** were analyzed using statistical tests indicated in the legends. We also used the Pearson's test to analyze correlations between plasma levels of ghrelin or desacyl-ghrelin and plasma levels and affinity (KD values) of corresponding IgG.

TABLE 1 | Plasma concentrations of acyl-ghrelin and desacyl-ghrelin in humans and rodents.

	Acyl-ghrelin (pM)		Desacyl-ghrelin (pM)		Acyl/desacyl-ghrelin ratio	
Mean ± SEM						
OB	24.88 ± 5.81	ns	137.7 ± 16.07	ns	1:6	↘$
AN	52.67 ± 7.14	↗$$$	249.1 ± 38.32	↗$$$	1:4	ns
Ctrl	25.65 ± 3.58		114.7 ± 29.24		1:3	
ob/ob	10.36 ± 0.96	↘#	75.17 ± 10.31	↘*	1:7	ns
Ctrl	13.02 ± 0.90		120.4 ± 17.32		1:8	
HFD	10.21 ± 0.95	ns	117.3 ± 9.75	↘**	1:10	ns
Ctrl	11.92 ± 1.45		196.5 ± 27.95		1:13	
Zucker	17.18 ± 1.59	ns	444.8 ± 33.86	↗*	1:25	ns
Ctrl	16.20 ± 4.38		242.0 ± 55.80		1:16	
ABA	21.58 ± 6.86	ns	1331.0 ± 87.52	↗$$	1:63	ns
FTR	17.82 ± 5.02	ns	1221.0 ± 120.7	↗$	1:71	↘$
Ctrl	9.14 ± 2.50		195.8 ± 22.50		1:17	
MTX	12.68 ± 2.21	↗**	698.8 ± 95.8	↗*	1:46	ns
Ctrl	7.44 ± 1.08		352.4 ± 27.79		1:46	

*Human patients: OB, obese; AN, anorexia nervosa. Rodent models of obesity: ob/ob mice; HFD, high fat diet-induced obese mice and Zucker rats. Rodent models of anorexia and starvation: ABA, activity-based anorexia in mice; FTR, feeding-time restriction in mice; and MTX, methotrexate-induced anorexia in rats. Ctrl, healthy human or rodent controls in corresponding row. Student's t-test or Mann–Whitney test, *$p < 0.05$; **$p < 0.01$; #$p < 0.10$. ANOVA with Tukey's posttest or Kruskal–Wallis with Dunn's posttest $^{\$}p < 0.05$, $^{\$\$}p < 0.01$, $^{\$\$\$}p < 0.001$.*

DETECTION OF GHRELIN-REACTIVE IgG

Ghrelin-reactive IgG were first shown to be naturally present in plasma of healthy humans and rodents in 2008 by Fetissov et al. (19). Further studies confirmed and extended this initial finding by including female patients with restrictive AN (20) and with hyperphagic obesity (13). Moreover, a study using plasma samples of a large number of healthy male ($n = 562$) and female ($n = 636$) adolescents also revealed an ubiquitous presence of ghrelin-reactive autoantibodies of both IgG and IgM classes, which mean plasma levels were slightly elevated in girls vs. boys (21). In rodents, ghrelin-reactive IgG were detected in both rats (15, 19) and mice (13, 14, 17). Since the rodents studies were performed only in males, presence of possible sex differences of ghrelin-reactive IgG was not explored. However, it is likely that similarly to humans they may also be increased in female rodents who naturally display elevated plasma levels of total IgG and IgM as well as increased levels of autoantibodies reactive with other neuropeptides, e.g., α-melanocyte-stimulating hormone (α-MSH) (22).

Ghrelin-reactive IgG were also measured in the hypothalamic and liver tissue of C57Bl6 mice, showing their levels at the limit of detection (13). However, if levels of ghrelin-reactive IgG in these tissues of lean mice were stratified according to their exposure to a restraint stress in an EchoMRI instrument, which they had undergone prior to tissue sample for the analysis of their body composition, an increase is observed in both tissues and a decrease in plasma of stressed animals. It suggests that ghrelin-reactive IgG may help transportation of ghrelin mobilized during stress from plasma to its tissue targets.

Desacyl-ghrelin-reactive IgG are also present in humans (13, 20) and rodents (15, 17). Although some part of desacyl-ghrelin-reactive IgG may bind to acyl-ghrelin, they should also have distinct paratopes, because levels of desacyl-ghrelin IgG are often higher than of acyl-ghrelin IgG in human plasma (13, 20) and also because they have different affinity kinetics properties (13). The exact epitopes responsible for plasma IgG binding to ghrelin and desacyl-ghrelin remain to be been studied.

Both "free" and "total" ghrelin- and desacyl-ghrelin-reactive IgG are detected in plasma of humans and rodents. While "free" IgG are measured in physiological buffer, the "total" levels are measured in a high salt buffer, which dissociate immune complexes typically resulting in increased "total" vs. "free" levels of IgG. Accordingly, increased levels of free/total ratios may reflect an increase in IgG available to bind ghrelin or desacyl-ghrelin (**Table 2**).

ORIGIN AND REGULATION OF GHRELIN-REACTIVE IgG

Ghrelin-reactive IgG are natural autoantibodies and, therefore, are produced by germ-like B-cells as a part of native immunity (23). As was postulated by Avrameas, natural autoantibodies may participate in a complex regulatory network, not necessarily

TABLE 2 | Plasma levels of IgG reactive with acyl-ghrelin and desacyl-ghrelin in humans and rodents.

	Acyl-ghrelin-reactive IgG				Desacyl-ghrelin-reactive IgG				Acyl-ghrelin-reactive IgG		Desacyl-ghrelin-reactive IgG	
	Free		Total		Free		Total		Free/total ratio		Free/total ratio	
Mean ± SEM												
OB	0.291 ± 0.050	ns	0.617 ± 0.054	↘$	0.563 ± 0.049	ns	1.250 ± 0.057	ns	1:2.1	↗$	1:2.3	ns
AN	0.288 ± 0.020	ns	0.753 ± 0.049	ns	0.643 ± 0.054	ns	1.364 ± 0.080	ns	1:2.5	ns	1:2.1	ns
Ctrl	0.264 ± 0.020		0.918 ± 0.103		0.584 ± 0.094		1.384 ± 0.101		1:3.2		1:2.4	
ob/ob	0.133 ± 0.025	↘*	0.098 ± 0.013	↘#	n.a.	n.a.	1:0.6	ns	n.a.			
Ctrl	0.207 ± 0.024		0.162 ± 0.028		n.a.	n.a.			1:0.8		n.a.	
HFD	0.504 ± 0.050	ns	0.450 ± 0.033	↘**	n.a.	n.a.	1:0.8	ns	n.a.			
Ctrl	0.589 ± 0.067		0.624 ± 0.059		n.a.	n.a.			1:0.9		n.a.	
Zucker	0.268 ± 0.062	↘*	0.235 ± 0.073	↘*	0.244 ± 0.048	↘#	0.241 ± 0.066	↘*	1:0.6	↗*	1:0.8	↗*
Ctrl	0.715 ± 0.206		1.061 ± 0.259		0.627 ± 0.165		0.975 ± 0.225		1:1.5		1:1.5	
ABA	0.258 ± 0.084	ns	0.340 ± 0.127	ns	0.226 ± 0.091	ns	0.366 ± 0.127	ns	1:1.5	↗£	1:1.4	ns
FTR	0.187 ± 0.045		0.459 ± 0.109		0.242 ± 0.062		0.501 ± 0.127		1:2.4		1:2.0	
Ctrl	0.172 ± 0.052		0.315 ± 0.043		0.238 ± 0.069		0.464 ± 0.080		1:1.9		1:2.0	
MTX	0.063 ± 0.016	↘*	0.041 ± 0.007	↘***	0.070 ± 0.015	↘*	0.031 ± 0.006	↘***	1:0.6	↗***	1:0.4	↗***
Ctrl	0.188 ± 0.043		0.319 ± 0.044		0.223 ± 0.048		0.271 ± 0.036		1:1.7		1:1.2	

*Human patients: OB, obese; AN, anorexia nervosa. Rodent models of obesity: ob/ob mice; HFD, high fat diet-induced obese mice and Zucker rats. Rodent models of anorexia and starvation: ABA, activity-based anorexia in mice; FTR, feeding-time restriction in mice; and MTX, methotrexate-induced anorexia in rats. Ctrl, healthy human or rodent controls in corresponding row. Student's t-test or Mann–Whitney test, *p < 0.05; **p < 0.01; ***p < 0.001; #p < 0.10. ANOVA with Tukey's posttest or Kruskal–Wallis test with Dunns' posttest, $p < 0.05; ABA vs. FTR £p < 0.05.*

TABLE 3 | Affinity kinetics of acyl-ghrelin-reactive IgG in humans and rodents.

	Acyl-ghrelin-reactive IgG					
	Ka (1/Ms)		Kd (1/s)		KD (M)	
Mean ± SEM						
AN	9.36E+04 ± 2.23E+03	ns	3.25E−03 ± 8.04E−04	↗$	4.86E−07 ± 1.20E−07	↗§§
OB	2.23E+04 ± 1.16E+04	↗$	1.86E−03 ± 3.04E−04	ns	1.91E−07 ± 1.48E−08	ns
Ctrl	6.84E+03 ± 1.19E+03		1.49E−03 ± 1.14E−04		2.54E−07 ± 2.81E±08	
ob/ob	1.01E+05 ± 9.46E+03	ns	1.81E−03 ± 7.81E−05	↘**	2.03E−08 ± 2.88E−09	↘*
Ctrl	8.89E+04 ± 9.08E+03		2.46E−03 ± 1.48E−04		3.02E−08 ± 3.57E−09	
HFD	1.68E+04 ± 4.87E+03	ns	1.58E−03 ± 3.22E−04	ns	1.17E−07 ± 1.18E−08	↘**
Ctrl	1.04E+04 ± 2.06E+03		2.02E−03 ± 2.82E−04		2.55E−07 ± 4.51E−08	
Zucker	1.01E+04 ± 1.54E+03	ns	1.22E−03 ± 1.61E−04	↘*	1.36E−07 ± 2.65E−08	↘*
Ctrl	7.93E+03 ± 6.53E+02		1.70E−03 ± 9.79E−05		2.24E−07 ± 2.50E−08	
ABA	4.72E+04 ± 8.12E+03	↗*£	5.11E−04 ± 1.07E−04	ns	1.24E−08 ± 2.83E−09	ns
FTR	2.03E+04 ± 6.62E+03	ns	2.51E−04 ± 1.32E−04	ns	1.38E−08 ± 5.61E−09	ns
Ctrl	1.48E+04 ± 5.58E+03		2.45E−03 ± 1.15E−04		1.52E−08 ± 6.15E−09	
MTX	2.00E+03 ± 6.30E+02	ns	1.29E−03 ± 2.28E−04	↗**	1.18E−07 ± 4.92E−07	ns
Ctrl	1.79E+03 ± 5.06E+02		5.97E−04 ± 1.92E−04		5.73E−07 ± 2.49E−08	

*Human patients: OB, obese; AN, anorexia nervosa. Rodent models of obesity: ob/ob mice; HFD, high fat diet-induced obese mice and Zucker rats. Rodent models of anorexia and starvation: ABA, activity-based anorexia in mice; FTR, feeding-time restriction in mice; and MTX, methotrexate-induced anorexia in rats. Ctrl, healthy human or rodent controls in corresponding row. ANOVA with Tukey's posttest or Kruskal–Wallis test with Dunn's posttest, $p < 0.05; AN vs. OB §§p < 0.01. Student's t-test or Mann–Whitney test vs. Ctrl *p < 0.05; **p < 0.01, vs. RFA £p < 0.05.*

directly related to the immune function but contributing to the homeostatic control (24). The production and affinity maturation of IgG are influenced by a variety of antigens and non-specific immune-stimulatory or inhibitory factors such as cytokines and steroid hormones. Moreover, increased plasma levels of ghrelin-reactive IgG in rats can be induced acutely by gastric electrical stimulation (25), suggesting that they can be stored and released upon a physiological stimulus. It is of interest that gastric electrical stimulation activated c-fos production in numerous lymphoid cells of the gastric mucosa surrounding the enteroendocrine ghrelin-synthetizing cells (26). Strong inhibition of ghrelin-reactive IgG levels in plasma is observed in rats after treatment with MTX, an immunosuppressive agent, which induces intestinal mucositis (15).

TABLE 4 | Affinity kinetics of desacyl-ghrelin-reactive IgG in humans and rodents.

	Desacyl-ghrelin-reactive IgG					
	Ka (1/Ms)		Kd (1/s)		KD (M)	
Mean ± SEM						
AN	1.04E+04 ± 1.02E+03	ns	2.26E−03 ± 3.86E−04	ns	8.61E−07 ± 7.17E−08	ns
OB	2.21E+04 ± 9.80E+03	ns	1.74E−03 ± 4.16E−04	ns	1.47E−07 ± 1.69E−08	ns
Ctrl	1.21E+04 ± 1.28E+03		1.65E−03 ± 1.50E−04		1.51E−07 ± 1.56E−08	
ob/ob	3.73E+03 ± 3.61E+03	↘#	7.23E−03 ± 8.10E−07	ns	9.59E−06 ± 3.76E−06	ns
Ctrl	1.89E+04 ± 9.33E+03		2.68E−05 ± 1.55E−05		4.13E−06 ± 3.38E−06	
HFD	2.72E+03 ± 1.74E+03	ns	1.34E−04 ± 1.30E−04	↘*	3.79E−08 ± 1.47E−08	↘#
Ctrl	1.09E+04 ± 4.71E+03		8.39E−04 ± 3.03E−04		1.38E−07 ± 4.72E−08	
Zucker	2.40E+04 ± 3.55E+03	ns	1.34E−03 ± 3.51E−04	↘*	6.78E−08 ± 2.30E−08	ns
Ctrl	2.85E+04 ± 4.24E+03		2.26E−03 ± 3.05E−04		9.88E−08 ± 2.21E−08	
ABA	1.94E+04 ± 8.06E+03	ns	1.12E−03 ± 2.09E−04	↗$$	9.20E−08 ± 2.34E−08	↗$
FTR	8.32E+03 ± 2.89E+03	ns	3.24E−04 ± 2.03E−04	ns	1.03E−07 ± 5.97E−08	ns
Ctrl	1.74E+04 ± 9.64E+03		2.07E−06 ± 1.20E−06		1.57E−08 ± 1.06E−09	
MTX	2.56E+03 ± 1.55E+03	ns	2.01E−04 ± 1.33E−04	ns	6.97E−08 ± 1.73E−08	↗*
Ctrl	1.71E+02 ± 1.33E+02		1.05E−06 ± 2.16E−07		2.07E−08 ± 5.46E−09	

*Human patients: OB, obese; AN, anorexia nervosa. Rodent models of obesity: ob/ob mice; HFD, high fat diet-induced obese mice and Zucker rats. Rodent models of anorexia and starvation: ABA, activity-based anorexia in mice; FTR, feeding-time restriction in mice; and MTX, methotrexate-induced anorexia in rats. Ctrl, healthy human or rodent controls in corresponding row. ANOVA with Tukey's posttest or Kruskal–Wallis test with Dunn's posttest, $p < 0.05; $$p < 0.01. Student's t-test or Mann–Whitney test, vs. Ctrl *p < 0.05; #p < 0.10.*

A particular role in production of IgG and other classes of Ig can be played by antigens derived from gut microbiota as a constitutive part of the gastro-intestinal tract in all animals (27). The gut bacterial antigens may also be involved in regulation of production and properties of ghrelin-reactive IgG. In fact, germ-free rats showed elevated plasma levels of ghrelin-reactive IgG (19), supporting a role of some intestinal antigens in tolerization toward ghrelin (28). The molecular basis linking microbial antigens with peptides may relate to the concept of molecular mimicry by cross-reactive autoantibody production (29). Indeed, sequence homology between ghrelin and several proteins from commensal gut microorganisms was shown, e.g., of seven consecutive amino acids present in a protein from *Enterococcus faecalis* (19). A role of molecular mimicry was recently validated as a mechanism underlying production of α-MSH-reactive IgG induced by caseinolytic peptidase B (ClpB) homolog protein from *Escherichia coli* (30). ClpB contains a discontinuous six amino acid homology with α-MSH; immunization of mice with ClpB generates α-MSH-cross reactive IgG (30). Plasma presence of the IgA class of ghrelin-reactive IgG also points to their origin triggered by luminal antigens (19).

Beside the stimulation of antigens from commensal gut microbiota, pathogenic and environmental microorganisms may possibly influence production of ghrelin-reactive IgG. In fact, plasma levels of ghrelin-reactive IgG in healthy adolescents correlate strongly with both IgG and IgM directed against influenza A virus (21). Although the initial *in silico* search for five consecutive amino acids sequence homology did not reveal matches between ghrelin and the Influenza viruses (19), a discontinuous eight amino acid homology for ghrelin is present in the PB1-F2 influenza A protein.

Another example of ghrelin-reactive IgG comes from studies of children with idiopathic short stature by the Lewinski group.

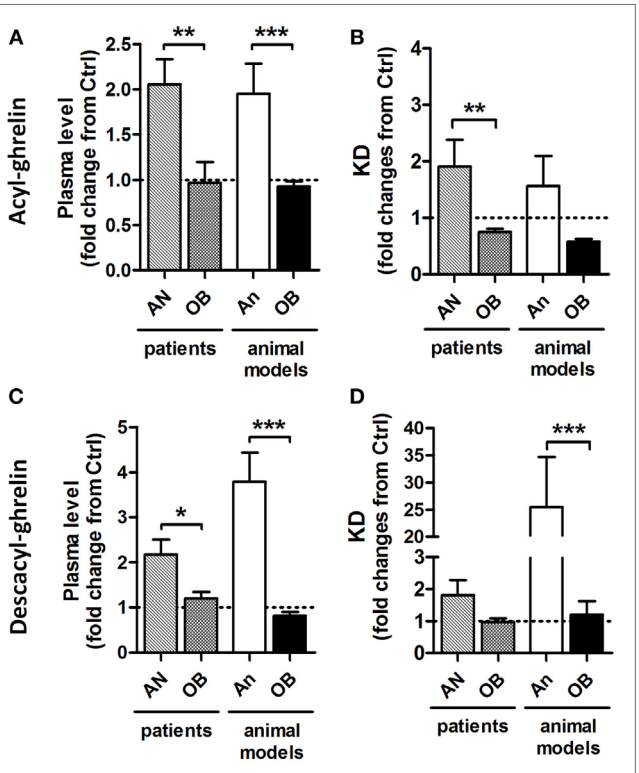


FIGURE 1 | Relative to control (Ctrl) values (1.0) changes in plasma ghrelin and desacyl ghrelin as well as affinity of their reactive IgG in patients and animal models of obesity and anorexia. Changes in plasma levels of acyl-ghrelin **(A)** and desacyl-ghrelin **(C)**. Changes in affinity (dissociation equilibrium constant, KD) of plasmatic IgG for acyl-ghrelin **(B)** and desacyl ghrelin **(D)**. AN, anorexia nervosa patients; An, animal models of anorexia; OB, obese patients or animals. Mann–Whitney tests ***$p < 0.001$, **$p < 0.01$, and *$p < 0.05$.

They showed that prevalence of high level IgG reactive with several peptide hormones, including ghrelin, orexin A, leptin, and α-MSH combined, was associated with an increased incidence of *Helicobacter pylori* and/or *Candida albicans* (31). A follow-up study showed that mean plasma levels of ghrelin-reactive IgG were also elevated in children with short stature, but they were not significantly different in patients with growth hormone deficiency (32). Since *H. pylori* is only residing in the stomach, which is the main source of ghrelin, it is of interest to explore possible role of *H. pylori* in production of ghrelin-reactive IgG. In fact, cure of *H. pylori* in humans was accompanied by increased plasma levels of ghrelin (33).

FUNCTIONAL ROLE OF GHRELIN-REACTIVE IgG

An established physiological role of IgG is to protect against infection including their ability to directly neutralize antigens and to trigger cell lysis *via* activation of the complement. While presence of IgG reactive with some peptide hormones have been detected in plasma of healthy humans (34), their possible functional role remained unknown, as previously reviewed (35). Ghrelin-reactive IgG were the first IgG revealing that they have a functional role in the peptide signaling by protecting ghrelin from degradation in plasma (13). In fact, as was shown by an *in vitro* assay, depletion of plasma from IgG resulted in an almost complete loss of acyl-ghrelin, while their reintroduction allowed its full recovery (13). Thus, the main functional role of ghrelin-reactive IgG appears to protect ghrelin from degradation.

Such protection did not reduce the orexigenic activity of ghrelin, because administrations of ghrelin together with plasmatic IgG, stimulated food intake and even enhanced it when IgG were derived from plasma of obese humans or mice (13). Such enhancing effect was explained by a slight, about three times, increase of IgG affinity for ghrelin and the evidence that such IgG are able to transport more active ghrelin in obese patients. Moreover, the KD values of ghrelin-reactive IgG remained in the micromolar range, which would preclude their competition with the nanomolar affinity of ghrelin receptor binding (36).

We show here that KD values of IgG for ghrelin correlate positively with plasma ghrelin concentrations in obese and AN patients as well as in *ob/ob* mice (**Figures 2A,B**). These correlations demonstrate that a decrease in IgG affinity for ghrelin (increase in KD) corresponds to higher ghrelin levels and suggest that properties of ghrelin-reactive IgG may at least partly underlie individual differences in plasma ghrelin levels. An inverse relation is unlikely, because increased antigen concentration should stimulate the affinity maturation of IgG. We also find that plasma levels of ghrelin-reactive free IgG as well as ratios of free/total IgG levels correlate positively with plasma ghrelin (**Figures 2C,D**). In contrast, ghrelin-reactive total IgG correlate negatively with plasma ghrelin (**Figure 2E**). Taken together, these correlations support a carrier role of plasmatic IgG for ghrelin.

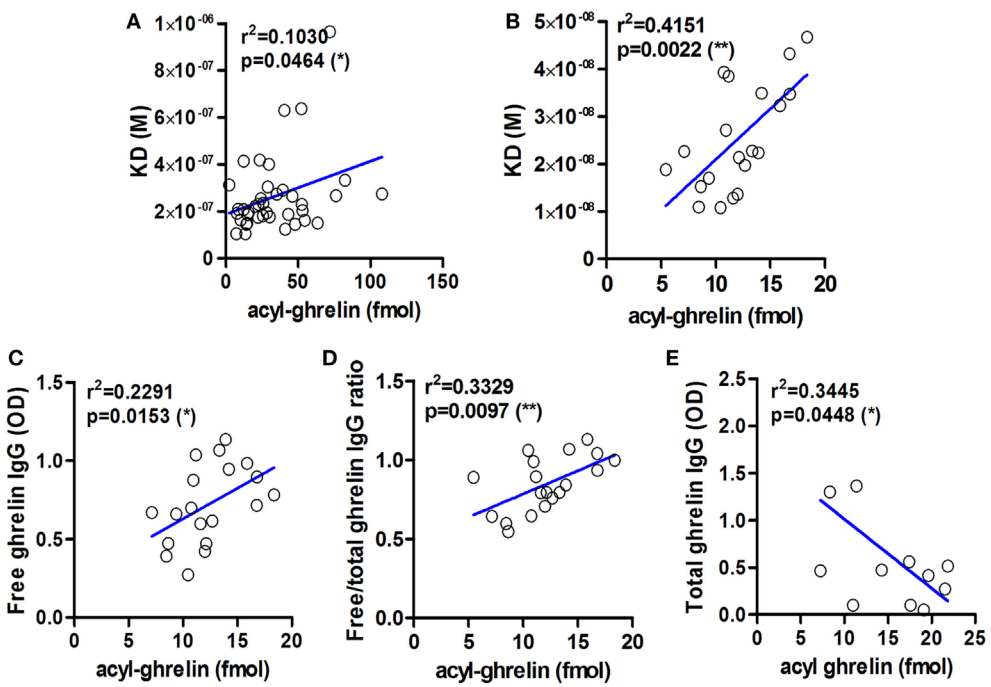


FIGURE 2 | Examples of correlations between affinity and levels of ghrelin-reactive IgG and plasma concentrations of acyl-ghrelin. Correlations between the values of dissociation equilibrium constants (KD) of plasmatic IgG reactive with ghrelin and acyl ghrelin in obese and anorectic patients and controls **(A)** and in *ob/ob* and lean mice **(B)**. Correlations between plasma levels of ghrelin-reactive free IgG **(C)** as well as ratios of their free/total levels **(D)** in ob/ob and lean mice. Correlations between plasma levels of ghrelin-reactive total IgG in Zucker rats **(E)**. *R*-squared and *p*-values for Pearson's correlation tests are shown, $^{**}p < 0.01$ and $^{*}p < 0.05$.

Considering increased functional effects of ghrelin in immune complex with IgG vs. ghrelin alone, the accompanying changes in plasma ghrelin levels during altered energy metabolism can be a "mirror" reflection of the ghrelin's functional activity. Accordingly, increased plasma levels of ghrelin, e.g., in AN patients and in animal models of anorexia (**Figure 1**), may signify ghrelin's inability to form stable immune complexes with IgG and, hence, may result in a functional deficiency of the ghrelin signaling or "ghrelin resistance." Such explanation may appear at first glance as paradoxical, but it gains further support from the corroborating data revealing an enhancing role of IgG in signaling by other peptide hormones such as α-MSH (37, 38).

Thus, the changes in IgG affinity kinetics resulting in a slight increase of affinity can be considered as a gain of function in ghrelin signaling. Taking in account important functional consequences of affinity changes in ghrelin-reactive IgG, it is clear that a simple measurement of their plasma levels is not sufficient and should be combined with the affinity kinetics analysis. Nevertheless, affinity changes should still be taken with caution as a putative biomarker, as long as we do not know if they involve or not any epitope changes that may prevent the availability of the ghrelin N-terminal, necessary for the GHSR1 binding. We do not yet know if presence of IgG in immune complex with ghrelin may play an allosteric role in ghrelin receptor activation, a phenomenon suggested for IgG reactive with α-MSH (37, 38).

To summarize these data, presence of IgG at normal levels and with physiological micromolar affinity for ghrelin in humans appears as a homeostatic factor in regulation of ghrelin signaling. Accordingly, any changes in these factors should lead to alteration of the ghrelin signaling with resulting effects on appetite and body weight regulation. Therefore, in order to facilitate the data interpretation toward an enhanced or diminished ghrelin signaling, the following algorithm can be proposed.

For the enhanced ghrelin signaling one of several changes can be observed:

- Increased plasma levels of ghrelin-reactive both free and total IgG;
- Increased micromolar affinity of IgG for ghrelin;
- Changes in affinity kinetics of IgG for ghrelin toward its increased binding including increased association rate (small Ka) and/or decreased dissociation rate (small Kd).

By the opposite, for the diminished ghrelin signaling, the following changes can be present:

- Decreased plasma levels of ghrelin-reactive both free and total IgG;
- Decreased micromolar affinity of IgG for ghrelin;
- Changes in affinity kinetics of IgG for ghrelin toward its decreased binding including decreased association rate (small Ka) and/or increased dissociation rate (small Kd).

While the functional role of ghrelin-reactive IgG with regard to ghrelin's orexigenic effect can be interpreted as facilitating, the corresponding role of IgG reactive with desacyl-ghrelin is uncertain. As discussed above, IgG may bind to the common central and the C-terminal parts of ghrelin and desacyl-ghrelin and, hence, may transport both types of peptides. In fact, absorption studies of ghrelin- and desacyl-ghrelin-reactive IgG confirmed that they can bind both peptides (20).

Although some studies showed potential anorexigenic effects of desacyl-ghrelin (39, 40), its functional role in the regulation of appetite remains uncertain including direct functional consequences of desacyl-ghrelin transport by IgG toward an unknown putative receptor (41). One alternative possibility is that desacyl-ghrelin may compete with acyl-ghrelin for protective IgG and, thereby, desacyl-ghrelin may have an anorexigenic effect by diminishing the ghrelin signaling *via* reduced formation of ghrelin/IgG immune complexes. Such possibility is of functional importance because starvation preferentially upregulates desacyl-ghrelin, e.g., its ratios to acyl-ghrelin can be increased from 1:20 in *ad libitum* fed mice to more than 1:70 in starved mice (**Table 1**) (17). Functional significance of the affinity kinetics changes in desacyl-ghrelin-reactive IgG is also difficult to interpret owning the same problem of the uncertain role of the desacyl-ghrelin peptide. Whether such changes are different from those obtained for acyl-ghrelin, it suggests that they may concern antibodies primarily targeting the desacylated N-terminal of ghrelin. It is, nevertheless, possible that decreased affinity of IgG for desacyl-ghrelin may be favorable for desacyl-ghrelin competition with ghrelin for ghrelin-protective IgG and, hence, may result in reduced orexigenic activity of ghrelin. Furthermore, we have seen that KD values of desacyl-ghrelin IgG correlate positively with plasma levels of desacyl-ghrelin in animals with anorexia. Significant correlations were also found between plasma levels of desacyl-ghrelin reactive IgG and desacyl-ghrelin in patients with obesity and AN. Such correlations suggest that, similar to acyl-ghrelin, desacyl-ghrelin plasma concentrations can also be regulated by desacyl-ghrelin-reactive IgG.

GHRELIN-REACTIVE-IgG IN OBESITY

Obesity is typically accompanied by increased food intake, suggesting a possible role of ghrelin in the mechanism of hyperphagia (42). Indeed, obese subjects are more sensitive than lean subjects to increase their food intake after ghrelin administration (43). A particular situation may exist in non-hyperphagic HFD-induced obesity in mice that are less sensitive to the ghrelin's orexigenic effect (44). In obese subjects, basal plasma levels of ghrelin measured as total ghrelin (acyl-ghrelin + desacyl-ghrelin) were found low (45, 46) or normal when measured selectively for acyl-ghrelin (13, 47, 48) (**Table 1**). Thus, increased food intake in common obesity cannot be related to increased ghrelin concentrations. There are, nevertheless, some exceptions showing elevated plasma ghrelin in hyperphagic obese patients with the Prader–Willi syndrome (PWS) (49) and hyperphagic obese Zucker rats (50, 51). However, our study showed that obese Zucker rats display elevated desacyl-ghrelin, while acyl-ghrelin remains normal (16) (**Table 1**).

IgG in obese patients showed their ability to better protect ghrelin from degradation by an *in vitro* test and to increase its orexigenic activity after intraperitoneal administrations in rats

(13). Such ghrelin's enhancing properties of IgG in obesity can be explained by its increased affinity, as discussed above. Similarly, increased affinity of IgG for ghrelin was found in genetic animal models of obesity including both *ob/ob* mice (13) and Zucker rats (16) (**Table 3**). Obesity in both rodent's models is due to the deficient leptin signaling and is characterized by hyperphagia. In mice that developed obesity after 2 months of HFD, IgG was also characterized by increased affinity for ghrelin (**Table 3**); however, these mice are not hyperphagic. In contrast to other animal models of obesity and to obese patients, HFD-obese mice also showed an increase in affinity of desacyl-ghrelin IgG (**Table 4**), but the significance of such change is not clear. The affinity kinetic properties of the association and dissociation rates leading to increased affinity were all different between humans and animal models of obesity including increased small *Ka* in obese patients, a decrease of small *Kd* in *ob/ob* mice and Zucker rats, and no significant changes in HFD obese mice (**Table 3**).

Although plasma levels of ghrelin-reactive IgG in obese patients and in all animal models reviewed here were slightly lower than in their non-obese controls (**Table 2**), the free/total ratios of ghrelin-reactive IgG were increased in patients and in Zucker rats (**Table 2**). Interestingly, a recent conference report revealed elevated plasma levels of ghrelin-reactive IgG in children with PWS (52). If confirmed, it will be the only so far known pathological conditions characterized by increased production of ghrelin-reactive IgG.

Taken together, these data suggest that obesity development leads to increased carrying properties of IgG for ghrelin in both obese humans and in animal models of obesity *via* increased affinity (**Figure 1**). Such changes in ghrelin-reactive IgG properties may represent a mechanistic factor underlying enhanced ghrelin signaling leading to hyperphagia and increased adiposity.

GHRELIN-REACTIVE-IgG IN ANOREXIA

Anorexia can be a primary problem in patient with eating disorders such as in restrictive AN, or it can be symptomatic during a chronic disease worsening its outcome. Ghrelin production and its plasma concentration are typically elevated during chronic starvation, accompanied or not by anorexia in both humans and

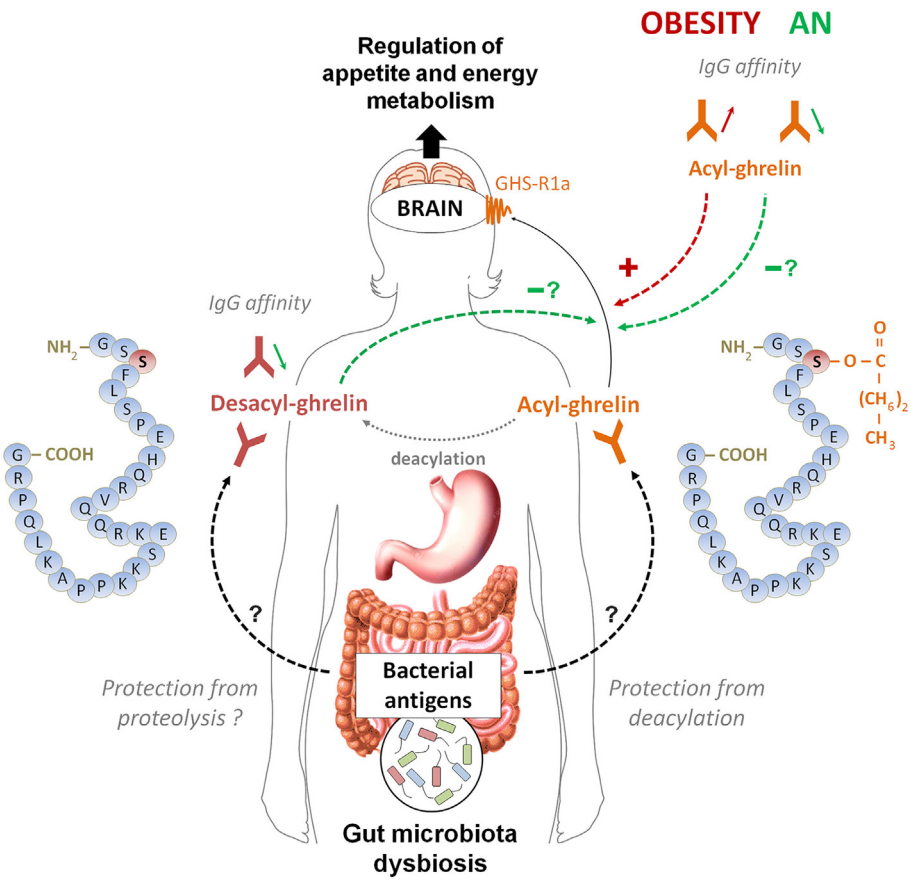


FIGURE 3 | A schematic model summarizing the postulated role of ghrelin-reactive IgG in ghrelin signaling in appetite control during normal and pathological conditions. According this model, acyl-ghrelin is protected by IgG from deacylation, therefore enhancing its orexigenic signaling to the brain. Changes in affinity of IgG for ghrelin in obese patients (increase) and in orexia nervosa patients (decrease) may enhance and diminish, respectively, ghrelin's orexigenic effects. Desacyl-ghrelin may lower orexigenic effect *via* competing with ghrelin for ghrelin-reactive IgG resulting in increased ghrelin degradation. Moreover, decreased affinity of IgG for desacyl ghrelin may favor its dissociation from immune complexes and completion with ghrelin. The origin of changes in affinity of ghrelin- and desacyl ghrelin-reactive IgG is currently unknown but may potentially depend on antigenic stimulation from dysbiotic gut microbiota associated with long-term nutritional modifications in anorexia and obesity.

experimental animals (13, 17, 53–56). Such elevation of plasma ghrelin suggests its homeostatic role in the long-term regulation of energy balance aimed at increased food intake, once food will be available (42). This homeostatic control is obviously not working in anorectic humans and rodents, who display functional "ghrelin resistance." A role of plasmatic IgG in mechanisms of "ghrelin resistance" during anorexia can be suggested based on changes in their properties in an opposite way to obesity. In fact, patients with AN display lower affinity than in controls (**Table 3**), and many AN patients display also low plasma levels of ghrelin-reactive IgG (20). In the ABA and MTX rodent models of anorexia, we did not observe low affinity of IgG for ghrelin but it was present for desacyl-ghrelin (**Tables 3** and **4**). Such decrease of affinity was detected only in ABA mice but not in FTR mice that were starved but did not develop spontaneous anorexia, suggesting a possible contribution of desacyl-ghrelin-reactive IgG in the anorectic phenotype. MTX-treated rats, which develop severe anorexia, were also characterized by strong inhibition of ghrelin-reactive IgG levels (15). As discussed above, lower levels and affinity of IgG for both ghrelin and desacyl-ghrelin may decrease ghrelin's orexigenic effects. Such possibility was not, however, experimentally validated.

Whether IgG may improve ghrelin's orexigenic effects was tested in the ABA model in mice, but the orexigenic effects of treatments were limited to the 3 h of daily feeding time, which was not sufficient to fully evaluate their enhancing roles (17). In any case, only IgG from obese mice were efficient to significantly improve ghrelin's orexigenic effect.

These data suggest that factors which specifically or non-specifically decrease plasma levels and affinity of ghrelin-reactive IgG may diminish ghrelin's orexigenic effects and, hence, will contribute to the mechanisms of anorexia *via* functional "ghrelin resistance."

CONCLUSION

The main conclusions that can be drawn after reviewing these data are the existence of a functional link between ghrelin-reactive IgG and plasma ghrelin levels as well as a physiological role of ghrelin-reactive IgG in modulating ghrelin's biological activities including conditions of altered energy balance. A summary **Figure 3** schematically illustrates the postulated link between ghrelin-reactive IgG and regulation of appetite whereas changes in IgG properties during obesity and anorexia may enhance or diminish ghrelin signaling, while desacyl-ghrelin may compete with ghrelin for IgG which protect ghrelin from deacylation.

Data analysis from patients and several animal models of altered energy balance shows that both obese humans and animals display lower levels of ghrelin-reactive IgG characterized by increased affinity. In contrast, patients with AN and anorectic rodents all show lower affinity of ghrelin- and desacyl-ghrelin-reactive IgG, respectively, the changes which were not observed in non-anorectic chronically starved mice. Such changes in properties of both ghrelin- and desacyl-ghrelin-reactive IgG in patients with obesity and anorexia suggest that they are not a direct consequence of the positive or negative energy balance *per se* but may be a result of some typical changes associated with altered regulation of energy metabolism impacting on the autoantibody production. Although the mechanisms of such common changes are currently unknown, they may potentially involve modifications of gut microbiota leading to altered production of ghrelin-like antigenic molecules. Indeed, typical, but not identical response of gut microbiota to starvation or an obesogenic diet may potentially explain similar autoantibody response including production of ghrelin-reactive IgG, which may contribute to the long-term regulation of host energy metabolisms by gut microbiota (57). Further studies are needed to identify potential ghrelin-mimetic antigens in gut microbiota and to analyze the effects of altered energy balance on their production.

AUTHOR CONTRIBUTIONS

SF wrote the manuscript, NL prepared the figures and tables, NL and RL analyzed the data and contributed to manuscript preparation.

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Conflict of Interest Statement: SF is a co-founder of TargEDys SA and NL and RL are currently its employees.

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Combination of Selective Immunoassays and Mass Spectrometry to Characterize Preproghrelin-Derived Peptides in Mouse Tissues

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Preproghrelin is a prohormone producing several preproghrelin-derived peptides with structural and functional heterogeneity: acyl ghrelin (AG), desacyl ghrelin (DAG), and obestatin. The absence of selective and reliable assays to measure these peptides simultaneously in biological samples has been a limitation to assess their real proportions in tissues and plasma in physiological and pathological conditions. We aimed at reliably measure the ratio between the different preproghrelin-derived peptides in murine tissues using selective immunoassays combined with a highly sensitive mass spectrometry method. AG-, DAG-, and obestatin-immunopositive fractions from the gastrointestinal tract of adult wild-type and ghrelin-deficient mice were processed for analysis by mass spectrometry (MS) with a Triple Quadrupole mass spectrometer. We found that DAG was predominant in mouse plasma, however it only represented 50% of total ghrelin (AG+DAG) production in the stomach and duodenum. Obestatin plasma levels accounted for about 30% of all circulating preproghrelin-derived peptides, however, it represented <1% of total preproghrelin-derived peptides production (AG+DAG+Obestatin) in the stomach. Assays were validated in ghrelin-deficient mice since neither ghrelin nor obestatin immunoreactivities were detected in their stomach, duodenum nor plasma. MS analyses confirmed that obestatin-immunoreactivity in stomach corresponded to the C-terminal amidated form of the peptide but not to des(1–10)-obestatin, nor to obestatin-Gly. In conclusion, specificity of ghrelin and obestatin immunoreactivities in gastrointestinal tissues using selective immunoassays was validated by MS. Obestatin was less abundant than AG or DAG in these tissues. Whether this is due to inefficient processing rate of preproghrelin into mature obestatin in gastrointestinal mouse tissues remains elusive.

Keywords: acyl ghrelin, desacyl ghrelin, obestatin, immunoreactivity, mass spectrometry

INTRODUCTION

Preproghrelin is a complex prohormone that, upon post-translational processing, leads to the production of several derived peptides with structural and functional heterogeneity. Ghrelin is a 28 amino acid peptide originating from the stomach (Kojima et al., 1999; Hosoda et al., 2000; Tomasetto et al., 2000) and identified as the endogenous ligand of the Growth Hormone Secretagogue Receptor (GHS-R; Howard et al., 1996). The addition of an acyl-group by the Ghrelin-O-Acyl-Transferase (GOAT; Yang et al., 2008), enables ghrelin (Acyl-ghrelin, AG) to stimulate GH secretion and appetite (Tolle et al., 2001, 2002). Another endogenous form of ghrelin is desacyl ghrelin (DAG) reported as the most abundant form in plasma (Hosoda et al., 2000). Its specific roles are to regulate glucose, lipid, and bone metabolism (Delhanty et al., 2013). Obestatin is a 23 amino acid amidated peptide derived from the same precursor as ghrelin. Originally isolated as the endogenous ligand for the GPR39 and described as an anorexigenic factor in rodents (McKee et al., 1997; Zhang et al., 2005), its physiological relevance has since been questioned (Zhang et al., 2005; Bresciani et al., 2006; Lauwers et al., 2006; Seoane et al., 2006; Yamamoto et al., 2007). Both DAG and obestatin interact pharmacologically with AG to modulate food intake, GH secretion or glucose metabolism through yet unidentified receptors (Hassouna et al., 2012; Delhanty et al., 2013; Gargantini et al., 2013). Previous studies found equimolar ratios of plasma AG and obestatin levels in the rat (Zhang et al., 2005; Zizzari et al., 2007), consistent with both peptides being processed from the same prohormone. However, evidence that tissue specific splicing variant encoding obestatin but not ghrelin exist in humans suggests that obestatin could also be produced independently of ghrelin (Seim et al., 2009).

With regard to the literature, many issues remain to be addressed concerning obestatin: its main source of production in the body and its abundance relative to ghrelin as well as its molecular form and way of processing. Obestatin was initially extracted from rat stomach and found in rat plasma (Zhang et al., 2005). In addition, obestatin immunoreactivity was detected in a number of human tissues using immunohistochemistry (Grönberg et al., 2008) and in cultured pancreatic islets *in vitro* (Granata et al., 2008). However, other studies failed to detect significant amounts of obestatin in rat plasma or stomach using radioimmunoassay (RIA) coupled to High Performance Liquid Chromatography (HPLC; Bang et al., 2007; Mondal et al., 2008).

The absence of selective and reliable assays to measure all three preproghrelin-derived peptides (AG, DAG, and obestatin) simultaneously in biological samples is an obstacle to further characterization of their specific physiological and pathophysiological functions. In this study, we developed selective immunoassays combined to a highly sensitive targeted mass spectrometry method in order to reliably measure and characterize the ratios of the different preproghrelin-derived peptides in mice. Preproghrelin gene deficient mice that do not produce AG or DAG (Hassouna et al., 2014) were used as negative controls to further validate the immunoreactivity and mass spectrometry assays.

MATERIALS AND METHODS

Animals

Dissections were performed on 7–12 weeks old preproghrelin deficient (*ghrl*−/−) mice and wild type (*ghrl*+/+) littermates backcrossed on the C57BL/6J genetic background as previously reported (Hassouna et al., 2014). Mice were housed in a room under controlled illumination (0700–1900 h) and temperature (22–24°C) and had free access to food and water. Offsprings were genotyped by PCR amplification of tail DNA. All experiments were carried out in accordance with the European Communities Council Directive (86/609/EEC) and were approved by the Animal Experimentation Committee of Paris Descartes University (agreement number 03422.02).

Peptides

Peptides used as standards in liquid chromatography (LC) were provided by NeoMPS (Strasbourg, France): rat/mouse acyl ghrelin (AG) and desacyl ghrelin (DAG), rat/mouse amidated obestatin (obestatin-NH_2), Des(1–10)-obestatin and obestatin-Gly (Sequences presented in Table S1 and Figure S1).

Dissection, Extraction, and Purification of Tissue Samples

Gastric epithelia, 1 cm proximal duodenum, small intestine and colon were collected and the tissues were extracted in 2N acetic acid during 10 min at 90°C, sonicated and frozen at −80°C for 24 h. The homogenate was centrifuged 20 min at 12,000 g at 4°C. Supernatants were lyophilized and further dissolved in phosphate assay buffer. Extracts were first filtered on a 10 kDa filter (Amicon Ultra, Millipore, Molsheim, France) then purified using SepPak C18 columns (Waters, Saint-Quentin-en-Yvelines, France). Briefly, the supernatants were loaded onto a SepPak C18 cartridge pre-equilibrated in 0.1% trifluoroacetic acid (TFA). The samples were desalted with aqueous TFA 0.1% and eluted with an acetonitrile gradient (10–100%).

Plasma Collection and Processing

Blood samples were collected from trunk blood on EDTA (1 mg/ml) and PHMB (0.4 mM final), a serine protease inhibitor and centrifuged at 1,000 g during 10 min at 4°C. Plasma were immediately acidified with HCl (0.1 M final) and stored at −80°C.

Hormone Assays

Immunoreactivities were measured in plasma, whole tissue and SepPak fractions (10–70 and 100%) with selective sandwich immunoassays for AG, DAG (SPIbio Bertin pharma, A05118 for the acyl form and A05117 for the desacyl form, Montigny-le-Bretonneux, France) and competitive assay for obestatin (Phoenix Pharmaceuticals, Burlingame, USA). Immunoreactive fractions were further analyzed by Mass Spectrometry (MS).

Mass Spectrometry

Mass Spectrometry (MS) analyses were performed in the Selected Reaction Monitoring (SRM) mode using a high pressure nanoLC (U3000 RSLC, Thermo Fisher Scientific) coupled to a triple quadrupole (QqQ) mass spectrometer (TSQ Vantage™, Thermo

Fisher Scientific, San Jose, CA, USA) in nano-ESI mode. Briefly, peptides were loaded and desalted on a C18 cartridge (C18 PepMap, 3 mm, 100 A°, 75 mm i.d., 2 cm length) using a loading buffer containing 0.05% aq TFA/acetonitrile 98:2 (v/v) at 10 μL/min. Peptides were then separated on a C18 analytical column (C18 PepMap, 2 mm, 100 A°, 75 mm i.d., 15 cm length) with a 60 min gradient from 99% A [0.1% aq formic acid/acetonitrile 98:2 (v/v)] to 50% B [0.1% aq formic acid/acetonitrile 10:90 (v/v)] at 300 nL/min. Standard was injected before each series of experiments. Blank runs were interposed until necessary to avoid peptide carry-over effects. QqQ parameters were set as follows: first and third quadrupole widths set at 0.7, scan time 200 ms/transition and total dwell time 3 s (method performed in unscheduled mode). The transitions for AG (retention time 29.1 min) were 553.1 (precursor MH_6^{6+}) → 513.3 (y_4); 641.4 (y_5); 712.4;(y_6); 809.5 (y_7) 906.5(y_8). The transitions for DAG (retention time 20.0 min) were 532.1 (precursor MH_6^{6+}) → 513.3 (y_4); 641.4 (y_5); 712.4; (y_6); 809.5 (y_7); 906.5(y_8). The transitions for obestatin-NH_2 (retention time 38.3 min) were 630.8 (precursor MH_5^{5+}) → 262.1(b_2); 416.2 (y_4); 553.3 (y_5); 681.4;(y_6); 972.5 (y_8). The transitions for obestatin-Gly (retention time 33.4 min) were 858.8 (precursor MH_3^{3+}) → 262.1 (b_2); 333.2 (b_3); 473.2 (y_5); 610.3 (y_6) 1029.5(y_9). The transitions for des(1–10)-obestatin (retention time 17.9 min) were 476.9 (precursor MH_3^{3+}) → 201.1 (b_2); 553.2 (y_5); 681.3;(y_6); 809.5 (y_7); 972.5(y_8) (Supplementary Table S1).

RESULTS

Preproghrelin-Derived Peptides Immunoreactivities in the Gastrointestinal Tract and Plasma

In adult mice, stomach is known to be the major source of ghrelin, however the main source of obestatin remains elusive. Accordingly, since ghrelin gene is widely expressed throughout the gastro-intestinal (GI) tract, we thus measured all preproghrelin-derived peptides in the different portions of the GI tract (stomach, duodenum, intestine, and colon) in mice using selective antibodies (Figure S1). Amongst all preproghrelin-derived peptides, AG and DAG were the most abundant forms found in stomach. In this tissue, AG represented 50%, DAG 50%, and obestatin <1% of all preproghrelin-derived peptides. Although their proportions varied in the different sections of the GI tract, ghrelin (AG and DAG) remained predominant compared to obestatin. In duodenum, AG represented 34%, DAG 38%, and obestatin 29% of all preproghrelin-derived peptides. Differences in proportions of obestatin relative to ghrelin may partly be due to the utilization of different techniques to detect specific immunoreactivities and revelation procedures (sandwich enzymoimmunoassays for ghrelin vs. competitive radioimmunoassay for obestatin). Whereas, AG and DAG levels were gradually decreasing from caudal to distal region of the GI tract, obestatin concentrations were slightly higher in the duodenum than in the stomach (**Table 1**). Both the intestine and colon produced small amounts of all three peptides (data not shown).

Previous studies using competition assays indicated that DAG accounts for 80–90% of total circulating ghrelin (Hosoda et al., 2000). In the present study, DAG accounted for nearly 80% of total circulating ghrelin (i.e., AG+DAG) and 60% of total circulating preproghrelin-derived peptides (i.e., AG+DAG+Obestatin; **Table 1**). Obestatin plasma levels represented about 30% of all circulating preproghrelin-derived peptides, which is much higher than its proportion in gastrointestinal tissues (**Table 1**).

Mass Spectrometry (MS) Analysis of Preproghrelin-Derived Peptides in *ghrl*+/+ and *ghrl*−/− mice

Selected Reaction Monitoring (SRM) method was set up using synthetic peptides AG, DAG, Obestatin-NH_2, Des(1–10)-Obestatin and Obestatin-Gly. The SRM method selected the best five transitions obtained by testing all the theoretic "y" and "b" ion fragments from MH_3^{3+} to MH_6^{6+} precursors (**Figure 1**).

TABLE 1 | Tissue content or concentrations, percentage and molar ratio of preproghrelin-derived peptides immunoreactivities in the stomach, duodenum, and plasma of *ghrl*+/+ mice (*n* = 6–10).

	Acyl ghrelin (AG)	Desacyl ghrelin (DAG)	Obestatin	AG/TG	DAG/TG	Obestatin/TG
STOMACH						
Tissue content (pmol/tissue)	184 ± 9	197 ± 19	0.65 ± 0.02		Molar ratio	
% of total preproghrelin-derived peptides	49 ± 3%	51 ± 3%	0.17 ± 0.01%	1/2	1/2	1/616
DUODENUM						
Tissue content (pmol/tissue)	1.32 ± 0.39	1.39 ± 0.42	1.00 ± 0.52		Molar ratio	
% of total preproghrelin-derived peptides	34 ± 6%	38 ± 10%	29 ± 12%	1/2	1/2	1/2.5
PLASMA						
Concentration (pmol/L)	103 ± 29	370 ± 44	210 ± 79		Molar ratio	
% of total preproghrelin-derived peptides	16 ± 4%	57 ± 5%	27 ± 8%	1/5	1/1.2	1/1.7

AG, Acyl ghrelin; DAG, Desacyl ghrelin; TG, Total Ghrelin (AG+DAG). Limits of detection are 0.03 pmol for AG and DAG and 0.08 pmol for obestatin. Data are expressed as Mean ± SEM, molar ratio or as percentages of total preproghrelin-derived peptides (AG+DAG+obestatin).

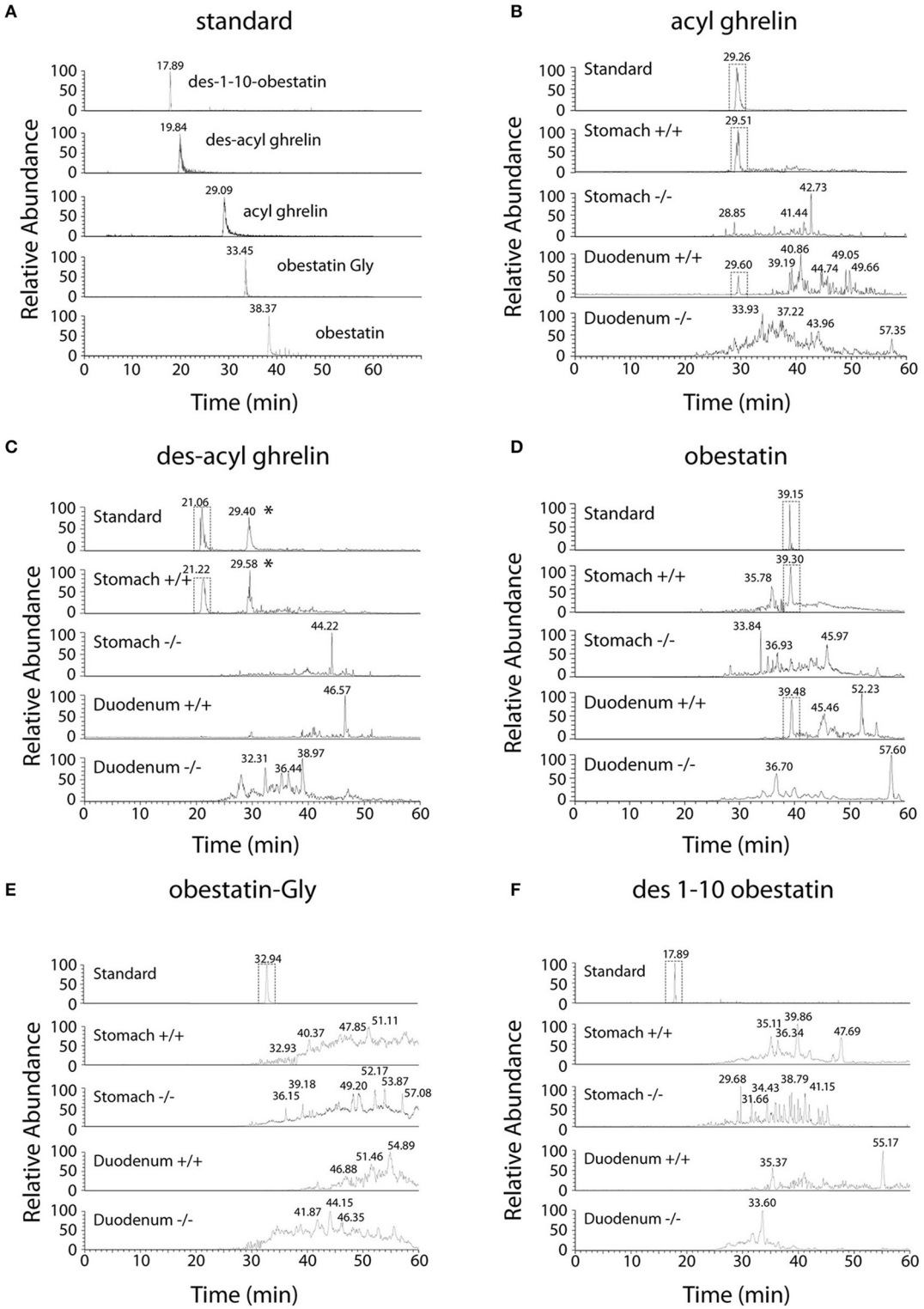


FIGURE 1 | Chromatographic profiles of SRM analyses related to preproghrelin-derived peptides in the stomach and duodenum from 60% chromatographic fractions in *ghrl*+/+ and *ghrl*−/− mice. SRM analyses of the synthetic peptide (standard) and tissue samples obtained from the fraction eluted with 60% acetonitrile: **(A)** standard, **(B)** acyl ghrelin, **(C)** des-acyl ghrelin, **(D)** obestatin, **(E)** obestatin-Gly, **(F)** des(1–10)-obestatin. Acyl ghrelin and obestatin-NH_2 were present in both the stomach and duodenum of *ghrl*+/+ mice but absent in the tissues of *ghrl*−/− mice. Des-acyl ghrelin was present in the stomach but not in the duodenum of *ghrl*+/+ mice and absent in the tissues of *ghrl*−/− mice. The star (*) denotes a peak in ghrelin chromatogram that is related to the "in source" neutral loss of the acyl group of acyl ghrelin converted partially acyl ghrelin to desacyl ghrelin after LC separation. Obestatin-Gly and des(1–10)-obestatin were absent in both the stomach and duodenum of *ghrl*+/+ and *ghrl*−/− mice. See experimental section for SRM method design.

The detection limits for AG and DAG was 10–50 fmol and for obestatin-NH_2 and its derivatives 1–5 fmol.

As preproghrelin-derived peptides are highly concentrated in stomach and duodenum, purified SepPak fractions from these tissue protein extracts from both *ghrl*+/+ and *ghrl*−/− mice were used for mass spectrometry. Preproghrelin-derived peptides immunoreactivities were retrieved in stomach and duodenum of *ghrl*+/+ mice but not in the same tissues in *ghrl*−/− mice (**Table 2** and data not shown). The residual immunoreactivity for obestatin found in two out of six *ghrl*−/− mice was unspecific, since it did not correspond to the correct specific masses in MS experiments (See below). To decrease the dynamic range and the complexity of the whole protein extract, samples were first depleted to conserve only peptides below 10 kDa. The resulting mixtures were further submitted to solid phase extraction on C18 stationary phase to remove hydrophilic species. According to standard synthetic peptides properties, the endogenous peptides of interest were expected to be eluted using 10–70% acetonitrile. The fractions were submitted to stepwise elution with successive 10% increments of acetonitrile. Immunoreactivity of each preproghrelin-derived peptides (AG, DAG, and Obestatin) was measured in each eluted fraction. In *ghrl*+/+ mice, 30, 40, and 60% acetonitrile-containing fractions presented the most intense immunoreactivity and were further analyzed by SRM LC-MS. The matched fractions obtained from *ghrl*−/− mice contained no immunoreactivity for all three preproghrelin-derived peptides and were also analyzed in SRM LC-MS.

Ghrelin and obestatin were detected by mass spectrometry in both *ghrl*+/+ mice stomach and duodenum, only in 60% acetonitrile fractions (**Table 3**). Thus LC-MS profiles of SRM analyses related to preproghrelin-derived peptides in stomach and duodenum were restricted to 60% acetonitrile fractions from *ghrl*+/+ and *ghrl*−/− mice (**Figure 1**). The antibody used in the obestatin immunoassay may detect other forms of obestatin, such as a truncated peptide, des(1–10)-obestatin, and a modified form of obestatin with additional glycine at the C-terminal position, obestatin-Gly. Thus, the presence of des(1–10)-obestatin and obestatin-Gly was also assayed by SRM in order to evaluate a possible antibody cross-reactivity. To develop a specific and sensitive detection method for the peptides of interest, a mix of AG, DAG, obestatin, Des(1–10)-obestatin and obestatin-Gly standards (100 fmol of each) was used to calibrate the SRM method before each analysis. Blanks were run to assure the absence of contaminations from standards before the analysis of samples. Comparison between the chromatographic profiles of SRM analyses related to the fractions eluted at 60% ACN of the stomach and duodenum tissues were compared between *ghrl*+/+ and *ghrl*−/− mice (**Figures 1B–F**). As further described below, only native obestatin-NH2 was present in the extracts analyzed (**Table 3**).

The three preproghrelin-derived peptides were present in stomach LC fractions of *ghrl*+/+ mice. In duodenum LC fractions of *ghrl*+/+ mice, AG, and obestatin were present while DAG was absent. As shown on **Table 3**, and **Figure 1**, SRM analyses further confirmed that obestatin-immunoreactivity in stomach and duodenum corresponded to the amidated (**Figure 1D**) form of the peptide but not to obestatin-Gly (**Figure 1E**) nor Des(1–10)-obestatin (**Figure 1F**). No MS signal for any preproghrelin-derived peptides was detected in stomach or duodenum of *ghrl*−/− mice. This further validated the specificity of the spectrometric signal detected (**Figures 1B–F**). In stomach and duodenum extracts from *ghrl*+/+ and *ghrl*−/− mice, extracted ion chromatograms related to DAG transitions (**Figure 1C**) presented two peaks instead of one, respectively associated to DAG retention time (21 min) and AG retention time (29 min). The "in source" neutral loss of the acyl group of AG converted partially AG to DAG after LC separation, so that DAG SRM signature can also be detected at AG retention time. On the opposite, when DAG and AG standards are analyzed separately (**Figure 1A**) no signals are detected at DAG retention time with AG transitions.

TABLE 2 | Preproghrelin-derived peptides immunoreactivities in the stomach and duodenum of *ghrl*+/+ and *ghrl*−/− mice.

	***Ghrl*+/+ (*n* = 6)**		***Ghrl*−/− (*n* = 6)**	
	Stomach (pg)	**Duodenum (pg)**	**Stomach (pg)**	**Duodenum (pg)**
Acyl ghrelin	606,100 ± 30,826	4,350 ± 1,301	UN	UN
Desacyl ghrelin	649,767 ± 64,027	4,587 ± 1,385	UN	UN
Total ghrelin	1,255,867 ± 67,509	8,937 ± 2,312	UN	UN
Obestatin	1,632 ± 57	2,600 ± 1,293	UN	710 ± 328*

*Acyl ghrelin, desacyl ghrelin, and obestatin immunoreactivities measured using selective assays are detected in the stomach and duodenum of ghrl+/+ mice (n = 6) but are undetectable in ghrl−/− mice (n = 6). Limits of detection are 10 pg for AG and DAG and 200 pg for obestatin. Data are expressed as Mean ± SEM. UN: Under detection limit. *Immunoreactivity is unspecific.*

DISCUSSION

Using a combination of selective immunoassays and highly sensitive mass spectrometry, we validate the presence of a specific immunoreactivity signal for amidated obestatin in protein extracts from stomach and duodenum and further characterize the ratio of the different preproghrelin-derived peptides in mouse gastrointestinal tract. Specific presence of each preproghrelin-derived peptide was validated by the lack of signal in preproghrelin deficient mice using both immunodetection and MS.

Until now, very few studies characterized all preproghrelin-derived peptides in murine tissues. This can be explained by the lack of sensitive and specific methods to simultaneously detect the three preproghrelin-derived peptides in a given biological sample. Moreover, their relative proportions in tissues and plasma remained unclear. Previous studies using competitive immunoassays reported equimolar ratios of acyl ghrelin and obestatin in rat plasma (Zhang et al., 2005; Zizzari et al., 2007) consistent with a model previewing both peptides obtained by the processing of the same prohormone, while ratios of 2:1–4:1 were reported in humans (Germain et al., 2009, 2010). The reason for such discrepancy is unclear but this could evoke alternative

TABLE 3 | SRM detection of preproghrelin-derived peptides in the different chromatographic fractions (30, 40, and 60% Acetonitrile) in the stomach and duodenum of *ghrl*+/+ mice.

Tissue	LC Fraction (%age acetonitrile)	Acyl ghrelin	Desacyl ghrelin	Obestatin-NH2	Obestatin-Gly	des(1–10) -obestatin
Stomach	30	−	+	−	−	−
	40	+	−	−	−	−
	60	+	+	+	−	−
Duodenum	30	−	−	−	−	−
	40	−	−	−	−	−
	60	+	−	+	−	−

Acyl ghrelin and obestatin-NH_2 were detected in the stomach and duodenum whereas, desacyl ghrelin was only detected in the stomach LC fractions of ghrl+/+ mice. MS-MS analyses further confirmed that obestatin-immunoreactivity in tissues is specific to the amidated peptide but not to obestatin-Gly nor des(1–10)-obestatin.

splicing and/or different processing mechanism in rodents and humans. Although one study demonstrated that obestatin is produced in gastrointestinal tract in humans (Grönberg et al., 2008), two other studies failed to identify significant amounts of obestatin in rat plasma or stomach by RIA coupled to HPLC (Bang et al., 2007; Mondal et al., 2008) in contrast with the original data from Zhang and collaborators in the rat (Zhang et al., 2005). Moreover, in the study by Mondal et al., the ratio of obestatin/ghrelin in gastric fundus of rats was 0.004%, which is far less than what is expected in plasma.

These inconsistent results raise many interrogations regarding the exact obestatin site of production, the abundance of the peptide, and the specificity of the signal measured, as well as specific differences between humans and rodents. To gain more knowledge on obestatin, we explored its presence in different murine tissues, including the gastrointestinal tract using immunological detection in association with MS analyses in order to identify the positive immune signals. Our data confirm that both forms of ghrelin as well as obestatin are produced in majority in the gastrointestinal tract in mice.

We show that in stomach, DAG represents 50% of total ghrelin production while in plasma, it accounts for about 60% of total preproghrelin-derived peptides and nearly 80% of total ghrelin. The latter result is in accordance with previous studies using competitive immunoassays which demonstrated that DAG accounts for 80–90% of total circulating ghrelin (Hosoda et al., 2000). Moreover, we find that while plasma obestatin levels represent about 30% of all circulating preproghrelin-derived peptides, obestatin is 500–1,000 times less abundant than total ghrelin in stomach.

Although we find equimolar concentrations of total ghrelin and obestatin in mouse plasma, the amount of obestatin in tissues is negligible compared to those of ghrelin. Several hypotheses may explain this observation. First of all, conditions of sampling, processing and storage may be inadequate to preserve immunoreactive obestatin. Furthermore, a low processing rate of obestatin from preproghrelin in the stomach cannot be excluded. Finally, the existence of different transcripts arising from the preproghrelin gene in a tissue-specific manner (Seim et al., 2009), including a human transcript that encodes obestatin but not ghrelin, also suggests that obestatin transcripts may be produced independently of ghrelin.

The specificity of the immunoreactive detection for all three preproghrelin-derived peptides in both stomach and duodenum was assessed by MS and further validated by the absence of signal in preproghrelin deficient mice (Hassouna et al., 2014). Residual immunoreactivity for obestatin was detected in duodenum of two out of six preproghrelin deficient mice. This is the result of an artifact as no MS signal confirmed that it was actually obestatin. Furthermore, we also confirmed by MS that the immunoreactivity detected in tissues was specific of the amidated form of obestatin. No MS signal for all preproghrelin-derived peptides was present in stomach or duodenum of *ghrl*−/− mice, confirming specificity of the assay.

In this study, we used a very sensitive mass spectrometer system. Indeed detection limits is estimated to be 10–50 fmol for AG and DAG and 1–5 fmol for Obestatin-NH_2 and its derivatives, allowing to detect very small amounts of the peptides. The specificity was verified by determining the transitions for each peptide and analyzing ions fragments. In the original study by Zhang et al. (2005), obestatin was extracted from rat stomach but its relative abundance as compared to ghrelin was not discussed. As far as we know, this is the first attempt to identify and quantify these peptides in mouse tissues and to validate the existence of a mature form of obestatin (Obestatin-NH2) in mouse gastrointestinal tract.

In conclusion, both forms of ghrelin and obestatin can be detected with very selective immunoassays coupled with MS in gastrointestinal tract in mice. In this tissue, obestatin appears to be far less abundant than AG or DAG. This could be the result of either a lower processing rate of proghrelin into mature obestatin in gastrointestinal tissues or degradation of the peptide during the different extraction/purification procedures. Whether the main source of obestatin production and/or processing is outside the gastrointestinal tract has to be further investigated.

AUTHOR CONTRIBUTIONS

RH: contributed to the conception and design of the work, performed experiments and analyses of data, participated to manuscript redaction. DG: contributed to reagents/materials/analysis tools, performed experiments, and analyses of data, participated to manuscript redaction. GC: contributed to the acquisition and analyses of MS data and participated to manuscript redaction. JL: contributed to the experiments and acquisition of data. OF: contributed to the experiments. CT: contributed to reagents/materials/analysis

tools and revised the manuscript. JV: contributed to reagents/materials/analysis tools and revised the manuscript. JE: contributed to the conception of the work and revised the manuscript. VT: contributed to the conception and design of the work, analyses, and interpretation of data, manuscript redaction.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fnins.2017.00211/full#supplementary-material

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REVIEW
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Development and Function of the Blood-Brain Barrier in the Context of Metabolic Control

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Under physiological conditions, the brain consumes over 20% of the whole body energy supply. The blood-brain barrier (BBB) allows dynamic interactions between blood capillaries and the neuronal network in order to provide an adequate control of molecules that are transported in and out of the brain. Alterations in the BBB structure and function affecting brain accessibility to nutrients and exit of toxins are found in a number of diseases, which in turn may disturb brain function and nutrient signaling. In this review we explore the major advances obtained in the understanding of the BBB development and how its structure impacts on function. Furthermore, we focus on the particularities of the barrier permeability in the hypothalamus, its role in metabolic control and the potential impact of hypothalamic BBB abnormities in metabolic related diseases.

Keywords: blood-brain barrier, neurovascular unit, development, hypothalamus, inflammation, obesity

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INTRODUCTION

Blood vessels transport and deliver nutrients to warrant organism function. In the brain, they specialized into a dynamic structure that provides a protective and homeostatic interface between the central nervous system (CNS) and the remainder of the body. In this sense, the blood-brain barrier (BBB) controls the passage of selected substances into the brain, while transporting toxic products back into circulation. This efficient permeable boundary allows maintenance of the homeostasis of the CNS milieu and correct function of brain circuits.

The functionality of the BBB depends on a strict architecture. The combination of non-fenestrated brain endothelial cells (BECs) lining the walls of the CNS blood vessels, together with pericytes, neurons and glia constitute the neurovascular unit (NVU) and confer integrity to the BBB.

In the ventromedial hypothalamus, the barrier has specialized to allow the dynamic passage of hormones and nutrients from the blood to the energy-sensing arcuate nucleus of the hypothalamus (ARC) and the export of newly synthesized hormones to the pituitary. At the level of the median eminence (ME), the barrier is formed by fenestrated capillaries that allow faster transport of substances into the nutrient-sensing hypothalamic nuclei lying adjacent to it. However, ME tanycytes, specialized radial glia cells lining the walls of the third ventricle, form a physical barrier to control the correct transport of nutrients and metabolic hormones into the brain parenchyma (Weindl and Joynt, 1973; Ganong, 2000; Mullier et al., 2010; Rodríguez et al., 2010). The metabolic state, as well as the consumption of saturated fatty acids, can damage this barrier and alter the nutrient sensing of tanycytes (Lee et al., 2012; Haan et al., 2013; Langlet et al., 2013b). Changes in the development of the BBB in the hypothalamus, especially in the vicinity of the ME, may predispose to obesity (Lee et al., 2012; Kim et al., 2016).

BBB STRUCTURE AND FUNCTION

In the last decade, there has been exponential progress in the understanding of how BBB structure and function work both in health and pathological conditions (Daneman and Prat, 2015; Banks, 2016). The first evidence for the existence of a barrier controlling the passage of substances from the circulating blood to and from the CNS dates back to 1885 when the German researcher Paul Ehrlich carried out experiments injecting Trypan Blue dye into the bloodstream of mice. Surprisingly, he noted that the dye could penetrate several tissues but not the brain and spinal cord (Ehrlich, 1885; **Figure 3B**). Years later, further studies performed by Goldmann (1909, 1913), Ehrlich's student, indicated that injection of the same dye directly into the brain showed the opposite as observed previously: the brains turned blue, whereas the peripheral tissues did not (**Figure 3B**). The term "blood-brain-barrier" was introduced by Lewandowsky (1900), based on experiments demonstrating that neurotoxic substances, e.g., cholic acids or sodium ferrocyanide, exhibited neurological symptoms only after intraventricular applications but not when injected into the bloodstream. Only in the late 1960s, with the development of electron microscopy, Reese and Karnovsky (1967) were able to visualize for the first time, at ultrastructural level, that the brain endothelial cells present unique cell-to-cell junctions, constituting a structural barrier that creates an almost impermeable frontier between the blood and CNS (Ribatti et al., 2006).

Anatomically, the BBB is comprised by a thin monolayer of BECs that are in intimate contact with vascular cells (pericytes and vascular smooth muscle cells), glial cells (astrocytes, microglia) and neurons. The crosstalk and molecular signaling between them are collectively known as the neurovascular unit (NUV) (Obermeier et al., 2013; Chow and Gu, 2015; Banks, 2016) (**Figure 1**). This close connection between different cells types within the NUV allows the BBB to properly perform its fundamental physiological functions.

Next, we present the main features of the cellular components of the BBB:

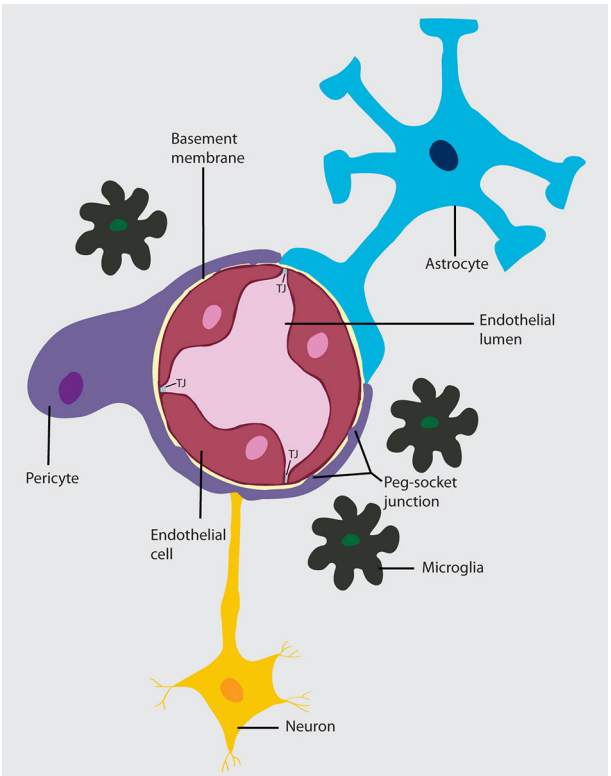


FIGURE 1 | Neurovascular unit (NVU). A layer of brain endothelial cells connected by tight junctions forms the blood-brain barrier. The intimate contact of these specialized endothelial cells with different cell types constitutes the NVU. A basement membrane embeds the brain endothelial cells, the pericytes, and astrocytes. In areas where the basement membrane is absent, brain endothelial cells and pericytes connect through peg-socket junctions. Astrocytes extend its end-feet and establish a close interaction with endothelial cells through transmembrane proteins, such as aquaporins. Astrocytes also connect with pericytes and neurons and together regulate BBB maintenance and function. The interaction of the cell components of the NVU with neurons and microglia can influence barrier function.

Brain Endothelial Cells

To sustain a more restrictive permeability, BECs enclose specific barrier properties, which differentiate them from peripheral endothelial cells (Andreone et al., 2015; Chow and Gu, 2015). One particular feature is the presence of junction complexes between adjacent BECs involving transmembrane proteins and multiple cytoplasmic adaptor proteins located on the apical and lateral sides of the plasma membrane.

The structural integrity of BBB is sustained mainly by tight junction (TJ) proteins and *adherens* junctions (AJ). Specifically, TJ are composed of claudin family members, occludin, junctional adhesion molecules (JAMs) and *zonulae occludens* (ZO-1, ZO-2, and ZO-3)—membrane-associated accessory proteins that connect the cytoplasmic tails of claudins and occludin to the actin cytoskeleton to sustain the TJ structure (Luissint et al., 2012; Tietz and Engelhardt, 2015).

The primary biological role of TJs is to establish a rigorous restriction of paracellular molecular diffusion, creating a high electrical resistance between the endothelial cells and ions and other polar solutes. Brain endothelial cell's AJs, ubiquitous in the vasculature, consist of cadherin proteins extended throughout the intercellular cleft that mediate cell-to-cell membrane adhesion and are anchored into the actin cytoskeleton by the scaffolding proteins alpha, beta and gamma catenin (Abbott et al., 2010; Blanchette and Daneman, 2015).

Although, the specific AJs function in the BBB is yet to be fully elucidated, it is known that they play an important role in the maintenance of TJs and the junctional complex by keeping the BECs together (Keaney and Campbell, 2015). In addition, brain endothelial cells display low rates of transcellular vesicular transport in comparison to peripheral endothelial cells, a process termed transcytosis (transport between the luminal and abluminal cell membranes). Still, transcytosis is the most common mechanism to selectively uptake macromolecules such as albumin, low-density lipoprotein and hormones, e.g., insulin and leptin (Holly and Perks, 2006; Xiao and Gan, 2013; Chow and Gu, 2015). As a result of this restrictive barrier, BECs

express several transporters and receptor proteins, as Glut-1, that selectively allow the entry of nutrients, neurotransmitters and other essential macromolecules into the brain parenchyma, at the same time that ensure the elimination of potentially metabolic waste and neurotoxic substances from the CNS into the blood (Keller, 2013; Keaney and Campbell, 2015).

Another important feature of the BECs is the low expression levels of leukocyte adhesion molecules (LAMs) such as E-selectin and Icam1, compared with ECs in non-neuronal tissues (Andreone et al., 2015; Daneman and Prat, 2015). This characteristic greatly restrains the number of immune cells that enter the CNS. In general, the physical barrier properties mediated by the junctional complex and the reduced transcytosis along with a highly selective cellular transport system illustrates how the brain endothelium orchestrate the regulation of the brain microenvironment.

Pericytes

Pericytes were first characterized by Eberth (1871) and Rouget (1873) in the 1870's and initially named "Rouget cells." Later, Zimmermann (1923) introduced the term pericyte due to their location in close proximity to the endothelial cells. Anatomically, pericytes are located at the abluminal surface of microvessels, embedded in a common basement membrane with the BECs, and exhibit elongated cytoplasmic processes that wrap the endothelium around (Trost et al., 2016). The CNS vasculature has significantly higher pericyte density/coverage compared with peripheral tissues, which correlates with the particular endothelial barrier properties. It is estimated that the ratio of pericytes to endothelial cells in CNS is 1:1-1:3, while this ratio appears to be from 1:10-1:100 in striated muscles (Shepro and Morel, 1993; Millis et al., 2013). Despite being separated by the basal membrane, in areas lacking a basement membrane (BM) BM, pericytes and BECs make direct peg-and-socket contacts, in which pericyte cytoplasmic fingers (pegs) are inserted into endothelial invaginations (pockets) (Winkler et al., 2011). Several different transmembrane junctional proteins are present at these contact points, including N-cadherin, forming key AJs between the two cell types; connexin-43 (CX43), hemichannels that form gap junctions enabling the exchange of nutrients, secondary messengers and ions; adhesion plaques composed predominately of fibronectin that anchor pericytes to endothelial cells (Berger et al., 2005; Zlokovic, 2008; Armulik et al., 2011; Winkler et al., 2011).

Pericytes play essential roles in the regulation of several neurovascular functions. They are critical during angiogenesis, participating in vessel formation, remodeling and stabilization (see developmental section) (Armulik et al., 2011; Winkler et al., 2011). *In situ* and *In vivo* studies evidenced that pericytes are modulators of capillary diameter and blood flow in response to changes in neural activity (Peppiatt et al., 2006; Fernández-Klett et al., 2010; Hall et al., 2014). However, Hill et al. (2015) demonstrate that capillary pericytes are not contractile *in vivo*. Thus, the active regulation of the capillary blood flow by pericytes remains controversial presumably due to the lack of proper pericyte definition and identification (Trost et al., 2016).

Apart of their importance in BBB development (see BBB development section), pericytes are involved in the formation and maintenance of the highly selected permeability of the BBB in adulthood and aging (Armulik et al., 2010; Bell et al., 2010; Daneman et al., 2010a). Pericyte deficiency promotes severe BBB dysfunction as a result of increased vascular permeability (directly correlated with absolute pericyte coverage, increased rates of transcytosis and paracellular transport due to reduced expression of various TJ and AJ proteins). Pericytes also guide astrocytic foot processes to surrounding CNS blood vessels and mediate the polarization of astrocytic end-feet, highlighting the interdependence among components of the NVU (Armulik et al., 2010; Keaney and Campbell, 2015).

Increasing evidence has shown that pericytes may constitute multipotent stem/progenitor cells that could differentiate into mesenchymal cells and into non-mesenchymal cell types, including glial and neuronal lineages under different *in vitro* conditions (Bribair et al., 2013). Until now, there is still a lack of studies describing that such differentiation occurs in the brain (Dore-Duffy and Cleary, 2011; Trost et al., 2016). Moreover, pericytes can control several aspects of the CNS immune response (reviewed in Rustenhoven et al., 2016).

Astrocytes

Due to the position of astrocytes in the NUV (ensheathing almost 90% of brain microvasculature), many studies have focused on the role of astrocytes in the maturation and maintenance of BBB (Correale and Villa, 2009). Indeed, astrocytes can induce barrier properties in non-CNS endothelial cells *in vivo* (Janzer and Raff, 1987).

Astrocytes, the most abundant cells in the brain, are spongiform-shaped glial cells that extend many branching cellular processes, including astrocytic end-feet that cover brain endothelial cells. Specifically, astrocytic end-feets establish a close interaction with BECs through transmembrane proteins anchoring, such as the water channel Aquaporin-4 (Aqp4) and the potassium channel KIR4.1, critical for CNS water homeostasis regulation (Cabezas et al., 2014). Furthermore, astrocytes communicate between each other through gap junctions forming an extensive glial syncytium that is associated with well-coordinated responses within large groups of cells (Theis et al., 2005; Alvarez et al., 2013). Astrocytes perform several functions critical to CNS physiology, including the regulation of blood flow in response to changes in neuronal activity which results in enhanced delivery of oxygen and glucose to the active brain region; the maintenance of ion and neurotransmitters concentration within the extracellular space for proper synaptic transmission; the modulation of synaptic and neural activity by the release of gliotransmitters, such as glutamate, ATP, D-serine, and GABA; and are also able to respond to local levels of nutrients, glucose uptake from blood vessels and the storage of glycogen, which is hydrolyzed to release lactate into the extracellular space when glucose is scarce (Sofroniew and Vinters, 2010; Cabezas et al., 2014).

One of the essential roles attributed to astrocytes is to regulate the nutrient availability, such as glucose, and also the access of some peripheral hormones, including leptin, ghrelin and GLP-1.

Their privileged anatomical position, in close proximity to the blood vessels and neurons, allows them to act as important metabolic sensors. Actually, hypothalamic astrocytes express specific transporters and receptors that are involved in the control of energy homeostasis. The astrocytic end-feet that enclose capillaries express the glucose transporter GLUT-1 that transports glucose into the CNS. The uptaken glucose is stored in form of glycogen, which is mobilized to release lactate to the neurons when glucose is not abundant, as in periods of energy deficit (Argente-Arizón et al., 2015; Chowen et al., 2016). Astrocytes also express the glucose transporter GLUT-2 that is important for glucosensing process and in the homeostatic control of circulating glucose levels and food intake (Marty et al., 2005; Stolarczyk et al., 2010). GLUT-2 inactivation results in overeating, providing evidence that glucose detection by GLUT2 contributes to the control of food intake by the hypothalamus (Bady et al., 2006; Stolarczyk et al., 2010). Leptin receptors are expressed in hypothalamic astrocytes and their physiological relevance was recently demonstrated. The conditional deletion of the leptin receptor in GFAP positive cells in mice decreased the number and length of astrocyte projections in hypothalamic neurons involved in feeding control, such as POMC and AGRP neurons of the arcuate nucleus. In addition, leptin-regulated feeding was diminished in mice with astrocyte-specific leptin receptor deficiency (Kim et al., 2014).

Regarding the contribution of astrocytes in BBB development, they do not appear to participate in this process at early embryonic stages (see development section) (Daneman et al., 2010b). Indeed, mature astrocytes produce factors that regulate BBB function and integrity. Astrocytes also secrete angiopoietin-1 and angiotensin that restrict BBB permeability by supporting efficient organization of TJs (Lee et al., 2003; Wosik et al., 2007; Siegenthaler et al., 2013). Thus, astrocytes are critical in sustaining BBB functionality and integrity as well as in the regulation of neuronal and synaptic activity.

Microglia

In addition to pericytes and astrocytes, the brain endothelium at the NUV is in close contact with immune cells. The two main immune cell populations within the CNS are perivascular macrophages and microglia.

Perivascular macrophages are monocytic lineage cells located outside of the CNS parenchyma. In fact, these cells reside in and circulate through the Virchow–Robin spaces, which lie between the BM around pericytes and at the surface of the glia interface of the brain vessels. The perivascular macrophage number is continuously maintained by replacement from blood-borne cells macrophages. They provide a first line of innate immunity due to their ability to quickly phagocytose particles from the cerebrospinal fluid (Daneman and Prat, 2015).

The brain parenchyma is populated by microglia, the most abundant CNS innate immune cells. Microglial cells, also referred as resident macrophages of the brain, are derived from progenitors in the yolk sac, which migrate into the CNS parenchyma during neonatal development. Microglia colonization comes first during BECs invasion into the brain (da Fonseca et al., 2014) and are shown to be involved in CNS vasculogenesis, promoting endothelial cell fusion to increase vascular complexity (Fantin et al., 2010), as well as decreasing paracellular permeability in cultured BECs (Zenker et al., 2003). Accordingly, these data suggest that the interactions between microglia and the brain endothelium could participate in the BBB formation and regulation (Correale and Villa, 2009; da Fonseca et al., 2014).

Generally, microglia displays two distinct morphological patterns. The steady-state population of microglia in the healthy brain exhibits a 'resting' phenotype, which is characterized by a small, and circular cell body with extensively ramified processes. As a part of their homeostatic functions, microglial cell bodies remain stationary, but their processes continuously scan the surrounding extracellular space and communicate directly with neurons, astrocytes, and blood vessels (Nayak et al., 2014). Microglia rapidly respond to injury signals or infection through an activated phenotype characterized by a morphological change into an amoeboid shape and alterations in signaling and gene expression in order to perform inflammatory functions (Lull and Block, 2010; Saijo and Glass, 2011).

Prolonged microglia activation and consequent chronic neuroinflammatory state in CNS might induce impairments in BBB integrity that, in turn, contribute to the progression of neurodegenerative diseases (reviewed in de Vries et al., 2012). Evidence from *in vitro* studies has shown that microglia activation may be related to BBB disruption. Sumi et al. (2010) demonstrated that the treatment of a rat brain microvascular endothelial cell/microglia co-culture system with lipopolysaccharide, a microglial activator, induced an increase in endothelial cell permeability and changes in the expression pattern of tight junction proteins. Another study using a similar *in vitro* co-culture system found that the tumoral necrosis factor α (TNF-α) released from activated microglia also increased the permeability of BECs, which could be blocked by a neutralizing antibody against TNF-α (Nishioku et al., 2010).

Although, these *in vitro* BBB models are important for unraveling the mechanisms involved in the crosstalk between microglia activation and BBB integrity, more studies are needed to improve our understanding on how these cell types interact *in vivo*.

BBB DEVELOPMENT

The development and differentiation of the BBB can be subdivided into three phases: i, angiogenesis; ii, differentiation; and iii, maturation (summarized in **Figure 2**).

The angiogenic phase begins early during neural tube development (as early as E9.0-E10.5 in mice). Endothelial cells from the perineural vascular plexus penetrate the neuroectoderm according to a vascular endothelial growth factor (VEGF) concentration gradient giving rise to immature brain vessels (Raab et al., 2004; Potente et al., 2011). VEGF is expressed by the neural progenitors of the ventricular neuroepithelium and serves, together with angiopoietin, as a major driving force for the migrating endothelial cells (EC) (Risau et al., 1986). VEGF deficiency is lethal (Shalaby et al., 1995; Carmeliet et al., 1996).

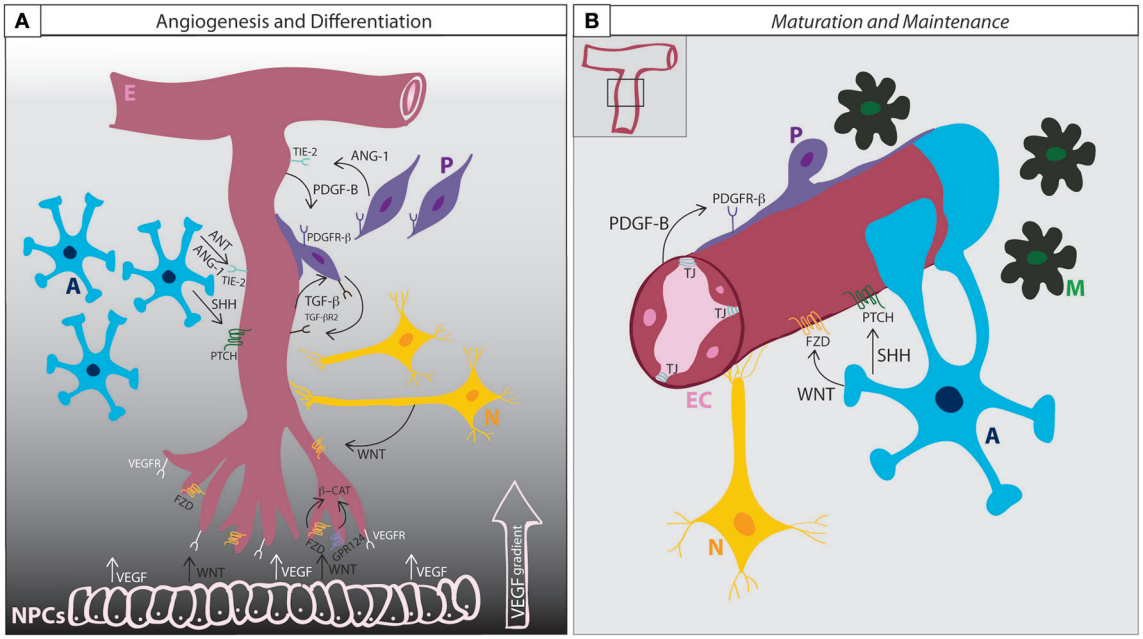


FIGURE 2 | BBB development and maintenance. (A) Angiogenesis and differentiation. VEGF secretion from neural progenitor cells induces endothelial cells penetration into the brain parenchyma according to a VEGF concentration gradient. Wnt ligands also secreted from the NPCs induces the migration of the endothelial cells and activate β-catenin signaling through the binding to Frizzled receptors, inducing the expression of BBB specific genes. GPR124, together with WNT, co-activates β-catenin signaling. Endothelial cells secrete PDGF-B and attract pericytes expressing PDGFR-β. The interaction between ECs and Ps induce the mutual expression of TGF-β and TGF-βR2. The activation of TGF-β signaling regulates basement membrane formation and the induction of Ang-1 expression in pericytes, that acting through the endothelial receptor Tie-2, enhance tight junction expression. Astrocytes release SHH, that when bound to PTCH receptor induces Shh signaling activation in ECs and contributes to BBB formation. Astrocytes also express ANT and ANG-1, which by limiting BBB permeability contributes to the maturation of BBB function. **(B)** Maturation and Maintenance. Pericytes and astrocytic end-feet cover the endothelium and secrete matrix proteins that will constitute the basement membrane. Astrocytes continue secreting SHH and WNT in order to maintain BBB functionality throughout life. A, astrocytes; E, endothelium; EC, endothelial cell; M, microglia; N, neuron; NPC, neural progenitor cell; P, pericyte; TJ, tight junction; Ang-1, angiopoietin-1; ANT, angiotensin; β-cat, beta-catenin; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; FZD, frizzled; GPR124, adhesion G protein coupled receptor A2; Shh, sonic hedgehog; Ptch, Patched 1; PDGF-B, platelet derived growth factor B; PDGFR-β, platelet derived growth factor receptor beta; TGF-β, transforming growth factor-beta; TGF-βR2, transforming growth factor beta receptor type-2.

Downstream VEGF signaling supports angiogenesis through endothelial cell proliferation, migration and survival (Olsson et al., 2006). Together with VEGF, neural Wnt signaling plays an important role in the development of the BBB (Daneman et al., 2009). Different Wnt ligands, including Wnt7a, Wnt7b, and Wnt3a are secreted by the neuroepithelium (Wang et al., 2012) and induce further ingression of ECs into the neural tissue, activating Wnt/B-catenin signaling in the newborn endothelial cells (Liebner et al., 2008; Daneman et al., 2009; Zhou and Nathans, 2014) that leads to induction of genes critical for the BBB formation and vascular patterning, such as glucose transporter Glut-1 (Stenman et al., 2008), death receptors DR6 and TROY (Tam et al., 2012) and tight junction proteins. Defects in Wnt/β-catenin signaling result in major vascular malformations and BBB breakdown (Stenman et al., 2008; Daneman et al., 2009; Wang et al., 2012). Retinoic acid, Notch signaling receptor tyrosine kinase, cadherins and ephrins also contribute to angiogenesis in the CNS (Adams and Alitalo, 2007; Dejana and Vestweber, 2013).

At about E15.5, the newly formed vessels continue to differentiate and mature as a complete NVU forming the basis for the BBB. During the differentiation phase (between E15.5 and E18.5 in mice), the brain barrier is structured properly by the induction of anti-angiogenic signals and the recruitment of pericytes and astrocytes to the newly formed vessels. Pericytes express the platelet-derived growth factor receptor- β (Pdgfr-β) and are directed to the endothelial developing capillaries that secrete Pdgf-B (Lindahl et al., 1997; Hellstrom et al., 1999). Pdgfr-β mouse mutants completely lack brain pericytes, and die as consequence of brain microhemorrhages (Lindahl et al., 1997; Lindblom et al., 2003; Tallquist et al., 2003); thus, pericyte recruitment to the developing endothelial capillaries is critical for the formation and maintenance of the BBB (Armulik et al., 2010; Bell et al., 2010; Daneman et al., 2010a). The lack of Pdgf-β or Pdgfr-β leads to erroneous TJ distribution and increased vascular permeability (Hellstrom et al., 2001).

Interactions between pericytes and the brain endothelial cells lead to the expression of transforming growth factor-β (Tgf-β) and its receptor (Tgf-βR2) by both cell types. TGF secretion induces cell adhesion through the production of cadherin-2 by the endothelial cells and the secretion of different extracellular matrix components that contribute to basement membrane (BM)

formation by the pericytes (Winkler et al., 2011). Notch and sphingosine-1-phosphate (S1P) signaling also contribute to the regulation of cadherin-2 expression in BECs (Winkler et al., 2011; Obermeier et al., 2013). Upon activation of Tgf-β signaling, pericytes produce Ang-1 that, by enhancing tight junctions formation, limit BBB permeability, and reduce the expression of leukocyte adhesion molecules (LAMs). Pdgf-β and Wnt/β-catenin signaling is also important during the differentiation of the BBB by the induction of transporters and increased expression of tight junctions. This provides integrity to the barrier (Liebner et al., 2008; Daneman et al., 2009; Tam et al., 2012; Wang et al., 2012; Zhou and Nathans, 2014; Andreone et al., 2015). In the BBB, Gpr124 has been described as a necessary endothelial receptor specifically in the CNS and function as a co-activator of Wnt/b-catenin signaling to mature the BBB (Kuhnert et al., 2010; Cullen et al., 2011; Zhou and Nathans, 2014).

Pericytes are apparently required to guide astrocytes toward the developing BBB (Armulik et al., 2010). Once recruited to the forming NVU, astrocytes are involved in limiting BBB permeability by the release of Sonic Hedgehog (Shh). The activation of Shh signaling leads to the expression of occludin and claudin5 and inhibition of chemokines and cell adhesion molecules in the endothelial cells, suggesting a role for astrocytes and Shh in maintenance of BBB functionality and immune surveillance (Alvarez et al., 2011; Obermeier et al., 2013; Siegenthaler et al., 2013). Recent studies have uncovered yet another important role for astrocytes in the production of retinoic acid, which is also necessary for the correct development of BBB vessels (Halilagic et al., 2007; Mizee et al., 2013).

Maturation and maintenance of the BBB is achieved by the persistence of tight junction proteins expression and their redistribution throughout the whole BBB structure. The production of TJs is regulated by Wnt signaling between astrocytes and BECs. Close contact between the endothelial cells, pericytes, astrocytes, and possibly neurons and microglia sustain BBB integrity and function as a stabilized neurovascular unit (reviewed in Obermeier et al., 2013; reviewed in Engelhardt and Liebner, 2014; reviewed in Zhao et al., 2015). The BBB is already formed and completely functional during late gestation in rodents and in the third trimester in humans (Bauer et al., 1995; Daneman et al., 2010a; Ben-Zvi et al., 2014). In the rat embryo, the BBB is already functional at E16 (Saunders et al., 2012). The exact time window remains elusive and is likely species dependent (Saunders et al., 2013; Hagan and Ben-Zvi, 2015).

There has been intense debate on the BBB features that provide its correct function. To date, it is believed that tight junctions are functional in sealing the space between BBB endothelial cells very early in development (Dziegielewska et al., 1979; Bondjers et al., 2006; Tam et al., 2012; Ben-Zvi et al., 2014) but only when the intracellular pathway of transcytosis is partially downregulated will the complete restrictive properties of the barrier become fully matured (Hagan and Ben-Zvi, 2015). Little is known about the functionality of transporters at the embryonic BBB, although some transporters, like Glut-1, are known to be expressed very early (Boado and Pardridge, 1990; Farrell and Pardridge, 1991; Bauer et al., 1995; Hagan and Ben-Zvi, 2015). A role of Mfsd2a in participating in the inhibition of vesicular trafficking and inducing a more sealed barrier during development was suggested recently (Ben-Zvi et al., 2014; reviewed in Hagan and Ben-Zvi, 2015). Mfsd2a mutants have a defective BBB even though presenting proper and functional tight junctions (Ben-Zvi et al., 2014). Thus, barrier transporting properties would be determined very early in development while the sealing function would be acquired gradually across development, first with the suppression of fenestrations, then with the appearance of functional TJs, and finally with the decrease of transcytosis by the expression of Mfsd2 (Ben-Zvi et al., 2014; Hagan and Ben-Zvi, 2015).

Alterations in barrier development and in TJ expression could lead to anomalies later in life as well as to increased predisposition to develop metabolic diseases. Maternal obesity increases BBB permeability in the offspring (Kim et al., 2016), leading to higher exposure to leptin and ghrelin. In consistence with that, overnutrition during early postnatal life alters brain sensitivity to ghrelin (Collden et al., 2014), suggesting that nutrient sensing control alterations during specific hypothalamic developmental time-points could contribute to metabolic defects in the adult life. These alterations can affect the programming of energy homeostasis circuits predisposing the offspring to the development of metabolic syndrome at early life and/or adulthood.

BBB IN THE HYPOTHALAMIC AREA

In some areas of the brain, the BBB is modified in order to allow the access of certain substances from systemic circulation into the central nervous system. This occurs particularly in periventricular areas creating a blood/spinal fluid interface (Bennett et al., 2009). In these regions, the BBB capillaries are highly fenestrated with less tight junctions between endothelial cells creating a more permeable barrier (Bennett et al., 2009). Seven periventricular regions display differential barrier properties and are collectively known as the circumventricular organs: i, sub-fornical organ; ii, organum vasculosum of the lamina terminalis; iii, pituitary; iv, area postrema; v, median eminence (ME); vi, subcomissural organ; and vii, pineal (Bennett et al., 2009; Szathmari et al., 2013). In these regions, in addition to the cells that classically form the BBB vessels, a differential type of radial glial cell, termed tanycytes are also found in the interface between the spinal fluid and the capillaries (Rodríguez et al., 2010). Because of its physical proximity with the hypothalamus, the BBB at the ME is of particular interest regarding whole-body energy homeostasis and metabolism.

The median eminence is adjacent to the arcuate nucleus (ARC) of the hypothalamus. This region harbors neurons that play important roles in the control of whole body metabolism (Velloso and Schwartz, 2011; Cavadas et al., 2016). Classically, two main neuronal populations control energy homeostasis in the ARC: i, NPY/AgRP neurons, which are active during fasting and provide orexigenic and anti-thermogenic signals; ii, POMC/CART neurons, which are active following food intake and provide anorexigenic and pro-thermogenic signals (Velloso and Schwartz, 2011; Cavadas et al., 2016). In addition,

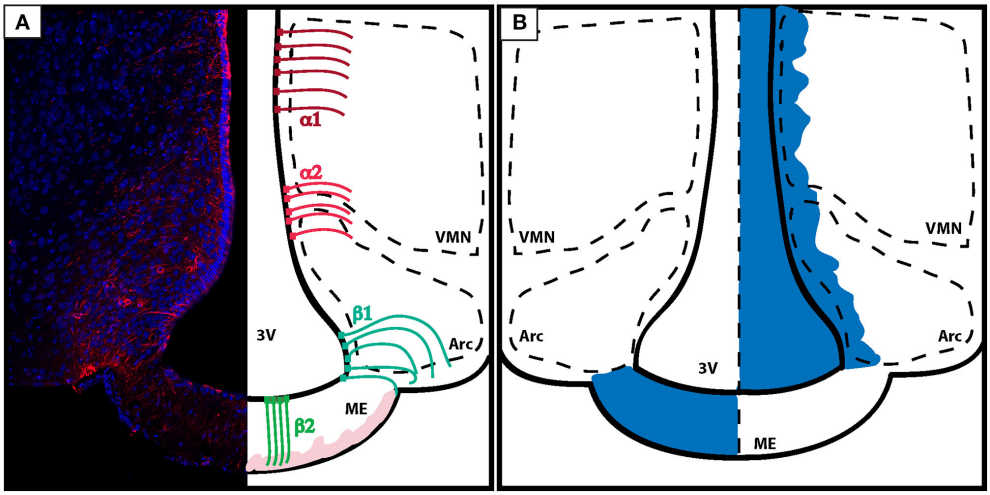


FIGURE 3 | Organization of the BBB in the energy-sensing hypothalamus. (A) Coronal section of the tuberal hypothalamus showing the distribution of tanycytes along the third ventricle wall. Left: vimentin staining (red) shows the projections of tanycytes to the brain parenchyma. Right: tanycytes line the third ventricle and can be classified according to location and function. α tanycytes don't possess barrier properties. α1 tanycytes (dark red) lye in the dorsal ventro-medial nucleus of the hypothalamus, while α2 tanycytes (light red) are found in between the ARC and the VMH. β tanycytes are located ventrally and function as gatekeeper cells controlling the passage of substances from the leaky ME to the ARC. β1 tanycytes (turquose) divide the ME from the ARC, while β2 tanycytes (green) are located in the ME, and characterized by processes with direct access to the blood capillaries. Blood capillaries are displayed in light pink. **(B)** Diagram showing how the diffusion of dyes injected peripherally do not penetrate the brain (exemplified here by the action of tanycytes lining the ME and the ARC) (left). On the other hand, dyes that are infused inside of the brain ventricles diffuse trhough the CSF and penetrate the brain parenchyma but do not pass the ME in the direction of the portal capillaries (right). VMN, ventro-medial nucleus; ARC, arcuate nucleus; ME, median eminence; 3V, third ventricle.

other distinct hypothalamic neuronal subpopulations have been described recently (Zhang and van den Pol, 2016; Campbell et al., 2017; Fenselau et al., 2017; Lam et al., 2017). Of note, a recent study has identified ARC neurons that express the dopamine-synthesizing enzyme, tyrosine hydroxylase (TH). These cells are involved in the integration of homeostatic and hedonic feeding signals and are potential targets for the treatment of obesity (Zhang and van den Pol, 2016).

Because ARC neurons are involved in the sensing of the nutritional status of the body, it would be expected that they were located in an anatomical area where the access to nutrients and energy-status signaling substances would be facilitated. In fact, studies have shown that the interface between the median eminence and the ARC is somewhat leaky to hormones and nutrients (Obici and Rossetti, 2003; Lam et al., 2005). For example, fatty acids present in the systemic circulation are not freely diffusible to most areas of the CNS (Mitchell and Hatch, 2011). In general, they rely on particular transport systems present in the structures of the BBB (Betsholtz, 2014). The proper function and distribution of these transport systems is of major importance in the development and physiology of the brain throughout life because most fatty acids that comprise the central nervous system phospholipids cannot be synthesized *de novo* in the brain, and thus, must be obtained from the systemic circulation (Mitchell and Hatch, 2011). However, in the ME specific conditions make circulating fatty acids more available to ARC neurons (Obici and Rossetti, 2003; Lam et al., 2005).

As early as the 1970's, studies have revealed that hormones involved in energy homeostasis and nutrients have particular properties to cross the BBB at the ME/ARC interface. Systemic insulin crosses the BBB by a saturable system (Woods and Porte, 1977) and concentrates mostly in the olfactory bulb and hypothalamus (Havrankova et al., 1981). Similarly, leptin reaches the brain by a saturable system, which is independent of insulin and is highly detectable in the choroid plexus, the ME and the ARC (Banks et al., 1996). A major advance in understanding how hypothalamic neurons are exposed to hormones and nutrients to respond to systemic variations in whole body energy status was obtained by the characterization of the presence of nutrient transporters in BBB endothelial cells. Beginning in the 1990's a series of studies have explored the particularities of how peptides can cross the BBB (Cashion et al., 1996; Pam et al., 1996; Banks et al., 1997). The BBB endothelial cells are also provided with transport systems for glucose (Pardridge et al., 1990); fatty acids (Obici and Rossetti, 2003; Lam et al., 2005) and aminoacids (Hawkins et al., 2006), and the variations in whole body energy status, such as in fasting/feeding or metabolic diseases can affect function of such transport systems (Kastin and Akerstrom, 2000; Kastin et al., 2001). This implies that the BBB at the ME/ARC interface plays a major role in controlling the way hypothalamic neurons are exposed to systemic factors involved in metabolism and nutrition. In this context, important advance in the field by exploring the mechanism involved in the regulation of the ME tanycytes has been provided over the last few years.

Tanycytes are originated from the radial bipolar glia around E17.5 in mice and have a morphology that is somewhere in between the aspects of an ependymal cell and an astrocyte; differing from the last by having a single basal projection directed

toward the parenchyma of the brain (Rodríguez et al., 2010; **Figure 3A**).

Mounting evidence suggests that, at least part of the selective leakiness of the BBB at the median eminence relies on the responsiveness of tanycytes to nutrients present in the bloodstream. Early studies evaluating hypothalamic tanycytes have demonstrated their role in the transport of substances between the third ventricle and ME (Wagner and Pilgrim, 1974). Also, tanycytes were shown to respond to hormone production and direct hormone delivery to certain anatomical sites and systemic circulation (Akmayev and Popov, 1977; Vallet et al., 1991). Interestingly, tanycytes respond rapidly to systemic stimulation, suggesting that this particular cell-type could play a role in the dynamic control of median eminence and adjacent areas exposure to systemic factors (Lichtensteiger et al., 1978). Recent studies have provided important progress in the understanding of the roles played by tanycytes in the ME and ARC (Langlet et al., 2013a,b).

The identification of leptin in the mid 1990's placed the ARC in the center of a complex system that controls body energy status (Zhang et al., 1994). With this concept in mind, researchers looked for a potential involvement of ME tanycytes as gatekeepers controlling the access of substances (particularly nutrients and hormones) that could modulate ARC-neuron function. However, it was only in 2011 that first evidence was provided showing that tanycytes are responsive to glucose fluctuations promoted by feeding (Frayling et al., 2011). Glucose leads to a powerful ATP-mediated Ca^{2+} release into the tanycytes, which in turn release ATP to adjacent cells. Therefore, it was proposed that by responding to glucose and releasing ATP, ME tanycytes could modulate the activity of ARC neurons (Frayling et al., 2011). Furthermore, ME tanycytes can transport leptin into the mediobasal hypothalamus (Balland et al., 2014). When leptin present in the bloodstream reaches the median eminence it activates leptin receptors expressed in the tanycytes. This in turn, activates the protein ERK that transduces the signals required for an appropriate transport of leptin through the cerebrospinal fluid into the ARC (Balland et al., 2014).

Interestingly, in order to respond to the constant changes in nutrient and hormone availability in the circulation, the plasticity of the median eminence tanycytes has proven remarkable. During the physiological cycles of fasting and feeding these cells undergo both morphological and functional changes (Langlet et al., 2013a). The decreased blood levels of glucose during fasting are capable of inducing changes in the structure of the interface between the blood and the ARC. At least in part, these morphological and functional changes are dependent of the expression of VEGF-A by the tanycytes (Langlet et al., 2013a).

In addition to glucose and leptin, fatty acids can also affect ME tanycytes. In diet-induced obesity, the amount of lipid droplets increase considerably in tanycytes (Hofmann et al., 2017). Moreover, the ratio between saturated and unsaturated lipids is modified suggesting that this cell type may also act as a sensor and gatekeeper for lipids in the median eminence/ARC interface (Hofmann et al., 2017).

CONCLUDING REMARKS

The BBB provides a physiological interface between the central nervous system and the systemic circulation allowing the entrance of nutrients and certain signaling molecules and restraining the entrance of microorganisms and particles that can harm the brain. Because hypothalamic neurons must sense the energy status of the body, the BBB at the median eminence specialized to a more permeable interface between the vasculature and the brain. Recent studies have demonstrated that tanycytes present in the interface between the ARC and the median eminence are very sensitive to nutrients. They can be rapidly modified in response to fast and fed states and also, can be disturbed by abnormal consumption of nutrients derived from the diet. In obesity and in metabolic conditions associated with the obese phenotype, hypothalamic neurons are affected by a local inflammatory response that is triggered by the excessive amount of fatty acids in the diet. Mounting evidence suggest that, at least in part, the anomalous activity of the hypothalamic tanycytes can play a role in the defective neuronal activity in obesity and associated conditions. Future studies should focus on the identification of mechanisms that may protect the tanycytes from diet-induced abnormalities and the impact of such protection in the progression of metabolic diseases.

AUTHOR CONTRIBUTIONS

RHT, NRVD, and LAV discussed the structure of the manuscript. NRVD wrote the aspects of the BBB structure and function and RHT wrote the developmental topic. LAV and AFSR wrote BBB specialization in the hypothalamus. RHT, NRVD, and LAV wrote the introduction, abstract, and conclusion remarks. RHT made the figures and figure legends.

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Effects of Fat and Sugar, Either Consumed or Infused toward the Brain, on Hypothalamic ER Stress Markers

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Protein-folding stress at the Endoplasmic Reticulum (ER) occurs in the hypothalamus during diet-induced obesity (DIO) and is linked to metabolic disease development. ER stress is buffered by the activation of the unfolded protein response (UPR), a controlled network of pathways inducing a set of genes that recovers ER function. However, it is unclear whether hypothalamic ER stress during DIO results from obesity related changes or from direct nutrient effects in the brain. We here investigated mRNA expression of UPR markers in the hypothalamus of rats that were exposed to a free choice high-fat high-sugar (fcHFHS) diet for 1 week and then overnight fed *ad libitum*, or fasted, or fat/sugar deprived (i.e., switched from obesogenic diet to chow). In addition, we determined the direct effects of fat/sugar on mRNA expression of hypothalamus UPR markers by intracarotic infusions of intralipids and/or glucose in chow-fed rats that were fasted overnight. Short term (1 week) exposure to fcHFHS diet increased adiposity compared to chow-feeding. Short term exposure to a fcHFHS diet, followed by mild food restriction overnight, induced hypothalamic ER stress in rats as characterized by an increase in spliced to unspliced X-box binding protein 1 mRNA ratio in hypothalamus of fcHFHS fed rats compared to chow fed rats. Moreover, infused lipids toward the brain of overnight fasted rats, were able to induce a similar response. Non-restricted *ad libitum* fcHFHS-diet fed or totally fasted rats did not show altered ratios. We also observed a clear increase in hypothalamic activating transcription factor 4 mRNA in rats on the fcHFHS diet while being *ad libitum* fed or when infused with intralipid via the carotic artery compared to vehicle infusions. However, we did not observe induction of downstream targets implying that this effect is a more general stress response and not related to ER stress. Overall, we conclude that the hypothalamic stress response might be a sensitive sensor of fat and energy status.

Keywords: ER stress response, hypothalamus, food restriction, fatty acids, sugar

INTRODUCTION

Obesity is the result of a mismatch between energy intake and energy expenditure. Although a sedentary lifestyle can contribute to obesity development, the consumption of sugar-sweetened beverages and high amounts of saturated fat in foods has been linked to the risk to become obese and develop metabolic disorders (Hall et al., 2012). Over the last decades, the intake of sugar-based beverages has clearly increased worldwide, and from recent surveys it has become clear that consumption of both sugar-sweetened beverages and saturated fats (especially from snack foods) exceeds recommended daily levels (Popkin et al., 2012). It is, therefore, of utmost importance to understand how fat and sugar affect energy balance.

The hypothalamus is an important brain area in regulating energy balance (Elmquist and Flier, 2004; Schwartz and Porte, 2005). Under normal conditions the hypothalamus senses whole body energy demands via nutrient, neuronal and hormonal signaling and adjusts feeding behavior and energy expenditure via the production of orexigenic and anorexigenic peptides. For example leptin, the hormone secreted by adipose tissue in proportion to fat mass, activates the release of α-melanocyte stimulating hormone [α-MSH; derived from pro-opiomelanocortin (POMC)] and inhibits the release of the orexigenic neuropeptide Y (NPY) and agouti related peptide (AgRP) (Sahu, 2011). During obesity, however, the function of intracellular organelles in AGRP/NPY or POMC neurons like the endoplasmic reticulum (ER) was shown to be impaired leading to disturbed leptin signaling and energy imbalance (Hosoi et al., 2008; Zhang et al., 2008; Ozcan et al., 2009; Cakir et al., 2013; Ramírez and Claret, 2015).

The ER is a complex membrane network responsible for the synthesis and folding of various transmembrane and secreted proteins (Westrate et al., 2015). Accumulation of toxic, misfolded proteins in the ER leads to ER stress and activation of the unfolded protein response (UPR). The UPR is a network of pathways controlled by three sensors; PKR-like ER kinase (PERK), the activating transcription factor 6 (ATF6) and the inositol-requiring protein-1 alpha (IREα). Activation of these pathways induces the expression of genes that lead to the expansion of the ER, reduction of protein translation and increase of protein folding capacity promoting cell survival or induces apoptosis (Walter and Ron, 2011; Lee and Ozcan, 2014). Upon PERK activation, activating transcription factor 4 (ATF4) mRNA is translated which increases the transcription of specific UPR target genes, including C/EBP homologous protein (CHOP) (Harding et al., 2000; Han et al., 2013). Immunoglobulin-heavy-chain-binding protein (BiP) mRNA is increased upon ATF6 activation and splicing of unspliced X box binding protein 1 to spliced XBP1 (sXBP1/usXBP1) occurs upon IREα activation (Yoshida et al., 2001). Both usXBP1 and sXBP1 were related to cell viability under ER stress conditions. Under severe ER stress, IREα activation can also lead to degradation of mRNAs and miRNAs and cell apoptosis via the c-Jun N-terminal kinase (JNK) pathway (Todd et al., 2008). DP5 (Death protein 5/harakiri) and FasL (Fas ligand) are genes expressed downstream of JNK indicating activation of the IREα—apoptotic pathway (Schenkel, 2004; Guan et al., 2006; Ma et al., 2007).

Long term high fat diet (HFD) feeding, resulting in profound body weight gain, induces hypothalamic ER stress characterized by increased protein levels of UPR markers like p-PERK, p-IREα, and phospho-eukaryotic initiation factor 2 alpha (p-eIF2α) (Ozcan et al., 2009; Cakir et al., 2013). Activation of UPR pathways was also reported when fatty acids (FA) [arachidic acid, palmitic acid, or ceramide] were directly supplied to the brain via intracerebroventricular (ICV) infusion in rodents (Milanski et al., 2009; Contreras et al., 2014) or when administered to murine neuronal cell lines (Choi et al., 2010) implying a more direct role for FA in hypothalamic ER stress induction. Although many of the HFD used also contain considerable amounts of sugar, the effects of sugar alone on the ER stress induction has not been studied so far.

The fact that long term HFD feeding, but also ICV infusions of FA, induce hypothalamic ER stress marker expression points to the idea that nutrients like FA can induce hypothalamic ER stress. However, it is not clear whether other nutrients or the increased adiposity reflecting a positive energy status or a combination of nutrients and energy status affects hypothalamic ER stress. We therefore determined whether overfeeding/fasting status and its interaction with diet-induced obesity affected genetic markers for ER stress in the hypothalamus. We exposed male Wistar rats to a free choice high-fat high-sugar (fcHFHS) diet or chow for 1 week followed by overnight *ad libitum* feeding, fat/sugar deprivation or fasting. In addition, we investigated if fat and sugar have a direct effect on the ER stress markers in the hypothalamus by intracarotic infusions of Intralipids (IL), IL and glucose, or glucose to the brain of overnight fasted lean rats. For both experiments, mRNA of different UPR markers was measured using RT-PCR as an indication for the induction of hypothalamic ER stress.

MATERIALS AND METHODS

Animals

Adult male Wistar rats (250–280 g, Charles River, Germany) were individually housed in a temperature controlled room (19 ± 1°C) on a 12 h light/ 12 h dark cycle (lights on at 7:00 a.m.). During the experiments animals had *ad libitum* access to water and standard laboratory chow (SDS, UK) unless stated differently. All the studies were approved by and performed according to the regulations of the Committee for Animal Experimentation of the Academic Medical Centre of the University of Amsterdam, Netherlands.

fcHFHS Diet Experiments

Three experiments were performed to study the effect of nutrient availability on the hypothalamic ER stress response and its interaction with obesity. In all experiments rats were on a fcHFHS diet or chow for 1 week. The fcHFHS diet consisted of *ad libitum* access to chow, tap water, 30% sugar water (1.0 M sucrose mixed from commercial grade sugar and water) and a dish of pure animal fat (beef tallow; Ossewit/Blanc de Boeuf, Vandermoortele, Belgium) (composition: 34% oleic acid, 25% plamitc, and 22%

stearic acid and 4% linoleic acid). After this period the three experiments differed in feeding regime the night before the end of the experiment (4:00 p.m. day 7–9:00 a.m. day 8).

- *experiment 1*: rats had *ad libitum* access to the control chow diet or to the fcHFHS diet.
- *experiment 2*: all rats received 10 g chow overnight (fat/sugar deprivation for rats on fcHFHS).
- *experiment 3*: all rats were fasted overnight.

Fat/sugar deprivation in our diet model is characterized by removing the fat/sugar components of the diet (i.e., saturated fat and sugar water) overnight. We previously showed that removing fat and sugar from the fcHFHS diet results in consumption of 10–15 g chow spontaneously without caloric compensation for the fat/sugar components of the diet (Pandit, 2015). To ensure equal intake overnight between fcHFHS and chow-fed rats we provided all animals with 10 g of chow. Food components of the diet were weighed 5 times a week and the amounts of components eaten were multiplied with the caloric value of each component to determine energy intake in kcals.

At the end of the experiment, rats were anesthetized via 30% CO_2/70% O_2 and decapitated between 9:00 a.m. and 11:00 a.m. Epididymal, mesenteric, subcutaneous and peritoneal fat were dissected and weighed, trunk blood collected and brains were removed and stored at −80°C until further analysis. Plasma leptin concentrations were determined by radioimmunoassay (Linco Research, Inc., St. Charles, MO, USA). Samples were assayed in duplicate. Amounts of sample, standards, label, antibody and precipitating reagent as described in the procedures of the assay were divided by 4. The detection limit was 0.5 ng/ml and the inter- and intra-assay coefficients were 8% or less.

TABLE 1 | Content of intralipid 20%.

Fatty acid	Amount (%)
Linoleic acid	52
Oleic acid	22
Palmitic acid	13
Linolenic acid	8
Stearic acid	4
Myristic acid	<1
Others	1

Source; Fresenius Kabi.

Intracarotic Infusion Experiment

Rats (n = 6–9 per group) underwent surgery under anesthesia induced with an i.p. injection of 80 mg/kg Ketamin (Eurovet Animal Health, Netherlands), 8 mg/kg Xylazin (Bayer Health Care) and 0.1 mg/kg Atropin (Pharmachemie, Netherlands). A silicon catheter was inserted in the carotid artery and directed toward the brain (according to the method of Steffens, 1969). The catheter was externalized at the vertex of the head and the animals were allowed to recover for 7 days.

To study the direct effect of fat and/or glucose on hypothalamic ER stress markers, NaCl (control), 20% IL [(Fresenuis Kabi); composition of IL is displayed in **Table 1**] or IL + 1% glucose (G) were infused via the carotid artery toward the brain (*experiment 4*). Another infusion experiment was performed to study the effect of glucose on hypothalamic ER stress response using glucose (1% in NaCl) or NaCl (*experiment 5*). All solutions were heparinized (0.04%) and Infusion rate was 5 µl/min for 2 h. One hour after the end of infusion the animals received a single shot of pentobarbital via the carotid artery (100–150 mg/kg BW) and were decapitated. Brains were removed and stored at −80°C for further analysis.

Brain Harvesting—Isolation of the Hypothalamus

Coronal brain slices of 250 µm were obtained from −0.96 to −4.36 mm Bregma (Rat brain atlas; Paxinos and Watson, 2007) and directly put in RNAlater solution (Ambion Life Technologies). The hypothalamic part in each section was isolated using syringe needles (0.4 × 19 mm, BD Microlance) and used for RT-PCR.

RNA Isolation—RT-PCR

One half of the hypothalamus was homogenized in lysis buffer provided with the "High Pure RNA isolation kit" (Roche

TABLE 2 | Primer sequences used for RT-PCR.

Primers	Forward 5′–3′	Reverse 5′–3′
ATF4	CTGAACAGCGAAGTGTTGGC	TCTGTCCCGGAAAAGGCATC
us-XBP1	GTCCGCAGCACTCAGACTAC	ATGAGGTCCCCACTGACAGA
s-XBP1	CTGAGTCCGAATCAGGTGCAG	ATCCATGGGAAGATGTTCTGG
DP5	ATGAAGCTGTGTTGCCGAGA	GCTTCAGTCCCACAGACTCC
FasL	TATCCTGGGGATCTGGTGCTA	TGCAGGCATTAAGGACCACT
Ddit3 (CHOP)	AGAGTGGTCAGTGCGCAGC	CTCATTCTCCTGCTCCTTCTCG*
Hspa (Bip)	TGGGTACATTTGATCTGACTGGA	CTCAAAGGTGACTTCAATCTGGG*
β-actin	CATGTACGTAGCCATCCAGGC	CTCTTTAATGTCACGCACGAT
Cyclophilin	ATGTGGTCTTTGGGAAGGTG	GAAGGAATGGTTTGATGGGT
HPRT	GCAGTACAGCCCCAAAATGG	AACAAAGTCTGGCCTGTATCCAA

**Oslowski and Urano (2011).*

Molecular Biochemicals, Manheim, Germany) and total RNA was isolated according to the manufacturer's instructions. RNA was quantified by spectrophotometry at 260 nm (Nanodrop 1000, Willmington, Delaware, USA) and cDNA synthesis was performed using the "Transcriptor First Strand cDNA synthesis kit" for RT PCR with oligo(dT) primers (Roche Molecular Biochemicals, Manheim, Germany). The mRNA levels of ER stress markers as well as Hypoxanthine-guanine phosphoribosyltransferase (Hprt), Cyclophilin A and β-actin were determined by RT-PCR using SensiFAST SYBR No-Rox mix (Bioline, Luckenwalde, Germany) at the Lightcycler 480 apparatus (Roche Molecular Biochemicals, Manheim, Germany). The primers were designed using "Primer Blast" (**Table 2**). Samples were baseline corrected and individually checked for their PCR efficiency using the "LC480 Conversion" and "LinRegPCR" software. Median efficiency was calculated for each assay and samples that differed more than 0.05 from the mean efficiency were excluded from statistical analysis. Specific gene expression was normalized to the geometric mean of three housekeeping genes; (Hprt × β-actin × Cyclophilin A)$^{1/3}$.

Statistical Analysis

Data are presented as Mean ± SEM. Outliers were detected using "Dixon's *Q* test" and excluded. Differences between diet groups were evaluated using *Student's t-test* [experiments 1–4, 5 (NaCl vs. G)] or ANOVA followed by *post-hoc* Tukey test (experiment 5, NaCl vs. IL vs. IL+G). Difference between groups was considered significant when $p < 0.05$. In order to determine the effects of low vs. high fat intake and low vs. high sugar intake a median split was performed whereby the median was calculated for fat or for sugar intake and the animals that consumed more than the median were depicted as high consumers and those that consumed lower as low consumers. All tests were performed using Graphpad Prism 6 (Graphpad software Inc., la Jolla, CA, USA).

RESULTS

One Week of fcHFHS Diet Does Not Induce Hypothalamic ER Stress Markers

Rats exposed to the fcHFHS diet for 1 week were hyperphagic as shown by increased caloric intake compared to those on chow (**Table 3**). As a result, % WAT/BW and plasma leptin levels were significantly higher in rats on the fcHFHS diet compared to rats on chow whereas ΔBW did not differ between the groups (**Table 3**).

One week of fcHFHS diet exposure significantly increased ATF4 mRNA expression (**Figure 1A**). However, mRNA expression of CHOP, a target gene of ATF4, and BiP, a target gene of ATF6, was not significantly changed in hypothalami of fcHFHS-fed rats compared to chow-fed controls (**Figures 1B,C**). Splicing of usXBP1 to sXBP1 as shown by the ratio sXBP1/usXBP1 (**Figure 1D**), as well as DP5 and FasL mRNA expression did not differ between the groups.

Overnight Fat/Sugar Deprivation Induces ER Stress in Animals Exposed to the fcHFHS Diet for 1 Week

ΔBW, % WAT/BW and leptin were still increased in fcHFHS-fed rats compared to chow-fed rats when overnight deprived of fat and sugar (**Table 3**), but no changes were observed in hypothalamic ATF4, CHOP, and BiP mRNA expression between the groups (**Figures 2A–C**; left column). However, sXBP1/usXBP1 mRNA was higher and DP5 mRNA was lower in the fcHFHS-fed group compared to the chow controls (**Figures 2D,E**; left column).

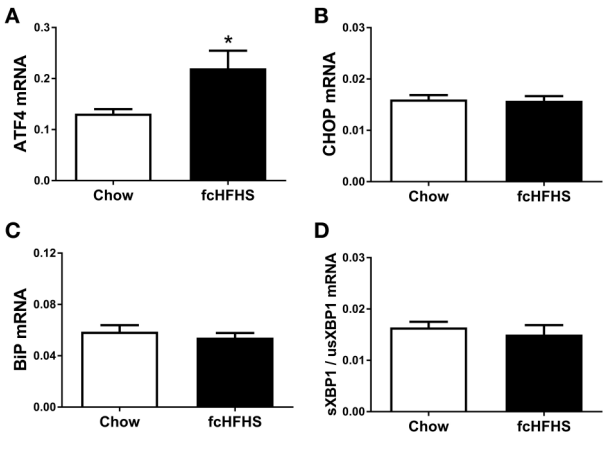


FIGURE 1 | ER stress markers in rat hypothalamus after 1 week *ad libitum* fcHFHS diet or chow. (A) ATF4 and **(B)** CHOP **(C)** BiP, and **(D)** sXBP1/usXBP1 mRNA expression. mRNA expression of specific genes was normalized to the geometric mean of three housekeeping genes. Significant differences between the fcHFHS and control group: *$p < 0.05$.

TABLE 3 | Characteristics of chow and fcHFHS animals.

Exp.	ΔBW (gr)		% WAT/BW		Leptin (ng/ml)		Average intake/day (Kcal)	
	Chow	fcHFHS	Chow	fcHFHS	Chow	fcHFHS	Chow	fcHFHS
1	34 ± 2.2	37 ± 2.3	2.58 ± 0.09	3.41 ± 0.05****	4.9 ± 0.8	7.6 ± 0.6**	76.0 ± 1.5	100 ± 2.5***
2	41 ± 3.4	52 ± 5.1	1.94 ± 0.13	2.81 ± 0.12***	2.01 ± 0.2	2.73 ± 0.3***	81.6 ± 4.4	122.1 ± 3.7****
3	51 ± 4.8	54 ± 3.7	2.20 ± 0.16	2.72 ± 0.16*	2.34 ± 0.3	3.24 ± 0.4**	88.0 ± 3.8	121.2 ± 5.5***

*Delta body weight, average food intake per day, % total white adipose tissue (WAT) relative to final body weight of animals and leptin plasma concentrations in experiment 1 (ad lib fed o/n), 2 (10 g chow fed o/n) and experiment 3 (fasted o/n) are shown as mean (n = 8) ± SEM. Total WAT represents the sum of mesenteric, peritoneal, subcutaneous and epididymal fat. Significant differences between the fcHFHS and chow control group: *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.*

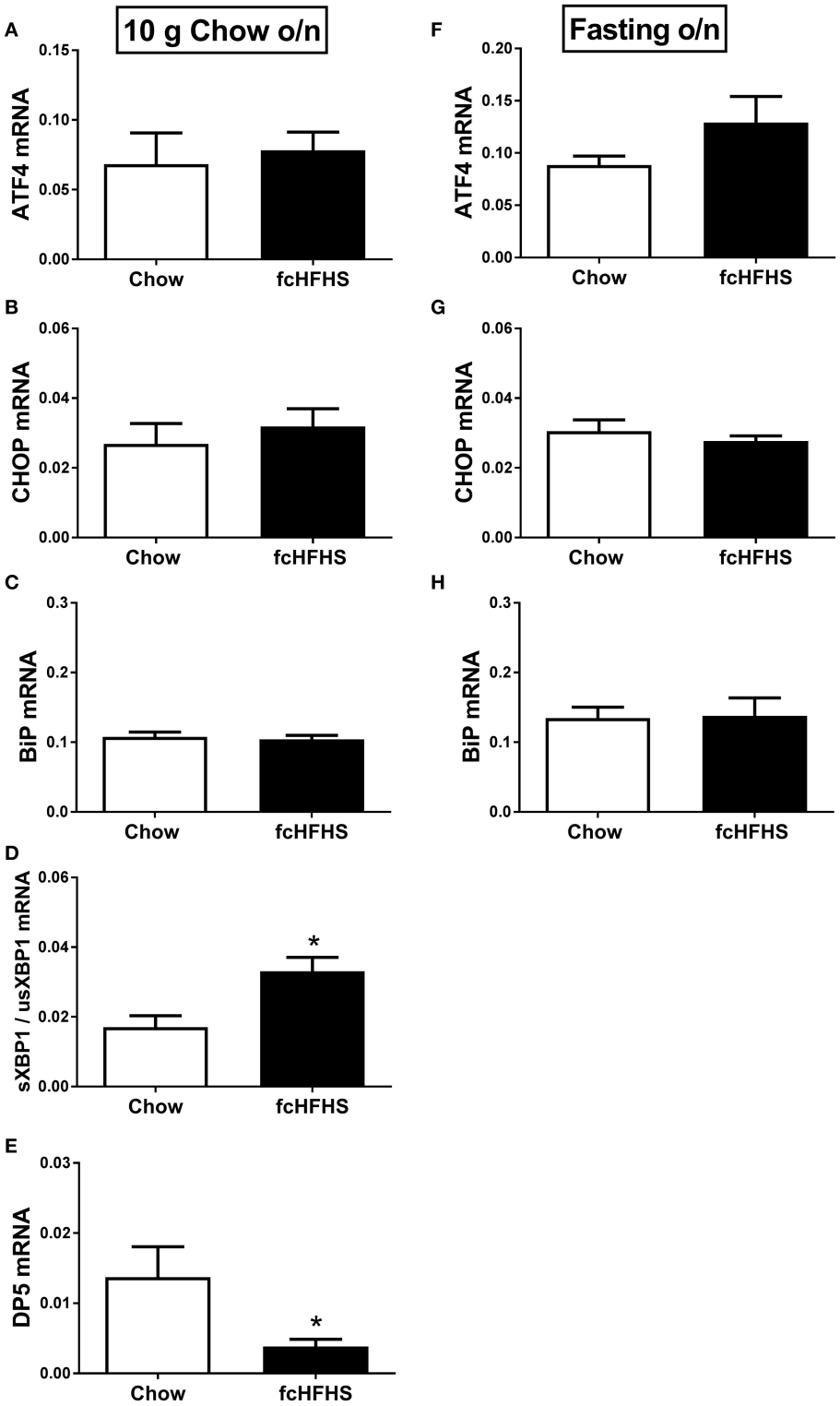


FIGURE 2 | The effect of overnight fat/sugar deprivation (10 g chow; left column) or fasting (right column) after 1 week fcHFHS diet or chow on the mRNA expression of hypothalamic (A,F) ATF4, **(B,G)** CHOP, **(C,H)** BiP, **(D)** sXBP1/usXBP1, and **(E)** DP5. mRNA expression of specific genes was normalized to the geometric mean of three housekeeping genes. Significant differences between the fcHFHS and control groups: $^*p < 0.05$.

No change in FasL mRNA expression was observed between the groups after removing fat and sugar overnight (not shown). Similarly, no differences in ATF4, CHOP, BiP, and FasL mRNA expression were observed between the groups after overnight fasting (**Figure 2**, right column). sXBP1/usXBP1 and DP5 mRNA expression were not detectable in the overnight fasted groups.

Fat Consumption Leads the Changes Induced in Stress Gene Markers

To determine to what extent fat or sugar contributed to the observed differences observed in hypothalamic mRNA, we divided the fcHFHS group according to fat and sugar intake using a median split forming either low or high fat consumers (LF or HF) or low or high sugar consumers (LS or HS). The median fat consumption was 10% out of total caloric intake. Rats that consumed <10% fat were assigned as LF consumers and those that consumed >10% as HF consumers. LF and HF consumers had similar BW, total caloric intake and chow intake, but fat consumption in HF group was significantly higher and sugar intake significantly lower compared to the LF group (**Table 4**). ATF4 mRNA expression tended to be higher ($p = 0.07$), in the hypothalamus of the HF consumers compared to the LF consumers, whereas sXBP1/usXBP1, DP5 and FasL mRNA expression was not different between the groups (data not shown). Similar analysis was performed for consumption of sugar over a week of fcHFHS exposure and median sugar consumption out of total caloric intake was 38%. No differences in ATF4, usXBP1, sXBP1/usXBP1, DP5, or FasL mRNA expression were observed between HS and LS consumers (data not shown).

To further investigate the direct effects of fat and sugar in the brain on hypothalamic ER stress markers, we infused IL, IL + glucose (ILG), glucose or saline (control) via the carotid artery directly to the brain of overnight fasted rats. IL and ILG increased hypothalamic ATF4 mRNA expression compared to saline but did not affect CHOP, DP5, and FasL mRNA (**Figures 3A–D**). In addition, IL infusion resulted in increased sXBP1/usXBP1 mRNA expression, and tended to increase BiP mRNA expression (**Figures 3E,F**). Glucose infusion did not have an effect on AFT4, sXBP1/usXBP1, and DP5 mRNA expression (**Figures 3G–I,K**), but reduced BiP and FasL mRNA levels compared to saline infusion (**Figures 3J,L**).

DISCUSSION

We showed that short term exposure to a fcHFHS diet, followed by mild food restriction overnight induces hypothalamic ER stress in rats as characterized by an increase in sXPB1/usXBP1 mRNA ratio in hypothalamus of fcHFHS fed rats compared to chow fed rats. Moreover, we showed that lipids, directly infused towards the brain of overnight fasted rats, are able to induce a similar response. As non-restricted *ad libitum* fcHFHS-diet fed or totally fasted rats do not show altered ratios, these data point to an interaction of lipid exposure to the brain and a negative energy balance in ER stress induction. In addition, we observed an increase in ATF4 mRNA when animals were *ad libitum* fed the fcHFHS diet which is abolished when rats are either provided with 10 g of chow overnight or totally fasted. In addition, high fat consumers on the fcHFHS diet have higher ATF4 mRNA, pointing to a direct role for fat intake in the increase in hypothalamic ATF4 mRNA. Indeed, ATF4 mRNA is also increased when animals are directly infused with lipids or lipids and glucose towards the brain, but not when glucose is infused alone.

An increase in sXPB1/usXBP1mRNA ratio implies activation of the IREα pathway (Yoshida et al., 2001; Calfon et al., 2002). The activation of IREα pathway due to HFD-feeding has been reported earlier. However, this was after long-term (8 or 20 weeks) exposure (Ozcan et al., 2009; Won et al., 2009). Here we showed for the first time that short term exposure to a fcHFHS diet is enough to induce activation of this pathway, but only under mild food restriction.

It is unclear why animals on a fcHFHS diet display this response only when food restricted while absent when animals are *ad libitum* fed overnight. One possibility might be that ER stress was induced via anabolic processes as illustrated by the observation that overnight fasting and subsequent refeeding increases sXBP1 mRNA and protein in the liver (Deng et al., 2013) and in the hypothalamus of mice (Williams et al., 2014). It is also possible that protective mechanisms play a role under *ad libitum* conditions. For example, we observed recently that 1 week fcHFHS diet increases beta- oxidation genes in the hypothalamus (Rijnsburger et al., 2016), and since enhanced beta-oxidation has been reported to counter palmitate-induced ER stress in *in vitro* models (McFadden et al., 2014), it is possible that we do not observe increased splicing of XPB1 under *ad libitum* feeding because of counter-active regulatory fatty acid oxidation. In line, we observed no changes in fatty acid oxidation genes after lipid infusion directly to the brain while it does induce XBP1 splicing (M. Rijnsburger, unpublished data). Further research is needed to determine the exact role of energy status and nutrient sensing on hypothalamic ER stress induction.

We observed a clear increase in hypothalamic ATF4 mRNA in rats on the fcHFHS diet while being *ad libitum* fed or

TABLE 4 | Characteristics of low fat and high fat consumers over 1 week of fcHFHS diet.

	Total (Kcal)	Chow (Kcal)	Fat (Kcal)	Sugar (Kcal)	BW (g)	% FAT/BW
HF	105 ± 2.3	50 ± 1.9	19 ± 1.40	36 ± 0.8	331 ± 1.8	3.26 ± 0.06
LF	98 ± 2.6	47 ± 3.0	6.9 ± 0.44**	49 ± 1.4**	331 ± 5.2	3.07 ± 0.08

*Average Total, Chow, Fat, or Sugar caloric intake as well as body weight and % Fat/BW of animals over 1 week on fcHFHS diet. High fat (HF) and low fat (LF) animals were determined using median split. The median of fat intake was 10% out of total caloric intake. Significant differences between the HF and LF group: **p < 0.01.*

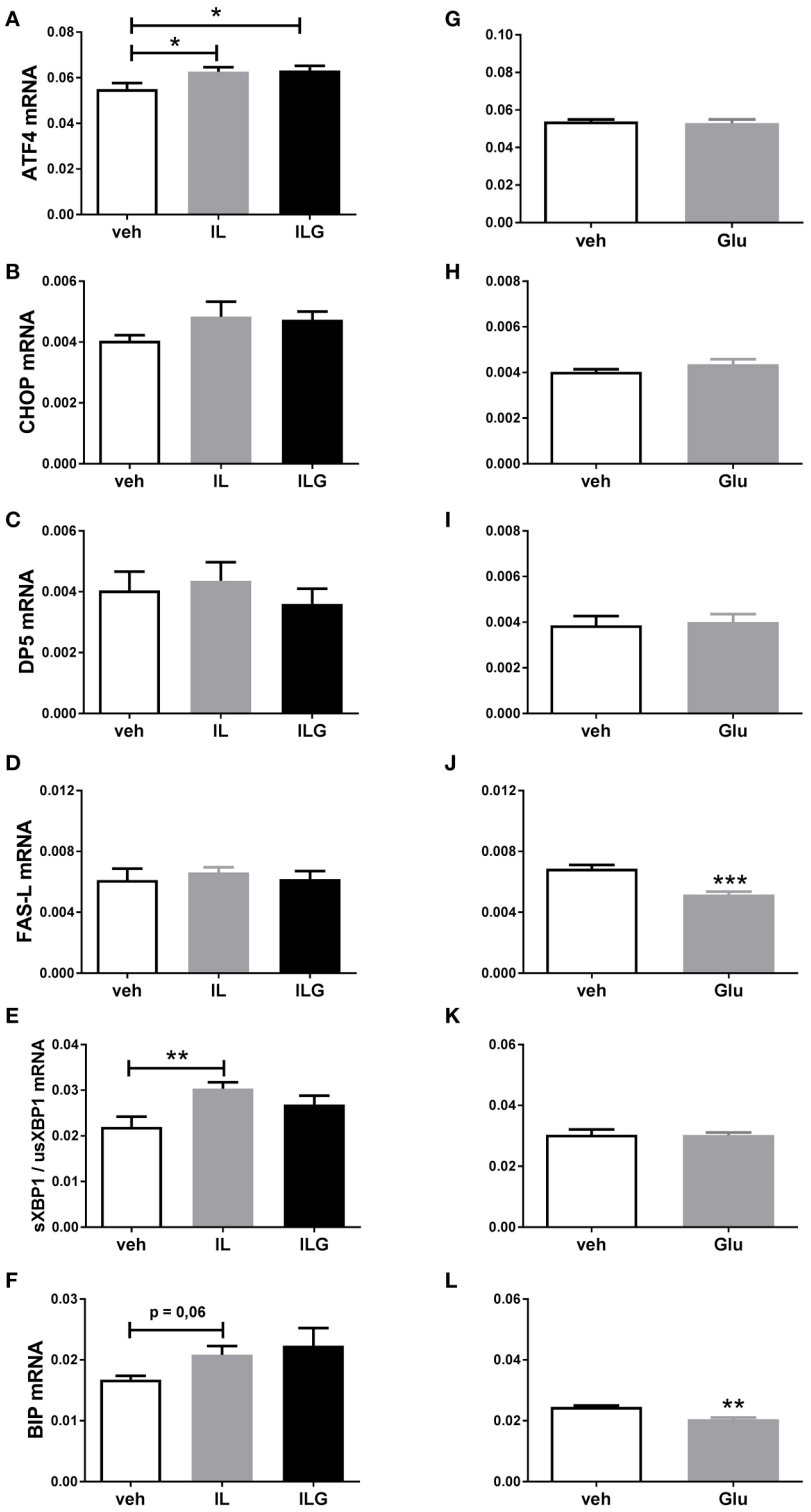


FIGURE 3 | The effect of central infusion of Intralipid (IL), Intralipid and Glucose (ILG) or Glucose on hypothalamic (A,G) ATF4, **(B,H)** CHOP **(C)**, **(C,I)** DP5, **(D,J)** FasL, **(E,K)** sXBP1/usXBP1, **(F,L)** Bip **mRNA expression in chow rats**. mRNA expression of specific genes was normalized to the geometric mean of three housekeeping genes. Significant differences between groups: $^{*}p < 0.05$, $^{**}p \leq 0.01$; $^{***}p < 0.001$.

when infused with intralipids via the carotic artery compared to vehicle infusions. However, we did not observe induction of downstream targets. It might be possible that increased ATF4 mRNA expression in our models is associated with other general stress related events within a cell i.e., amino-acid or glucose deprivation (Siu et al., 2002) or less protein availability (which has been shown in muscle to result in UPR activation; Deldicque et al., 2010). An additional effect of the fcHFHS diet is reduced chow—and thus protein—intake compared to chow controls, it is well possible that this might be the cause for the changes in ATF4 mRNA expression observed in our study. It is unclear at this point whether this increase in ATF4 mRNA will result in increased ATF4 protein. Interestingly, overexpression and inhibition of ATF4 in the hypothalamus induces hepatic insulin resistance and improves hepatic insulin sensitivity, respectively (Zhang et al., 2013). In addition, a recent study showed that ATF4 deletion in AGRP neurons of the hypothalamus specifically protects against high fat diet induced weight gain and insulin resistance (Deng et al., 2016). Together, this suggests an important role for hypothalamic ATF4 in the regulation of energy metabolism. Interestingly, we previously reported hepatic insulin resistance in rats on a fcHFHS diet (Diepenbroek et al., 2017), which could well be related to the observed increase in hypothalamic ATF4 mRNA.

Under severe ER stress, IREα activation can also lead to cell apoptosis via the JNK pathway (Todd et al., 2008) and to activation of apoptotic genes (Urano et al., 2000; Guan et al., 2006; Kim et al., 2006; Ma et al., 2007). We therefore measured DP5 and FasL mRNA, target genes of JNK pathway (Schenkel, 2004; Guan et al., 2006; Ma et al., 2007). However, 1 week fcHFHS diet did not result in activation of apoptotic gene expression. It could well be that this is due to the duration of the diet, as exposure to the diet for a longer period induces apoptosis accompanied by obesity and other metabolic disturbances (Ozcan et al., 2009; Won et al., 2009).

Interestingly a decrease in DP5 mRNA expression was observed in the fcHFHS group after overnight mild food restriction (10 g chow intake). That might be related to the increase in sXBP1 mRNA under the same conditions. Since both are regulated by IREα activation, the observed alterations suggest a shift from cell death related pathways to the induction of cell survival mechanisms. Like DP5, FasL mRNA can be also induced upon IREα/JNK activation during neuronal apoptosis (Le-Niculescu et al., 1999; Schenkel, 2004; Chen et al., 2015). However, no change was observed in FasL expression indicating that FasL does not play a major role in our experimental setting. In addition, a glucose infusion directly to the brain lowered FasL mRNA expression when compared to a vehicle control infusion, however what this means physiologically remains to be determined.

In summary, a fcHFHS diet and overnight fat and sugar availability affects the mRNA expression of hypothalamic ER stress related UPR markers. Overall, the UPR markers seemed to be a sensitive sensor of fatty acid availability as well as nutrient load. More studies are necessary to define the exact role of nutrients in induction of UPR intermediates that play a role in cellular metabolism and viability.

AUTHOR CONTRIBUTIONS

EB, AB, and Sl designed experiments. EB, MR, LE, UU, and Sl performed experiments. EB, AB, and Sl prepared the manuscript. WS, MR, LE, and UU edited the manuscript. The entire study was supervised by AB and Sl.

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REVIEW
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Lipid Processing in the Brain: A Key Regulator of Systemic Metabolism

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Metabolic disorders, particularly aberrations in lipid homeostasis, such as obesity, type 2 diabetes mellitus, and hypertriglyceridemia often manifest together as the metabolic syndrome (MetS). Despite major advances in our understanding of the pathogenesis of these disorders, the prevalence of the MetS continues to rise. It is becoming increasingly apparent that intermediary metabolism within the central nervous system is a major contributor to the regulation of systemic metabolism. In particular, lipid metabolism within the brain is tightly regulated to maintain neuronal structure and function and may signal nutrient status to modulate metabolism in key peripheral tissues such as the liver. There is now a growing body of evidence to suggest that fatty acid (FA) sensing in hypothalamic neurons *via* accumulation of FAs or FA metabolites may signal nutritional sufficiency and may decrease hepatic glucose production, lipogenesis, and VLDL-TG secretion. In addition, recent studies have highlighted the existence of liver-related neurons that have the potential to direct such signals through parasympathetic and sympathetic nervous system activity. However, to date whether these liver-related neurons are FA sensitive remain to be determined. The findings discussed in this review underscore the importance of the autonomic nervous system in the regulation of systemic metabolism and highlight the need for further research to determine the key features of FA neurons, which may serve as novel therapeutic targets for the treatment of metabolic disorders.

Keywords: lipid metabolism, brain, liver, energy homeostasis, hypothalamus

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INTRODUCTION

Metabolic disorders, particularly aberrations in lipid homeostasis, such as obesity, type 2 diabetes mellitus (T2D), non-alcoholic fatty liver disease, and hypertriglyceridemia, often manifest together as the metabolic syndrome (MetS) (1). Despite major advances in our understanding of the pathogenesis of these disorders, the prevalence of the MetS continues to rise (2). Since MetS constitutes an increased risk to cardiovascular morbidity and mortality (3), a more detailed understanding of the common causes, and integration between these disorders of energy homeostasis, is necessary to identify novel therapeutic targets and interventions that may halt the development of severe metabolic disease.

It is becoming increasingly apparent that the central nervous system (CNS) is a major contributor to the regulation of systemic metabolism and lipid balance. In the CNS, the nutritional status of the body is constantly being surveyed and assessed by key energy-sensing regions of the brain, such as the hypothalamus. Key nuclei within the hypothalamus, such as the ventromedial nucleus (VMH), arcuate nucleus (ARC), dorsomedial hypothalamic nucleus (DMH), and the paraventricular nucleus

(PVN), integrate signals to elicit peripheral responses, such as changes in feeding behavior, fuel mobilization, energy utilization, and energy storage (4). These nuclei detect both nutrients and nutritionally regulated endocrine factors, such as insulin (5), ghrelin (6), melanocortin (MC) (7), and leptin (8), in order to regulate feeding and energy balance. Here, in this review, we will focus on the mechanisms involved in lipid sensing in the brain and its emerging influence on systemic metabolism.

HOW DO LIPIDS ENTER THE BRAIN?

Lipids and lipid intermediates are essential components of the structure and function of the brain. In fact, the brain has the second highest lipid content behind adipose tissue, and brain lipids constitute 50% of the brain dry weight (9). However, unlike adipose tissue, which largely stores FAs as triglycerides for subsequent utilization and mobilization to other metabolic tissues, the brain is thought to mainly utilize acylated lipids to generate phospholipids for cell membranes (9). The FA composition of the brain is unique and is rich in long-chain polyunsaturated fatty acids (LC-PUFAs), particularly arachidonic acid (AA), eicosapentaenoic acid, and docosahexaenoic acid (DHA). Although some FAs can be synthesized *de novo*, essential FAs must be transported into the brain from the systemic circulation. And contrary to previously held theories, recent data suggest that this is a dynamic process, with up to 8% of LC-PUFAs actively being turned over daily and being replaced by plasma-derived FAs (10). Indeed, a number of studies have shown that FAs are able to cross the blood–brain barrier (BBB) and enter neurons. For example, radiolabeled FAs that are injected into the carotid artery of rats can be traced to neuronal cells (10). In addition, brain perfusion studies in rats have shown that radiolabeled palmitate is readily incorporated into cerebral phospholipids and neutral lipids and has a similar rate of uptake when delivered *via* whole rat plasma or a synthetic saline containing physiological levels of albumin. This suggests that FAs cross the BBB and that albumin may be important in this process (11). Even though these studies convincingly demonstrate FA uptake, they do not address the mechanism of transport.

How FAs enter the brain remains an unanswered fundamental question. Although we cannot rule out the possibility that FAs could passively diffuse across the BBB, several studies highlight the role of FA transporters in this process. The membrane localized FA transport proteins (FATP1 and FATP4) appear to be the predominant FA transport proteins expressed in the BBB based on human and mouse expression studies, whereas the FA translocase/CD36 plays a prominent role in the transport of FAs across human brain microvessel endothelial cells (12). Furthermore, cytosolic-localized fatty acid-binding protein 5 has an important function in FA transport across cultured brain microvascular cells (13). Although still under active investigation, is it plausible that some transporters may also exhibit specificity toward particular FAs. For example, the major facilitator superfamily d 2 a, which is exclusively expressed in the endothelium of the BBB, has been shown to selectively transport DHA in the form of the partially hydrolyzed phospholipid, lysophosphatidylcholine, otherwise known as lysolecithin (14).

NEURONAL UPTAKE OF LIPIDS AND FAs

Neuronal uptake of FAs remains an important yet poorly understood process. However, it is possible that once FAs have traversed the BBB, FA transporters may also play a key role in facilitating FA uptake into neurons. For example, dissociated neurons from the VMH express both FATP1 and CD36 (15). Specifically, important studies using fura-2 calcium imaging and fluorometric imaging plate reader membrane potential dye, in addition to pharmacological manipulations have shown that while neurons of the VMH and ARC respond to oleic acid (OA, C18:1 n-9) (15, 16), this response is lost when CD36 is depleted in the VMH using an adeno-associated viral (AAV) vector expressing CD36 short hairpin RNA (17). Since CD38 is an established gustatory lipid sensor, it is also plausible that other lipid sensors involved in the chemoreception of long-chain fatty acids (LCFAs)/omega-3 fatty acids (ω-3 FAs), such as GPR120, are also involved in neuronal lipid sensing. However, although GPR120 is functionally active in immortalized hypothalamic neurons and mediates the anti-inflammatory actions of the ω-3 FA, DHA (18), its role in neuronal lipid sensing *in vivo* has not been determined. In addition, FABP3, which is localized in neurons, facilitates brain AA but not palmitic acid (C16:0) uptake and trafficking into specific brain lipid pools (19).

It is also becoming more widely accepted that neurons may receive metabolic support from glial cells in a variety of forms. The "astrocyte–neuron lactate shuttle" has been postulated, whereby astrocytes metabolize glucose to release lactate, which is then taken up by the neuronally expressed monocarboxylate transporter, providing a supplemental energy source for neurons (20). Moreover, lipid metabolism in astrocytes plays a key role in FA sensing, since FA oxidation is thought to occur predominantly in the astrocyte rather than the neuron. When the levels of FAs are increased, as in the case of a high-fat diet (HFD), astrocyte-mediated lipid oxidation results in elevated ketone levels in the brain. Conversely, changes in ketone abundance within energy-sensing regions of the hypothalamus are able to modify energy homeostasis. Specifically, reduced ketone production within the VMH and ARC signals a decrease in HFD intake, and ketone production can override glucose and FA sensing in VMH neurons (21, 22). Astrocytes may play a key role in the regulation of energy balance by sensing LCFAs as metabolic signals. For example, hypothalamic but not cortical astrocytes have a high capacity for the oxidation of LCFAs (23), and this flux may be AMP-activated protein kinase (AMPK) dependent. Both neurons of the VMH and astrocytes have been shown to express many of the FA transporters, including FATP1, FATP4, and CD36. Most recently, the fatty acid bind protein 7 (FABP7) was shown to be important for astrocyte–neuron lipid homeostasis. Mice lacking FABP7 develop neuropsychiatric disorders such as schizophrenia, which may at least in part be due to aberrant dendritic spine morphology, and decreased spine density compared to WT mice (24). Moreover, transplantation of WT astrocytes into FABP7 KO mice partially attenuated cognitive impairments (24). Once transported into the cell, LCFAs are esterified by acyl-CoA-binding protein (ACBP), a protein that is ubiquitously expressed in tissues with high lipid turnover. Interestingly, ACBP is expressed in the hypothalamus

and may play a role in the LCFA sensing by hypothalamic astrocytes (25).

Astrocytes are also critical for the regulation of cholesterol homeostasis in the neuron. Cholesterol is an essential component of neuronal physiology during both development and adulthood and is independently tightly regulated in the brain, largely due to the existence of the BBB (26). Cholesterol depletion in neurons impairs vital functions, including synaptic vesicle exocytosis, neuronal activity, and neurotransmission and results in synaptic loss and neurodegeneration (27, 28). Clinically, deficits in cholesterol homeostasis in the CNS manifest as severe primary neurological disorders such as Neimann–Pick C disease and Parkinson's disease (26). It is thought that astrocytes are a major site of lipoprotein synthesis and assembly in the brain (29). Of particular relevance is apolipoprotein E (ApoE), a 39-kDa protein that is highly expressed in the brain, surpassed only by hepatic ApoE production (30).

Apolipoprotein E-containing lipoproteins have been extensively studied and are known to have several major functions. For example, ApoE-containing high-density lipoprotein (HDL)-like lipoproteins are secreted by astrocytes and are taken up into neurons *via* low-density lipoprotein receptors. This transfer of key lipids and cholesterol facilitates axonal extension and neuronal survival and requires the presence of sphingomyelin in the ApoE-containing lipoprotein particle (31, 32). ApoE does not only play a major role in glia-neuronal lipid metabolism but also acts as a ligand for multiple receptors in neurons, which interact with a number of downstream physiological processes (33). In particular, a number of recent studies have shown that brain ApoE is an important regulator of peripheral energy homeostasis. In rats, intracerebroventricular (ICV) ApoE injections significantly decreased food intake, whereas infusion of ApoE antiserum stimulated feeding, therefore suggesting that ApoE may be important satiety factor in the hypothalamus (34). In support, exogenous ApoE treatment has been shown to activate phosphatidylinositol-3-kinase (PI3K)/Akt signaling, and PI3K inhibition by LY294002 attenuates both ApoE-induced signaling and satiation (35).

Apolipoprotein E has three major isoforms (ApoE2, ApoE3, and ApoE4), which have varying effects on lipid homeostasis and neuronal function. While both ApoE2 and ApoE3 preferentially associate with phospholipid rich HDL-like particles, ApoE4 prefers large triglyceride-rich VLDL particles (36). Interestingly, ApoE4 has been repeatedly implicated in the pathogenesis of Alzheimer's disease (AD) and may bind to the Aβ peptide leading to impair Aβ clearance (37). Current hypothesis suggests that the proteolytic cleavage products of ApoE4 may have a central role in AD pathology, since AD patients have markedly increased levels of these cleavage products compared to controls (38).

These findings suggest that lipoprotein metabolism in the CNS may be key to lipid homeostasis and nutrient sensing; however, to date, the molecular mechanisms underlying these processes remain largely unknown. Nonetheless, we have recently shown that lipoprotein lipase (LPL), the rate-limiting enzyme in the hydrolysis of lipoprotein-derived FAs, may facilitate the uptake of FAs into dissociated hypothalamic neurons (39). Moreover, mice with a specific neuronal LPL deficiency exhibit a defect in nutrient sensing and become hyperphagic, relatively inactive and obsessed compared to WT control mice (40). Interestingly, these mice are also polyunsaturated fatty acid (PUFA) deficient in the hypothalamus (40), and in the hippocampus, where LPL deficiency is also associated with alterations in learning and memory and synaptic function (41). In further support, a specific deletion of LPL in the hippocampus also leads to increased weight gain and decreased activity *via* a ceramide-dependent pathway (42). We have also shown that while mice lacking pan-neuronal LPL develop obesity, this obesity is not exacerbated on an HF diet, or rescued by a PUFA enriched diet, further highlighting the importance of LPL in lipid sensing and body weight regulation (43). These data highlight the potential role of LPL as a mechanistic link between brain lipid uptake, neuronal lipoprotein metabolism, and nutrient sensing; however, the precise role of LPL in lipid sensing in both neurons and glia requires further investigation.

HYPOTHALAMIC FA METABOLISM

The evidence for facilitated FA uptake into neurons is limited, which may be due to the long-held view that neurons do not derive much of their energy supply from lipids. However, neurons do express many molecular components of lipid catabolism pathways, suggesting that lipid utilization is a critical process to neuronal function. For example, neurons of the VMH express enzymes involved in the intracellular metabolism of FAs, including long-chain acyl-CoA synthase (ACS), carnitine palmitoyltransferase-1a and 1c (CPT-1a and 1c), and uncoupling protein-2 (UCP2), and enzymes involved in *de novo* lipogenesis, such as fatty acid synthase (FAS) (15). In addition, the nuclear receptor peroxisome proliferator-activated receptor (PPARγ), a key factor in lipid metabolism that can be activated by endogenous lipid ligands to promote adipogenesis and insulin sensitivity, is predominantly expressed in neurons of the hypothalamus (44), including the ARC.

Activation of these intracellular metabolic pathways in hypothalamic cells in response to FAs provides further support of the role of lipid metabolism and sensing in hypothalamic neurons. For example, UCP2, which increases proton leak from the respiratory electron transport chain, may act as a metabolic switch from glucose metabolism to mitochondrial FA oxidation in hypothalamic NPY/AgRP neurons during fasting (45). Interestingly, UCP2 likely mediates the actions of ghrelin, which is increased upon fasting, on the activation of NPY/AgRP neurons. While ghrelin increases palmitate-induced mitochondrial respiration, this is not observed in UCP2−/− mice. Moreover, NPY/AgRP neurons of UCP2−/− mice do not show the ghrelin-induced increase in mitochondrial biogenesis (45). In normal circumstances, increased FA oxidation increases reactive oxygen species (ROS), which are then scavenged by UCP2 *via* enhanced proton leak. However, in UCP2−/− mice, these ROS levels remain increased, supporting the hypothesis that ghrelin-triggered ROS production promotes UCP2 activity and mRNA expression to further promote ROS scavenging (45). These studies also suggest that ROS may be involved in neuronal lipid metabolism and activation. In further support, suppression of ROS activates NPY/AgRP neurons to promote feeding, whereas activation of ROS activates proopiomelanocortin (POMC) neurons to reduce

feeding. Although further studies are warranted, it is likely that neuronal ROS accumulation may intrinsically link neuronal substrate metabolism to feeding behavior and systemic energy balance (46).

AMP-activated protein kinase may also act as a cellular energy sensor within neurons to link neuronal lipid metabolism to systemic lipid metabolism and energy balance. AMPK is widely expressed in the ARC, PVN, and VMH of the hypothalamus and is able to sense intracellular energy status by the AMP/ATP ratio and the level of adipokines (e.g., leptin and ghrelin) [see Ref. (47) for a comprehensive review]. Activated AMPK responds to the cellular energy status and can switch cellular metabolism toward catabolic processes that produce ATP and away from anabolic processes that consume ATP (48). In response to glucose, hypothalamic AMPK activity is inhibited leading to the activation of acetyl-CoA carboxylase (ACC) and the generation of malonyl-CoA from glucose-derived acetyl-CoA, with downstream effects on food intake (described in more detail below) (49). Similar to peripheral tissues, malonyl-CoA is thought to inhibit CPT-1a and LCFA oxidation in the brain (49). Specifically, glucose inhibits palmitate oxidation *via* AMPK in hypothalamic neurons (23). Hypothalamic AMPK may also regulate downstream lipid metabolism through brown adipose tissue (BAT) thermogenesis. A number of recent studies have suggested that hypothalamic AMPK is involved in the autonomic regulation of BAT thermogenesis, specifically the SNS. For example, central administration of 3,3′,5′-triiodothyronine (T_3) within the VMH stimulates a thermogenic response associated with decreased AMPK activity in the VMH and elevated sympathetic firing in BAT (50). Whether this effect is observed following neuronal glucose (or lipid) sensing remains to be seen. Nonetheless, this interesting topic has recently been reviewed in detail (51).

AMP-activated protein kinase also plays a key role in the hypothalamic response to leptin in the context of high-fat feeding. While leptin results in reduced food intake and reduced hypothalamic AMPK activity, these effects were not observed in diet-induced obese mice (52). Hypothalamic phospho-AMPK is also modulated by the FA composition of the diet and is dependent on brain region and metabolic status (53). Interestingly, AMPK regulates the activity of a number of enzymes involved in the synthesis of complex lipids that are critical for optimal brain function and metabolism. For example, long-term AMPK stimulation blunted FA-mediated induction of serine palmitoyl transferase and the synthesis of ceramides *de novo* and has thus been shown to protect against fatty acid-mediated apoptosis in the astrocyte (54). Similarly, AMPK is highly expressed in neurons due to their high-energy demands and can promote neuronal survival during periods of glucose deprivation (55). Recently, neuronal AMPK has also been implicated in the pathogenesis of AD. AMPK activity can reduce sphingomyelin levels, inhibit Aβ generation, and reduce amyloid precursor protein (APP) distribution in lipid rafts, whereas deletion of AMPKα2 increases sphingomyelin and APP distribution in lipid rafts (56).

There is growing evidence to suggest that the accumulation of FA metabolites may signal nutrient status and thus may be critical to central lipid sensing, and the modulation of systemic metabolism. For example, upon entry into the neuron, LCFAs are esterified to LCFA-CoA by ACS. It is thought that this accumulation of FA derived LCFA-CoA triggers a lipid-sensing mechanism to inhibit hepatic glucose production (HGP) and to maintain systemic glucose homeostasis (57). In support, direct inhibition of ACS in the hypothalamus disrupts the accumulation of hypothalamic LCFA-CoA, and in turn disrupts the inhibitory effect on hepatic gluconeogenesis, resulting in dysregulated glucose production (58).

In metabolic tissues, intracellular LCFA-CoAs enter the mitochondria *via* CPT-1, where they are then subject to FA β-oxidation. Importantly, the liver isoform of CPT-1, CPT-1a, is prevalent in the hypothalamus, and inhibition of hypothalamic CPT-1a causes an increase in intracellular LCFA-CoA, which triggers a satiation signal, leading to reduced systemic glucose production and food intake (59). However, the neurocircuitry is complex since the role of CPT-1a may vary between hypothalamic nuclei. For example, VMH-selective overexpression of CPT-1a causes over feeding, a phenotype which can be reversed with the CPT-1 specific inhibitor etomoxir (60). In support, long-term overexpression of permanently activated CPT-1a using a viral AAV vector injected into the VMH of rats, leads to hyperghrelinemia, increased food intake, increased body weight, hyperglycemia, and insulin resistance (61). In contrast, CPT-1a expression in the ARC does not have the same effect on the central regulation of feeding (60). Interestingly, the brain also expresses a neuron-specific isoform of CPT-1, CPT-1c, which is found in the endoplasmic reticulum (ER) of key energy-sensing nuclei of the hypothalamus (ARC) and has also been repeatedly implicated in the modulation of systemic metabolism (62–64). Specifically, CPT-1c KO mice have reduced body weight and food intake compared to control mice (62, 63). CPT-1c does not have a typical acyltransferase activity, and thus its precise molecular function is less well understood. Nonetheless, data from recent studies suggest that the orexigenic action of ghrelin is associated with increased hypothalamic (C18:0) ceramide levels, an effect that is blunted in CPT-1c KO mice (65). While the mechanism linking increased ceramide levels to energy balance remain an active area of research, recent studies have demonstrated that ceramides induce hypothalamic lipotoxicity and ER stress, which leads to sympathetic inhibition, reduced BAT thermogenesis, weight gain, and hepatic steatosis (66). In addition, recent metabolomic analysis of the brains of CPT-1c KO mice shows reduced levels of oxidized glutathione, suggesting that CPT-1c may play a role in neuronal oxidative metabolism (67). In addition, these mice show suppressed endocannabinoid levels, which offers an alternative yet consistent mechanism to account for the suppressed food intake observed in CPT-1c KO mice (67). In addition, to their established role in appetite modulation, endogenous endocannabinoids may serve as functional neuromodulatory lipids, derived from neuronal phospholipids, which once secreted undergo lipid catabolism within glial cells. A detailed description of endocannabinoid signaling is beyond the scope of this manuscript but has been previously reviewed in depth (68).

CPT-1 has been suggested to act downstream of malonyl-CoA in the hypothalamic control of feeding (60). This is particularly pertinent to FA metabolism in hypothalamic neurons since CPT-1 activity is inhibited by malonyl-CoA (69), and thus

elevated malonyl-CoA leads to an accumulation of LCFA-CoA (70), which has been previously referred to as a satiety signal. Indeed, manipulation of the key enzymes involved in malonyl-CoA metabolism, including ACC, FAS, and malonyl-CoA decarboxylase (MCD), have all been shown to have major effects on food intake and peripheral metabolism (71). For example, ACC, which catalyzes the carboxylation of acetyl-CoA to malonyl-CoA, is expressed in the ARC of the hypothalamus, where it is increased following central leptin treatment resulting in elevated malonyl-CoA and reduced food intake (72). Similarly, inhibition of FAS activity by central administration of the pharmacological FAS inhibitor C75 has been shown to reduce food intake (73) and to elevate malonyl-CoA levels (74). MCD, which is important for malonyl-CoA degradation, is also important for regulating lipid intermediates and systemic metabolism. Overexpression of MCD in the mediobasal hypothalamus (MBH) chronically reduces malonyl-CoA levels and causes rapid increases in food intake and weight gain (75). In addition, MBH expression depleted both malonyl-CoA and LCFA-CoA and was sufficient to induce hepatic insulin resistance in the presence of hyperlipidemia (75). These studies begin to highlight the importance of hypothalamic sensing of circulating lipids in the maintenance of hepatic metabolism and systemic glucose homeostasis. However, the physiological and molecular mechanisms that integrate the brain–liver axis remain unresolved and an active area of investigation.

HYPOTHALAMIC LIPID SENSING AND HEPATIC METABOLISM

Early studies by Obici and colleagues, where OA was administered into the brain *via* ICV injection, were among the first to demonstrate the profound central effects of LCFAs on peripheral metabolism. Interestingly, the short chain FA octanoic acid (C8) did not have the same effect. Moreover, these studies showed that central OA administration could lower plasma insulin and glucose levels under basal physiological conditions. To determine the mode of blood glucose reduction, pancreatic euglycemic clamps were performed during ICV infusion of OA. ICV OA administration leads to a marked decline in glucose production compared to basal levels (59). However, when clamps were performed in the presence of ICV OA and sulfonylurea, a potent inhibitor of neuronal K_{ATP} channels, the profound effect of central OA on HGP was blunted, suggesting that OA acutely enhances hepatic insulin action *via* the activation of K_{ATP} channels in the hypothalamus (59). Further studies support this notion and have shown that pharmacological activation of K_{ATP} channels in the hypothalamus (*via* hypothalamic diazoxide administration) suppresses HGP (76). These observations, taken together with the findings from CPT-1 inhibition studies, strongly suggest that the accumulation of LCFA-CoAs is able to suppress HGP (70). Recent studies have suggested that this system may actually be even more complex and have shown that the effect of LCFAs on hepatic metabolism may be FA specific. For example, while bilateral infusion of OA into the MBH results in a significant reduction in HGP, palmitic acid (PA, C16:0) has a lesser effect, and linoleic acid has no effect compared to vehicle control (LA, C18:2 n-6) (77). These data suggest that mono-unsaturated fatty acids are a more potent suppressor than saturated fatty acids or PUFAs; however, the mechanisms underlying these differential effects of FAs on hepatic glucose metabolism remain to be determined.

In addition to the central regulation of HGP, hypothalamic nutrient sensing may also be a key regulator of hepatic lipid homeostasis. The liver maintains lipid homeostasis through tightly coordinated synthesis and secretion of triglyceride-rich lipoproteins (VLDL-TG), lipogenesis, and FA oxidation. Interestingly, infusion of the orexigenic neuropeptide Y directly into the third ventricle of the hypothalamus has been shown to increase hepatic VLDL-TG secretion, which may be part of the physiological response to fasting when lipids become the main energy source and NPY neurons in the ARC of the hypothalamus are activated (78). Thus, VLDL-TG secretion may be a response by the autonomic nervous system (ANS) to mobilize lipids during a period of relative nutrient deficiency. In addition, sympathetic denervation prevented the increase in the VLDL-TG secretion in the fasted state, whereas total denervation, or parasympathetic denervation did not, suggesting that the central regulation of hepatic lipid mobilization during fasting may be largely mediated through the sympathetic nervous system (78). In further support, sympathetic hepatic denervation also prevented the stimulatory effect of NPY on VLDL-TG secretion (78). This is in contrast to glucose sensing in the MBH (79), and glycine in the dorsal vagal complex (DVC) (80), which is thought to signal sufficient nutrient status and inhibit VLDL-TG secretion, possibly through the parasympathetic nervous system.

In addition to NPY, a number of studies have also shown that MC expressing neurons of the hypothalamus may also regulate hepatic lipogenesis and TG metabolism. Central administration of MTII, a synthetic MC3/4 receptor agonist, has been shown to reduce hepatic lipogenic gene expression in mice (81) and decrease hepatic TG content in rats (82), suggesting that increased MC signaling can inhibit hepatic VLDL-TG production. In support of this notion, central administration of an MC3/4 receptor antagonist, markedly increased liver TG content in rats, strongly suggesting that hepatic lipogenesis was increased (7).

Melanin-concentrating hormone (MCH) is also involved in the neuronal circuits that modify autonomic outflow to the liver and white adipose tissue. MCH-deficient mice are hyperphagic and lean when fed a normal diet (83), are resistant to age associated insulin resistance (84), and when fed an HFD they are resistant to obesity and hepatic steatosis (85). Recent key studies have also shown that genetic activation of MCH specifically in the LH triggers hepatic lipid accumulation and lipoprotein uptake (86).

This neurocircuitry is relevant to the pathogenesis of obesity, since numerous models of obesity and diabetes are characterized by elevated NPY. In a recent study, ICV administration of NPY or a selective NPY Y1 receptor agonist was shown to robustly elevate key genes involved in MUFA synthesis, such as stearol-CoA desatrate-1, and PL remodeling, such as ribosylation factor-1 (ARF-1) and lipin-1 (87). Importantly, these effects were attenuated following sympathetic denervation of the liver,

supporting a model in which central NPY modifies hepatic PL and VLDL *via* the sympathetic signaling to the liver (87). Although these findings suggest that central lipid sensing may be implicated in the regulation of hepatic lipid homeostasis (see **Figure 1**), the direct mechanisms remain elusive. Nonetheless, a number of recent studies highlight the role of central FAs in hepatic lipid homeostasis. For example, central administration of PA resulted in impaired leptin signaling and pro-inflammatory response in the MBH and PVN (88). Furthermore, this was coupled with blunted leptin-induced changes in hepatic gluconeogenesis, glucose transportation, and lipogenesis (88). In a recent report, Yue and colleagues have shown that infusion of OA directly into the MBH activates a PKC-δ to K_{ATP} channel axis, which suppresses VLDL-TG secretion in rats (89). Moreover, this signaling requires DVC and hepatic innervation, highlighting a novel MBD-DVC neurocircuitry that mediates MBH FA sensing and hepatic lipid homeostasis (89). These findings are one step closer to the development of novel therapies that lower VLDL-TG secretion and restore lipid homeostasis in metabolic disorders; however, there is considerably more to learn regarding differential function of other hypothalamic neurons and their role in the autonomic regulation of hepatic metabolism.

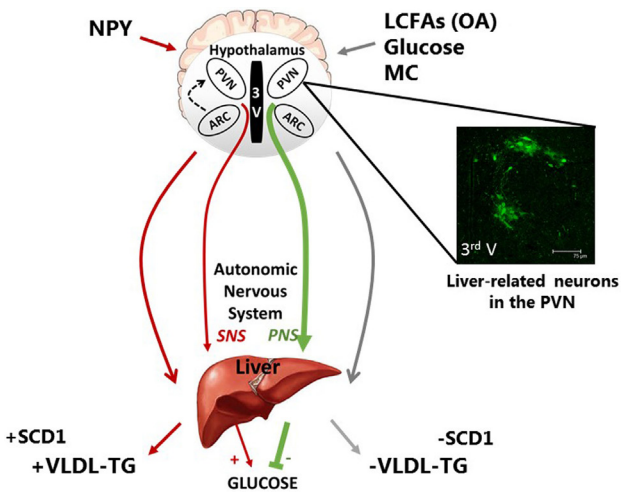


FIGURE 1 | Schematic representation of the neuronal regulation of hepatic carbohydrate and lipid metabolism. Neurons of discrete hypothalamic nuclei including the paraventricular nucleus (PVN) and arcuate nucleus (ARC) within the mediobasal hypothalamus respond to neuronal inputs to regulate hepatic glucose and very low density lipoprotein (VLDL-TG) metabolism. The accumulation or application of long-chain fatty acids (LCFAs), such as oleic acid (OA), glucose, and melanocortin (MC) signaling act through the autonomic nervous system to signal nutritional plenty a reduced requirement for energy substrate mobilization, by inhibiting VLDL-TG secretion and hepatic glucose production (HGP). This is at least in part due to reduced stearol-CoA desatrate-1 (SCD1) expression. In contrast, hypothalamic NPY is indicative of hunger and nutritional deficiency and can increase energy substrate mobilization *via* an increase in VLDL-TG and SCD1 expression and HGP. The features of these fatty acid and nutritionally sensitive neurons are unclear; however, pseudorabies virus-152 labeling supports the notion that liver-related neurons in the PVN exist and may be involved in this autonomic regulation of hepatic metabolism.

LIVER-RELATED NEURONS IN THE HYPOTHALAMUS

Recent findings support the existence of liver-related neurons in key hypothalamic nuclei; therefore, we can speculate that these liver-related neurons are the link between central lipid sensing and hepatic lipid metabolism. The control of hepatic functions by the ANS is well known. In general, beside the abovementioned examples, sympathetic stimulation of the liver enhances endogenous glucose production and glycogenolysis, while the parasympathetic nerves are responsible for inhibiting glucose production and promoting glucose storage (90–92). Preganglionic neurons are located in the spinal cord and brainstem, respectively, and transmit the information through the sympathetic and parasympathetic nerves. These autonomic motor neurons receive information from preautonomic neurons, which are located in higher brain areas and crucial for integration of brain signals (93, 94). Therefore, identifying the location of liver-related neurons and determining their cellular and molecular properties would be crucial to the understanding of brain–liver circuit.

Within the hypothalamus, the ARC, VMH, DMH, LH, and PVN are well recognized for their involvement in the regulation of a variety of metabolic functions of the body, and in particular the control of hepatic metabolism (95–101). Electrical and chemical stimulation have been feasible methods to establish direct connections between the hypothalamus and sympathetic neurons in the spinal cord or parasympathetic neurons in the brainstem and to demonstrate the importance of the autonomic control in hepatic functions (98, 102–105). On the other hand, establishing the location of premotor inputs to the sympathetic and parasympathetic motor neurons has been more challenging. Anterograde and retrograde tracers in combination with histochemical studies provided valuable information on the connections between the brain and liver (106), and the development of retrograde viral tracers opened new avenues to dissect the brain–liver pathway. Currently, transsynaptic neurotropic viruses are very valuable tools for identification of synaptic connections and neural networks. Among the neurotropic viruses, pseudorabies viruses (PRVs) are often used for circuit analysis and revealing organization of the nervous system (107–110). PRVs, such as PRV-152, an attenuated viral strain driving the expression of EGFP, are reliable and effective transsynaptic tracers, and numerous publications reported consistent organ-specific labeling of neurons (111–115). The spread of PRV-152 is strictly retrograde across synapses, and the virus is not capable of assembling in axons or glia; therefore, labeling of neurons not specific to the liver is unlikely, as has been shown (110, 116–118).

Polysynaptic neural connections between the brain and liver were identified in rodents using PRVs (118–120). PRV labeling was observed in the spinal cord of rats 3 days following inoculation, whereas at this time point no labeling was detected in parasympathetic nuclei (119). Labeled neurons in the dorsal motor nucleus of the vagus (DMV) were observed 4–5 days after inoculation of the liver. At this time point, liver-related neurons were also detected in the brainstem including the ventrolateral medulla, NTS, raphe pallidus, and few neurons were identified in the hypothalamic PVN and LH (119). Longer survival time

provided labeling of liver-related neurons in nuclei connected to the PVN including the medial preoptic area, anterior hypothalamic area, and ARC. PRV-labeled cells were also present in the VMH, suprachiasmatic nuclei, central amygdala, and bed nucleus of stria terminalis (119). This study demonstrated that PRV provides reliable identification of polysynaptic sympathetic and parasympathetic pathways to the liver (113, 115, 118, 120).

Despite the identification of liver-related neurons in the CNS, labeling with PRV does not distinguish between sympathetic and parasympathetic liver-related neurons in higher brain areas. In order to identify sympathetic- or parasympathetic liver-related hypothalamic neurons in rats, PRV inoculation was combined with hepatic sympathectomy or parasympathectomy (121, 122). Following sympathetic denervation of the liver, parasympathetic liver-related neurons were identified in the brainstem DMV and nucleus ambiguus (122). Medium length survival time (~4 days) resulted in labeling of additional brainstem areas (e.g., NTS, area postrema) and hypothalamic nuclei including PVN, LH, DMH, ARC, and others (122). Retrograde labeling following parasympathectomy identified pre-sympathetic liver-related neurons in the PVN, medial preoptic area, anterior hypothalamic area, DMH, ARC, VMH, and SCN (121). These studies revealed that preautonomic PVN neurons project either to sympathetic or parasympathetic division, and the segregation of the neurons exists in other hypothalamic areas including LH and SCN suggesting functional specialization of preautonomic neurons controlling liver function. The segregation of the autonomic divisions was also supported by the observation that stimulation of PVN resulted in hyperglycemia largely due to sympathetic activation of the liver (121). On the other hand, we have to note, that viral injection into peripheral organs causes infection of nerve terminals innervating both vascular and non-vascular tissues (123). PRV injected into the liver is taken up by nerve endings near hepatocytes and sinusoidal cells, and we cannot distinguish between neurons innervating liver function or hepatic vasculature at the injection site. Therefore, it is likely that the PRV-labeled pre-sympathetic neurons contribute to both vasomotor and non-vasomotor sympathetic innervation of the liver.

Our current knowledge regarding the role and cellular properties of liver-related hypothalamic neurons is somewhat limited. Earlier studies suggested that the VMH is involved in the sympathetic control of the liver, whereas the LH plays role in the parasympathetic control of the liver (91, 124, 125). The PVN was also shown as an important, integrative center for the regulation of sympathetic and parasympathetic pathways to the liver (121, 126–128). Hypothalamic action of metabolic signals including insulin, leptin, and FAs has been shown to control glucose homeostasis (58, 59, 76, 129–133); however, the cellular properties, the phenotype, or the involved neural circuits underlying the central control of hepatic functions are less defined.

Hypothalamic nuclei are heterogeneous containing different types of neurons, which are able to control multiple organ systems; therefore, identification of pathway-related neurons is crucial. Our laboratory used the retrograde PRV tracing technique, discussed above, to identify liver-related neurons in the PVN and to reveal synaptic properties of liver-related neurons in control and hyperglycemic conditions (113). The studies determined that liver-related neurons receive transient receptor potential vanilloid type 1 (TRPV1) containing inputs. TRPV1 is a non-selective cation channel and has been linked to the development and progression of type 1 diabetes mellitus and T2D (134), and recent reviews have discussed its role in diabetes mellitus and obesity in further detail (135, 136). The TRPV1-dependent excitation of liver-related PVN neurons was diminished in a hyperglycemic, insulin-deficient mouse model. Both *in vivo* and *in vitro* insulin replacement restored the TRPV1-dependent excitatory neurotransmission *via* PI3-kinase, PKC, and/or TRPV1 trafficking (113). Similarly, TRPV1 was shown to play role in the regulation of excitatory neurotransmission to motor neurons in the DMV and stomach-related neurons in the PVN (137, 138). Furthermore, potential interaction between leptin signaling and TRPV1 has been proposed in the brainstem (114). In addition, there is limited information about the neurochemical phenotype of liver-related neurons. A study by Stanley and coworkers identified liver-related hypothalamic neurons using PRV and showed that a subpopulation of liver-related neurons co-localized with oxytocin and CRH in the mouse PVN (118). In the LH, a subset of MCH and orexin neurons was shown to be liver related. In the ARC, a subpopulation of POMC neurons but not NPY-expressing neurons were labeled with PRV, indicating that they are part of the brain–liver pathway (118).

Despite the scientific advances in our understanding of liver-related neurons, their precise role in the autonomic regulation of hepatic lipid metabolism remains to be determined. Although there is clear evidence that increased sympathetic outflow to the liver may increase VLDL-TG production, resulting in increased systemic FA availability, it remains to be determined whether the liver-related neurons of the hypothalamus are indeed FA sensitive, or indeed whether the FA-sensing neurons of the hypothalamus are liver related. It is plausible to suggest that liver-related neurons that direct hepatic triglyceride metabolism may express key lipid processing factors involved in lipid transport, e.g., CD36, FATP1 and/or TG metabolism, e.g., LPL. However, a more detailed understanding of these fundamental autonomic processes is needed in order to identify novel therapeutic targets that may halt the development of lipid-related disorders.

SUMMARY, IMPLICATIONS, AND INTERVENTIONS

The findings summarized in this review highlight the role of central lipid sensing in the regulation of systemic metabolism, including food intake, body weight, and hepatic glucose and lipid metabolism. Since increased HGP is a key factor in the development of glucose intolerance, understanding the neuronal mechanisms that drive the central regulation of HGP may be key to the development of novel therapeutic strategies that prevent hyperglycemia and the development of type 2 diabetes. In addition, our increased understanding of the hypothalamic control of TG metabolism highlights the potential utility of specifically modulating sympathetic nervous system activity toward the liver reduces VLDL-TG secretion and combat hypertriglyceridemia (139). The clinical relevance

of this strategy is highlighted by a recent north European human cohort, in which elevated sympathetic nervous system activity was associated with features of the MetS, such as elevated circulated VLDL-TG (140). While current interventions for hypertriglyceridemia are aimed at reducing circulating FAs by increasing FA uptake from the plasma, an alternative approach to effectively lower TG could be though decreasing VLDL-TG production by the liver. This is the case with GLP-1 receptor agonists, which have been shown to decrease both hepatic lipogenesis (141) and VLDL-TG production (142). Based on the literature outlined in this review, interventions targeting specific liver-related or FA-sensitive neurons in of the hypothalamus could have a similar impact on systemic metabolism, and we recommend that the identification of the features of these neuronal populations should be the subject of intensive research focus.

AUTHOR CONTRIBUTIONS

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Lipoprotein Lipase Expression in Hypothalamus Is Involved in the Central Regulation of Thermogenesis and the Response to Cold Exposure

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Lipoprotein lipase (LPL) is expressed in different areas of the brain, including the hypothalamus and plays an important role in neural control of the energy balance, including feeding behavior and metabolic fluxes. This study tested the hypothesis that hypothalamic LPL participates in the control of body temperature. We first showed that cold exposure induces decreased activity and expression of LPL in the mouse hypothalamus. We then selectively deleted LPL in the mediobasal hypothalamus (MBH) through an adeno-associated virus approach in LPL-floxed mice and generated MBHΔ^{Lpl} mice with 30–35% decrease in hypothalamic LPL activity. Results showed a decrease in body temperature in MBHΔ^{Lpl} mice when compared with controls at 22°C. Exposure to cold (4°C for 4 h) decreased the body temperature of the control mice while that of the MBHΔ^{Lpl} mice remained similar to that observed at 22°C. MBHΔ^{Lpl} mice also showed increased energy expenditure during cold exposure, when compared to controls. Finally, the selective MBH deletion of LPL also increased the expression of the thermogenic PRMD16 and Dio2 in subcutaneous and perigonadal adipose tissues. Thus, the MBH LPL deletion seems to favor thermogenesis. These data demonstrate that for the first time hypothalamic LPL appears to function as a regulator of body temperature and cold-induced thermogenesis.

Keywords: lipoprotein lipase, cold exposure, hypothalamus, brown adipose tissue, browning of white adipose tissue

INTRODUCTION

Adaptation to change in ambient temperature, especially cold exposure, is one of the mechanisms essential to the survival of mammals. This adaptation involves numerous signals of neural or hormonal origin and acute responses or acclimatization to prolonged cold exposures (1). Acute responses include vasoconstriction or shivering. The hypothalamus is a key area of central nervous system (CNS) involved in the adaptation to cold exposure. Indeed, afferent signals from the skin are sensed by the preoptic area of the anterior hypothalamus. The measurement of c-fos expression also suggests a role for the dorsomedial, medial, and the ventromedial hypothalamus after cold exposure in rats (2). In addition, several studies have also highlighted a role for arcuate (ARC) or ventromedial (VMH) nuclei in this adaptation (3, 4). Peripheral adaptation to cold

exposure involves the activation of the sympathetic nervous system (SNS) and hypothalamic–pituitary–thyroid axis (5, 6), including an increase in type 2 iodothyronine deiodinase (Dio2) expression that activates thyroxine (T4) to 3,3′,5-triiodothyronine (T3) (7). These signals combine to activate catabolic pathways with release of energy substrates such as free fatty acids (FFA) into the bloodstream from white adipose tissue (WAT). FFA can then serve to fuel shivering thermogenesis in muscle (8). In contrast, triglycerides (TG) stored in the brown adipose tissue (BAT) are also mobilized as FFA *via* activation of the SNS, however, these FFA are used primarily within the BAT itself as a fuel to generate heat through uncoupling of oxidative phosphorylation (8). Furthermore, browning of WAT during cold exposure can also be an adaptive mechanism to produce thermogenesis accompanied by increased expression of beige adipocyte markers such as Dio2, PR domain containing 16 (PRDM16), cell death-inducing DNA fragmentation factor α-subunit effector 1 (Cidea), and carnitine palmitoyl transferase-1b (Cpt1b) (9–11). In Humans, short-term cooling increases serum FFA, TG, and total cholesterol concentration (12). Short-term cooling also enhances large very low-density lipoprotein, small low-density lipoprotein, and small high-density lipoprotein particle number (12). Hydrolysis of TG during cold exposure is mainly due to lipoprotein lipase (LPL) that supplies FFA for nonshivering thermogenesis. Short-term cold exposure (i.e., 4–8 h) increased LPL activity in BAT in rats (13), Djungarian hamsters (14), and mice (15). Of note, other lipases such as patatin like phospholipase domain containing 2 [Pnpla2, also known as adipose triglyceride lipase (ATGL)] and hormone-sensitive lipase (HSL) are also affected by cold exposure (16). Interestingly, this cold-induced rise of LPL activity is specific to BAT, while cold exposure decreases epididymal WAT LPL activity in rats (17). Finally, LPL is strongly expressed in the CNS especially in regions involved in the control of the energy balance (18). We have previously shown that a decrease in the expression of LPL in hippocampus (19) or hypothalamus (20) led to the development of obesity and dysregulation of energy homeostasis. Thus, it was tempting to investigate whether hypothalamic LPL was involved in regulation of body temperature depending on different ambient conditions, such as moderate hypothermia relative to thermoneutrality (22°C) or cold (4°C). The passage from 22 to 4°C represents an adaptive challenge requiring increased thermogenic needs (21). To that end, mice deficient for LPL in mediobasal (ARC + VMH) hypothalamus (MBHΔ^{Lpl}) were generated by bilateral injections of adeno-associated virus (AAV)-cre in the mediobasal hypothalamus (MBH) of $Lpl^{lox/lox}$ male mice, leading to a 30–35% decrease in hypothalamic LPL activity as previously published (20). The results obtained from these mice suggest that MBH LPL dampens cold-induced thermogenesis and its deletion promotes cold adaptation.

MATERIALS AND METHODS

The experimental protocol was approved by the institutional animal care and use committee of the Paris Diderot University (CEEA40) and the agreement # 03752.02 was given to the project.

Animal Models

Ten-week-old $Lpl^{lox/lox}$ male mice (Jackson laboratory, strain B6.129S4-Lpltm1Ijg/J, no. 006503) and wild-type littermates were used as controls. They were housed individually in stainless steel cages in a room maintained at 22 ± 1°C with lights on from 07:00 a.m. to 07:00 p.m. They were given a standard laboratory diet (proteins 19.4%, carbohydrates 59.5%, lipids 4.6%, vitamins, and minerals 16.5%) and water *ad libitum*.

Viral Production

An AAV Cre-GFP was used in order to induce genetic recombination within the hypothalamus in $Lpl^{lox/lox}$ mice. The plasmid CBA.nls myc Cre.eGFP expressing the myc-nls-Cre-GFP fusion protein was kindly provided by Richard Palmiter (University of Washington, Seattle, WA, USA). Adeno-associated viruses of the serotype 2/9 (AAV2/9) (6×10^{11} vg/ml and 1.7×10^{8} pi/µl) known to have a neuronal tropism (22) were produced by the viral production facility of the UMR INSERM 1089 (Nantes, France).

Surgical and Stereotactic Procedures

At 10 weeks of age, mice were anesthetized with isoflurane and received an i.p. injection of 180 µg/kg buprenorphine hydrochloride (Axiance, Pantin, France) analgesic before being placed on a stereotaxic frame. AAVs were injected bilaterally into the MBH (stereotactic coordinates are relative to bregma: x ± 0.5 mm; y −1.64 mm; z −5.7 mm) at a rate of 0.20 µl/min for a total of 0.5 µl per side, in $Lpl^{lox/lox}$ mice (MBHΔ^{Lpl} mice) and in wild-type C57Bl6/J used as controls (MBH^{Lpl} mice). At the end of surgical procedures, mice received an i.p. injection of 50 µg/kg ketoprofen (Mérial, Lyon, France). Intraperitoneal probes (Anipill, Caen, France) were placed in animals during a laparotomy, to measure their body temperature. Animals had 7 days postsurgery to recover.

Indirect Calorimetry

Animals were individually housed in a cage with lights on from 7 a.m. to 7 p.m. and an ambient temperature of 22 ± 0.5 or 7°C (see Cold Exposure). All animals were acclimated to their cages for 48 h before experimental measurements. Data were collected every 15 min. Animals were analyzed for total energy expenditure (kcal/h), oxygen consumption and carbon dioxide production (VO_2 and VCO_2, where V is the volume), respiratory exchange rate (RER = VCO_2/VO_2), food intake (g), and locomotor activity (beam breaks/h) using calorimetric cages with bedding, food and water *ad libitum* (Labmaster, TSE Systems GmbH, Bad Homburg, Germany). The instrument combined a set of highly sensitive feeding and drinking sensors for automated online measurement. To allow measurement of ambulatory movements, each cage was embedded in a frame with an infrared light beam-based activity monitoring system with online measurement at 100 Hz; the detection of movement operated efficiently in both light and dark phases, allowing continuous recording. Gas ratio was determined using an indirect open-circuit calorimeter (23), which monitored O_2 and CO_2 concentrations by volume at the inlet ports of a tide cage with an airflow of 0.4 l/min,

with regular comparisons to an empty reference cage. Total energy expenditure was calculated according to the Weir equation, using respiratory gas exchange measurements (24). The flow was previously calibrated with O_2 and CO_2 mixture of known concentrations (Air Liquide, S.A. France). Fatty acid oxidation was calculated from the following equation: fat ox (kcal/h) = energy expenditure × (1 − RER/0.3) according to Bruss et al. (25). Animals were monitored for body weight and composition at the beginning and end of the experiment. Data analysis was carried out with Excel XP using extracted raw values of VO_2 consumption, VCO_2 production (ml/h), and energy expenditure (kcal/h). Subsequently, each value was expressed either as a function of total body weight or total lean tissue mass extracted from the EchoMRI analysis.

Cold Exposure

Mice were exposed to cold at 10 days postsurgery. For the acute cold exposure, animals were exposed to 4°C for 4 h in a refrigerated bench, closed by a Plexiglas plate. Additional mice were exposed to 7°C for 24 h in the calorimetric cages described above.

Body Temperature Measurement

Body temperature was measured every 15 min by telemetry with intraperitoneal probes (Anipill, Caen, France). Data were collected by an electronic monitor and transferred to a computer with Anipill Software (Anipill, Caen, France) and were then extracted to an Excel XP file (Microsoft France, Issy-les-Moulineaux, France) before analysis.

BAT Temperature Measurement

Skin temperature surrounding BAT was recorded with an infrared camera (B335: Compact-Infrared-Thermal-Imaging-Camera; FLIR; West Malling, Kent, UK) and analyzed with a FLIR-Tools-Software (FLIR; West Malling, Kent, UK) as previously described (26, 27). For each image, the area surrounding BAT was delimited and the average temperature of the skin area was calculated as the average of two pictures/animal.

Tissue Collection

Brain tissues were dissected following the Glowinski and Iversen technique (28); hypothalamus, hippocampus, striatum, and cerebral cortex were immediately frozen. In addition, subcutaneous (WATsc) and gonadal white adipose tissue (WATgon) and intrascapular BAT were also collected and immediately frozen for further measurement of mRNA of gene of interest.

LPL Activity Assay

Heparin-releasable LPL activity was assayed in brain regions using a Roar LPL activity assay kit (RB-LPL, Roar Biomedical, Inc.). Briefly, tissues were lysed in 500 µl of assay buffer (150 mM NaCl, 10 mM Tris, 2 mM EDTA, pH 7.4) and incubated for 45 min at 37°C with an equivalent volume of heparin (100 U/ml). After incubation, samples were centrifuged for 10 min at 3,000 *g*, and aliquots (10 µl for mice) of the aqueous phase deposited on LPL substrate emulsion in 96-well black microplates (VWR International, # 25227-304) and incubated for 1 h at 37°C. Finally, fluorescence was read using a fluorimeter (370 nm excitation/450 nm emission), and compared to a standard curve made using known concentrations of prehydrolyzed LPL substrate. LPL activity was expressed as µmoles of FFA produced per minute per gram of tissue.

Real-Time Quantitative PCR

Total RNA was isolated from the hippocampus dissected at day 28 at the end of the experimentation using RNeasy Lipid Tissue mini kit (Qiagen, Courtaboeuf, France). Real-time quantitative PCR was carried out in a LightCycler 480 detection system (Roche, Meylan, France) using the Light-Cycler FastStart DNA Master plus SYBR Green I kit (Roche). The mRNA transcript level for each gene was normalized against the mean of 2 HKG: rpL19 and TBP, which we have previously shown to be unaffected by LPL inhibition (**Table 1**: primers sequences).

Statistical Analysis

Data are expressed as mean ± SEM. Statistical analysis were performed using Student's test or ANOVA followed by two-by-two Bonferroni *post hoc* test (GraphPad software). $p < 0.05$ was considered significant. Representative bar graph is expressed as mean ± SEM.

RESULTS

Cold Exposure Decreases Hypothalamic LPL Activity in Control Mice

There was no effect of nutritional status (i.e., fed or fasted) on hypothalamic LPL activity at either ambient temperatures of 22 or 4°C (**Figure 1A**). In contrast, in fed and fasted mice, exposure

TABLE 1 | Primers used for RT-qPCR.

Gene	Forward primers	Reverse primers
Rpl19	gggcaggcatatgggcata	ggcggtcaatcttcttggatt
Tbp	ggggagctgtgatgtgaagt	ccaggaaataattctggctca
Abhd5	atctttggagcccgatcct	cttctggctgatctgcatacac
Pnpla2	tgaccatctgccttccaga	tgtaggtggcgcaagaca
Plin5	Agctggagcccgtgtcta	gggcatccactgcattct
Lipe HSL	gcctggcaaaatctgagg	gcctccgtggatgtgaac
Lpl	tttgtgaaatgccatgacaag	cagatgctttcttctcttgtttgt
CerS1	ggagctactgcgcttacctg	ccatgcctgacctccagt
Clock	ccagtcagttggtccatcatt	tggctcctaactgagctgaaa
Bmal1	cgactcattgatgccaagac	tgcacttcattctacagaacagaga
Cidea	ttcaaggccgtgttaagga	cctttggtgctaggcttgg
Cpt1b	Ctcctttcctggctgaggt	gatctggaactgggggatct
Prdm16	Cctaaggtgtgcccagca	caccttccgcttttctaccc
Dio2	tggcatgccctgtaggtt	tgagaagtctggtacatcagcaa
Ucp1	ggctctgcaggagtccgaagt	ggcgtgagtgcaagaacaaaa
Ucp2	tttgggtttggttgtttttga	ggaggcagatgcaggtagat
Ucp3	ctgcctgtccatcagttcaa	ccagtcctaacaattccctaaaga
AdRβ3	cagccagccctgttgaag	ccttcatagccatcaaacctg

Rpl19, ribosomal protein L19; Tbp, TATA-box binding protein; Abhd5, α/β hydrolase domain containing 5; Pnpla2, patatin-like phospholipase domain containing 2 (ATGL); Plin5, perilipin5; Lipe HSL, lipase hormone sensitive; Lpl, lipoprotein lipase; CerS1, ceramide synthase1; Clock, clock circadian regulator; Bmal1, brain and muscle Arnt-like protein-1; CIDEA, cell death-inducing DNA fragmentation factor alpha subunit-like effector A; Cpt1b, carnitine palmitoyltransferase 1B; PRDM16, PR/SET domain 16; Dio2, type 2 iodothyronine deiodinase; UCP, uncoupling protein; AdRβ3, β3-adrenergic receptor.

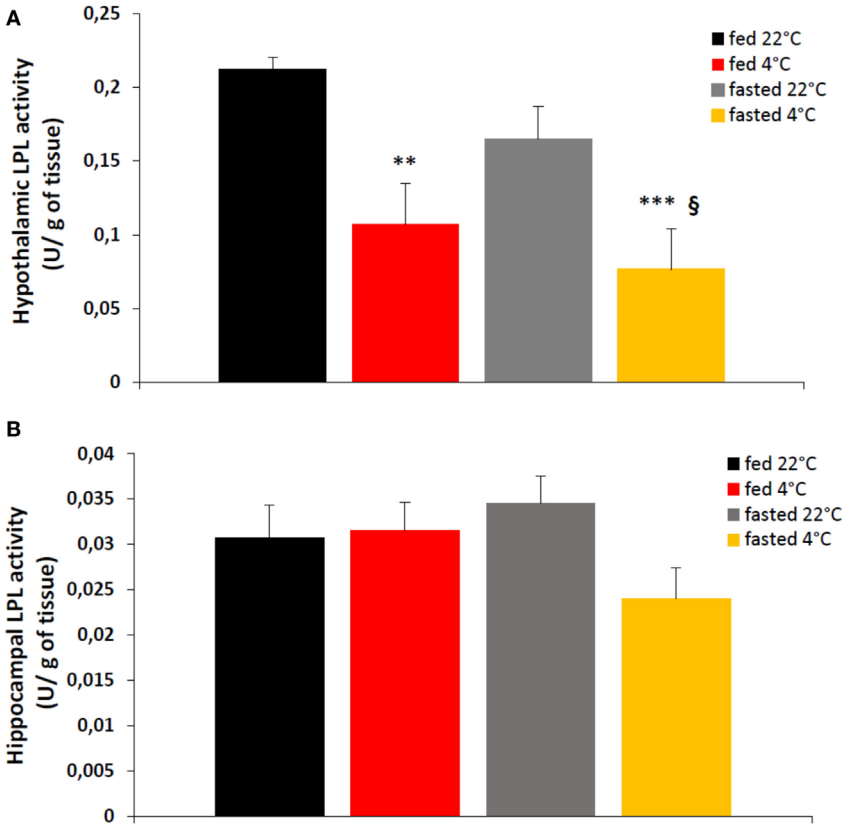


FIGURE 1 | An acute cold exposure (4 h at 4°C) decreases significantly enzymatic activity of lipoprotein lipase (LPL) in hypothalamus **(A)** but not in hippocampus **(B)**. LPL activity is expressed in enzymatic units (U, μmol/min) per g of tissue. Data are mean ± SEM, $n = 6–8$ for each group. $^{*}p < 0.05$ and $^{***}p < 0.001$ vs. mice "fed 22°C", $^{§}p < 0.05$ vs. "fasted 22°C" mice.

after 4 h at 4°C, there was a significant decrease of ~50% in hypothalamic LPL activity when compared with LPL activity at 22°C (**Figure 1A**). Of note, neither nutritional status nor ambient temperature exposure affected hippocampal LPL activity (**Figure 1B**).

Cold Exposure-Induced Changes in Brain Gene Expression in Control Mice

Cold exposure induced changes in the expression of genes involved in lipid metabolism in several brains areas (**Figure 2**). LPL mRNA expression was decreased in response to cold exposure in the hypothalamus, as well as hormone sensitive lipase (Lipe HSL) and abhydrolase domain containing 5 (Abhd5) mRNA. Conversely, acute cold exposure significantly increased the expression of hypothalamic perilipin 5 (Plin 5). Abdh5 and Plin5 are cofactors of the Pnpla2 (also known as ATGL) which catalyzes the first step of intracellular TG hydrolysis (16). No change was observed in the expression of other hypothalamic genes involved in lipid metabolism. In the hippocampus and in the cerebral cortex, cold exposure significantly increased the expression of the perilipin 5 gene, as in the hypothalamus, but also of Pnpla2 (ATGL). No change in expression of mRNAs for lipases or their cofactors was seen in the striatum. For clock genes, the expression of hypothalamic Bmal1 and Clock and striatal Bmal1 mRNA was decreased, while hippocampal Bmal1 expression was increased after cold exposure.

MBH LPL Depletion Dysregulates Body Temperature and Energy Metabolism

As shown in **Table 2** and **Figure 3A**, MBHΔ^{Lpl} mice had a significantly lower basal temperature at 22°C when compared with controls mice. However, after 4 h of exposure to 4°C, MBHΔ^{Lpl} mice maintained their body temperature, whereas body temperature fell by 1.2°C during the first hour of cold exposure in control MBH^{Lpl} mice and remained lower than that in MBHΔ^{Lpl} mice for the remainder of the 4 h exposure period. Figure S1 in Supplementary Material shows the temperature time courses for MBH^{Lpl} and MBHΔ^{Lpl} mice with their respective controls maintained at 22°C during 4 h. The thermogenic images over intrascapular BAT pads (**Figures 3B–E**) and their quantification (**Figure 3F**) appear to correlate with comparable changes in core body temperature measured with the intraperitoneal probes: MBHΔ^{Lpl} mice maintained their BAT temperature after 4 h at 4°C, whereas BAT temperature significantly dropped in controls. The expression of uncoupling protein 1 (UCP1) mRNA was upregulated by 4 h of cold exposure in both MBH^{Lpl} and MBHΔ^{Lpl} mice (**Figure 3G**), and there was no difference

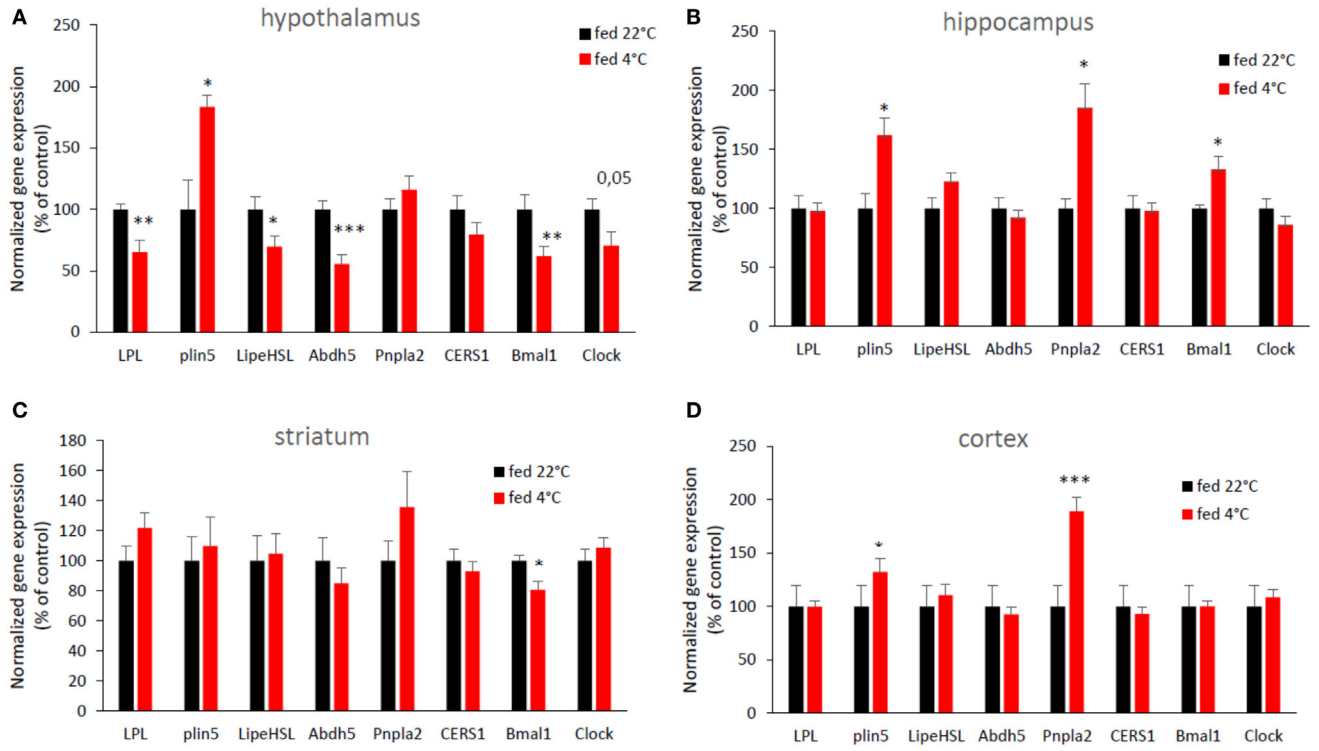


FIGURE 2 | An acute cold exposure modifies the expression of several genes involved in lipid metabolism and circadian clock. mRNA expression in **(A)** hypothalamus, **(B)** hippocampus, **(C)** striatum, and **(D)** cerebral cortex. Data are mean ± SEM and show change of expression rate relative to mRNA for mice at 22°C. $n = 8–10$ for each group. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ vs. "fed 22°C" group.

TABLE 2 | Comparison of physiological parameters of MBH^{Lpl} vs. MBHΔ^{Lpl}.

	MBH^{Lpl}	MBHΔ^{Lpl}
Body weight (g)	33.07 ± 0.63	31.45 ± 0.88
Body temperature before cold exposure (°C)	37.02 ± 0.12	36.26 ± 0.10***
Body temperature after cold exposure (°C)	35.58 ± 0.21	36.34 ± 0.15*
Temperature change (°C)	−1.31 ± 0.14	+0.02 ± 0.11***
Weight of BAT at 22°C (g)	0.11 ± 0.026	0.15 ± 0.025
Weight of BAT at 4°C (g)	0.094 ± 0.015	0.16 ± 0.021*

Data are mean ± SEM, n ≥ 6 for each group.
$^{*}p < 0.05$ vs. MBH^{Lpl}.
$^{***}p < 0.001$ vs. MBH^{Lpl}.

in UCP2 and UCP3 expressions in MBHΔ^{Lpl} mice compared to controls. In MBH^{Lpl} mice, expression of β3-adrenoreceptor mRNA was significantly downregulated by the cold exposure, whereas it remained higher in MBHΔ^{Lpl} mice than their 4°C controls (**Figure 3G**).

During 24 h cold exposure in metabolic cages, food intake was similar in the two groups (Figure S2 in Supplementary Material), and the locomotor activity was decreased during the daylight (cf. **Figure 4A**) whereas energy expenditure normalized to lean mass was significantly increased in MBHΔ^{Lpl} mice (**Figure 4B**). A shown in **Figure 4C**, these mice presented a trend toward carbohydrate-oriented metabolism before the nighttime, as demonstrated by the increased RER, and consequently a lower rate of fatty acid oxidation (**Figure 4D**).

The Expression of "Beige" Genes Is Increased in WAT of MBHΔ^{Lpl} Mice

Beige adipocytes share some common characteristics with brown adipocytes and have a thermogenic potential. In the subcutaneous adipose tissue, some genes involved in WAT thermogenic function ("beige" markers; CPT1b and PRDM16) were significantly increased in basal conditions (22°C) and remained higher compared than controls after a cold exposure. Moreover, Dio2 gene expression was higher at 22°C in MBHΔ^{Lpl} mice (**Figure 5A**). In gonadal WAT, PRDM6 trended to be more expressed in MBHΔ^{Lpl} mice compared to controls at 22°C, and DiO2 expression showed a ninefold increase following cold exposure (cf. **Figure 5B**).

DISCUSSION

In the present study, we first demonstrated that hypothalamic LPL mRNA expression and activity were regulated as a function of ambient temperatures between 22 and 4°C. Indeed, cold exposure induced a selective decrease in both LPL activity and gene expression in hypothalamus with no change in mRNA expression in the hippocampus, striatum, or cerebral cortex when temperature dropped from 22 to 4°C. It has already been reported that the expression of the brain LPL was regulated by the 72 h fasted or fed states (29), but to our knowledge it is the first time that a regulation as a function of the ambient temperature is described. We and others previously reported that brain LPL

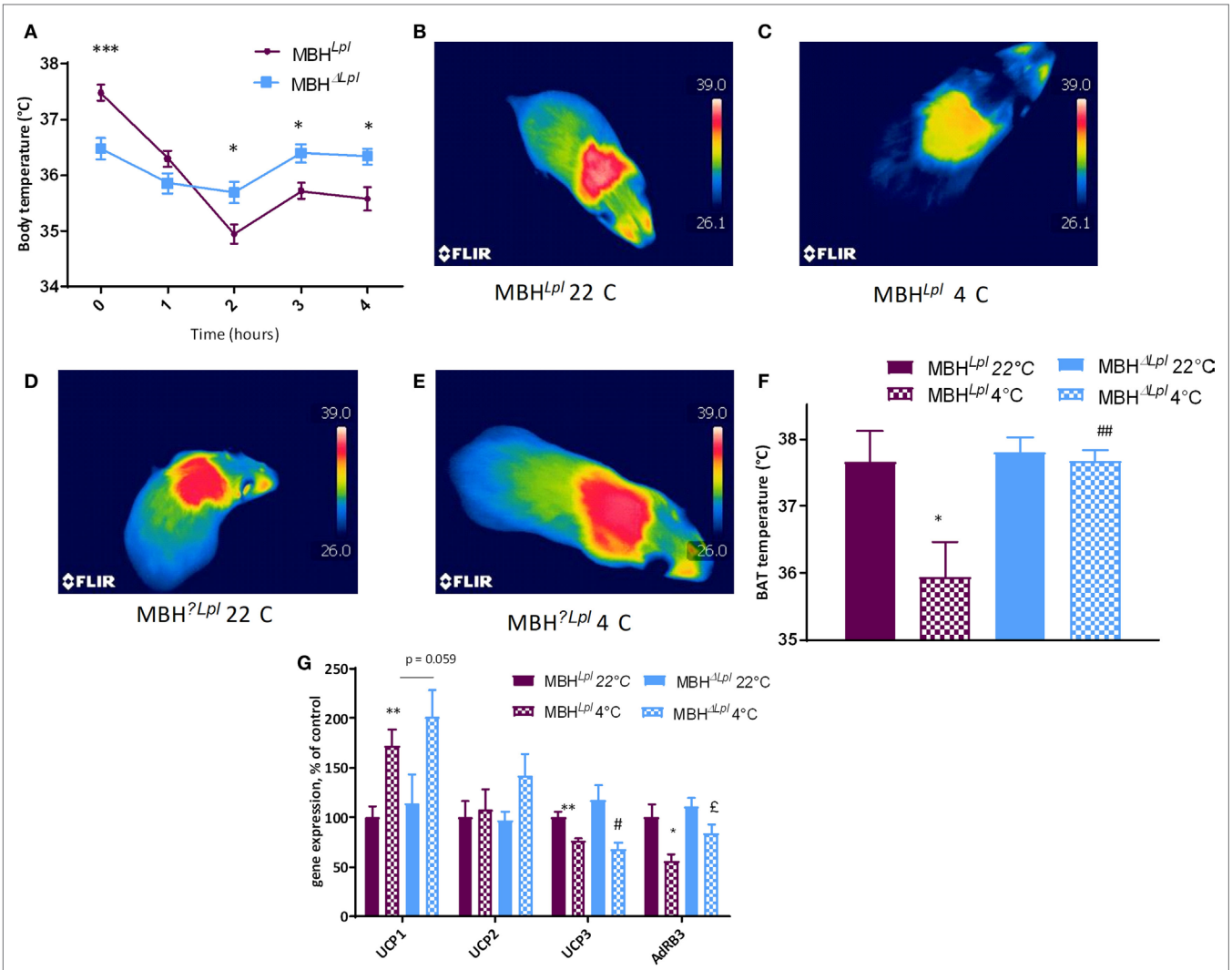


FIGURE 3 | An acute exposure to cold decreases body and skin temperature of MBH^{Lpl} mice but not of MBHΔ^{Lp} **(A)**. Thermic images of brown adipose tissue (BAT) temperature of MBH^{Lpl} at 22°C **(B)** and at 4°C **(C)** shows a decrease during the acute cold exposure whereas BAT temperature of MBHΔ^{Lpl} at 22°C **(D)**, is maintained at 4°C **(E)**. The value of BAT temperature for MBH^{Lpl} and MBHΔ^{LPL} mice at 22°C and at 4°C is presented in a global histogram, showing a decrease of BAT temperature at 4°C for MBH^{Lpl} whereas MBHΔ^{Lpl} mice present no difference at 4°C **(F)**. Measurement of mRNA expression in BAT **(G)** for uncoupling proteins (UCPs) show no difference between MBH^{Lpl} and MBHΔ^{Lpl} at 22 or at 4°C. mRNA expression of AdRβ3 is increased at 4°C in MBHΔ^{LPL} group compared to MBH^{Lpl}. Data are mean ± SEM ($n = 6$). *$p < 0.05$, **$p < 0.01$ vs. MBH^{Lpl} mice at 22°C, £$p < 0.05$ vs. MBH^{Lpl} mice at 4°C.

regulated energy balance. Wang et al. showed that neuronal LPL deficiency promoted obesity and maladaptive responses to environmental challenges (30–32). In addition, we reported that specific deletion of hypothalamic LPL induced body weight gain without hyperphagia, but with increased food efficiency in mice (20). Such increased body weight gain was partly explained by an early decrease in locomotor activity, a trend toward lower energy expenditure at night, and a preferential use of carbohydrate rather than lipid metabolism (20).

In the present study, after having shown that LPL in the hypothalamus was regulated by ambient temperature, it seemed important to investigate whether partial invalidation of LPL in the hypothalamus could therefore influence cold adaptation in mice in order to establish a cause-and-effect relationship between the expression of the enzyme and body temperature. It is well known that the control of BAT thermogenesis is regulated by the CNS (33, 34), and numerous studies have highlighted the role of the hypothalamus and the SNS in such control (4, 27, 35, 36). Besides the preoptic area of the hypothalamus, which is considered as a major coordinator of thermoregulation since it receives inputs from skin and visceral thermoreceptors, other nuclei participate in thermoregulation such as the paraventricular, the ARC and the VMH [review by Labbe et al. (4)]. Although the characterization of the neurons controlling thermogenesis remains unsolved, data pointed toward a role for the melanocortin system in stimulating BAT thermogenesis (3). In the ARC, NPY/AgRP neurons inhibit this system while POMC neurons activate it. In the VMH, steroidogenic factor 1 (SF1) neurons are known to affect BAT thermogenesis (37), by integrating both AMPK and mTOR signaling (27, 38, 39). We showed here that

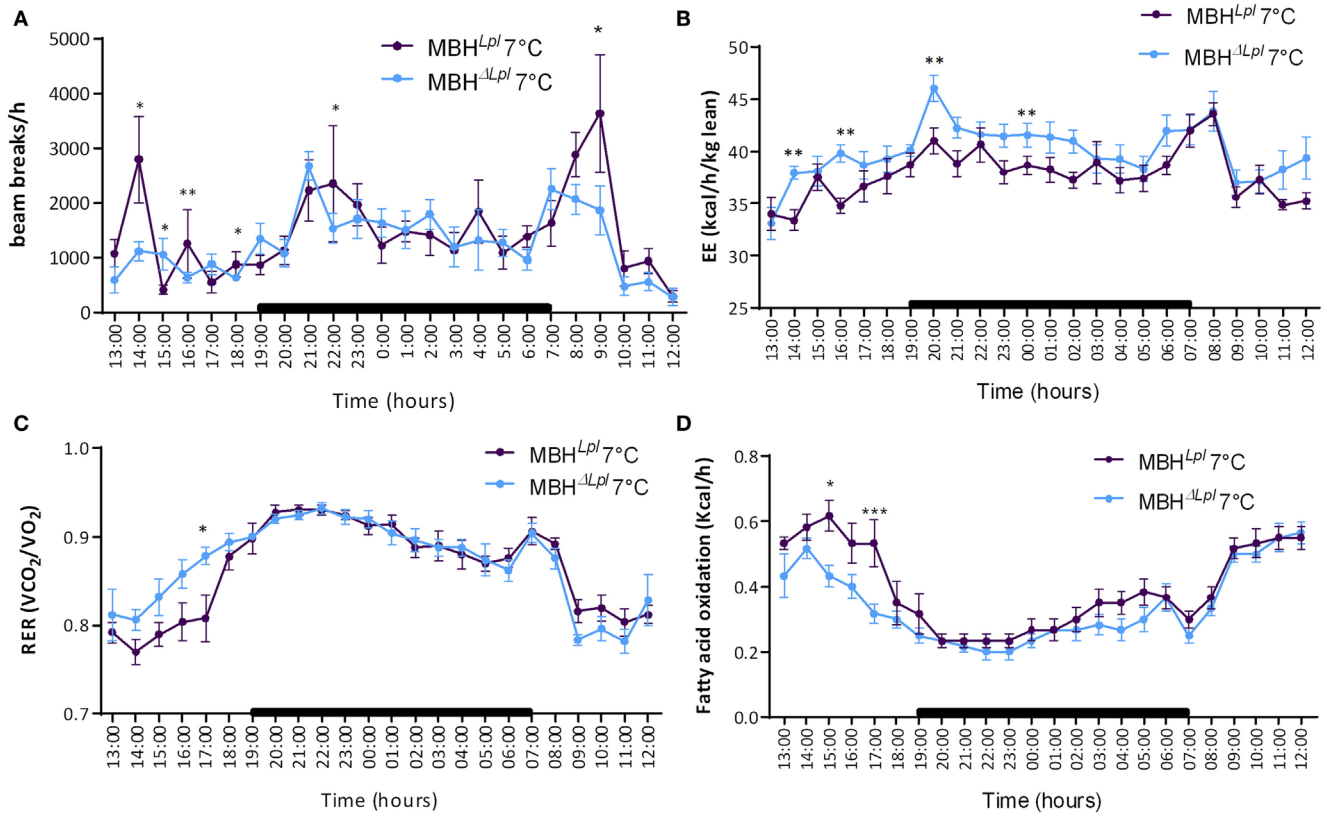


FIGURE 4 | Calorimetry and locomotor activity for MBH^{LPL} and MBHΔ^{LPL} mice during 24 h of cold exposure at 7°C. **(A)** Locomotor activity. **(B)** Energy expenditure normalized per lean body mass. **(C)** Respiratory exchange rate (RER, VCO_2/VO_2). **(D)** Fatty acids oxidation rate. Data are mean ± SEM (n = 5–6). $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ vs. MBH^{Lpl} mice.

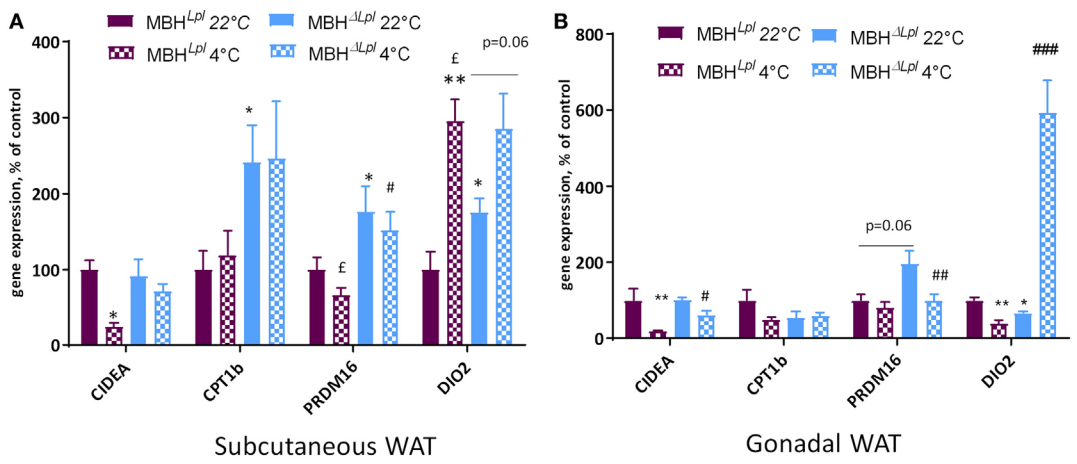


FIGURE 5 | Depletion of lipoprotein lipase in the mediobasal hypothalamus (MBH) increases mRNA expression of genes responsible for "beigeing" in white adipose tissues (WATs). **(A)** mRNA expression in subcutaneous WAT. **(B)** mRNA expression in gonadal WAT. Data are mean ± SEM and show change of expression rate relative to mRNA for mice at 22°C. n = 4–6 for each group. $^*p < 0.05$, $^{**}p < 0.01$ vs. MBH^{Lpl} mice at 22°C, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$ vs. MBH^{Lpl} mice at 4°C, $^{£}p < 0.05$ vs. MBHΔ^{Lpl} mice at 22°C.

LPL deficiency in the hypothalamus induced a dysregulation of thermogenesis in mice at both normal vivarium temperature of 22 and at 4°C. Specifically, MBHΔ^{Lpl} mice had a significant lower core body temperature compared to controls when the ambient temperature was 22°C. This result seemed to be consistent with our previous observations (i.e., sparing fatty acids and decrease

of locomotor activity) (20). As mentioned above, we observed a decrease in both LPL expression and activity in the hypothalamus in normal mice during cold exposure. This result could suggest that the physiological decrease of LPL activity in the hypothalamus, in response to decreased ambient temperature plays a role in adaptive thermogenesis. In accordance with this hypothesis, when the hypothalamic LPL was permanently depleted, the animals presented higher energy expenditure and were able to defend their body temperature more successfully than controls when exposed at 4°C for 4 h. At mild temperature corresponding to their basal housing conditions (22°C), LPL depleted animals displayed a significantly lower body temperature (0.76°C), which could be interpreted as an attempt of saving energy for the long-term. The subsequent question might be to understand how a drop in hypothalamic LPL activity could induce this change in energy flow and control body temperature. It can be hypothesized that a decrease in hypothalamic LPL activity and thus a consequent local decrease in TG hydrolysis and FFA availability could be sensed by specialized neurons (18, 40) as a "thrifty" signal and a need for metabolic adaption. By specifically decreasing LPL in MBH, we therefore mimicked such a "thrifty behavior" that was observed during cold exposure (41). Because MBHΔ^{Lpl} mice had already displayed some metabolic features of cold exposure, the actual exposure to cold (at least during a short period) did not induce a greater decrease in their body temperature, whereas this occurred in the control mice (decreased body temperature from 37 to 35°C). This adaption could be explained by a modulation of the circadian clock, as it has been shown that transcription of genes involved in the metabolism can be regulated by the circadian clock. For example, Clock and Bmal1 genes favor transcription of Lpl (42). Interestingly, we showed that exposure to cold decreased the expression of Bmal1 and Clock. The downregulation of Bmal1 and Clock mRNA could partly explain the decrease observed for LPL mRNA. Furthermore, a recent study suggested the existence of an independent circadian clock in the VMH (43). Exposure to cold could have an impact on hypothalamic circadian clock and thus on LPL expression. Finally, this study by Orozco-Solis et al. showed that a mouse model KO for Bmal1 in Sf1 neurons of the VMH was associated with increased BAT temperature throughout the diurnal cycle. Therefore, a decrease of Bmal1 expression during cold exposure in MBHΔ^{Lpl} compared to MBH^{Lpl} could contribute to explain the preservation of BAT temperature.

Partial deletion of hypothalamic LPL also resulted in changes in mRNA expression of some marker genes of adipocyte function as well as thermogenesis-related genes. We measured some of these genes of interest in subcutaneous and gonadal adipose tissues. Interestingly, MBHΔ^{Lpl} mice that were exposed to mild temperature showed a significant increase in PRDM16 expression in WATsc and a tendency in WATgon when compared with controls. PRDM16 is a critical transcriptional regulator of thermogenesis and the increase of PRDM16 in WATsc of MBHΔ^{Lpl} mice could be interpreted as an attempt to adapt to their decreased body temperature at an ambient temperature of 22°C. It has been reported that adipocyte-specific deletion of PRDM16 markedly inhibited beige adipocyte function in subcutaneous fat following cold exposure or β3-agonist treatment (44). Thus, browning may occur in WATsc of MBHΔ^{Lpl} mice as an adaptation to chronically lowered body temperature. However, it must be pointed out that such adaptation was not sufficient to bring the body temperature back to that observed in the control mice. This can be explained by the fact that other parameters were also affected by the hypothalamic deletion of LPL (20).

Interestingly, mRNA expression of Dio2, the enzyme that activates T4 to 3,3′,5-triiodothyronine (T3) was also significantly elevated in WATsc of MBHΔ^{Lpl} mice compared to controls exposed to 22°C. Dio2 is also known as being a thermogenesis-related gene (9). As for PRDM16, the local increase in Dio2 mRNA could be interpreted as an adaptation to the decrease in body temperature through increased local production of T3. During cold exposure, mRNA expression of Dio2 in WATsc was significantly increased in controls to reach a value similar to MBHΔ^{Lp} mice. As mentioned, exposure to cold induced a decrease in the body temperature of control mice (from 37 to 35°C) while that of MBHΔ^{LPL} mice remained constant compared to exposure to mild. During cold exposure, PRDM16 expression remained significantly higher in WATsc of MBHΔ^{LPL} mice compared to controls. In WATgon, Dio2 was significantly upregulated in MBHΔ^{Lp} mice exposed to 4°C as compared with their controls. This could be related to the fact that they managed to maintain their body temperature: the upregulation of browning markers, already present at 22°C, may in part explain the absence of significant fluctuation in body temperature during exposure to cold. In particular, de Jesus et al. showed that increased Dio2 expression in BAT played a key role for local production of T3, even in a situation where serum concentrations of both T3 and T4 remained constant during cold stress (24 h at 4°C) (7). Such a result highlighted a key role for local production of T3 through activation of Dio2.

Finally, skin temperature surrounding BAT was recorded as an indirect index of thermogenesis and BAT function. In control mice, skin temperature decreased when mice were exposed to cold whereas it remained unchanged compared to mild temperature exposure for MBHΔ^{Lpl}. This could be related to the decrease in body temperature that we also observed. However, in both groups UCP1 mRNA expression was increased during cold temperature when compared to mild exposure. Again, this difference in response between the two groups in the face of cold exposure suggests that the deficiency of LPL in the hypothalamus regulated energy homeostasis and especially thermogenesis.

In conclusion, we demonstrate here for the first time, that LPL in hypothalamus is a negative regulator of thermogenesis. Normal adaptation to a drop-in temperature involves a decrease in hypothalamic LPL activity that promotes cold-induced thermogenesis. A chronic decreased in LPL activity in hypothalamus could impair the regulation of body temperature in response to changes in ambient temperature. More generally, a defect in the central sensing of lipids could compromise the survival of individuals during winter and more particularly when a lack of food also occurred.

ETHICS STATEMENT

The experimental protocol was approved by the institutional animal care and use committee of the Paris Diderot University (CEEA40), and the agreement # 03752.02 was given to the project.

AUTHOR CONTRIBUTIONS

ML, SL, CC-G, and CM designed experiments. EL, RD, NK, CC, and CC-G performed experiments. EL, CC-G, and CM prepared the manuscript. RD, CC, and ML edited the manuscript. The entire study was supervised by CC-G and CM.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fendo.2018.00103/full#supplementary-material.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Sweet Taste Receptor Serves to Activate Glucose- and Leptin-Responsive Neurons in the Hypothalamic Arcuate Nucleus and Participates in Glucose Responsiveness

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The hypothalamic feeding center plays an important role in energy homeostasis. In the feeding center, whole-body energy signals including hormones and nutrients are sensed, processed, and integrated. As a result, food intake and energy expenditure are regulated. Two types of glucose-sensing neurons exist in the hypothalamic arcuate nucleus (ARC): glucose-excited neurons and glucose-inhibited neurons. While some molecules are known to be related to glucose sensing in the hypothalamus, the mechanisms underlying glucose sensing in the hypothalamus are not fully understood. The sweet taste receptor is a heterodimer of taste type 1 receptor 2 (T1R2) and taste type 1 receptor 3 (T1R3) and senses sweet tastes. T1R2 and T1R3 are distributed in multiple organs including the tongue, pancreas, adipose tissue, and hypothalamus. However, the role of sweet taste receptors in the ARC remains to be clarified. To examine the role of sweet taste receptors in the ARC, cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) in isolated single ARC neurons were measured using Fura-2 fluorescent imaging. An artificial sweetener, sucralose at 10^{-5}–10^{-2} M dose dependently increased $[Ca^{2+}]_i$ in 12–16% of ARC neurons. The sucralose-induced $[Ca^{2+}]_i$ increase was suppressed by a sweet taste receptor inhibitor, gurmarin. The sucralose-induced $[Ca^{2+}]_i$ increase was inhibited under an extracellular Ca^{2+}-free condition and in the presence of an L-type Ca^{2+} channel blocker, nitrendipine. Sucralose-responding neurons were activated by high-concentration of glucose. This response to glucose was markedly suppressed by gurmarin. More than half of sucralose-responding neurons were activated by leptin but not ghrelin. Percentages of proopiomelanocortin (POMC) neurons among sucralose-responding neurons and sweet taste receptor expressing neurons were low, suggesting that

majority of sucralose-responding neurons are non-POMC neurons. These data suggest that sweet taste receptor-mediated cellular activation mainly occurs on non-POMC leptin-responding neurons and contributes to glucose responding. Endogenous sweet molecules including glucose may regulate energy homeostasis through sweet taste receptors on glucose-and leptin-responsive neurons in the ARC.

Keywords: feeding, sweet taste receptor, sucralose, glucose, leptin, POMC

INTRODUCTION

The feeding center in the hypothalamus plays an important role in energy homeostasis. Neurons in the feeding center are activated or suppressed by the molecules reflecting peripheral energy status, including hormones such as ghrelin, leptin and insulin, and nutrients such as glucose, free fatty acids, and amino acids. These neuronal responses are integrated by intracellular signaling and the brain circuit; as a result, feeding and metabolism are controlled (Williams and Elmquist, 2012). Therefore, nutrient sensing is important for the control of energy balance.

There are two types of glucose-sensing neurons in the hypothalamic arcuate nucleus (ARC; Routh et al., 2014). One is the glucose-excited (or glucose-responsive) neurons, which are activated by high concentrations of glucose and inhibited by low concentrations of glucose. They are thought to be satiety neurons. The other is glucose-inhibited (glucose-sensitive) neurons, which are activated by low concentrations of glucose and inhibited by high concentrations of glucose. Glucose-inhibited neurons are thought to be orexigenic neurons. Regarding the mechanism of glucose-sensing in the hypothalamic neurons, several molecules have been proposed, which include Na^+, K^+ ATPase, AMP-activated protein kinase, ATP-sensitive K^+ channel, and glut2 (Oomura et al., 1974; Ibrahim et al., 2003; Bady et al., 2006; O'Malley et al., 2006; Claret et al., 2007; Routh et al., 2014; Kurita et al., 2015). However, the precise mechanisms underlying glucose-sensing in the hypothalamus are not fully understood.

Glucose-receptor hypothesis was first proposed some decades ago, and recent progress has suggested the sweet taste receptor as the candidate molecule for glucose receptor (Kojima et al., 2015). The sweet taste receptor is a heterodimer of taste type 1 receptor 2 (T1R2) and taste type 1 receptor 3 (T1R3), and this receptor senses multiple sweet taste molecules. In the body, there are variety of sweet taste molecules including carbohydrate, amino acids, polypeptides, proteins, glycosides, and glycerol. T1R2 and T1R3 are distributed throughout multiple organs including the tongue, intestine, pancreas, brain, testis, and lung (Ren et al., 2009; Li, 2013). Recent studies have shown that sweet taste receptors in the pancreatic β cells are necessary for the first phase of glucose response (Nakagawa et al., 2009, 2015). In the brain, the sweet taste receptor is abundantly expressed in the hypothalamus including ARC and the expression level of sweet taste receptor in the hypothalamus is affected by metabolic conditions (Ren et al., 2009; Herrera Moro Chao et al., 2016). However, the role of the sweet taste receptor in the regulation of ARC cellular activities is not understood. Here, we explored the potential contribution of sweet taste receptor to glucose-responses in ARC neurons that could be implicated in energy homeostasis.

MATERIALS AND METHODS

Materials

Sucralose, leptin, and nitrendipine were purchased from Sigma (Sigma-Aldrich, St. Louis, MO). ω-Contoxin GIVA and ghrelin were purchased from Peptide Institute (Osaka, Japan). All of the other chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan).

Animals

All mouse care and experimental procedures were approved by the Institutional Animal Care and Experimentation Committee at Gunma University. Mice were kept at room temperature (20–24°C) with a 12 h light/dark cycle. All mice used in this study were backcrossed to C57B6J more than eight generations. Regular chow (CLEA Rodent diet CE-2; CLEA Japan, Tokyo, Japan) and water were provided *ad libitum*. POMC-GFP mice were kindly provided by Dr. Jeffrey Friedman (Pinto et al., 2004).

Preparation of Single Neurons from ARC

Single neurons were prepared according to procedures reported previously (Kohno et al., 2003, 2007) with slight modifications. Briefly, mice were anesthetized with 10% pentobarbital (10 μl/g) injected intraperitoneally (IP) and decapitated. Their brains were taken out and brain slices containing the entire ARC were prepared. The entire ARC was excised from the left and right sides. The dissected tissues were washed with 10 mM HEPES-buffered Krebs-Ringer bicarbonate buffer (HKRB) containing 1 mM glucose. They were then incubated in HKRB supplemented with 20 U/ml papain (Sigma-Aldrich, P4762), 0.015 mg/ml DNase II (Sigma-Aldrich, D-4138), and 0.75 mg/ml BSA (Sigma-Aldrich, A2153) for 16 min at 36°C. This was followed by gentle mechanical trituration. Then, the cell suspension was centrifuged at 100 × g for 5 min. The pellet was resuspended in HKRB and distributed onto coverslips. The cells were kept in moisture-saturated dishes for up to 4 h at RT.

Measurements of $[Ca^{2+}]_i$ in Single ARC Neurons

Cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) was measured by ratiometric fura-2 microfluorometry in combination with digital imaging, as previously reported (Kohno et al., 2003, 2007). Briefly, after incubation with 2 μM fura-2/AM (Dojindo, Kumamoto, Japan, F016) for 45 min, the cells were mounted in a chamber and superfused with HKRB at 1 ml/min at 33°C.

Fluorescence images due to excitation at 340 and 380 nm were detected every 10 s with a cooled charge-coupled device camera (ORCA-R2 C10600, Hamamatsu Photonics, Hamamatsu, Japan), and the ratio image was produced by an Aquacosmos (Hamamatsu Photonics). The data were obtained from single cells identified as neurons by previously reported procedures (Kohno et al., 2003, 2007); briefly, they have a relatively large diameter ($\geq$10 μm), and their cell bodies are clear and round on phase-contrast microscopy. Cells with astrocyte-like flat morphology were excluded. The data were obtained from cells that met these criteria for neurons.

Criteria for $[Ca^{2+}]_i$ Responses and Expression of Results

Agents were administered in the superfusion solution. When increases in $[Ca^{2+}]_i$ took place within 5 min after adding agents and their amplitudes were 0.25 or larger, they were considered to have responded. In the studies using inhibitor and blockers, when the amplitude of $[Ca^{2+}]_i$ responses with drug treatment was 40% or smaller than that of $[Ca^{2+}]_i$ responses without drugs, inhibition was judged to have occurred. Each experiment was based on data prepared from at least 3 mice. A total of 896 neurons were examined.

Immunohistochemistry

Mice were anesthetized with 10% pentobarbital (10 ml/kg, IP). Then, mice were perfused transcardially with saline and 10% formalin (Wako, 062-01661). Brains were taken out and post-fixed overnight at 4°C. Then, they were transferred to phosphate-buffered saline (PBS, pH 7.4) containing 20% sucrose. The brains were frozen and kept at −80°C until sectioning. Coronal sections were cut at 25 μm using a cryostat (1:5 series). Sections were collected in, transferred to a cryoprotectant solution, and stored at −30°C.

Double immunostaining of T1R2 and POMC were performed as follows. Sections were rinsed in PBS and then blocked with 3% normal donkey serum (NDS; Abcam, Cambridge, UK, ab7475) diluted in PBS containing 0.25% Triton X-100 for 30 min. Next, sections were incubated in goat anti-T1R2 antibody (Santa Cruz Biotechnology, Dallas, TX, SC-22456, 1:50) and rabbit anti-POMC antibody (Phoenix Pharmaceuticals, H-029-30, 1:500) diluted in blocking solution overnight. After rinsing in PBS, sections were incubated with Alexa 488 donkey-anti-goat IgG (Thermo Fisher Scientific, A-11055, 1:400) and Alexa 594 goat-anti-rabbit IgG (Thermo Fisher Scientific, A-21207, 1:400) diluted in 3% NDS for 40 min.

Double immunostaining of T1R3 and POMC were performed as follows. Sections were rinsed in PBS and then blocked with 1.5% normal goat serum (NGS; Rockland Immunochemicals, Gilbertsville, PA, D204-00-0050) diluted in PBS containing 0.25% Triton X-100 for 30 min. Next, sections were treated with streptavidin solution and biotin solution (Vector laboratories, Burlingame, CA, SP-2002) for 15 min, respectively. They were then incubated in rabbit anti-T1R3 antibody (Abcam, ab65423, 1:1000) diluted in Can Get Signal immunostain solution A (Toyobo, Osaka, Japan, NKB-501) overnight. Sections were rinsed in PBS and incubated with biotinylated goat anti-rabbit IgG (Vector Laboratories, VA-1000, 1:400) for 40 min and incubated with ABC reagent (Vector Laboratories) for 40 min. Sections were rinsed in 0.1 mM Tris-HCl buffer (pH 7.5) containing 0.15 mM NaCl and 0.05% Tween 20, and they were blocked with 0.05% blocking reagent (PerkinElmer, Waltham, MA). They were then treated with biotinyl tyramide (PerkinElmer, NEL700001KT, 1:50) for 5 min. After rinsing in 0.1 mM Tris-HCl buffer (pH 7.5) containing 0.15 mM NaCl and 0.05% Tween 20, sections were incubated with streptavidin-Alexa 488 conjugate (Thermo Fisher Scientific, Waltham, MA, S-11223, 1:500) diluted in blocking reagent for 40 min. After a rinse in PBS, sections were incubated with anti-POMC antibody (1:500) diluted in Can Get Signal immunostain solution A overnight. After rinsing in PBS, sections were incubated with Alexa 594 goat-anti-rabbit IgG (Thermo Fisher Scientific, A-21207, 1:400) diluted in 1.5% NGS for 40 min.

Slices were mounted on slides and coverslipped with mounting medium (Vector Laboratories, H-1200). Fluorescence images were acquired with a BZ-9000 (Keyence, Tokyo, Japan). Confocal florescence images were acquired with a confocal laser-scanning microscope (FV10i, Olympus, Tokyo, Japan).

Intracerebroventricular (ICV) Administration of Sucralose and c-Fos and POMC Immunohistochemistry

ICV administration was performed as reported previously (Sasaki et al., 2010) with slight modification. Mice were anesthetized with 10% pentobarbital (7 μl/g, ip) and 5% xylazine (10 μg/g, ip) and guide cannula (C315G, Plastics One, Roanoke, VA) was implanted to the right lateral ventricle, and secured to the skull with dental cement (QUICK RESIN, SHOFU, Kyoto, Japan) and adhesion bond (Loctite 454, Henkel, Dusseldorf, Germany). The cannula tip was located 0.2 mm caudal and 1.0 mm right to bregma and 2.5 mm below the skull. More than 1 week after the surgery, mice that had recovered to 90% of their preoperative weight were used for injection. PBS or 0.085 mg sucralose diluted in PBS (0.5 μl) were injected. Thirty min after injection, mice were perfused transcardially with saline and 10% formalin.

Double immunostaining of c-Fos and POMC were performed as follows. Sections were rinsed in PBS, and then treated with 0.3% H_2O_2 diluted in PBS for 15 min. Sections were then blocked with 1% BSA and 2% NGS diluted in PBS containing 0.25% Triton X-100 for 30 min. Next, sections were incubated in rabbit anti-c-Fos antiserum (Merck Millipore, PC38, 1:25,000) diluted in blocking solution overnight. After rinsing in PBS, sections were incubated with biotinylated goat anti-rabbit IgG (Vector Laboratories, VA-1000, 1:400) for 40 min and incubated with ABC reagent (Vector Laboratories) for 40 min. Sections were rinsed in PBS and 0.175M sodium acetate buffer (pH 5.6), and color was developed with a nickel-diaminobenzidine solution (10 g/liter nickel ammonium sulfate, 0.2 g/liter DAB, and 0.006% H_2O_2 in sodium acetate buffer). After rinsing in PBS, sections were treated with 0.3% H_2O_2 diluted in PBS for 15 min. Sections were then blocked with blocking solution. Next, sections were incubated in anti-POMC antibody (1:4000) diluted in blocking solution overnight. After rinsing in PBS, sections were incubated with biotinylated goat anti-rabbit IgG (1:400) for 40 min and

incubated with ABC reagent (Vector Laboratories) for 40 min. Sections were rinsed in PBS and 0.1 mM Tris-HCl buffer (pH 7.5) containing 0.15 mM NaCl, and color was developed with a diaminobenzidine solution.

Statistical Analysis

Data are presented as mean ± SEM. Statistical analyses were performed using IBM SPSS Statistics 23. Unpaired Student's *t*-test was used to evaluate differences, with values of $P < 0.05$ considered significant.

RESULTS

Sucralose Dose-Dependently Increased $[Ca^{2+}]_i$ in ARC Neurons

To observe the effect of sweet taste molecules on ARC neurons, an artificial sweetener, sucralose, in a concentration range from 10^{-6} to 10^{-2} M was administered to single ARC neurons. Sucralose at 10^{-6} M increased $[Ca^{2+}]_i$ in 1 of 56 neurons (2%); at 10^{-5} M in 16 of 136 neurons (12%); at 10^{-4} M in 40 of 248 neurons (16%); at 10^{-3} M in 33 of 229 neurons (14%); and at 10^{-2} M in 35 of 229 neurons (15%), showing a concentration-dependent effect (**Figures 1A,B**). In a neuron presented in **Figure 1A**, sucralose at 10^{-5} M induced a small $[Ca^{2+}]_i$ increase, and at 10^{-4} M a sustained $[Ca^{2+}]_i$ increase with a larger amplitude. Peak fura-2 ratio amplitudes of sucralose-response were concentration dependent. Peak fura-2 ratio amplitudes of response to sucralose at 10^{-5}, 10^{-4}, 10^{-3}, and 10^{-2} M were significantly higher than the peak amplitude of basal fura-2 oscillation level (**Figure 1C**).

Sucralose-Induced $[Ca^{2+}]_i$ Increases Were Inhibited by Sweet Taste Receptor Inhibitors, under Ca^{2+}-Free Condition, and by L-Type Calcium Channel Blocker

The second administration of sucralose induced repeated $[Ca^{2+}]_i$ increases in 16 of the 20 single-ARC neurons (80%; **Figures 2A,F**). The second addition of sucralose in each neuron was performed in the presence of drugs or in the absence of Ca^{2+}. In the presence of sweet taste receptor inhibitor, gurmarin (Imoto et al., 1991; Shigemura et al., 2008), at 3 μg/ml suppressed sucralose-induced $[Ca^{2+}]_i$ increase in 8 of 10 neurons (80%; **Figures 2B,F**). Peak amplitudes of sucralose-induced $[Ca^{2+}]_i$ increase in the presence of gurmarin were significantly decreased compared with those of control (**Figure 2G**). These data suggest that the sucralose-induced $[Ca^{2+}]_i$ increases in ARC neurons are mediated by the sweet taste receptor. Under extracellular Ca^{2+}-free condition (added with no Ca^{2+} and 0.1 M EGTA), the $[Ca^{2+}]_i$ increase in response to sucralose was abolished in all of 12 neurons (100%; **Figures 2C,F**). The sucralose-induced $[Ca^{2+}]_i$ increase was suppressed by an L-type Ca^{2+} channel blocker, nitrendipine, at 2 μM in 6 of 10 neurons (60%; **Figures 2D,F**) and peak amplitudes of sucralose-induced $[Ca^{2+}]_i$ increase in the presence of nitrendipine were significantly decreased (**Figure 2G**). In contrast, an N-type Ca^{2+} channel blocker, ω-conotoxin GIVA at 500 nM, failed to affect the sucralose-induced $[Ca^{2+}]_i$ increase in most [7 of 8 (80%)] of the sucralose-responding neurons (**Figures 2E–G**). These data suggest that the sucralose-induced $[Ca^{2+}]_i$ increase in ARC neurons depends on sweet taste receptor and extracellular Ca^{2+} influx, especially through the L-type Ca^{2+} channel.

Activation in Response to High-Concentration of Glucose is Mediated by Sweet Taste Receptor

The relationship between sucralose-response and glucose response was examined. Among 22 neurons that responded to sucralose at 10^{-4} M, 12 neurons (55%) exhibited $[Ca^{2+}]_i$ increases in response to elevating the glucose concentration from 1 to 10 mM, the response used to judge the glucose-excited neurons (**Figures 3A,C**). On the other hand, among 22 neurons that increased $[Ca^{2+}]_i$ in response to elevating glucose, 12 neurons (55%) exhibited $[Ca^{2+}]_i$ increases in response to sucralose at 10^{-4} M (**Figures 3A–C**). Notably, there were no sucralose-responding neurons in glucose-inhibited neurons that decreased $[Ca^{2+}]_i$ in response to elevating the glucose

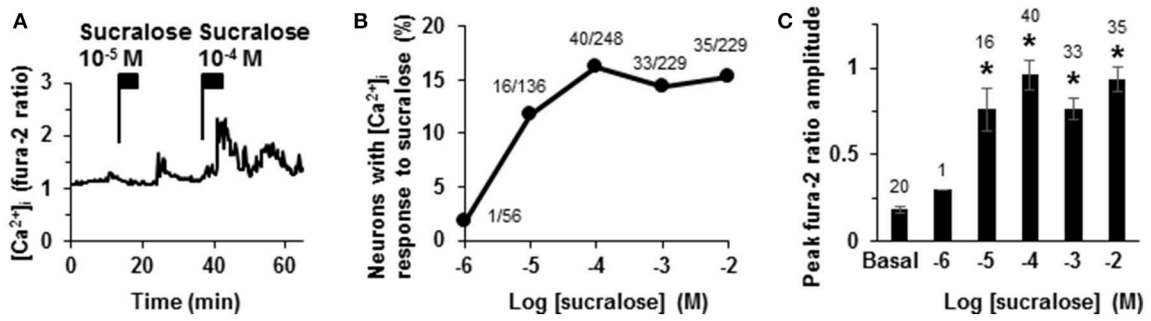


FIGURE 1 | Increase of cytosolic calcium concentration ($[Ca^{2+}]_i$) in ARC neurons after the administration of sucralose. (A) Sequential addition of sucralose at 10^{-5} and 10^{-4} M increased $[Ca^{2+}]_i$ in an ARC neuron. **(B)** Response is expressed as the percentage of neurons that exhibited $[Ca^{2+}]_i$ increases in response to sucralose. The numbers above each point indicate the number of neurons that responded over the number of neurons examined. **(C)** Peak amplitudes of basal oscillation and sucralose-induced $[Ca^{2+}]_i$ increases are expressed. The numbers above each point indicate the number of neurons examined. Mean ± SEM. Unpaired Student's *t*-test was performed to compare peak amplitudes with basal oscillation peak. *$p < 0.001$.

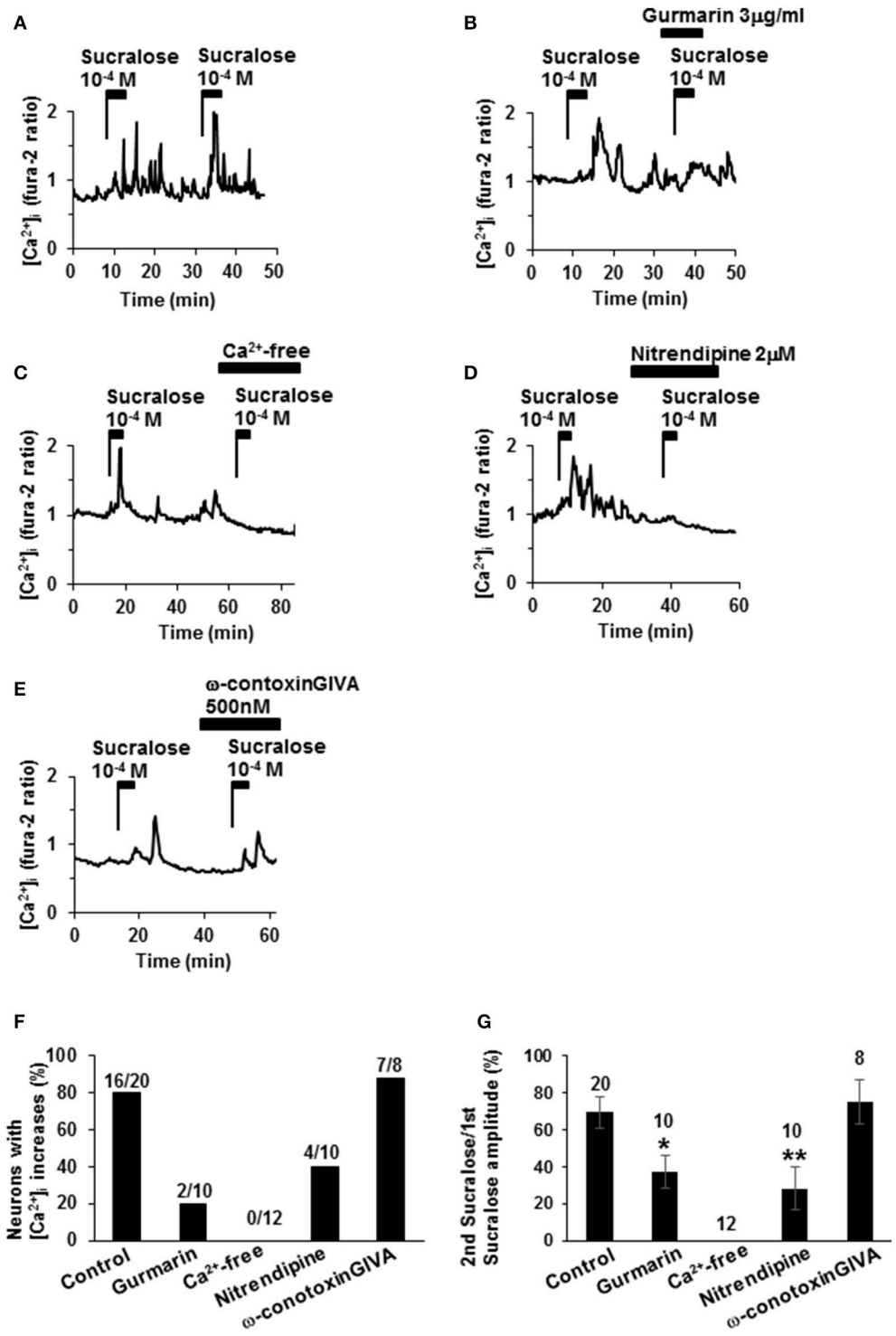


FIGURE 2 | Mechanisms underlying sucralose-induced $[Ca^{2+}]_i$ increases. (A) Administration of sucralose at 10^{-4} M twice induced repeated $[Ca^{2+}]_i$ increases in an ARC neuron. **(B)** Sucralose-induced $[Ca^{2+}]_i$ increases in an ARC neuron were suppressed by gurmarin, an inhibitor of the sweet taste receptor. **(C)** Sucralose-induced $[Ca^{2+}]_i$increases were abolished under a Ca^{2+}-free condition. Sucralose-induced $[Ca^{2+}]_i$ increases were markedly inhibited by nitrendipine, a blocker of L-type Ca^{2+} channel, at 2 μM **(D)**, while it was unaltered by ω-conotoxin GVIA, a blocker of N-type Ca^{2+} channel, at 500 nM **(E)**. **(F)** Incidence of response under control and test conditions is expressed as the percentage of cells that exhibited $[Ca^{2+}]_i$ responses to the second addition of sucralose in each cell. The numbers above each bar indicate the number of neurons that responded over the number of neurons examined. **(G)** The amplitude of sucralose-induced $[Ca^{2+}]_i$ increases under control and test conditions is expressed. The numbers above each point indicate the number of neurons examined. Unpaired Student's *t*-test was performed to compare amplitudes with control. $^*P < 0.05$, $^{**}P < 0.01$.

concentration. These data indicate that more than half of sucralose-responding neurons are glucose-excited neurons, while none of them are glucose-inhibited neurons. These data emphasize the anorexigenic property of sucralose-responding neurons. To explore the contribution of sweet taste receptor to glucose responses, the effect of gurmarin on response to high-glucose was examined. When glucose concentration was increased from 1 to 10 mM, continuous increases of $[Ca^{2+}]_i$ were observed in 11 out of 11 glucose-excited neurons (100%; **Figures 3D,F**). The administration of gurmarin at 3 μg/ml suppressed $[Ca^{2+}]_i$ increases induced by 10 mM glucose in 8 out of 12 neurons (67%; **Figures 3E,F**). Area under the curve of fura-2 ratio amplitudes in the presence of gurmarin was significantly decreased compared to those in the absence of gurmarin (**Figure 3G**). These data suggest that excitation induced by high-concentration of glucose is mediated in part by sweet taste receptor.

Sucralose-Responding ARC Neurons Also Responded to Leptin but Not to Ghrelin

To further characterize sucralose-responding neurons in ARC, responsiveness to a satiety hormone, leptin, and an orexigenic hormone, ghrelin, (Nakazato et al., 2001; Coppari et al., 2005; Kohno et al., 2007) was examined. Among 25 neurons that responded to sucralose at 10^{-4} M, 13 neurons (52%) exhibited $[Ca^{2+}]_i$ increases in response to leptin at 10^{-10} M (**Figures 4A,B**). Among 11 neurons that responded to sucralose at 10^{-4} M, only 2 neurons (18%) exhibited $[Ca_{2+}]_i$ increases in response to ghrelin at 10^{-10} M (**Figures 4C,D**). The high overlapping with leptin-responding neurons and low overlapping with ghrelin-responding neurons suggest that sucralose-responding neurons are primarily the neurons implicated in negative energy balance. However, it should be noted that sucralose-responding neurons also include considerable number of non-leptin-responding neurons and ghrelin-responding neurons.

Sucralose Increased $[Ca^{2+}]_i$ in Non-POMC Neurons and a Few of POMC Neurons

Proopiomelanocortin (POMC) neurons are major satiety neurons in the ARC. We examined if POMC neurons were large population of sucralose-responding neurons. Among 14 neurons that responded to sucralose at 10^{-4} M, 2 neurons (14%) were POMC-GFP neurons and 12 neurons (86%) were non-POMC neurons (**Figures 5A,C**). Among 13 POMC-GFP neurons, 2 neurons (15%) exhibited $[Ca^{2+}]_i$ increase in response to sucralose at 10^{-4} M and 11 neurons (85%) did not exhibit $[Ca^{2+}]_i$ increase (**Figures 5A,B,D**). Thus, the majority of sucralose-responding neurons are non-POMC neurons, and only a small part of the POMC neurons responded to sucralose.

Expression of Sweet Taste Receptor on POMC Neurons

Colocalization of T1R2 or T1R3 on POMC neurons was examined. T1R2-immunoreactivity was observed on 31.7 ± 3.0 % ($n = 3$ brains) of POMC-immunoreactive (IR) neurons (**Figures 6A–D**). Among T1R2-IR neurons in the ARC, 20.7 ± 4.7 % ($n = 3$ brains) were POMC-IR neurons (**Figures 6A–C,E**). T1R3-immunoreactivity was observed on 17.8 ± 2.8 % ($n = 3$ brains) of POMC-IR neurons (**Figures 7A–D**). Among T1R3-IR neurons in the ARC, 20.5 ± 4.6 % ($n = 3$ brains) were POMC-IR neurons (**Figures 7A–C,E**). The colocalization was confirmed by confocal microscopy (**Figures 6F–H, 7F–H**). These

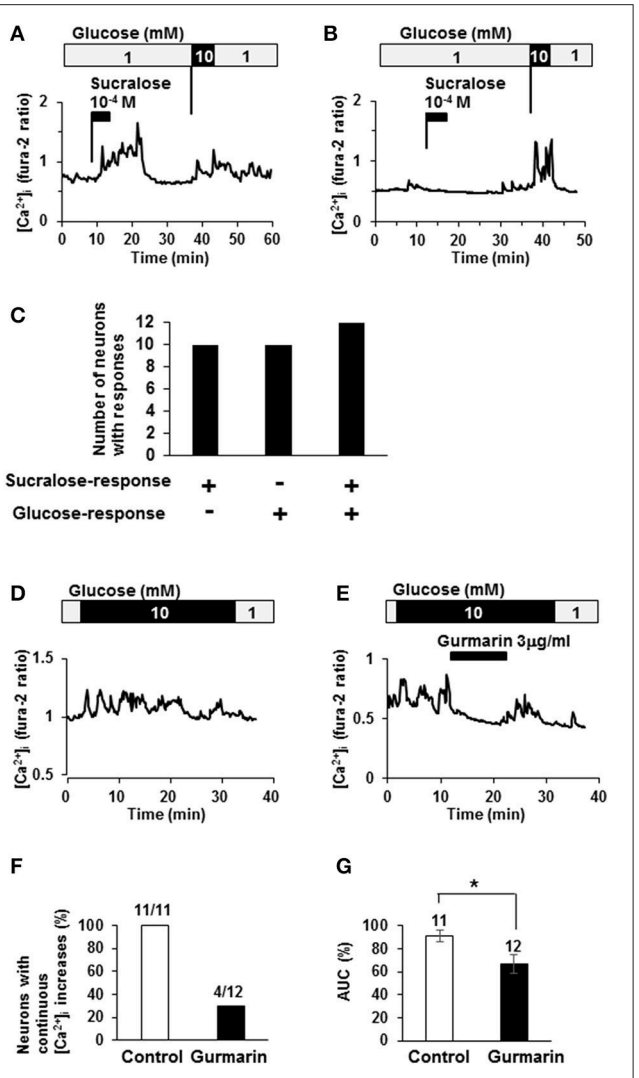


FIGURE 3 | Relationship between sucralose-response and glucose-sensing. (A) An ARC neuron exhibited $[Ca^{2+}]_i$ increases in response to sucralose and raising glucose concentration from 1 to 10 mM. **(B)** An ARC neuron did not exhibit $[Ca^{2+}]_i$ increase in response to sucralose but did in response to raising glucose concentration from 1 to 10 mM. **(C)** The number of the ARC neurons that responded to sucralose and/or glucose at 10 mM. + responding neurons, – non-responding neurons. **(D)** An ARC neuron exhibited continuous $[Ca^{2+}]_i$ increase in response to 10 mM glucose. **(E)** In the presence of gurmarin at 3 μg/ml, 10 mM glucose-induced $[Ca^{2+}]_i$ increase was inhibited. **(F)** Percentage of neurons with continuous $[Ca^{2+}]_i$ increases in response to 10 mM glucose in the absence or presence of gurmarin. **(G)** Area under the curve (AUC) of fura-2 ratio amplitude in the absence or presence of gurmarin. The numbers above bar indicate the number of neurons examined. *$P < 0.05$ (Unpaired *t*-test).

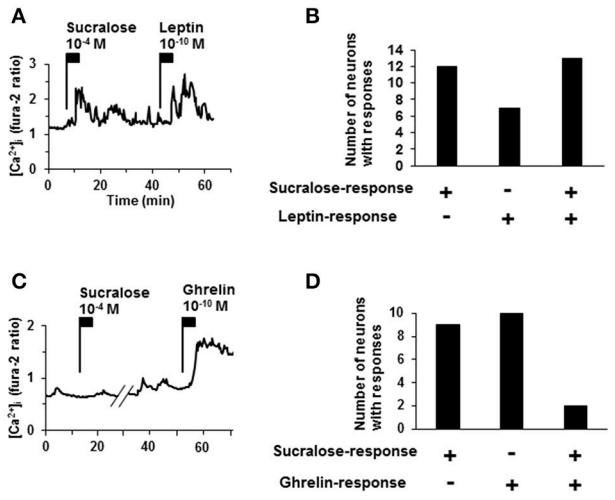


FIGURE 4 | Relationship of sucralose-response to leptin- and ghrelin-response. (A) An ARC neuron exhibited $[Ca^{2+}]_i$ increase in response to both sucralose at 10^{-4} M and leptin at 10^{-10} M. **(B)** The number of responding neurons to sucralose and/or leptin. **(C)** An ARC neurons exhibited $[Ca^{2+}]_i$ increase in response to ghrelin at 10^{-10} M but not to sucralose at 10^{-4} M. **(D)** The number of the ARC neurons that responded to sucralose and/or ghrelin. + responding neurons, – non-responding neurons.

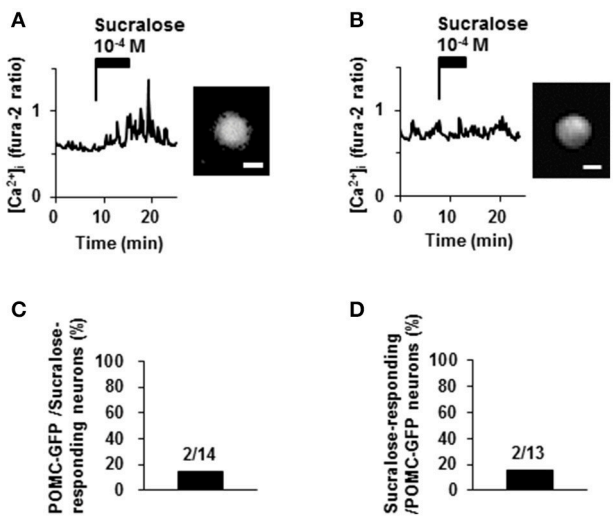


FIGURE 5 | Effect of sucralose on POMC-GFP neurons. (A) A neuron with POMC-GFP fluorescent (right) exhibited $[Ca^{2+}]_i$ increase in response to sucralose (left). **(B)** Another neuron with POMC-GFP fluorescent (right) did not increase $[Ca^{2+}]_i$ in response to sucralose (left). **(C)** Percentage of POMC-GFP neurons among sucralose-responding neurons in ARC. **(D)** Percentage of sucralose-responding neurons among POMC-GFP neurons. The numbers above bar indicate the number of neurons examined. Scale bar 10 μm.

data indicate that the sweet taste receptor is expressed on ARC neurons including a part of the POMC neurons, but non-POMC neurons are major property of sweet taste receptor expressing ARC neurons.

Expression of c-Fos after ICV Administration of Sucralose

Using c-Fos protein as a marker of neuronal activation, activation of ARC neurons following ICV administration of 0.085 mg of sucralose was observed. The number of c-Fos-IR ARC neurons were significantly increased in sucralose-administered mice [266.7 ± 50.5 % ($n = 3$ brains)] compared to PBS-administered mice [105.7 ± 12.1 % ($n = 3$ brains); **Figures 8A–E**]. The number of ARC neurons that were IR to both c-Fos and POMC trended to be increased in sucralose-administered mice [33.3 ± 4.3% ($n = 3$ brains)] compared to PBS-administered mice [18.7 ± 3.9 % ($n = 3$ brains); $P = 0.07$; **Figure 8F**]. The percentage of POMC-IR neurons among c-Fos-IR neurons (**Figure 8G**) and the percentage of c-Fos-IR neurons among POMC-IR neurons (**Figure 8H**) were not altered after sucralose administration. These data indicate that the sucralose in the brain activates ARC neurons, which partly contain POMC neurons.

DISCUSSION

In this study, we found that an artificial sweetener, sucralose, at 10^{-5}–10^{-2} M increases $[Ca^{2+}]_i$ in ∼15% of ARC neurons through the sweet taste receptor-mediated pathway. Sucralose-induced $[Ca^{2+}]_i$ increases were dependent on the extracellular Ca^{2+} influx at least partly through the L-type Ca^{2+} channel. A large part of sucralose-responding ARC neurons were high-glucose- and leptin-responsive neurons. In addition, response to high concentration of glucose was contributed by sweet taste receptor. Low percentage of POMC neurons were included in sucralose responding neurons, c-Fos expressing neurons, and sweet taste receptor-expressing neurons in the ARC. These findings indicate that sweet taste receptor mediated neuronal activation occurs mainly in high-glucose-and leptin- responsive non-POMC neurons in the ARC, which may contribute to the neuronal activation in response to high-glucose. These mechanisms could be implicated in feeding regulation and energy homeostasis.

There are variety of natural and artificial sweet taste molecules in the body and in food, including carbohydrates, numerous amino acids, metabolites, peptides, glycoside, and artificial sweeteners. Carbohydrates are common natural sweet taste molecules and work as an energy source after being catabolized in each cell. Sucralose is barely catabolized and is thereby non-nutritive. In addition, the sweetness intensity of sucralose is much higher than sucrose (Knight, 1994). To purely observe the effect of the sweet taste molecules and to exclude their nutritive effects, sucralose was used in this study. Indeed, most effects of sucralose were suppressed by the inhibitor of the sweet taste receptor, suggesting that sucralose works as a sweet taste molecule but not as fuel for cellular metabolism.

We found that sucralose at 10^{-5}–10^{-2} M induces $[Ca^{2+}]_i$ increases in ARC neurons. Similarly, it is reported that sucralose at 10^{-3}–5×10^{-2} M induces $[Ca^{2+}]_i$ increases in MIN6 cells, a mouse pancreatic β-cell line (Nakagawa et al., 2009). Additionally, sucralose at 2×10^{-4}–5×10^{-3} M induces GLP-1 secretion from NCI-H716 cells, a human enteroendocrine L cell

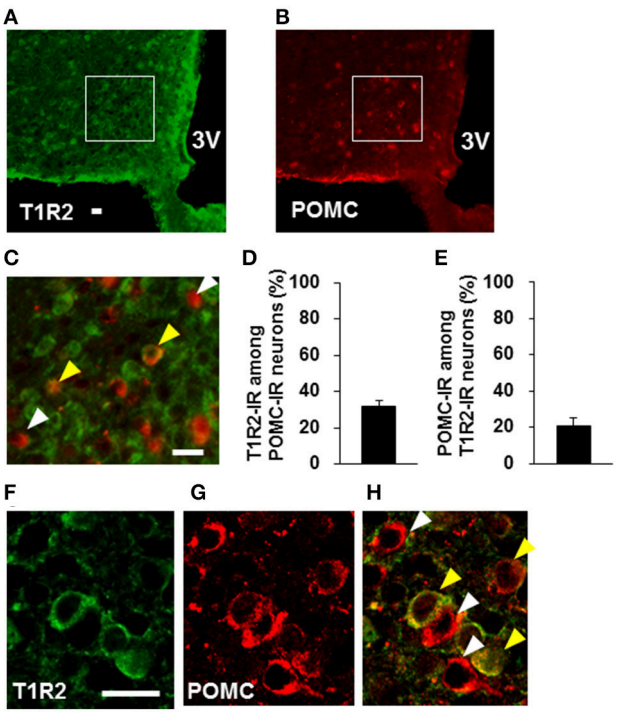


FIGURE 6 | Distribution of T1R2 on POMC neurons in the ARC. T1R2 **(A)** and POMC **(B)** immunofluorescent images and their merged image with high magnification **(C)**. **(D)** Percentage of T1R2-imunoreactive (IR) neurons among POMC-IR neurons in the ARC ($n = 3$ brains). **(E)** Percentage of POMC-IR neurons among T1R2-IR neurons in the ARC ($n = 3$ brains). Confocal images of T1R2 **(F)** and POMC **(G)** immunofluorescent and their merged image **(H)**. White arrowhead indicates non-colocalizing POMC-IR neuron, and yellow arrowhead indicates a colocalizing neuron. Scale bar; 20 μm. Graphs show mean ± SEM.

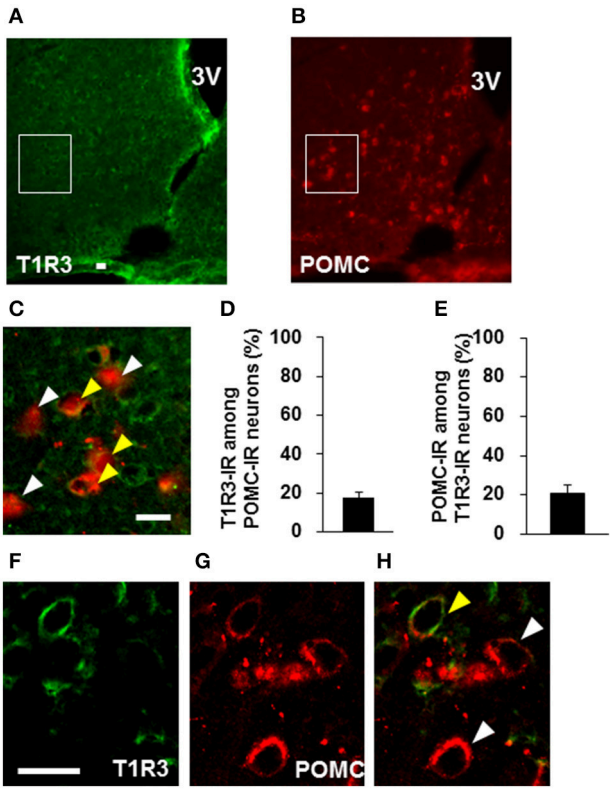


FIGURE 7 | Distribution of T1R3 on POMC neurons in the ARC. T1R3 **(A)** and POMC **(B)** immunofluorescent images and their merged image with high-magnification **(C)**. **(D)** Percentage of T1R3-imunoreactive (IR) neurons among POMC-IR neurons in the ARC ($n = 3$ brains). **(E)** Percentage of POMC-IR neurons among T1R3-IR neurons in the ARC ($n = 3$ brains). Confocal images of T1R3 **(F)** and POMC **(G)** immunofluorescent and their merged image **(H)**. White arrowhead indicates non-colocalizing POMC-IR neuron, and yellow arrowhead indicates a colocalizing neuron. Scale bar; 20 μm. Graphs show mean ± SEM.

line (Jang et al., 2007). These data support the assumption that sucralose may induce the excitement of cellular activity in a sweet taste receptor-dependent manner. Higher sensitivity of ARC neurons to sucralose could be related to the lower concentration of sweet taste molecules, such as glucose, in the brain compared to the periphery. Glucose concentration in the brain is estimated to be lower than that in blood (Routh et al., 2014).

Sucralose-induced $[Ca^{2+}]_i$ increases in ARC neurons were dependent on extracellular Ca^{2+} influx, especially through L-type Ca^{2+} channel. Of note, sucralose-induced intracellular signaling could just be a part of the signals that may be induced at the downstream of sweet taste receptors. In pancreatic β-cells and intestinal GLP-1-secreting cells, a variety of sweeteners induce diverse patterns of intracellular signals (Nakagawa et al., 2013; Ohtsu et al., 2014).

In this study, we found sweet taste receptor contributes to the response to high-concentration of glucose. This is similar to role of sweet taste receptor in the pancreatic beta cells where sweet taste receptors serve as glucose receptors (Nakagawa et al., 2009; Hamano et al., 2015; Kojima et al., 2015). RNA expression levels of T1R2 and T1R3 are altered in response to the change of glucose concentration and obesity (Ren et al., 2009; Herrera Moro Chao et al., 2016). Glucose-sensing mediated by sweet taste receptor could be affected by whole body energy status. High-glucose responses are also contributed by some other molecules including AMP-activated protein kinase and ATP-sensitive K^+ channel (Ibrahim et al., 2003; Claret et al., 2007). In fact, we found that in 33% of glucose-excited neurons, the responses to high-glucose were not altered by the inhibitor of sweet taste receptor, suggesting that high-glucose responses are mediated by sweet taste receptor and other signaling mechanisms. These distinct mechanisms are segregated but could interact with each other. Further studies are required to clarify the precise mechanisms of high-glucose response.

While high-glucose response was suppressed by sweet taste receptor inhibitor in 67% of glucose-excited neurons, sucralose response was observed only in 55% of glucose-responding neurons. This 12% of the difference could be due to the differences between glucose and sucralose as a ligand of sweet taste receptor. The characteristics of sweet taste receptor that can induce multiple downstream signaling cascades depending on ligands (Nakagawa et al., 2013) may have caused the difference

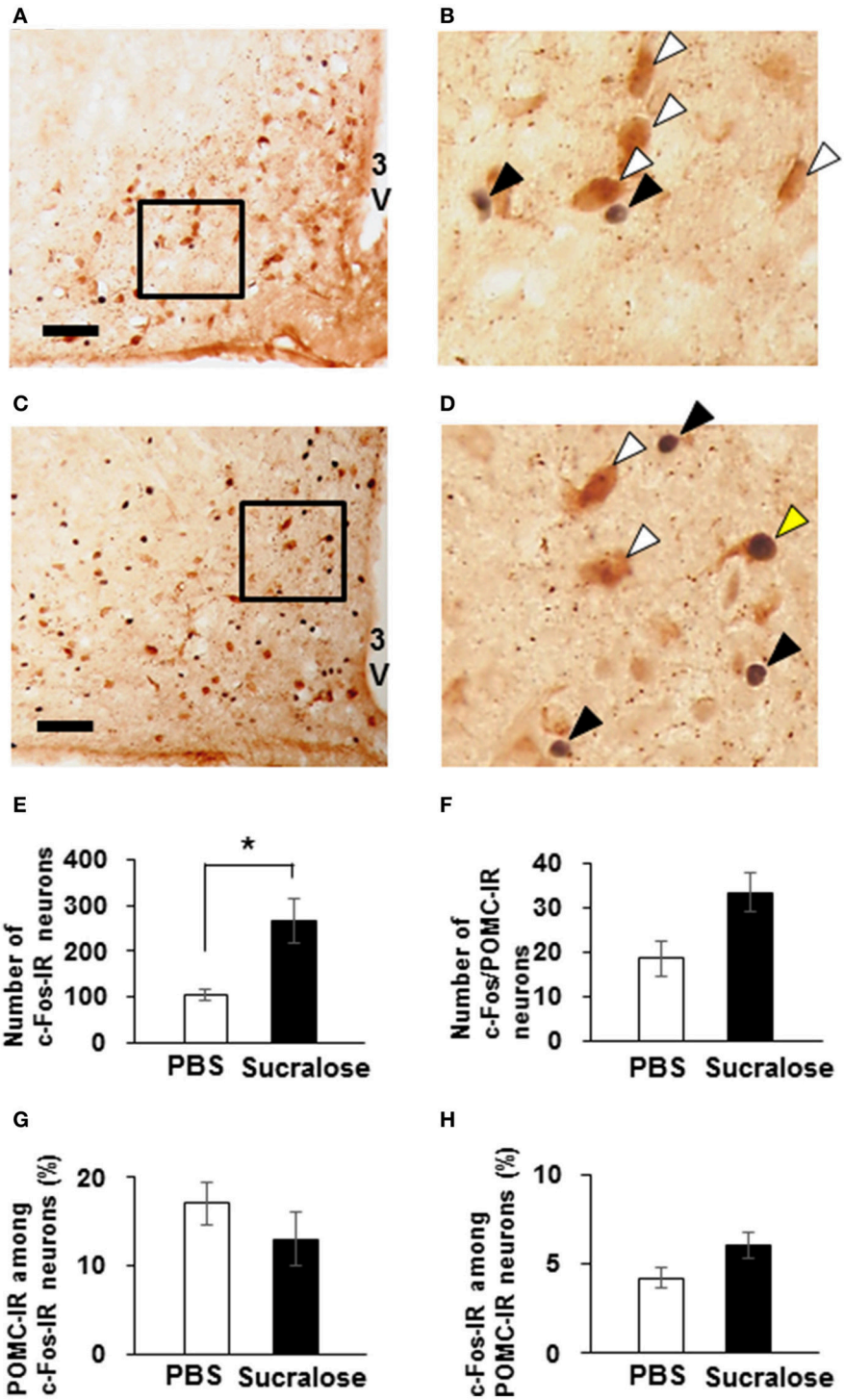


FIGURE 8 | Expression of c-Fos on POMC neurons after intracerebroventricular (ICV) administration of sucralose. Mice ($n = 3$) were given a single ICV administration of PBS **(A,B)** or 0.085 mg of sucralose **(C,D)** and killed 30 min later. c-Fos-IR neurons (black) and POMC-IR neurons (brown) were detected in the ARC. Scale bar 100 μm. Black arrowhead, c-Fos-IR neuron, white arrowhead, POMC-IR neurons, yellow arrowhead, neuron immunoreactive to both c-Fos and POMC. **(E)** The number of c-Fos-IR ARC neurons 30 min after ICV administration of PBS or 0.085 mg of sucralose. **(F)** The number of ARC neurons that are immunoreactive to both c-Fos and POMC 30 min after ICV administration of PBS or 0.085 mg of sucralose. **(G)** Percentage of POMC-IR neurons among c-Fos-IR neurons. **(H)** Percentage of c-Fos-IR neurons among POMC-IR neurons. Graphs show mean ± SEM. $^*P < 0.05$ (Unpaired *t*-test).

between glucose and sucralose in inducing sweet taste receptor-mediated responses.

In this study, 45% of sucralose-responding neurons were not glucose-excited neurons, and also 18% of sucralose-responding neurons were ghrelin-responding neurons. These data suggest that wide variety of neurons could be regulated by sweet taste receptor. Not only glucose but other sweet taste molecules might also be ligands of sweet taste receptor on these neurons. There are several other sweet molecules that can be potential ligands of sweet taste receptor, including amino acids and metabolites, such as glycerol. Comprehensive search of endogenous ligand of sweet taste receptor in the ARC is required for better understanding.

More than half of sucralose-responding neurons also responded to leptin and high concentrations of glucose, but not ghrelin. The concentration of ghrelin in blood rises preprandially while glucose and leptin levels rise post-prandially (Cummings et al., 2001). Ghrelin induces food intake and high-concentration of glucose and leptin induce satiety (Cummings et al., 2001; Nakazato et al., 2001; Routh et al., 2014). These effects are largely mediated by direct actions of ghrelin on neuropeptide Y/AgRP neurons in ARC and by direct excitatory actions of glucose and leptin on satiety neurons including POMC neurons in ARC (Nakazato et al., 2001; Kohno et al., 2003; Coppari et al., 2005). Therefore, the role of ARC neurons in feeding regulation can be suspected by their responsiveness to these key molecules. Sucralose-responding neurons largely responded to both leptin and high-glucose. Inversely, only a minor fraction of sucralose-responding neurons responded to ghrelin. These data implies that the majority of sucralose-responding neurons are those functioning post-prandially or in fed states, which could control feeding, energy metabolism, and glycemia. While POMC neurons are known to be major satiety neurons in the ARC (Williams and Elmquist, 2012), only 14% of sucralose-responding neurons and only 13% of c-Fos-IR neurons were POMC neurons. These results suggest the minor and major contributions of POMC and non-POMC neurons, respectively, to the sweet taste receptor-medicated activation of ARC. This is consistent with previous reports that substantial fraction of glucose-excited neurons in ARC are non-POMC neurons (Wang et al., 2004; Claret et al., 2007; Fioramonti et al., 2007; Parton et al., 2007). While it is well-known that leptin activates POMC neurons in the ARC, leptin's action in the ARC is not limited on POMC neurons. Leptin receptor and leptin-induced immediate signals are observed in ARC neurons including non-POMC neurons (Elias et al., 2000; Lima et al., 2016). There could be unidentified and/or uncharacterized satiety neurons in the ARC. For example, a satiety hormone, nesfatin-1, is localized in ARC neurons other than POMC neurons (Foo et al., 2008). Neurochemical properties of sucralose-responding neurons in the ARC remain to be further characterized.

It is reported that only a small percentage of orally administrated sucralose are absorbed from the intestinal tract in rats, and that most of intravenously administrated sucralose is excreted in urine and feces within 24 h in rats (Sims et al., 2000). It is not yet known if the portion of blood sucralose penetrates the blood-brain barrier (BBB), and the effect of orally ingested sucralose on the hypothalamus is unknown (Schiffman and Rother, 2013). BBB in the ARC is morphologically different from other area (Shaver et al., 1992; Rodríguez et al., 2010; Ciofi, 2011) and is compromised in mice from obese mother (Kim et al., 2016). These characteristics of BBB in the ARC might increase the permeability of sucralose. To clarify the potential side effect of sucralose consumed with foods and to explore the therapeutic application of sucralose, further studies are required.

In this study, we found that the sweet taste receptor is implicated in activation of high-glucose- and leptin- responsive neurons in the ARC, the majority of which are non-POMC neurons. Further, approaches including genetically modified mouse and non-antibody staining could uncover the physiological role and localization of sweet taste receptor in the ARC. Sweet taste receptors may play an important role in coupling systemic factors to neuronal activity and energy homeostasis.

AUTHOR CONTRIBUTIONS

DK and TY designed research; DK and MK performed experiments and data analysis; YN, IK, and TK contributed to theoretical discussions. DK and TY wrote the paper.

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REVIEW
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Central Amino Acid Sensing in the Control of Feeding Behavior

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Dietary protein quantity and quality greatly impact metabolic health *via* evolutionary-conserved mechanisms that ensure avoidance of amino acid imbalanced food sources, promote hyperphagia when dietary protein density is low, and conversely produce satiety when dietary protein density is high. Growing evidence supports the emerging concept of protein homeostasis in mammals, where protein intake is maintained within a tight range independently of energy intake to reach a target protein intake. The behavioral and neuroendocrine mechanisms underlying these adaptations are unclear. While peripheral factors are able to signal amino acid deficiency and abundance to the brain, the brain itself is exposed to and can detect changes in amino acid concentrations, and subsequently engages acute and chronic responses modulating feeding behavior and food preferences. In this review, we will examine the literature describing the mechanisms by which the brain senses changes in amino acids concentrations, and how these changes modulate feeding behavior.

Keywords: food intake, amino acids, protein, leucine, brain, appetite

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INTRODUCTION

Over the past 20 years, a large number of studies have refined our understanding of how neuroendocrine networks detect internal energy availability and modulate behavioral circuits controlling energy intake to maintain energy homeostasis (1). Food intake is also driven by factors independent of internal energy balance. This is well illustrated by the contribution of the sensory and hedonic value of a diet to the control of energy intake independently of energy homeostasis (2). In addition, the need for specific macronutrients or nutrients can affect appetite and food choices, but the mechanisms underlying how individual macronutrients influence feeding behavior or how appetite for specific macronutrients/nutrients influences energy intake remain unclear.

Ensuring sufficient consumption of protein is essential for growth, reproduction, and species survival (3). Animals, from insects to mammals, have evolved mechanisms to ensure quantitatively and qualitatively adequate protein intake (3, 4). Detection of lack or abundance of single amino acids can have profound acute and chronic effects on feeding behavior and food preference (5, 6). In addition, within a certain range, dietary protein content is a determinant of total

Abbreviations: eIF2, eukaryotic initiation factor 2α; AAA, aromatic amino acids; AgRP, agouti-related peptide; APC, anterior piriform cortex; ATP, adenosine triphosphate; BBB, blood–brain barrier; BCAA, branched-chain amino acids; BCAT, branched-chain amino acid transferase; BCKDH, branched-chain ketoacid dehydrogenase; CNS, central nervous system; EAA, essential amino acids; GABA, γ-aminobutyric acid; GCN2, general control non-derepressible 2; HP, high protein; icv, intracerebroventricular; KIC, α-ketoisocaproic acid; KO, knock out; LP, low protein; MBH, mediobasal hypothalamus; mTOR, mammalian target of rapamycin; NPY, neuropeptide Y; NTS, nucleus tractus solitarii; OVN, overnight; POMC, pro-opio melanocortin; PVH, paraventricular nucleus of the hypothalamus; TCA, tricarboxylic acid; WT, wild type.

energy intake (7). Moderately low-protein diets are associated with an increase in energy intake, adjusted to match minimum requirements for nitrogen and essential amino acids (EAA) (8). Conversely, high-protein diets reduce energy intake, presumably to prevent excessive amino acid levels potentially toxic for the brain (9). This remarkable bidirectional adjustment of energy intake based on dietary protein content has been proposed to target a protein intake of 15% across multiple species from insects to humans (10) and supports the idea that protein intake is regulated by homeostatic mechanisms somewhat independent of energy intake or intake of carbohydrate and fat.

In this review, we will examine the literature exploring how the brain monitors internal amino acid availability and how this central detection modulates food intake. We will not discuss in detail the peripheral mechanisms by which amino acids are sensed and how these mechanisms may interact with the brain to control food intake (8, 11).

DIET-INDUCED CHANGES IN BRAIN AMINO ACID CONCENTRATIONS

The unique morphological and functional properties of mammalian cerebral endothelial cells that form the blood–brain barrier (BBB) allow the brain to be protected from toxins and sheltered from variations in blood composition, presumably providing the central nervous system (CNS) with an optimal chemical environment for cerebral functions. Amino acid homeostasis is particularly critical in the brain, as a number of non-EAA – L-glutamate, L-aspartate, L-cysteine, L-homocysteine, glycine, alanine, and taurine – can act directly as neurotransmitters when released at the synapse, while other amino acids, L-tyrosine and L-tryptophan, serve as precursors for neurotransmitters, the catecholamines, and serotonin, respectively. In addition, branched-chain amino acids (leucine, isoleucine, and valine – BCAA) serve as precursors for the neurotransmitter glutamate and pathologically high BCAA concentrations, as seen in Maple Syrup Disease, cause excessive glutamatergic signaling and neurological symptoms (9).

Four facilitative saturable amino acid carriers have currently been identified to be expressed on the luminal side (blood side) of the mammalian BBB, maintaining intra-cerebral levels of amino acids within a narrow range to about 10% of plasma levels (12–14). However, the idea that all amino acids are non-specifically buffered to that fraction is challenged by discoveries demonstrating selective transport of amino acids across the BBB. This was first suggested by the observation that following arterial delivery of radiolabeled amino acids in rats, brain uptake of essential neutral amino acids is 5- to 10-fold greater than that of non-EAA (15, 16). Molecular support for this observation indicates that the system L1 amino acid transporter, which carries most EAA including branched-chain (leucine, isoleucine, and valine – BCAA) and aromatic (phenylalanine, tyrosine, and tryptophan – AAA) amino acids, is the predominant amino acid transport system expressed in the brain endothelium (13, 17, 18). In addition, the luminal and abluminal (brain side) membranes of the brain endothelium are functionally distinct, as sodium-dependent amino acid transport systems are present exclusively on the abluminal membrane, providing the BBB with a mechanism to actively export amino acids against the concentration gradient (13, 19). Thus, the BBB expresses transport systems that allow selective import and exports of amino acids and active regulation of brain extracellular amino acid composition.

BCAA and AAA compete for transport through system L1, and consequently, the blood ratio of BCAA to AAA levels is a major determinant of brain extracellular and cerebrospinal amino acid composition (9). Decreased plasma BCAA levels, as seen in specific contexts including endurance training or adaptation to ketogenic diets, are associated with decreased brain BCAA levels, increased uptake of tryptophan and tyrosine into brain, and increased synthesis of serotonin and catecholamines (20, 21). Conversely, increased plasma BCAA levels are reflected in brain BCAA levels, and negatively impact brain AAA uptake and serotonin synthesis (9, 13). Such changes have been reported in contexts associated with chronic increases in BCAA levels including diabetes or maintenance on a high-protein diet (12, 22–25) but also acutely following food ingestion (26). In fact, while the plasma level of most amino acids remains relatively stable in the postprandial period, BCAA levels rapidly and transiently rise as they largely escape first-pass splanchnic metabolism (27–29), resulting in a rapid increase in brain BCAA levels (26, 30–32). This observation led to the hypothesis that circulating BCAA levels may represent a signal of postprandial protein availability that regulates various anabolic functions modulated by dietary proteins. Consistently, BCAA have been shown to mediate protein-induced transcription, insulin secretion, and protein synthesis (33–35). Likewise, brain BCAA levels may mediate protein-induced modulations of centrally controlled functions, including appetite and metabolism, as discussed below.

Only a few studies have explored the regional differences in the kinetics of brain amino acid uptake following meal ingestion. Microdialysis studies in rats demonstrated that after oral gavage of a balanced amino acid mix or ingestion of a 50% protein meal, there were consistent increases in the concentrations of BCAA in the lateral and periventricular nuclei of the hypothalamus within 20–40 min of meal consumption, while the concentrations of the majority of other amino acids remained unchanged, with the exception of methionine and tyrosine that consistently increase in these brain regions following a meal (36–38). Whether this effect is specific to these brain regions or also occurs in other areas of the brain, including other hypothalamic nuclei, remains to be determined. In contrast, regional differences in the concentration of BCAA and AAA following the ingestion of amino acid imbalanced diets have been described. In this context, while the concentration of the limiting amino acid decreases in discrete sites, including the pyriform cortex, locus coeruleus, and the nucleus of the solitary tract, hypothalamic areas are protected from this deficiency (39–41). Collectively, these data indicate that amino acid concentrations in the brain are not a simple reflection of the plasma amino acid profile but vary selectively in discrete sites under specific dietary contexts.

CENTRAL DETECTION OF ESSENTIAL AMINO ACID DEVOID OR UNBALANCED DIETS

The marked reduction in energy intake and growth of animals maintained on diets containing very low protein amounts (<8% in rats; <5% in mice) or imbalanced EAA ratios was first described over 100 years ago (42). Seminal studies from Harper and colleagues demonstrated that the anorectic response to imbalanced amino acid diets is the cause rather than the consequence of growth failure, supporting a direct role for dietary amino acids in the regulation of food intake (43–46). Analysis of the behavioral responses to the ingestion of a diet devoid in one EAA indicated that the initial rapid anorectic response is followed by the onset of a learned conditioned taste aversion and the development of a specific appetite for the limiting amino acid (5, 47–51). These two latter adaptations have been associated with chronic changes in feeding-regulating circuits and are secondary to acute neuronal amino acid sensing, as reviewed in (52).

The rapid initial aversive response to EAA-devoid diets, manifested by a decrease in meal size and an increase in inter-meal interval, occurs within 20–40 min following feeding onset and is dependent on acute amino acid interoception. This response is independent of food sensory stimuli or peripheral signals (53–55) and instead relies on direct neuronal sensing of EAA imbalance by the anterior piriform cortex (APC). This assertion is supported by the following observations: (i) APC lesions prevent rats discriminating between AA containing and AA devoid diets (56, 57), (ii) concentrations of the limiting EAA in the APC rapidly fall after the introduction of the devoid diet (41, 48), through competition at the capillary endothelial amino acid transport system (58–60), and (iii) replacement of the limiting EAA into the APC *via* microinjections rapidly increases intake of a diet deficient in that EAA (49, 57, 61, 62). Importantly, this aversive response was shown to be independent of diet palatability and novelty (63). Thus, the APC is both necessary and sufficient to produce rapid hypophagia in response to EAA imbalanced diets.

Neurophysiological and neuroanatomical evidence further indicate that local APC EAA sensing initiates the response to EAA-devoid diets and engages neurocircuits connected to hypothalamic, pontine, and hindbrain feeding-regulating networks (**Figure 1A**). Neurons in the APC have been shown to be excited by the absence of threonine or the presence of the amino alcohols, which cause tRNA uncharging (61, 64). Changes in local interneuron interactions, at least in part *via* decreased local inhibitory GABAergic tone (65), cause a change in output signal from glutamatergic APC neurons (66) – their excitatory output is potentiated when GABAergic inhibitory control is lost. Tracing studies from the APC identified the projection targets of APC EAA sensing neurons [reviewed in Ref. (52)], but the functional

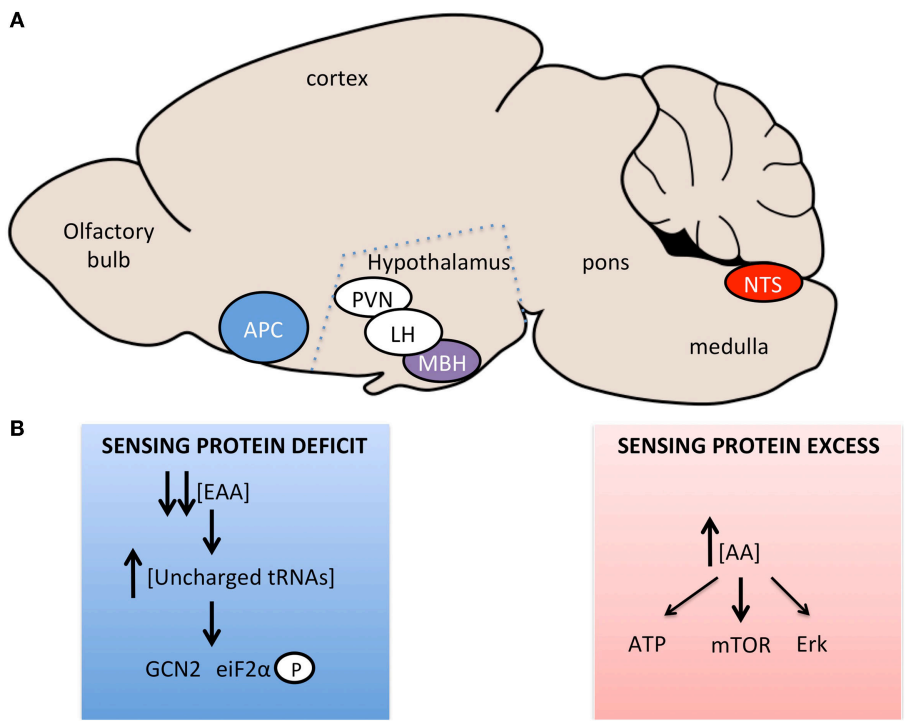


FIGURE 1 | **Brain regions and signaling pathways involved in central amino acid sensing**. **(A)** Sagittal representation of the rodent brain with amino acid sensing brain regions identified. Blue – regions sensing amino acid imbalanced diets or very low-protein diets, Red – regions sensing increases in amino acid concentrations, Purple – regions involved in sensing bidirectional changes in amino acid concentrations, White – regions involved in neurocircuitory of amino acid sensing, which are not primary sensing sites. **(B)** Signaling pathways implicated in central sensing of amino acid deficit or excess.

relevance of the targets in the acute aversion to EAA deficiency has not been directly addressed. Two hypothalamic regions have been implicated in this acute response: the VMH and the LH, both rapidly activated in response to a lysine-deficient meal according to fMRI assessments in rats (67). Norepinephrine and dopamine levels are rapidly increased in these regions under these conditions (39, 62), providing some neurochemical insights into the circuits engaged from APC EAA imbalance chemodetection. Clearly, the precise circuits engaged downstream from the APC in mammals to produce the rapid aversion to EAA imbalance remain partially characterized, and novel circuit mapping tools would prove useful to decipher these circuits.

Work by two independent groups demonstrated that the rapid detection of dietary EAA deficiency within the APC occurs *via* a GCN2-dependent mechanism in mice; this pathway is also required for rejection of for EAA imbalanced diets in drosophila (68). The GCN2 pathway is an evolutionarily conserved pathway identified in yeast to mediate the detection of amino acid deficiency (69, 70). When cellular amino acid levels fall, uncharged tRNAs accumulate in the cell, bind to GCN2 that displays kinase activity toward eiF2α (eukaryotic initiation factor 2α), causing a global suppression of translation, but increased transcription of starvation relevant transcripts (**Figure 1B**). Evidence supporting a role for this pathways in the aversion to unbalanced EAA diet was obtained using GCN2 knockout mice, in which the rapid aversion to unbalanced diets is markedly blunted (71, 72) and downstream signaling, increased eiF2α phosphorylation (73), is absent (71, 72). In addition, direct injection of amino alcohols (that cause tRNA uncharging and GCN2 activation), into the APC of rats fed a normal diet, caused a suppression of feeding but had no effect when mice were on a diet devoid of the amino acid for which the matched amino alcohol was injected (71). This effect is specific to EAA, with proline and serine amino alcohols having no effect on feeding.

However, a recent study challenged these findings and failed to observe a rapid GCN2-dependent hypophagic response to threonine and leucine deficient diets (74, 75). Mice switched from a control to a leucine or threonine-devoid diet did not display a rapid hypophagic response during the first 3 h of feeding the novel diet but did show a hypophagic response after this time point. This delayed hypophagia was GCN2 independent. This latter observation is not necessarily in opposition with previous reports, as GCN2 signaling in the APC is restored within 2 h following the ingestion of an imbalanced diet and is not involved in the longer term hypophagic response to EAA imbalanced diets (76). However, the lack of acute aversion to the imbalanced diets in the Leib study contrasts with the rest of the literature. These discrepancies could be explained by diverging experimental paradigms (duration of fast, amino acid composition of the baseline diet) that may affect the production or kinetics of central EAA imbalance (77). In paradigms evidencing a rapid aversive response to EAA imbalance diets, a rapid fall in the APC concentration of the limiting amino acid occurred in the same time course as the production of the hypophagic response, within 40 min of diet exposure (41, 48, 78), and correlated with the activation of the GCN2 pathway in the APC (71, 72). In the Leib study, plasma concentrations of the missing amino acid and APC GCN2 signaling 1 h after diet exposure were unchanged, suggesting that the EAA imbalance failed to reach the APC during early exposure to the diet in these conditions.

More recently, the mediobasal hypothalamus has been proposed to be a primary sensing site of EAA deficiency (79). After an overnight fast, a leucine devoid diet caused an increase in eIF2α phosphorylation in the MBH within 40 min of diet consumption. Adenoviral-mediated knockdown of GCN2 in the MBH blunted the anorectic response to a leucine deficient diet over an hour of diet consumption. In addition, icv L – leucinol injection increased eiF2α phosphorylation selectively in the MBH and was sufficient to inhibit feeding in WT mice, but not GCN2 KO mice. Importantly, L – leucinol did not activate eiF2α signaling in the APC, demonstrating (i) the specificity of the protocol to target the MBH alone and (ii) the sufficiency of the MBH to initiate an aversive response to a leucine deficient diet. However, in earlier studies, no changes in the concentration of the limiting amino acid were found in three hypothalamic nuclei studied (39), suggesting changes in amino acid concentrations are not uniform across the brain. While the hypothalamus is clearly involved downstream of the APC in the anorectic response to AA deficient diets (80–82), further work will be needed to explore if the MBH is required for primary sensing of EAA deficiency.

While there is controversy about the mechanism and site(s) of central amino acid sensing in this context (75, 77), the ability of central amino acid absence to regulate feeding behavior is clear, and further work will be required to clarify these mechanisms and the neural circuits involved.

CENTRAL SENSING OF AMINO ACID ABUNDANCE AND THE CONTROL OF FEEDING BEHAVIOR

Early work from the Mayer and Harper labs identified that dietary supplementation with amino acids, in particular L-leucine, induces a hypophagic response comparable to that seen following adaptation to high-protein diets in rats (83, 84). Panskeep and Booth suggested that hypothalamic amino acid sensing may contribute to this anorectic response and reported that direct administration of a balanced mixture of amino acids into the hypothalamic parenchyma reduces food intake within 1 h following the injection (85). Subsequently, several groups confirmed that administration of physiologically relevant amounts of leucine into the 3rd ventricle or discrete brain nutrient-sensing regions of fasted rodents reduces energy intake during the subsequent refeeding period (26, 86–88). This anorectic response is not produced by other branched-chain amino acids or any aromatic amino acids (26, 87), is not accompanied by the development of conditioned taste aversion (26, 86), persists for 24 h, and produces a significant decrease in body weight gain (26, 86), as summarized in **Table 1**. Brain leucine levels are increased following a meal, and brain leucine administration reduces food intake, suggesting that brain amino acid levels may constitute a signal of energy and/or protein availability detected by brain nutrient-sensing regions that modulate homeostatic feeding-regulatory circuits.

TABLE 1 | Effects of centrally administered amino acids on food intake.

Paper	Species	Conditions	Time of injection	Route	Amino Acid	Dose	Food intake
(86)	Rat	24 h fast	Before onset of dark cycle	3rd icv	Leucine	1.1 μg	↓
(88)	Rat	24 h fast	–	3rd icv	RPMI Amino Acid Mix	1 μg Leucine, and other EAA	↓
					Leucine	1, 3, 10 μg	↓
(89)	Rat	6 h fast	5–6 pm (onset of dark cycle)	3rd icv	Leucine	197 ng, 787 ng, 1.6 μg	No effect of 197 ng, others lowered feeding
(26)	Rat	OVN fast	1 h before onset of dark cycle	MBH	Leucine	56 ng	↓
	Mouse	6 h fast	1 h before onset of dark cycle	MBH	Leucine	28 ng	↓
					Valine	28 ng	No effect
(90)	Rat	OVN fast	1 h before onset of dark cycle	NTS	Leucine	28 ng	↓
					Valine	28 ng	No effect
(87, 91)	Rat	24 h fast	2 h prior to lights off	3rd icv	Leucine	10 ug	↓
	Rat	24 h fast	2 h prior to lights off	3rd icv	Tryptophan, Methionine, Lysine, Threonine, Serine	10 ug	No effect
	Rat	No fast LP diet	2 h prior to lights off	3rd icv	Leucine	10 μg	↓
(92)	Mouse	OVN fast	After overnight fast	3rd icv	Leucine	2.2 μg	No effect
(93)	Mouse	6 h fast	1 h before onset of dark cycle	NTS	Leucine	14 ng	↓

Leucine abundance is detected in the brain through neurochemically diverse and neuroanatomically distributed networks modulating feeding behavior. This is supported by the observation that both the mediobasal hypothalamus and the nucleus of the solitary tract in the caudomedial brainstem contain leucine-sensing cells that can modulate feeding behavior in response to leucine abundance (**Figure 1A**). Although these two apparently redundant sensing sites produce similar behaviors when injected with leucine, further studies are necessary to understand how they may differently be engaged in physiological contexts. Within each of these sensing sites, the population of leucine-sensing cells is heterogeneous. In the mediobasal hypothalamus, POMC neurons but also non-POMC neurons are activated in response to local leucine administration (26). Likewise, in the NTS, leucine-sensing cells are diverse and comprise POMC neurons, catecholaminergic neurons, and maybe as yet uncharacterized cell types (90). However, this is based on the sole use of c-FOS as a marker of neuronal activation, and we know very little about the electrophysiological response of these cells to leucine. Based on c-FOS studies, only a sub-population of cells within the MBH or NTS respond to leucine (26, 90), but what makes a cell leucine-sensitive or not is an open question, and whether leucine-sensing cells share a common molecular signature remains to be determined. To date, only arcuate POMC neurons have been shown to depolarize in response to leucine bath application in slice preparations, and the underlying mechanisms remains to be characterized (26, 92) (**Figure 1B**).

The detailed analysis of the feeding response to increased leucine levels within the hypothalamus or brainstem further supports the idea that leucine sensing engages multiple behavioral output circuits to control food intake. Indeed, meal pattern analyses show that the reduction in food intake measured in food-deprived rats and mice who received brain injections of leucine results from the alteration of various components of the feeding sequence: increased first meal latency (and therefore decreased orexigenic tone), decreased meal size and decreased meal frequency. One circuit implicated in the acute reduction in meal size following hypothalamic leucine sensing involves hypothalamic POMC neurons, their melanocortinergic projections to PVH oxytocin neurons and oxytocinergic projections to the nucleus of the solitary tract in the caudomedial brainstem (26). However, pharmacological inhibition of this circuit is neither sufficient to suppress the rapid reduction in first meal latency nor entirely blunt the anorectic response to leucine, suggesting that other circuits are involved in the overall behavioral response to brain leucine detection. One possible candidate to mediate the immediate increase in first meal latency following leucine hypothalamic administration in fasted rodents is AgRP neurons. This neuronal population is critical to the development of hunger and foraging during food deprivation (94, 95). Consistent with this possibility, leucine was found to regulate AgRP expression in hypothalamic GT1-7 cells (88). However, whether leucine can rapidly affect electrical or synaptic activity of AgRP neurons is unknown, and more generally, how changes in extracellular concentrations of leucine can rapidly and more chronically affect neuronal activity remains poorly characterized.

Activation of mTORC1 has been consistently associated with brain leucine sensing in the regulation of feeding (86). mTORC1 is an evolutionary-conserved signaling pathway that couples nutrient and growth factor sensing in the control of protein synthesis, growth, cell cycle progression and other processes (96). Within 30 min following discrete injections of leucine into the rodent MBH or NTS, phosphorylation of one of the major effector of mTORC1, p70 S6 kinase 1 (S6K1), increases in the respective site (26, 90). In rats, co-administration of rapamycin, a specific mTORC1 inhibitor, blunts leucine's effect on meal size and early hypophagia, implicating activation of mTORC1 in the mechanisms underlying leucine-sensing in hypothalamic and brainstem feeding-regulating circuits. Further evidence in support for a role of mTOR in the regulation of feeding indicates that inhibition of MBH or Dorsal Vagal Complex mTOR signaling with rapamycin in fed rodents rapidly drives feeding and increases meal size

(26, 86, 90). Conversely, constitutive activation of S6K1 inhibits feeding *via* a specific decrease in meal size in both the MBH and the Dorsal Vagal Complex. These data support a role for endogenous mTOR signaling in the control of meal size and foraging behavior. Interestingly, activation of neuronal TOR signaling has also been implicated in the control of feeding in drosophila. In this invertebrate, activation of p70 S6K1 signaling in neurons that control feeding produces hypophagia in fasted larvae, whereas its down-regulation produces increased foraging and feeding in fed larvae, and these responses engage neuropeptide Y-like signaling (97). Collectively, these data indicate that mTOR in discrete nutrient-sensing neurons is an evolutionary-conserved regulator of feeding behavior.

To better characterize the neurochemical populations of the MBH in which mTOR signaling is important in the control of feeding and metabolism in mice, Smith et al. generated mice with germline deletion of S6K1 from either POMC or AgRP neurons (92). Unexpectedly, a thorough metabolic phenotyping revealed that both lines had normal food intake, feeding behavior and energy expenditure under various experimental conditions. S6K1 deletion in both POMC and AgRP neurons reduced neuronal excitability, and reduces synaptic strength in AgRP neurons, resulting in impaired POMC and AgRP tone in these mice, but these alterations are not sufficient to produce a phenotype. This latter observation is consistent with reports showing that germline loss of AgRP neurons fail to affect energy balance, which supports the idea that feeding-regulatory circuits are plastic during development (98). This raises the possibility that S6K1 loss of function is compensated for in these lines. Further investigations are required to clarify the role of S6K1 in AgRP and POMC neurons in the regulation of energy balance in adult rodents.

As mentioned above, increased mTOR signaling is not sufficient to account for decreased meal frequency following MBH or NTS leucine administration (26, 90). We hypothesized that intracellular leucine metabolism, leading to ATP production, may contribute to neuronal leucine sensing in a mechanism analogous to the pancreatic and brain glucose sensing mechanism (99–101). Leucine undergoes intracellular metabolism, producing α-ketoisocaproic acid (KIC) and isovaleryl – CoA, respectively by branched-chain amino acid transferase (BCAT) and branched-chain ketoacid dehydrogenase (BCKDH), leading to the production of TCA cycle intermediates and ATP production that could contribute to leucine sensing in the MBH. Consistently, KIC injection into the mouse MBH suppressed feeding but through a specific decrease in meal number, without affecting meal size, suggesting that this mechanism is not recruited for the acute effects of leucine on foraging and meal size (26). Moreover, injection of an activator of BCKDK, resulting in an increase in the metabolism of leucine, caused a suppression of food intake, due to specific reduction in meal number. In addition, data obtained in mice bearing a whole body deletion of BCATm, expressed in astrocytes in the CNS and peripheral tissues, suggest that long-term high BCAA levels in the brain affect dietary preferences (102). This deletion produces high brain leucine concentrations. Although energy intake does not differ between the KO and the controls, these mice have a higher preference for a low BCAA diet over a normal diet, indicating that chronic abundance of BCAA in the brain induces changes in food choices that do not require BCAA transamination.

To our knowledge, leucine is the only EAA that has been found to produce an anorectic response when administered alone (**Table 1**). Orexin/hypocretin neurons, an orexigenic neuronal population in the lateral hypothalamus, have been shown to respond specifically to non-EAA using c-FOS staining and slice electrophysiology (103). The depolarization of these neurons in response to non-EAA was mediated by System A amino acid transporters and a suppression of the hyperpolarizing activity of K_{ATP} channels. Unlike in the MBH, leucine did not activate these neurons, as assessed by c-FOS immunohistochemistry and electrophysiology. The sensing of non-EAA was shown to be mTOR independent. Whether this sensing mechanism modulates feeding behavior remains to be established.

DIETS WITH VARYING PROTEIN CONTENT: THE PROTEIN LEVERAGE HYPOTHESIS

As discussed above, a number of species avoid and develop conditioned aversion for diets very low in protein or with imbalanced EAA compositions. In contrast, marginally low-protein diets (LP, 8–10% of energy as protein in rats) with balanced EAA profiles induce a hyperphagic response restoring nitrogen and EAA intakes which, together with various metabolic adaptions, are sufficient to enable growth (87, 91, 104–108). Conversely, high-protein diets (HP, 20–70% of energy as protein) produce a sustained decrease in energy intake that is not caused by taste aversion even at very high-protein levels (109, 110). These bidirectional behavioral responses to shifts in dietary protein content are evolutionary conserved from insects to humans (111), and protein intake has been proposed to be regulated independently of energy intake (7, 112). In the following section, we will highlight the literature describing the role of brain amino acid sensing in the bidirectional regulation of energy intake in response to changes in dietary protein content.

Response to Low-Protein Diets

Rats develop hyperphagia (starting day 2) following transition to a 10% protein diet (87, 113), but little is known about the behavioral components of this response, and to our knowledge, meal pattern analysis has not been performed in this context. Plasma levels of most EAA drop in the first 24 h after shift to a LP diet, but this response is only transient (87), and consistently, brain levels of amino acids, including BCAA and AAA, are not affected by LP feeding (12, 87). However, plasma and brain levels of BCAA and AAA fail to increase in response to food ingestion in rats adapted to a LP diet (87), suggesting that the inability to detect amino acids in the postprandial period could reduce satiety and contribute to hyperphagia. Consistent with this hypothesis, icv administration of leucine produces anorexia in LP-fed rats (87). To directly test the role of brain BCAA sensing in the hyperphagic response to LP diets, Morrison et al. measured energy intake in rats fed LP diets supplemented with leucine or BCAA (87). None of these treatments suppressed the

hyperphagic response to LP diets, but confirmation that they produce increases in brain BCAA levels is missing. The authors went on and treated LP-fed rats with icv chronic amino acid infusions (87). They found that icv amino acid infusions could only partially blunt LP-induced hyperphagia, suggesting that the hyperphagic response to LP diets primarily involves peripheral amino acid sensing sites. These data argue against a role for direct brain amino acid detection in LP-induced hyperphagia but are not sufficient to rule out a contribution of brain amino acid sensing is this adaptation, as a lack of dynamic changes in BCAA or leucine levels in discrete nutrient-sensing regions, typically in the postprandial period, may contribute to reduced satiety in this model.

Data from the same group implicate hepatic FGF21 production in the metabolic and feeding responses to LP diets (91, 114). FGF21 circulating levels are dramatically increased in response to LP diets in mice, rats, and humans (91), and remarkably, *Fgf21* knockout mice are protected against the metabolic effects of LP diets (114). Using *Gcn2* knockout mice, the authors provided evidence suggesting that hepatic GCN2 responds to LP diets and promotes FGF21 production and release. These data clearly implicate FGF21 signaling in the metabolic adaptations to protein restriction, but whether brain amino acid sensing contributes to these responses remains to be clarified (**Figure 1B**). Interestingly, mTOR signaling interacts with GCN2 signaling (115, 116) and bidirectionally regulates FGF21 production (117), suggesting that multiple amino acid sensing pathways may orchestrate the overall metabolic and feeding responses to LP diets.

Response to High-Protein Diets

At the other end of the spectrum, high-protein diets have been consistently shown to reduce energy intake in multiple species, from flies to humans (7, 111). When rats previously adapted to a normal-protein diet are offered a HP diet, they immediately decrease their food intake and progressively but incompletely re-increase food intake on the following days (118–121). Gut amino acid sensing and recruitment of local vagal afferents have been implicated in these responses, as reviewed in Ref. (11, 122). However, vagal afferents are not sufficient to produce the anorectic response to HP diets, as subdiaphragmatic vagotomy does not abolish the ability of a HP diet to reduce food intake (123). Brain detection of increased amino acid levels, particularly BCAA, may therefore also contribute.

Meal pattern analysis of rats exposed to a HP diet indicates that the initial important anorectic response to HP diets is mainly driven by a transient decrease in meal size, whereas after adaptation to the diet, decreased meal frequency is primarily accounting for the reduced energy intake (109). Ropelle et al. implicated activation of hypothalamic mTOR signaling in this response and found interestingly that although adaptation to a HP diet induced a chronic increase in brain leucine concentrations, hypothalamic mTOR signaling was only transiently increased (89). Thus, transient increase in hypothalamic mTOR signaling could account for the transient decrease in meal size during early exposure to HP feeding. In contrast, increase hypothalamic leucine catabolism may underlie the chronic decrease in meal frequency. However, there is currently no direct evidence to support this interpretation, and research in this field would significantly progress with the identification of a central mechanism specifically mediating the identification of protein abundance. Likewise, little is known about the neurocircuits involved in the hypophagic response to HP diets. Expression of *Pomc*-, *Npy*-, and *Agrp*-feeding neuropeptides is altered in response to a HP diet in rats (89), but these findings have been recently challenged (124), leaving open the contribution of melanocortinergic feeding circuits in the hypophagic response to increased dietary protein intake.

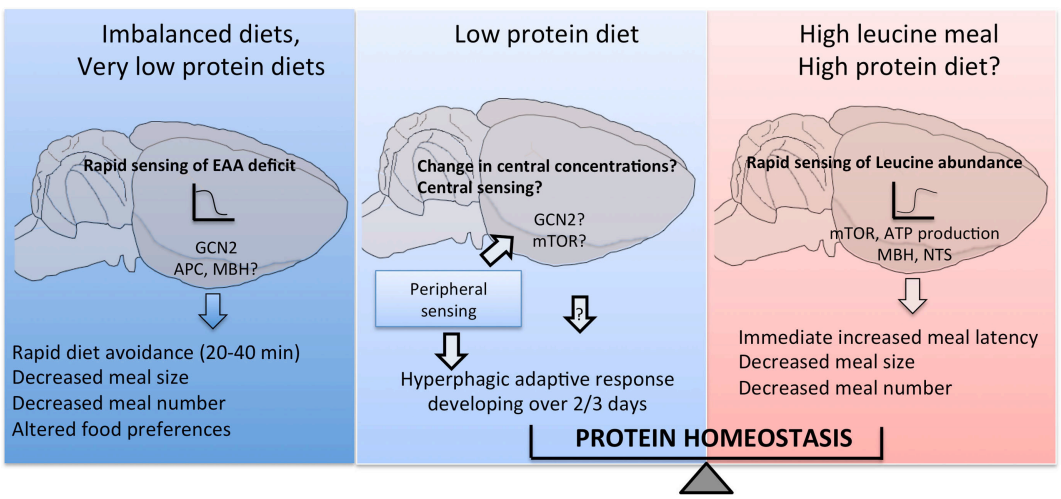


FIGURE 2 | **Central detection of amino acid availability in the regulation of feeding behavior**. Discrete cell populations in the brain monitor extracellular amino acid availability through a variety of mechanisms and engage downstream feeding-regulatory circuits to prevent or limit the ingestion of diets with imbalanced essential amino acid content, produce hyperphagia if dietary protein content is low and conversely reduce food intake if dietary protein content is high.

CONCLUSION

Collectively, the data reviewed here support a role for a distributed network of discrete brain regions in primary amino acid sensing in the control of multiple behavioral responses to changes in dietary amino acid intake, as summarized in **Figure 2**. Many gaps remain to be filled to complete our understanding of these processes, and a key poorly described step is the intracellular coupling of intracellular amino acid availability to neuronal electrical and synaptic activity, as highlighted in **Figure 1B**. Does this coupling rely on specific intracellular components that could perhaps represent a unique molecular signature of amino acid sensing neurons, making these cells amenable to molecular genetics? Identifying a molecular marker for amino acid sensing neurons would prove extremely useful in the characterization of neurocircuits engaged downstream from primary brain sensors to regulate feeding behavior.

Brain amino acid sensing also modulates feeding behavior on a chronic basis and in some cases affects food preferences. The neurobiological substrates supporting these chronic changes are poorly characterized, and how dietary amino acids may affect the remodeling of central circuits regulating energy balance is unknown.

Central amino acid sensing has also been implicated in the regulation of metabolism and glucose homeostasis. Studies in flies and rodents suggest that decreased protein intake accounts for the beneficial metabolic effects of caloric restriction (3, 4, 125). Further work is required to determine how central amino acid sensing processes implicated in the control of feeding behavior may also coordinate metabolic effectors of energy balance and possibly mediate the beneficial effects of caloric restriction of metabolic health and lifespan.

Lastly, although we did not discuss in depth the evidence supporting the concept of protein homeostasis, understanding how distributed peripheral and central amino acid sensors monitor amino acid quantitative and qualitative availability to adjust feeding behavior is a long-term challenge in the field. The characterization of this "homeostatic" control could help identify novel targets for the prevention or treatment of obesity, potentially uncoupling the beneficial satiety effects of protein from their deleterious consequences on metabolic health.

AUTHOR CONTRIBUTIONS

Both authors contributed to the preparation of the manuscript.

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METHODS
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Food Seeking in a Risky Environment: A Method for Evaluating Risk and Reward Value in Food Seeking and Consumption in Mice

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Most studies that measure food intake in mice do so in the home cage environment. This necessarily means that mice do not engage in food seeking before consumption, a behavior that is ubiquitous in free-living animals. We modified and validated several commonly used anxiety tests to include a palatable food reward within the anxiogenic zone. This allowed us to assess risk-taking behavior in food seeking in mice in response to different metabolic stimuli. We modified the open field test and the light/dark box by placing palatable peanut butter chips within a designated food zone inside the anxiogenic zone of each apparatus. We then assessed parameters of the interaction with the food reward. Fasted mice or mice treated with ghrelin showed increased consumption and increased time spent in the food zone immediately around the food reward compared to *ad libitum* fed mice or mice treated with saline. However, fasted mice treated with IP glucose before exposure to the behavioral arena showed reduced time in the food zone compared to fasted controls, indicating that acute metabolic signals can modify the assessment of safety in food seeking in a risky environment. The tests described in this study will be useful in assessing risk processing and incentive salience of food reward, which are intrinsic components of food acquisition outside of the laboratory environment, in a range of genetic and pharmacological models.

Keywords: behavior, food seeking, open field test, light dark box test, reward, risk

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INTRODUCTION

In a free-living situation, organisms always face a trade off in procurement of food. They must evaluate the risk of obtaining food in an unsafe environment, against the reward value of the food itself once obtained. This calculation must necessarily be dynamic and quickly recalculated when changes to either the environment or the nutritional needs of the organism occur. This flexible value attached to the food reward can be termed the "incentive salience" of the reward. The incentive salience (i.e., the importance) of the food is heavily influenced by physiological state (Lockie and Andrews, 2013). Studies of feeding behavior in laboratory animals, generally focused on mice, do not tend to take this balance into account, with most relying on a simple measurement of food consumed in the safe home cage environment. This largely removes the food-seeking behavioral

component of food consumption, which is ubiquitous in animals outside of those kept as pets. Even humans in the current "obesigenic" environment must engage in food seeking behavior with some inherent risk, such as driving to the grocery store or restaurants. Studies that measure only food intake in a cage may miss deficits in the complete feeding behavioral spectrum, as they are not equipped to measure them. We have set out to address this issue by establishing and optimizing a set of simple and cost-effective behavioral tasks, using traditional behavioral equipment, designed to evaluate the incentive salience of a food reward in a risky environment.

Previous models of risk have approached this idea differently, using an operant conditioning paradigm and either a variable reward schedule or choice between a small, safe reward, and a large, "risky" reward—where "risky" is defined as an intermittent reward schedule for that lever (Leblond et al., 2011). This falls short of modeling "risk" as loss of a potential reward does not result in punishment or loss of personal resources. This task was redesigned to include a possible foot shock delivered along with the food reward (Simon and Setlow, 2012). These variations are informative regarding decision making in reward seeking, but fall short of modeling environmental risk *per se* as the animal can choose to avoid the risky lever entirely. Current mouse risk/reward paradigms do not incorporate the intrinsic risk associated with reward or food seeking in natural environments, such as injury and predation risk (Anselme, 2015). Others have previously used a modified light/dark box task with food included in the light zone (Teegarden and Bale, 2007; Cottone et al., 2012; Liu et al., 2016) as a measure of food seeking in a risky environment, and in this paper we seek to expand, validate and standardize this idea. Considering the light/dark box, like most common tests of unconditioned anxiety, relies upon the evolutionarily conserved fear of predation (Crawley, 2006), we supposed that this could be exploited to model ecologically relevant risk processing in food-seeking behavior in mice.

We have modified several standard behavioral tests of anxiety-like behavior to include a food reward element in the anxiogenic zone. We have experimentally validated these tests in a number of paradigms designed to alter the motivational state of the animal and/or the incentive salience of the reward. We fasted mice for 16 h or administered exogenous ghrelin, as both interventions are well-known to enhance food consumption, food seeking, and lever pressing in operant conditioning tasks (Tang-Christensen et al., 2004; Overduin et al., 2012; Skibicka et al., 2012). To examine the tests' sensitivity to acute metabolic state, we also included a group that had been fasted for 16 h and then administered IP glucose. Previous studies have shown that glucose can inhibit feeding in fasted mice, and that blood glucose levels can influence reported hunger levels in humans (Mayer, 1953; Bady et al., 2006), so we hypothesized that these mice should behave like fed mice.

METHODS

Animals

C57black/6 male mice were obtained from Monash Animal Services at 8 weeks of age. For modified light/dark box and open field they were housed in pairs at 22°C in a 12:12 light/dark cycle. They were fed standard laboratory chow (Specialty Feeds, Glen Forrest, WA). Palatable food rewards were Reece's peanut butter chips (The Hersey Company, Hershey, PA, USA), referred to throughout as PB chips. All mice were exposed to the PB chips three times in the home cage before the test day to avoid neophobia and to familiarize mice with the taste and caloric content of the chips. All experiments were performed in the last 4 h of the light phase. For fasting experiments, food was removed at 1 h after the onset of the dark on the previous day and testing was conducted in the last 4 h of the light cycle on the test day. Food was returned immediately upon completion of the behavioral task.

Drugs

Ghrelin (Rat, SC1356, lot number HF40076B, PolyPeptide group, Strasbourg, France) was administered at 0.3 mg/kg, in saline, and injected IP at 10 ml/kg. This dose was previously determined by dose response to be the lowest to reliably increase feeding in a 2 h window (Lockie et al., 2015). Ghrelin or saline vehicle were given 5 min before mice were placed in the apparatus. Glucose was given at 2.25 g/kg in 10 ml/kg of distilled water, not saline, to limit hypertonicity of the injection. Control mice for glucose injections received saline, not distilled water, avoid hypotonicity of the injection. Glucose was given 10 min before mice were placed in the apparatus.

Behavioral Tasks

Modified Open Field

The open field arena consisted of a circular area with a larger than normal diameter of 800 mm to increase the open field and enhance anxiety. PB chips were placed in an equilateral triangle within the center zone of the open field, with each PB chip being 280 mm from the external wall. PB chips of known weight were secured to the floor of the arena with Blu-Tack (Bostic). All other parameters were as per usual open field protocols, as follows. After injections, each mouse was placed in the same area of the perimeter of the open field and allowed to explore the space without interruption for 10 min. The trial was filmed for later analysis. After 10 min, the mouse was removed from the arena and placed back in the home cage with *ad libitum* access to food and water. The PB chips were removed and weighed and the apparatus cleaned with mild soap and water and allowed to dry.

Modified Light/Dark Box

The light dark box apparatus consisted of a two-chambered box, with a large, white zone (480 × 300 mm) and a smaller black zone (150 × 300 mm). Both zones were open to the light at the top. A single, previously weighed PB chip was secured in the center of the center of light zone with Blu-Tack, 240 mm from the entry to the dark zone. Mice were injected, then placed in the dark side of the apparatus. They were allowed 10 min to explore the apparatus undisturbed, and trials were taped for later analysis.

Modified Elevated Plus Maze

The elevated plus maze apparatus was a standard mouse plus maze with two open and two closed arms, with each arm being 50

× 300 mm, and a center zone 50 × 50 mm. Each open arm was baited with a PB chip 150 mm from the center zone, which was fixed to the arm with Blu-Tack. Mice were placed in the center zone facing the same open arm each time. They were allowed to explore the apparatus for 6 min and trials were filmed for later analysis.

Video Analysis

All behavior tests were filmed and video analysis was done later using CleverSys TopScan software. Zoning for software analysis included a "food zone," which was the area immediately surrounding the PB chip. For images of sample arenas, see Supplementary Figure 2. The food zone was always 78 mm in diameter, with the PB chip at the center of the zone. Mice were tracked from the center of the body and time in food zone was defined as center of the body within the 78 mm zone. Individual bouts were defined as the mouse entering and then leaving the zone, with the length of the bout being defined as the amount of time the mouse was continuously tracked within that zone. Latency was defined as time to approach one of the PB chips after introduction into the arena. For the reward-baited open field, approach of any one of the three pellets was considered the initial entry into the food zone.

Validation Parameters and Statistics

We were aiming to see data variation consistent with our results from standard versions of open field, elevated plus maze, and light/dark box, typically needing around 15/group to achieve an alpha of <0.05 for an effect size of ~ 0.8. We required the tests to be similarly sensitive to the non-modified open field, light dark box, and elevated plus maze. We intend to use these tests to screen genetically modified mice, and so we require a large to very large effect size to minimize animal numbers used in these tests, meaning values of Cohen's *d*-values of 0.8–1.2. Accepting an α of 0.05 and a β of 0.8, we need a sample size in the range of 10–21 mice/group, calculated using G*power (Faul et al., 2007). All statistics were performed using Graphpad prism software. Independent measures *t*-tests were used to compare results, with an alpha level of <0.05 being considered significant.

RESULTS

Validation of Tests Comparing Fasted and Ad Lib Fed Mice

We started with three common tests of "anxiety" in mice, the open field, the light/dark box, and the elevated plus maze. We used fasted mice to validate the concept, as fasting is a well-established method to induce motivated responding and food intake (Toth and Gardiner, 2000). We considered a number of aspects of experimental design that might impact upon test performance. To this end, we performed our tests close to the end of the light cycle, when mice are naturally becoming more alert and hungry. We considered the distance between the safe zone and the food reward, which we felt needed to carefully balance the anxiogenic nature of the open zone, with the motivational pull of the PB chip reward. We found that in general, we needed group sizes of 12–15 mice to see significant results for time spent in the food zone and amount of PB chip consumed in the experiments we performed. **Figure 1** shows the results of the three tests. Mice fasted for 18 h spent significantly more time in the food zone (**Figures 1A,E**) and ate significantly more of the PB chip (**Figures 1D,H**) in both the open field and the light dark box tests. We did not see any differences in latency to approach to food zone (**Figures 1B,F**) or number of bouts in the foods zone (**Figures 1C,G**) for either test. In the elevated plus maze, we did not see any differences in time spent in the food zone, which was overall very low, or in any other parameter (**Figures 1I–K**). Variability of results was particularly high for this test. We concluded that the elevated plus maze was a less suitable test for these modifications than the open field and light dark box, so did not pursue this test further. We then set out to validate our modified open field and light dark box test in additional experimental conditions of modified metabolic and motivational states.

Ghrelin Treatment Increases PB Chip Consumption and Time in Food Zone

Similarly to fasting, ghrelin has been well-described to increase both food consumption and motivated responding in operant tasks (Dickson et al., 2011). We administered ghrelin or saline to fed mice and repeated the baited open field and light/dark box as for the fasted mice. In both the light dark box (**Figure 2A**) and the open field (**Figure 2E**), we saw a significant increase in time spent in the food zone in the ghrelin-treated mice. This was supported by increased consumption of the PB chip in ghrelin treated mice (**Figures 2D,H**), in line with the well-known orexigenic actions of ghrelin. We did not see differences in latency to approach the food zone (**Figures 2B,F**) or number of bouts in the food zone (**Figures 2C,G**) in either test, suggesting that there were no differences in baseline anxiety-like behavior in ghrelin and vehicle-treated mice. Interestingly, we saw significant increase in locomotor activity in ghrelin-treated mice in the light/dark box only (Supplementary Figure 1C), which was not seen in the open field test.

Glucose Pretreatment Reduces PB Consumption and Food Zone Time in Fasted Mice

In order to validate that energy status was driving the motivated behavior in the anxiogenic environment, we wondered if acutely mitigating the energy deficit in fasted mice with IP administration of glucose would reduce interest in the PB chip. We found that, indeed, giving glucose to fasted mice 10 min before testing significantly reduced the amount of time fasted mice spent in the food zone in both the light/dark box (**Figure 3A**) and the open field (**Figure 3E**). Additionally, it significantly reduced the amount of PB chip consumed in both tests (**Figures 3D,H**). Again, no differences were noted in either latency to approach (**Figures 3B,F**) or number of bouts in the food zone (**Figures 3C,G**).

Generally, we did not see differences in time spent in the anxiogenic (center of the open field or light side of the light dark box) or anxiolytic zones (perimeter zone of the open field or

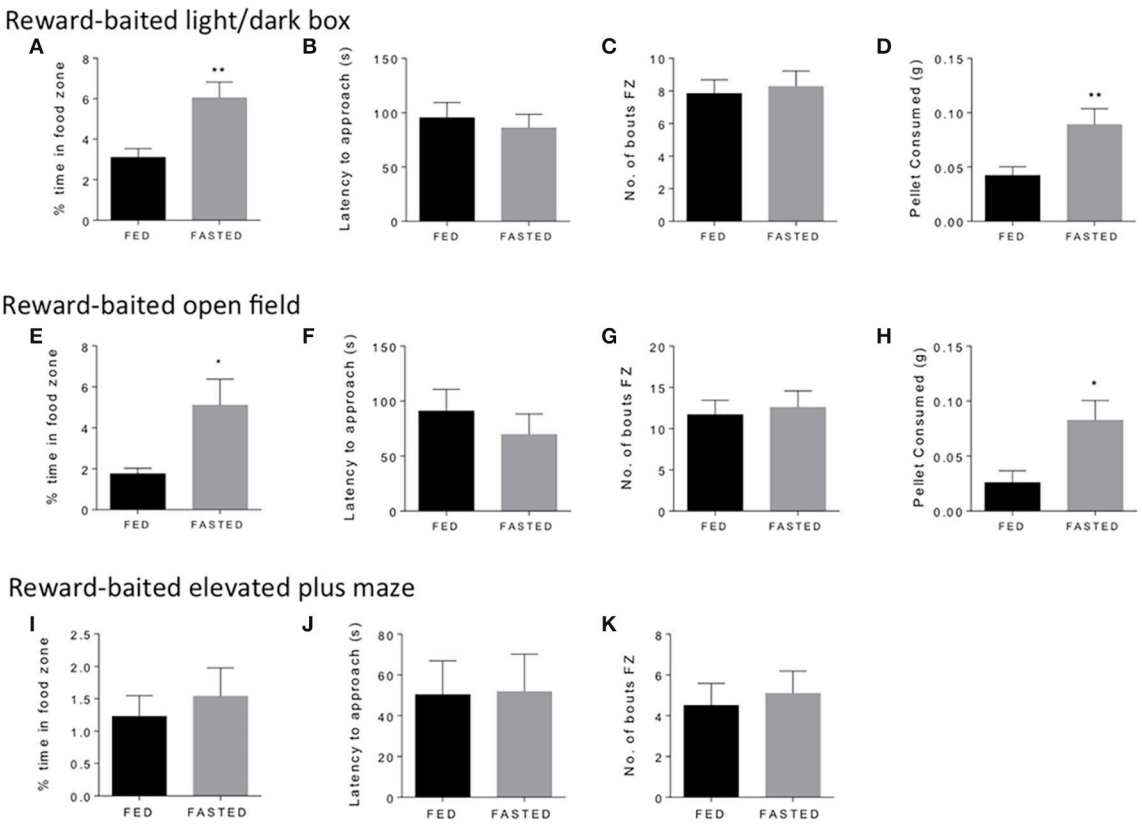


FIGURE 1 | Fasting increases time in food zone and consumption of peanut butter chip reward. Comparison of *ad libitum* fed and 18 h fasted mice in the reward-baited light/dark box **(A–D)**, $n = 18$; reward-baited open field test **(E–H)**, $n = 12$; and reward-baited elevated plus maze, **(I–K)**, $n = 20$–25. FZ, food zone; $^{*}p < 0.05$, $^{**}p < 0.01$, independent measures *t*-test.

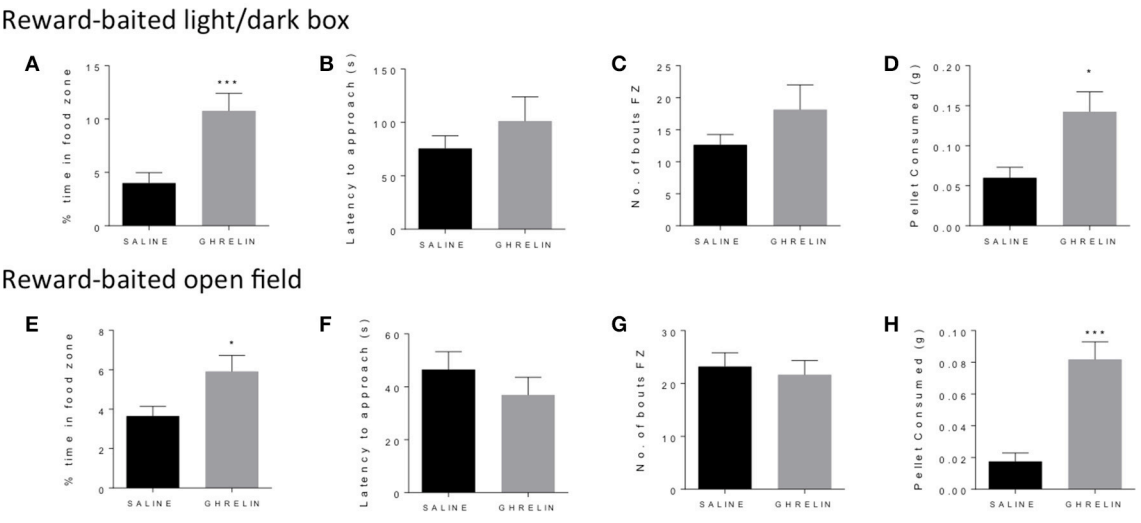


FIGURE 2 | Ghrelin treatment increases time in food zone and consumption of peanut butter chip reward. Comparison of saline treated and ghrelin treated mice in the reward-baited light/dark **(A–D)**, $n = 8$; and reward-baited open field test **(E–H)**, $n = 16$–18. FZ, food zone; $^{*}p < 0.05$, $^{***}p < 0.001$, independent measures *t*-test.

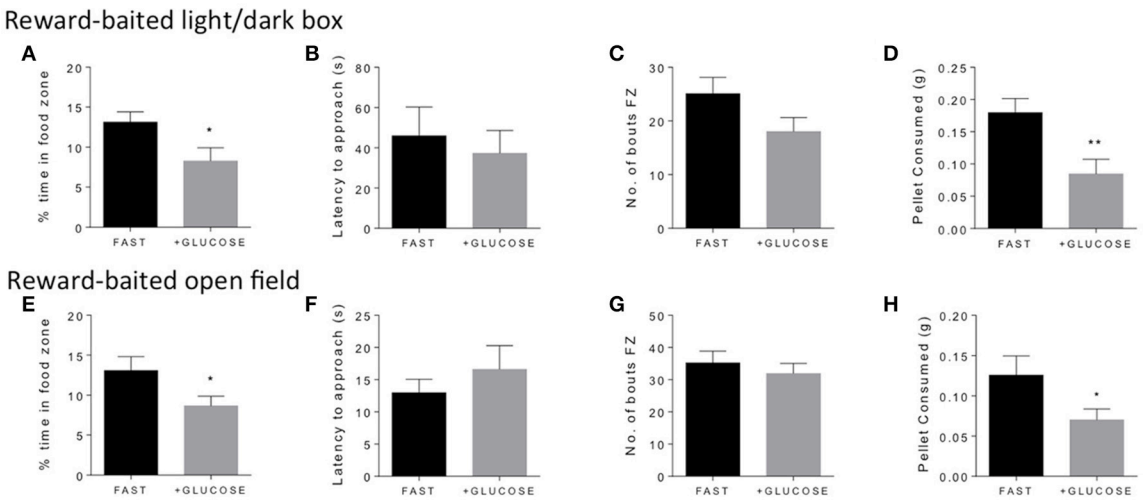


FIGURE 3 | Glucose treatment in fasted mice decreases time in food zone and consumption of peanut butter chip reward. Comparison of 18 h fasted, saline treated, and 18 h fasted, glucose treated mice in the reward-baited light/dark **(A–D)**, $n = 15$–16; and reward-baited open field test **(E–H)**, $n = 12$–14. FZ, food zone; $^{*}p < 0.05$, $^{**}p < 0.01$, independent measures *t*-test.

dark side of the light/dark box; Supplementary Figure 1). The exceptions to this were decreased time spent in the perimeter zone for fasted mice compared to fed mice in the open field (Supplementary Figure 1K) and increased time in the perimeter zone for fasted mice treated with glucose compared to fasted mice treated with saline (Supplementary Figure 1Q). Both these results reflect the increased time the fasted group spent in the food zone, and do not represent a true anxiety based result. With the exception of the already mentioned ghrelin-treated group we did not see alterations in locomotor activity between groups (Supplementary Figure 1).

DISCUSSION

Food consumption in laboratory rodents is almost exclusively measured in the context of *ad libitum* feeding in a home-cage environment. This fails to adequately model the reality of food seeking in a natural environment where attainment of food necessarily involves risk taking. We designed a series of experiments in which the mouse understands the value of the reward, but not the risk required to obtain the reward. To validate these tests, we manipulated the metabolic state to influence the decision to commit to food seeking in the novel, anxiogenic environment. We specifically set out to address a hole in the current battery of tests used to assess feeding and food reward, and attempted to design tests that would incorporate ecologically relevant risk processing, as currently available tests do this poorly (Anselme, 2015). To this end, we have described and validated two tests, which have innate perceived risk to the mouse, are sensitive to metabolic state, and are repeatable. These two tests are the reward-baited open field, the reward-baited light/dark box, which have the additional advantage of being cheap, time-effective and using standardized equipment readily available to most labs. We attempted to modify the elevated plus maze in a similar manner, by baiting the open arms with PB chips, but this test proved difficult to validate. It showed high variability within groups and low levels of investigation/consumption of the BP chips. We concluded the elevated plus maze apparatus was not appropriate for this modification to assess risk in food seeking.

We found no differences in time spent in the light zone of the light/dark box or center zone of the open field for any experimental condition, which demonstrates that mice explore the novel anxiogenic space similarly, regardless of metabolic state. This is an important parameter to measure to preclude the possibility that the experimental variable being tested influences anxiety state. Increases in anxiety will alter perceived risk and may confound assessment of the motivational/rewarding aspects of interaction with the PB chip. In two experiments, we report a difference in time spent in the perimeter zone of the open field, with the more metabolically replete (ad lib fed or glucose-treated) mice showing increased time in this space. This is reflective of the increased time the fasted mice spend in the food zone, and indicates greater motivation for the food reward rather than an altered anxiety state (Lockie and Andrews, 2013).

Locomotor activity was similar between all groups except for ghrelin-treated mice in the light/dark box. Ghrelin has previously been shown to increase locomotor activity in the absence of food (Jerlhag et al., 2006), although others have demonstrated that it has the opposite effect in the presence of food (Tang-Christensen et al., 2004). We might have expected, given the presence and increased consumption of the PB chip in this group, to have reduced locomotor activity in the ghrelin treated mice. We suggest that this result is due to an interaction between ghrelin signaling and the novelty and anxiogenic nature of the arena, as ghrelin has been shown to reduce anxiety in the face of stress (Spencer et al., 2012).

The results were most uniform in the ghrelin-treated experiment, and least uniform in the fasted mice treated with glucose, which likely reflects the greater innate physiological variability of the interventions. Ghrelin targets key neuronal

populations that influence food seeking and motivation. Hypothalamic AgRP neurons are highly ghrelin sensitive and are active during fasting. Artificial activation of these neurons results in increased motivation to obtain food, increased consumption of food (Aponte et al., 2011; Krashes et al., 2011) and increased risk taking behavior around food acquisition (Jikomes et al., 2016).

One criticism of the current models of risk/reward decision making in mice is that the organism's survival is not endangered by bad choices, because at worst "bad" choices lead to loss of reward, not punishment (Anselme, 2015). Negative consequences, or at least concrete fear of them, is required to generate real awareness of risk within the organism, as is clearly demonstrated in food seeking and foraging choices undertaken in the wild (Holmes, 1984; Anderson, 1986). This can be done by punishment intrinsic to experimental procedure, or fear of natural consequences, like starvation, or predation. We used a larger than normal open field to exploit and exacerbate the natural fear of predation in open spaces (Holmes, 1984; Anderson, 1986), which underlies the traditional open field and light/dark box tests (Crawley, 2006), and force the mouse to make a calculated decision about the value of a known palatable food reward situated in the anxiogenic zone. This value ascribed to the PB chip following this assessment by the mouse can be termed the incentive salience of the reward, and should be sensitive to the metabolic state of the mouse. If this was the case, we would expect the lure of the PB chip to be greater in those mice experiencing metabolic need, and that these mice would make "riskier" choices about approaching and consuming the PB chip. Indeed, we demonstrated that hunger or exogenous ghrelin increased time spent eating and total amount consumed of the chip, while fasted mice injected with glucose immediately prior to the test behaved like fed mice. This indicates that the metabolic state of the mouse, while in the test situation, greatly influences its behavioral choices. Others have demonstrated that manipulation of AgRP neurons is capable of directing similar behavioral choices, driving food consumption in the presence of foot shocks (Jikomes et al., 2016) or directing more risky foraging behavior (Padilla et al., 2016). While we did not directly assess the role of AgRP neurons in our experiments, given that they are active in the fasted state (Yang et al., 2011), are targeted by ghrelin (Nakazato et al., 2001), and are glucose sensitive (Claret et al., 2007), we propose AgRP neurons are likely involved in mediating these behavioral changes.

Importantly, we do not see differences in other measures of exploratory or anxiety-like behavior such as time spent in anxiogenic zone or latency to approach PB chip. This demonstrates that we are not impacting these behaviors by altering metabolic state, but having a focused effect on consumptive behavior. Several times we saw a significant difference in time spent in the non-anxiogenic zone (open field perimeter zone or dark zone in light dark box), with the non-metabolically challenged mice showing increased time in this space. This is likely to be the reciprocal of the decreased time spent in the food zone during these tests. This indicates that mice are spending a similar amount of time exploring the anxiogenic zone, but without a pressing metabolic need to be in the food zone they prefer to spend time in the non-anxiogenic zone. This reflects the preference mice have for safer spaces when there is no incentive to leave them.

If we understand hunger, or high ghrelin, as a signal of food resource scarcity, the risk of starvation becomes more salient to the mouse than the risk of predation. In the fed state, there is no resource pressure as food has been constantly available. Fasting or administering ghrelin creates perceived resource pressure on the food supply, and amplifies the perceived consequences of forgoing the PB chip out of fear. Therefore these mice are more likely to spend time in the unsafe zone consuming a known high-energy food source, as the cost of forgoing this opportunity is greater than the fear of predation. Fed mice are much more conservative in their risk/reward calculation because they have no immediate metabolic pressure to contend with. We used highly palatable PB chips, however some experiments may require use of standard chow or other less well-liked food rewards to avoid ceiling effects on food exploration and consumption.

Others have used modified light/dark box to assess food consumption in an aversive environment (Teegarden and Bale, 2007; Cottone et al., 2012; Liu et al., 2016). Each of these previous uses of the modified light dark box comprise of a food reward placed in the light compartment and then investigated both time spent in the light compartment and time spent consuming food. An increased amount of time spent in the light compartment has universally been described as "risk-taking behavior," however what consumption of the food under those circumstances means is more controversial. Cottone describes it as "compulsive-like eating" based on the premise that food intake is usually suppressed when a rat faces adverse circumstances like exposure in an open arena. However, arguing against "compulsive" eating is the fact that ad lib fed mice do not consume much of the PB chip, compared to fasted or ghrelin-treated mice. We feel that rather than representing "compulsivity" in fasted and ghrelin-treated mice, food consumption in an aversive environment is rather a logical response to a metabolic need, which is more pressing than the risky environment of the open arena. This paper offers a standardized and validated protocol for use of a reward-baited light/dark box, in addition to a reward-baited open field test. Using both tests in conjunction provides comprehensive assessment of risk vs. reward seeking in mice.

Food seeking in the modern human situation rarely has this perceived level of risk associated with it, none the less it is not risk free. Aside from the basic risks associated with driving or walking to the grocery store, there are social risks around food selection and consumption, which can make eating psychologically taxing. Perhaps the point here though, is not that we are modeling the human situation, but rather that we are trying to better model the natural food seeking-obtaining-consuming cycle of wild animals, which necessarily contains motivational, reward, and risk-taking elements. We propose that this test battery will be a useful addition to standard food intake measurements, and standard behavior tests to assess deficits in global processing of food attainment in genetic and other mouse models.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of Monash University Animal Ethics

Committee guidelines. The protocol was approved by the Monash University Animal Ethics Committee (MARP 2014 026).

AUTHOR CONTRIBUTIONS

ZBA, designed research and drafted manuscript. SHL, designed research, performed research, analyzed data, and drafted manuscript. CM, performed research and analyzed data. SR, performed research and analyzed data. NG, Performed research and analyzed data.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fnins.2017.00024/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Prebiotics Supplementation Impact on the Reinforcing and Motivational Aspect of Feeding

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Energy homeostasis is tightly regulated by the central nervous system which responds to nervous and circulating inputs to adapt food intake and energy expenditure. However, the rewarding and motivational aspect of food is tightly dependent of dopamine (DA) release in mesocorticolimbic (MCL) system and could be operant in uncontrolled caloric intake and obesity. Accumulating evidence indicate that manipulating the microbiota–gut–brain axis through prebiotic supplementation can have beneficial impact of the host appetite and body weight. However, the consequences of manipulating the implication of the microbiota–gut–brain axis in the control motivational and hedonic/reinforcing aspects of food are still underexplored. In this study, we investigate whether and how dietary prebiotic fructo-oligosaccharides (FOS) could oppose, or revert, the change in hedonic and homeostatic control of feeding occurring after a 2-months exposure to high-fat high-sugar (HFHS) diet. The reinforcing and motivational components of food reward were assessed using a two-food choice paradigm and a food operant behavioral test in mice exposed to FOS either during or after HFHS exposure. We also performed mRNA expression analysis for key genes involved in limbic and hypothalamic control of feeding. We show in a preventive-like approach, FOS addition of HFHS diet had beneficial impact of hypothalamic neuropeptides, and decreased the operant performance for food but only after an overnight fast while it did not prevent the imbalance in mesolimbic markers for DA signaling induced by palatable diet exposure nor the spontaneous tropism for palatable food when given the choice. However, when FOS was added to control diet after chronic HFHS exposure, although it did not significantly alter body weight loss, it greatly decreased palatable food tropism and consumption and was associated with normalization of MCL markers for DA signaling. We conclude that the nature of the diet (regular chow or HFHS) as well as the timing at which prebiotic supplementation is introduced (preventive or curative) greatly influence the efficacy of the gut–microbiota–brain axis. This crosstalk selectively alters the hedonic or motivational drive to eat and triggers molecular changes in neural substrates involved in the homeostatic and non-homeostatic control of body weight.

Keywords: food intake, hedonic and motivational component, dopaminergic system, prebiotic, reward

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INTRODUCTION

Obesity and corollary pathologies, such as dyslipidemia, diabetes, and cardiovascular diseases are spreading in both developed and developing countries as a result of increased accessibility to energy-dense food associated with a general decrease in physical activity and energy expenditure (1). Whereas some genetic loci were clearly identified and extensively studied as monogenic causes for obesity, it is widely accepted that the metabolic syndrome is in essence a multifactorial disease that encloses a complex network of molecular, cellular, and physiologic alterations (1, 2). Understanding the complex pathology of the metabolic syndrome will be critical in shaping effective preventive and therapeutic strategies. However, despite the encouraging results obtained through pharmacological and surgical interventions, no effective anti-obesity treatment with long-lasting effects on body weight is nowadays available.

Proper energy balance is insured by the ability of the central nervous system to integrate nervous and circulating signals that reflect nutritional status to produce adaptive metabolic and behavioral responses aiming at maintaining body weight within a physiological narrow range (3). In addition, the rewarding aspects of energy-dense food largely depends on dopamine (DA) release from dopaminergic neurons in the ventral tegmental area that project to limbic regions, notably the prefrontal cortex and the nucleus accumbens (NAcc) (4, 5). This neural substrate referred as to mesolimbic "reward" circuit is instrumental in the encoding of both the volume of desire, i.e., "motivation" and the hedonic aspect, i.e., "liking" in food rewards (6).

Hence, the complex behavioral sequence leading to food intake results from the integration of metabolic needs but also reinforcing aspects of food. Multiple lines of evidence suggest that high-fat feeding and obesity *per se* can promote long-lasting adaptations in both hypothalamic and limbic regions thus leading to increased vulnerability to over-consume energy-dense food. In turn, such vulnerability can promote aberrant behaviors (7, 8) in which the reward becomes the primary driving force to consume energy-dense food (9–12). For instance, emerging theories suggest that chronic exposure to palatable food might impair the proper encoding of reward and, similar to drug of abuse, lead to desensitization of the DA mesolimbic system, and ultimately promote craving and addictive-like consummatory behavior (4, 6).

However, despite similar exposure to palatable and hypercaloric food, the development of eating-habits dissociated from actual homeostatic needs does not occur in every individual, suggesting differential degrees of vulnerability. In that regard, the microbiota–gut–brain axis has recently emerged as a key regulator of brain structures involved in stress-like responses (13, 14) together with resilience to high-fat-induced body weight gain (15). Dietary prebiotic such as the soluble fibers fructo-oligosaccharides (FOS) represents selectively fermented compounds that promote changes in the activity and composition of the gut microbiota, that are associated with a wide spectrum of beneficial effects including reduced appetite (16–20), decreased body weight (15), improved glucose metabolism (15), dampened susceptibility to stress (21, 22), and improved learning discrimination in rodents (23).

These observations suggest that prebiotic manipulation of the microbiota–gut–brain axis could directly impact both homeostatic and non-homeostatic control of food intake. However, the behavioral and molecular consequences of prebiotic supplementation onto the reinforcing and motivational components of food seeking have hitherto been largely unexplored.

In the current study, we investigated how administration of FOS could oppose-in a preventive-like approach or reverse-in a curative approach the behavioral and molecular adaptations induced by high-fat high-sucrose exposure and their consequences on food preference and motivation for food seeking.

MATERIALS AND METHODS

Animals and Diets

Ten-weeks-old male mice C57Bl/6J (25–30 g, Janvier, Le Genest Saint Isle, France) were housed in stainless steel cages in a room maintained at 22 ± 1°C with light from 7:00 a.m. to 7:00 p.m. Food (Safe, Augy, France) and water were given *ad libitum* unless otherwise stated. C57Bl/6J were split in six groups (n = 12/group). The first four groups were exposed, respectively, during 2-months to a control diet (Ctrl, 3,438 kcal/kg, protein 19%, lipid 5%, carbohydrates 55%, reference #U8959 version 63 Safe, Augy, France), a control diet enriched in fructo-oligosaccharide (Ctrl-FOS, 3,438 kcal/kg, protein 17%, lipid 8%, carbohydrates 49%, oligofructose 10%), a high-fat high-sugar diet (HFHS, 4,362 kcal/kg, proteins 20%, lipid 23%, carbohydrate 37%, reference #U8954 version 14 Safe, Augy, France), and an HFHS diet enriched in fructo-oligosaccharide (HFHS-FOS, 4,362 kcal/kg, proteins 18%, lipid 20%, carbohydrate 34%, oligofructose 10%). The last two groups were subjected to a 2-months HFHS diet and split in two groups (n = 12) that received during the following 2-months a "control" diet (HFHS/Ctrl) and a control diet enriched in fructo-oligosaccharide (HFHS/Ctrl-FOS). Groups supplemented with FOS will also be referred in the text by preventive (Ctrl-FOS, HFHS-FOS) or corrective (HFHS/Ctrl-FOS) effects of FOS. All animal experiments were performed with approval of the Animal Care Committee of the University Paris Diderot-Paris 7 and according to European directives.

Body Composition Analysis

Mice were monitored for body weight and composition at the beginning and the end of the experiment. Body mass composition (lean tissue mass, fat mass, free water, and total water content) was analyzed using an Echo Medical systems' EchoMRI (Whole Body Composition Analyzers, EchoMRI, Houston, TX, USA), according to manufacturer's instructions.

Measurement of Food Intake and Food Preference

Analyses were performed in an automated online measurement system using high sensitivity feeding and drinking sensors and an infrared beam-based activity monitoring system (Phenomaster, TSE Systems GmbH, Bad Homburg, Germany).

Mice were evaluated for food preference when exposed to HFHS and control chow diet (CTRL). Food preference was measured during six short sessions of 2 h (days 1–6) using animals as their own controls.

Operant Conditioning System

Operant responding performance was performed as previously described (24). Computer-controlled operant conditioning was conducted in 12 identical conditioning chambers equipped with a swiveling infusion device (Phenomaster, TSE Systems GmbH, Bad Homburg, Germany). Each chamber contains an operant wall with a food cup, two levers located 3 cm lateral to the food cup, with the left lever designated the active lever (for food pellet delivery). Mice are maintained at 90% of initial body weight to facilitate initial learning and performance of a fixed ratio (FR1) operant learning task. The reinforcer was a single 20-mg peanut butter flavored sucrose tablet (TestDiet, Richmond, VA, USA).

Operant training was carried out over six consecutive days with two overnight fix ratio of 1 (FR) and then four consecutive days with one 2-h trial of FR1 per day. At the conclusion of the 6-days operant training period, animals were given four trials to lever press for sucrose under a progressive ratio of 3 (PR), lever press requirement for each subsequent reinforcer increased by 3 with an initial requirement of 3 lever press ($r = 3N + 3$; $N =$ reinforcer number).

The PR schedule requires the mouse to perform an increasing number of lever presses for each consecutive reward, the number of rewards received (also called breakpoint) was used to assess motivation or effort to work for a food reward.

At the end of the experiment, animals were sacrificed, brain, liver, cecum tissues, and plasma collected.

Gut Microbiota Analysis

At the end of the experiment, the total cecum content was collected and weighed before storage at −80°C. Metagenomic DNA was extracted from the cecal content using the QIAamp DNA stool mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Quantitative PCR (qPCR) for total bacteria, *Bifidobacterium* spp., *Lactobacillus* spp., *Akkermansia muciniphila*, *Roseburia* spp., and *Bacteroides-Prevotella* spp. were performed by using Mesa Fast qPCR™ (Eurogentec, Seraing, Belgium). Real-time PCRs were performed with the StepOnePlus™ real-time PCR system and software (Applied Biosystems, Den Ijssel, The Netherlands). The primers sequences were described previously (15, 25). Cycle threshold of each sample was then compared with a standard curve (performed in triplicate) made by diluting genomic DNA obtained from BCCM/LMG (Ghent, Belgium) or DSMZ (Braunshweig, Germany). Prior to isolating the DNA, the cell counts were determined by BCCM/LMG or DSMZ, respectively; fivefold serial dilution of *Bifidobacterium animalis* BCCM/LMG 18900 for *Bifidobacterium* spp., *Bacteroides fragilis* BCCM/LMG 10263 for *Bacteroides-Prevotella* spp., *Lactobacillus acidophilus* DSM 20079 for *Lactobacillus* spp., *A. muciniphila MucT* (ATTC BAA-835, DSMZ22959) for *A. muciniphila*, *Roseburia intestinalis* (DSMZ 14610) for *Roseburia* spp., and *Lactobacillus acidophilus* DSM 20079 for total bacteria.

Isolation of Total RNA and Quantitative RT-PCR

From all groups, total hypothalamic RNA was extracted and analyzed by qRT-PCR for agouti-related protein (AgRP), neuropeptide Y (NPY), pro-opiomelanocortin (POMC), and cocaine and amphetamine-regulated transcript (CART), and total nucleus accumbens (NAcc) RNA was extracted and analyzed by qRT-PCR for dopamine transporter (DAT), dopamine receptor D1 (DR1), dopamine receptor D2 (DR2), dopamine beta hydroxylase (DBH), and tyrosine hydroxylase (TH).

Total RNA was isolated as described previously (9). We retro transcribed 1 µg RNA using Superscript II (Invitrogen). Real-time quantitative (qRT-PCR) analyses were performed with 25 ng cDNA and 250 nM sense and antisense primers (Eurogentec) in a final reaction volume of 25 µl by using qPCR Core Kit (Eurogentec) and the MyiQ real-time PCR detection system (Bio-Rad). Specific primers were designed using Primer Express software (version 1.0, Applied Biosystems) and primers sequences and housekeeping gene (HKG) are listed below. Relative quantification of hypothalamic and NAcc RNA for each gene was calculated after normalization to HKG by using the comparative Ct method.

Primers	Sequences
POMC	Forward: 5′-AGTGCCAGGACCTCACCA-3′ Reverse: 5′-CAGCGAGAGGTCGAGTTTG-3′
NPY	Forward: 5′-CCGCTCTGCGACACTACAT-3′ Reverse: 5′-TGTCTCAGGGCTGGATCTCT-3′
AgRP	Forward: 5′-CGGAGGTGCTAGATCCACAGA-3′ Reverse: 5′-AGGACTCGTGCAGCCTTACAC-3′
CART	Forward: 5′-CGAGAAGAAGTACGGCCAAG-3′ Reverse: 5′-CTGGCCCCTTTCCTCACT-3′
DAT	Forward: 5′-GCCCTACCTGCTCTTCATGC-3′ Reverse: 5′-GGATGACAGTGAAGCCCACA-3′
DR1	Forward: 5′-TCTGGTTTACCTGATCCCTCA-3′ Reverse: 5′-GCCTCCTCCCTCTTCAGGT-3′
DR2	forward: 5′-TGAACAGGCGGAGAATGG-3′ Reverse: 5′-CTGGTGCTTGACAGCATCTC-3′
DBH	Forward: 5′-ATCTCCATGCATTGCAACAA-3′ Reverse: 5′-AGGCTGCAGATTCCACTCAC-3′
TH	Forward: 5′-GGTATACGCCACGCTGAAGG-3′ Reverse: 5′-TAGCCACAGTACCGTTCCAGA-3′
RPL19 HKG	Forward: 5′-GGGCAGGCATATGGGCATA-3′ Reverse: 5′-GGCGGTCAATCTTCTTGGATT-3′
H1A HKG	Forward: 5′-AGAAGAACAACAGCCGCATC-3′ Reverse: 5′-TGCACCAGTGTGCCTTTATT-3′
HPRT HKG	Forward: 5′-GTTGGATACAGGCCAGACTTTGTTG-3′ Reverse: 5′-GATTCAACTTGCGCTCATCTTAGGC-3′
CYCLOA HKG	Forward: 5′-ACGCCACTGTCGCTTTTC-3′ Reverse: 5′-GCAAACAGCTCGAAGGAGAC-3′

Statistical Analysis

Displayed values are mean ± SEM. Variance equality was analyzed by a paired *t*-test (GraphPad Prism 6®). Unless otherwise stated, comparisons between groups were carried out using analysis of variance (ANOVA, GraphPad Prism 6®). A *P*-value of less than 0.05 was considered statistically significant.

RESULTS

Preventive vs Corrective Prebiotic Supplementation Differentially Impact Body Weight and Gut Microbiota Composition

Four groups were subjected to a 2-months long preventing-like approach of prebiotic supplementation in which two groups received regular chow diet with or without soluble fibers FOS (Ctrl or Ctrl-FOS groups) while two last groups received a similar treatment but were raised on palatable high-fat high-sucrose diet with or without FOS (HFHS or HFHS-FOS groups) (**Figure 1A**). An additional two animal cohorts were dedicated to explore the corrective potency of prebiotic supplementation onto metabolic and behavioral changes induced by a 2 months exposure to HFHS diet. In this setting, 12 weeks-old C57Bl/6J male mice were first raised on HFHS diet for 2-months then shifted onto Ctrl or Ctrl-FOS diet and will be referred as to HFHS/Ctrl or HFHS/Ctrl-FOS, respectively (**Figure 1A**). Based on the extensive literature in the field, we chose a 10% FOS enrichment since it was described to promote metabolic benefits (26–28) together with improvement in learning discrimination and improved cognitive performances (23, 29).

While average body weight did not differ among groups before the treatment, a significant increase in body weight was reached after HFHS but not HFHS-FOS exposure (**Figures 1B,E,H**). Body weight gain was mostly attributable to adipose tissue as revealed by body composition analysis (**Figures 1C,D,F,G**; Figures S1A–D in Supplementary Material). In HFHS/Ctrl and HFHS/Ctrl-FOS groups, the switch of HFHS to control diet stopped diet-induced body weight gain (**Figure 1H**) in similar way between FOS and non-FOS-treated group (**Figure 1H**; Figure S1E in Supplementary Material).

Prebiotic supplementation is known to change the composition and activity of specific gastrointestinal microbiota (30, 31). Therefore, we decided to investigate if specific bacteria were modified following our diets. qPCR analysis of the cecum bacterial content revealed that the prevalence of *Bifidobacterium* spp., *A. muciniphila*, and to a lesser extent *Lactobacillus* spp. was significantly increased but only in condition in which both prebiotics and HFHS were combined either simultaneously or when FOS supplementation followed HFHS exposure. It is worth to mention that other bacterial families, such as *Roseburia* spp. and *Bacteroides* spp., were not affected by dietary regimens (**Figures 1I,J**). However, when mice were exposed to Ctrl or Ctrl-FOS diets, we could not identify significant changes in the microbiota ecosystem (**Figures 1I,J**).

Timing in Prebiotic Supplementation Is Instrumental in the Beneficial Impact on Hedonic and Motivational Component Feeding

Previous data suggest that modifications in the microbial diversity may influence food choice and tropism in the host and participate to satiety responses by regulating the gut–brain axis (16, 30, 32). After nutritional manipulation aimed at either preventing or correcting any metabolic and behavioral changes induced by HFHS exposure, the six groups were subjected to a two-food choice paradigm consisting in a seven daily consecutive 2 h-limited access to both Ctrl and HFHS diet followed by an overnight exposure to food choice (**Figure 2A**). Short-term access to two-food choice aimed at deciphering the preference to select and consume palatable over chow pellets in a non-fasted condition. While Ctrl-fed animals displayed a tropism for HFHS over control chow diet (CTRL), Ctrl-FOS maximized their consumption of HFHS starting from the first session (**Figures 2B–D**). During the overnight exposure to food choice, a similar pattern was observed with increased HFHS consumption in Ctrl and Ctrl-FOS animals with an overall 90% preference for the palatable diet (**Figure 2E**).

Interestingly both cohorts exposed to HFHS diet (HFHS and HFHS-FOS) displayed minimal consumption of palatable diet when given the choice on a short period of time (**Figures 2B–D**), in agreement with the reduction in palatable food preference observed in animal fed with high-fat diet (33). This result contrasted, however, with a large preference for HFHS over CTRL in the overnight access in both HFHS and HFHS-FOS groups (**Figure 2E**). In addition, FOS supplementation in HFHS-FOS led to increased tropism for palatable diet compared to HFHS group (**Figure 2E**). These results indicate that mice exposed to diets supplemented with FOS (Ctrl or HFHS) show increased preference for palatable food (**Figures 2B–E**).

In sharp contrast with the lack of preventive action, when prebiotics were added in the diet after a 2 months HFHS exposure (HFHS/Ctrl-FOS), we observed a strong corrective action of FOS on both 2-h time-restricted and overnight palatable diet intake (**Figures 2F–I**).

Food preference and seeking strongly rely on dopamine whose release within the mesocorticolimbic (MCL) system participate in driving the motivational and reinforcing values of food reward (6, 34–36). Hence, we next sought to behaviorally dissect the consequences of FOS supplementation on the motivational drive to obtain food rewards. After nutritional manipulation, the different cohorts underwent through an operant conditioning task to obtain food rewards. Animals were first subjected to fixed ratio (FR) reinforcement schedule in which a single lever press triggers the delivery of a palatable food pellet. Once mice have reached their discriminatory ability between active and inactive lever, they are shifted to a progressive ratio (PR) in which the number of lever presses required to obtain a reward increases progressively (**Figure 3A**). If a subject abnormally inflates the reinforcing aspect of a reward it will be more likely to exert effort to obtain it. Alternatively, if the perceived value of the reward is abnormally diminished, the willingness to engage in effortful behavior to obtain it will be reduced. In all conditions, operant responding for sucrose pellets reward was evaluated on mice gradually food restricted to 90% of body weight or following acute overnight in fed or fasting condition (**Figure 3A**).

Here again, the behavioral output of prebiotic supplementation was different according to the timing (preventive or corrective) of FOS addition in the diet. Ctrl and Ctrl-FOS groups had a similar profile with enhanced operant responding for food reward compared to both HFHS and HFHS-FOS group

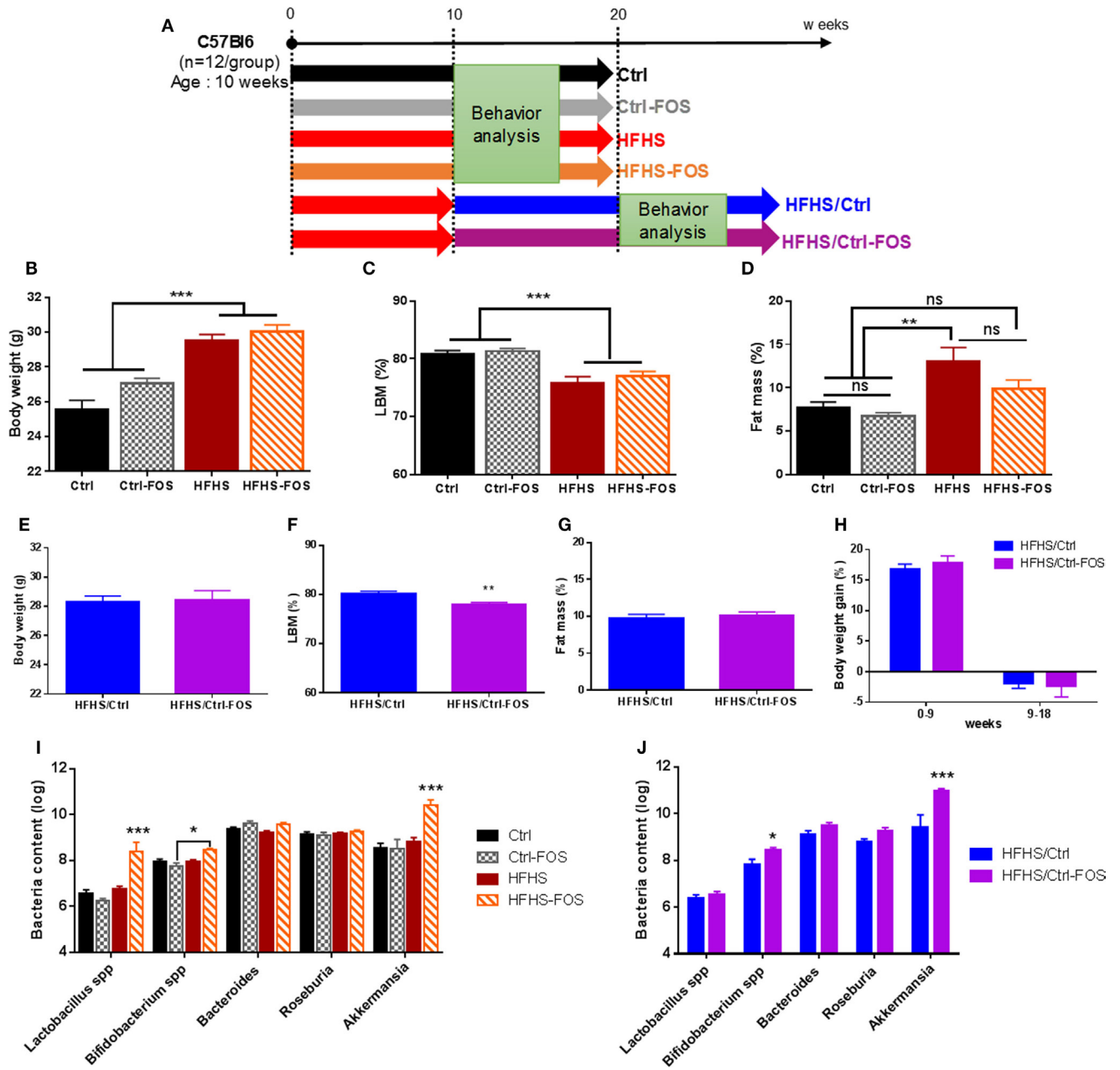


FIGURE 1 | **(A)**. Experimental design of diet manipulation in which mice were fed, respectively, regular chow (Ctrl, black), high-fat high-sucrose diet (HFHS, red), or diet enriched in fructo-oligosaccharides (FOS) (Ctrl-FOS, gray and HFHS-FOS, orange) in a protective approach. Two additional groups were fed an HFHS and switched onto either regular chow diet (HFHS/Ctrl, blue) or regular chow enriched in FOS (HFHS/Ctrl-FOS, purple). **(B–H)** Body weight and body composition evolution during diet manipulation in the six different groups. **(B,E)** Body weight evolution, **(C,D,F,G)** body composition in **(C,F)** lean body mass (LBM) and **(D,G)** fat body mass in Ctrl (black), Ctrl-FOS (gray), HFHS (red), HFHS-FOS (orange), HFHS/Ctrl (blue), and HFHS/Ctrl-FOS (purple). **(I,J)** Targeted metagenomics analysis of cecum bacterial content in animal fed Ctrl (black), Ctrl-FOS (gray), HFHS (red), and HFHS-FOS (orange) and **(J)** after switch on Ctrl or Ctrl-FOS diet in HFHS/Ctrl (blue) and HFHS/Ctrl-FOS (purple). Data are expressed as mean ± SEM of 12 mice in each group. Significant differences from a one-way ANOVA **(B–D)**, paired *t*-test **(F)** and a two-way ANOVA **(I,J)**, *Bonferroni Post hoc* test are shown (*$P < 0.005$, **$P < 0.001$, ***$P < 0.0005$).

(**Figure 3B**). These results are consistent with the previous studies showing attenuated operant performance in high-fat fed animals (37). Surprisingly, FOS addition did not alter the number of collected rewards (**Figure 3B**), active lever press (**Figure 3C**), and discriminatory capacity between active and inactive lever (**Figures 3D,J**) in animal exposed to Ctrl diet (Ctrl and Ctrl-FOS) under both chronic and acute fasting-induced body weight loss (**Figures 3E,F,H**). However, FOS supplementation on HFHS diet decreased the motivational drive to collect food reward under conditions of drastic energy deprivation (**Figures 3G,I**).

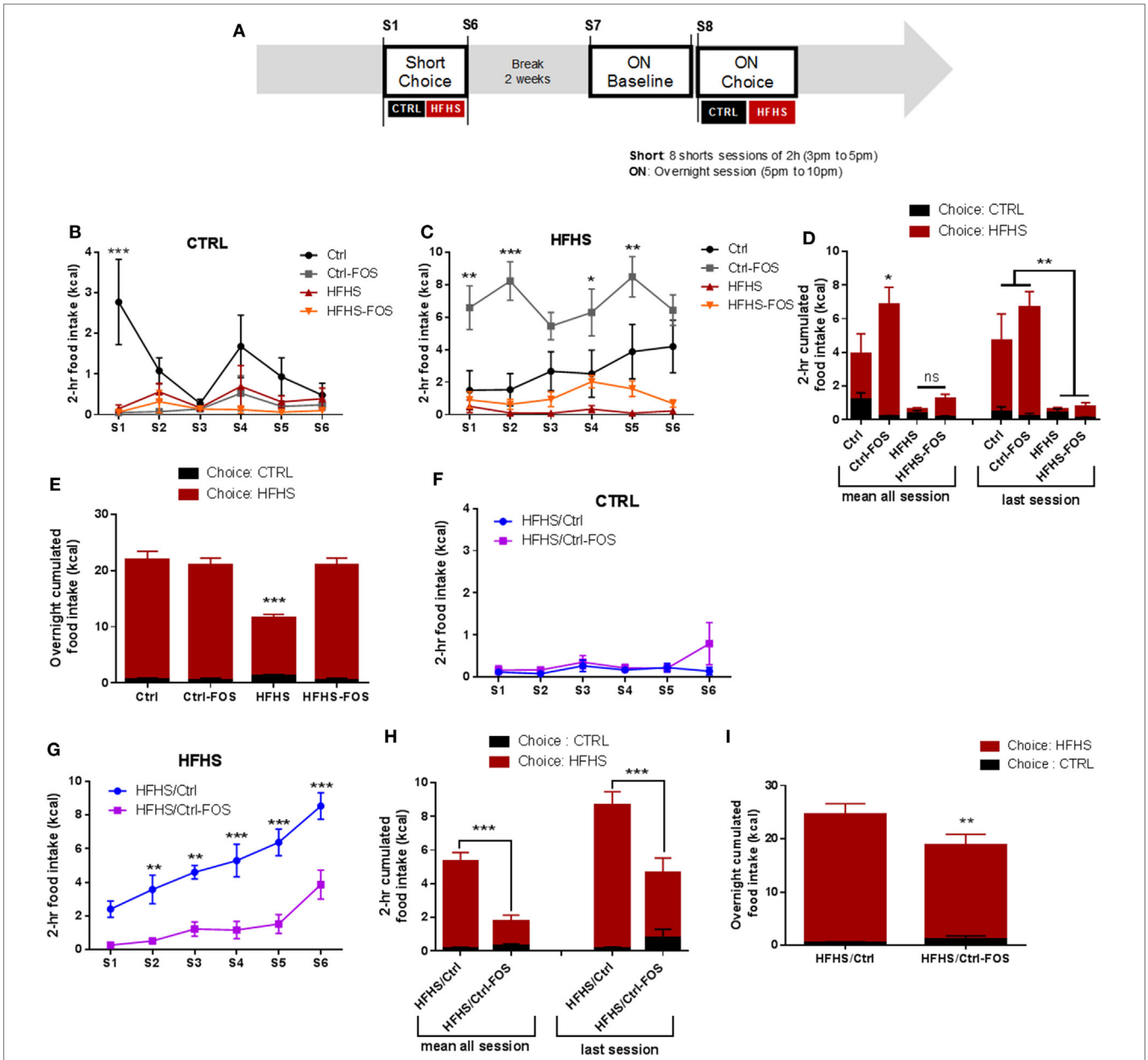


FIGURE 2 | **(A)** Experimental design for food tropism analysis using short or overnight access to a two-food choice protocol using regular chow diet or palatable HFHS diet for six mice per group. Regular chow diet [CTRL **(B,D,F,H)**] or HFHS intake **(C,D,G,H)** during seven sessions consisting of a 2-h two-food choice access, **(D,H)** representative tropism for CTRL *vs* HFHS as average cumulated intake through all session or during the last session, **(E,I)** representative tropism for CTRL vs HFHS as average cumulated intake overnight in Ctrl (black), Ctrl-fructo-oligosaccharides (FOS) (gray), HFHS (red), HFHS-FOS (orange), HFHS/Ctrl (blue), and HFHS/Ctrl-FOS (purple). Data are expressed as mean ± SEM of six mice in each group. Significant differences from a two-way ANOVA, *Bonferroni Post hoc* test (*$P < 0.005$, **$P < 0.001$, ***$P < 0.0005$) **(B–I)**.

A similar experimental design was carried out to evaluate operant conditioning in animals previously fed with an HFHS diet and shifted under Ctrl or Ctrl-FOS diets (HFHS/Crtl, HFHS/Ctrl-FOS).

While a corrective property of FOS supplementation was evident in palatable diet intake and preference (**Figures 2F–I**), HFHS/Crtl and HFHS/Ctrl-FOS exhibited identical performance in every aspect of operant response for food reward in both tested conditions (chronic or acute food deprivation) (**Figures 3K–Q**).

These results highlight that prebiotics might exert a distinct and specific action onto the hedonic "liking," and motivational "wanting" drive to consume palatable food as well as feeding response to energy deprivation. Furthermore, our data support the notion that time-dependent exposure to prebiotics may be instrumental in their action onto reward-seeking behavior.

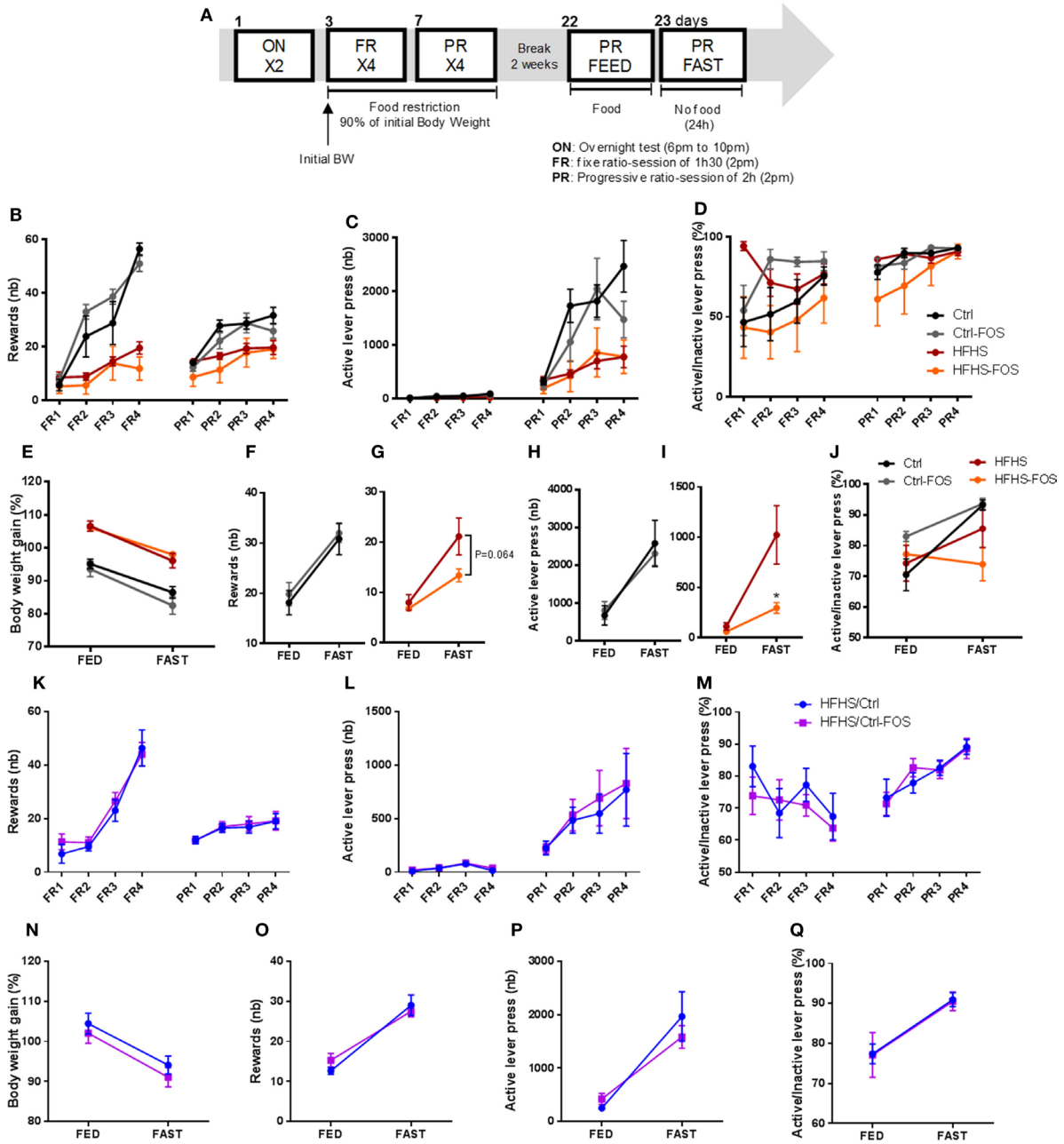


FIGURE 3 | **(A)** Experimental design for the operant responding performance assessment for six mice per group. **(B,K)** Reward number, **(C,L)** active lever press, **(D,M)** ratio between the active and inactive lever presses in **(B–D)** Ctrl (black), Ctrl-fructo-oligosaccharides (FOS) (gray), HFHS (red), **(K–M)** HFHS-FOS (orange), HFHS/Ctrl (blue), and HFHS/Ctrl-FOS (purple) during a 90% body weight reduction. **(E,N)** body weight change, reward number **(F,G,O)**, active lever press **(H,I,P)** or active vs inactive lever press ratio **(J,Q)** in response to an overnight fast in Ctrl (black), Ctrl-FOS (gray), HFHS (red), HFHS-FOS (orange), HFHS/Ctrl (blue), and HFHS/Ctrl-FOS (purple). Data are expressed as mean ± SEM of six mice per group. Significant differences from a two-way ANOVA, *Bonferroni Post hoc* test are shown (*$P < 0.005$) **(I)**.

Timing in Prebiotic Supplementation Is Instrumental in Molecular Adaptation in Mesolimbic and Hypothalamic Structures

The action of feeding results from the ability of the brain to properly integrate circulating signals of hunger and satiety together with food-related cues coding for palatable and rewarding values (38). The hypothalamus–brainstem axis, by primarily encoding metabolic needs, is regarded as the key neural network in the homeostatic control of body weight whereas the dopaminergic system is mainly involved in encoding the rewarding and reinforcing values of food seeking. Hence, hypothalamic–brainstem

circuit is typically referred as to homeostatic while mesolimbic circuit are referred as to non-homeostatic regulation of feeding (38, 39). Interestingly, obesity and high-fat feeding have been shown to provoke various adaptive changes in both MCL and hypothalamic structures which could account for the toxic effect of energy-dense food (8, 10, 40–43).

We therefore explored how time-dependent prebiotic manipulation modulated the molecular adaptations of dopaminoceptive and hypothalamic structures in response to high-fat feeding. mRNA were extracted from the NAcc and hypothalamus and analyzed for expression of genes involved in dopamine synthesis and signaling, i.e., DAT, DBH, DR1, DR2, and TH in the NAcc, while genes encoding neuropeptides involved in melanocortin signaling and body weight regulation i.e., NPY, agouti-related protein (AgRP), POMC, and cocaine and amphetamine-regulated transcript (CART) in the hypothalamus (**Figures 4A,B**).

In a preventive approach, the effect of FOS addition to Ctrl or HFHS diets was evident in hypothalamic expression of energy-related neuropeptides but failed to alter expression of dopamine-associated genes in the NAcc. Both Ctrl and Ctrl-FOS displayed similar levels of mRNA encoding DAT and DR2 that were significantly higher than those observed in either HFHS or HFHS-FOS (**Figure 4A**). In the hypothalamus, however, while exposure to energy-dense food led to increased mRNA contents for NPY and AgRP in HFHS group, prebiotic supplementation induced a significant decrease in NPY expression (**Figure 4B**). These results are in agreement with published observations showing that high-fat diet and/or obesity result in decreased expression of DR2 and DAT (7, 8) and, on the one hand, increased expression of hypothalamic orexigenic peptides (44, 45). Our results show that, in a preventive-like approach, FOS supplementation partially restores hypothalamic expression of orexigenic peptides but fails to correct the modifications induced by energy-dense food in the NAcc (**Figures 4A,B**).

Surprisingly, FOS enrichment had an opposite consequence onto hypothalamic peptides on mice exposed to Ctrl or HFHS

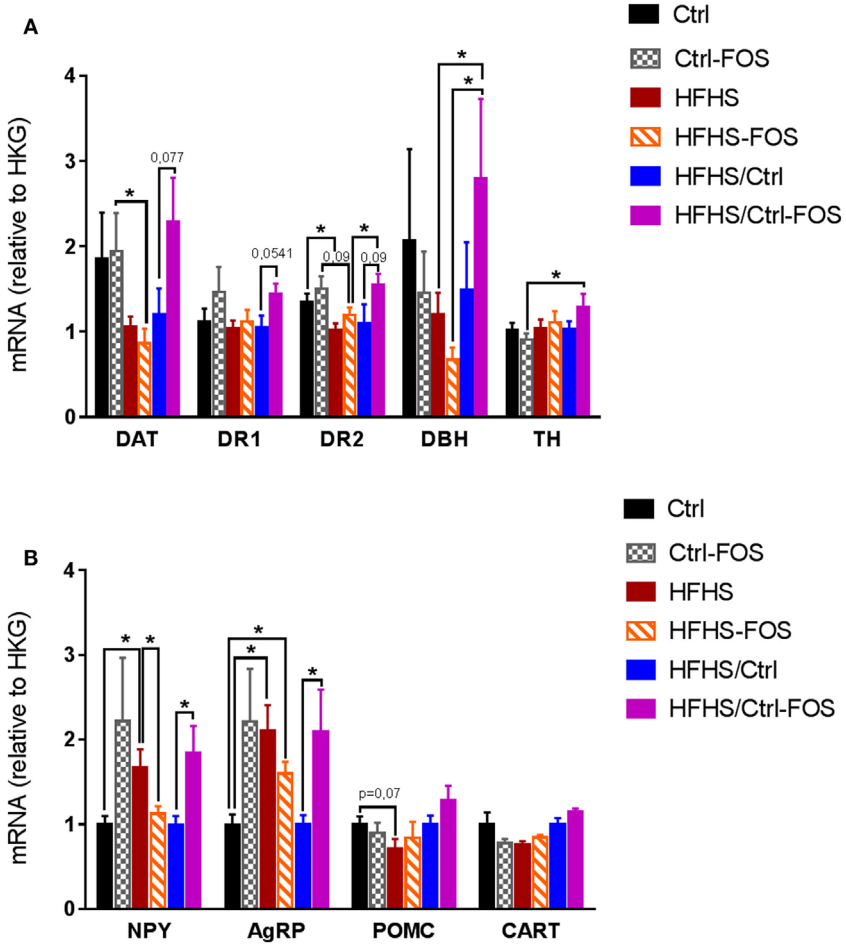


FIGURE 4 | Real-time PCR analysis of mRNA content **(A)** in the nucleus accumbens, for gene encoding for dopamine transporter (DAT), dopamine receptor 1 (DR1), dopamine receptor 2 (DR2), dopamine beta hydroxylase (DBH), and tyrosine hydroxylase (TH) and **(B)** in the hypothalamus for neuropeptide Y (NPY), Agouti-related protein (AgRP), Pro-opiomelanocortin (POMC), and cocaine and amphetamine-regulated transcript (CART) in Ctrl (black), Ctrl-fructo-oligosaccharides (FOS) (gray), HFHS (red), HFHS-FOS (orange), HFHS/Ctrl (blue), and HFHS/Ctrl-FOS (purple). Expression level corresponds to a ratio relative to housekeeping gene (HKG). Data are expressed as mean ± SEM of eight mice in each group. Significant differences from a two-way ANOVA, *Bonferroni Post hoc* test (*$P < 0.005$) **(A,B)**.

diets. Ctrl-FOS diet led to increases in both orexigenic peptides NPY and AgRP compared to Ctrl group while the same nutritional manipulation operated onto HFHS diet led to a decrease of these peptides (**Figure 4B**). In both conditions, the modulation of NPY and AgRP were not counterbalanced by a change in the expression of the anorectic transcripts for POMC or CART (**Figure 4B**).

This result provides a molecular underpinning supporting the relative hyperphagia observed in Ctrl-FOS compared to Ctrl animals in food choice condition (**Figures 2C,D**). In the same food choice paradigm, FOS enrichment in HFHS diet led to increased consumption of palatable diet in an overnight session (**Figure 2E**) which point toward a decorrelation between hypothalamic decrease in NPY, AgRP and food reward seeking.

In a corrective-like approach, however, prebiotic supplementation to Ctrl diet in animal previously exposed to HFHS diet for 2 months fully restored Nacc level of DAT, DR2, and DBH (**Figure 4A**). However, while the shift onto Ctrl diet was *per se* sufficient to restore normal hypothalamic levels for NPY and AgRP, this benefic action was counterbalanced by FOS addition which was associated to sustained level of both orexigenic peptides (**Figure 4B**).

Altogether our results show that the molecular adaptations induced by high-fat feeding in brain structures that govern food intake in response to either metabolic demand or reward can be restored or opposed by prebiotic supplementation. Importantly, the timing in FOS supplementation together with the nature of the diet in which FOS is introduced with have critical impact on the direction by which prebiotic will operate the adaptive changes in MCL or hypothalamic structures and ultimately predict the ability of prebiotic to change food tropism and reward-seeking behavior.

DISCUSSION

While metabolic needs are primarily encoded in the hypothalamus, the reinforcing value of food encompasses a multisensory component including flavors and texture which ultimately modulates the release of DA in the MCL system. In modern society, calorie-dense foods are widely available and were associated with the progression of obesity together with the development of compulsive eating in which reward-driven eating behaviors are bypassing homeostatic regulation of nutrients intake (4, 46, 47). The microbiota–gut–brain axis has emerged as a pivotal player in appetite control as well as reward-driven behavior (48).

In the present study, we described the impact of prebiotic supplementation onto various components of food reward-seeking behavior, gut microbiota ecosystem and molecular adaptation in both hypothalamic and mesolimbic structures. We used food choice paradigm associated with operant conditioning to lever press for food reward in order to dissect out how FOS supplementation could prevent or correct the consequence of chronic exposure to palatable, energy-rich diet onto the hedonic and motivational component of food seeking behavior. We manipulated the timing of FOS introduction in the diet using, first, a preventive-like approach in which animals were exposed to CTRL diet or HFHS diet with or without FOS supplementation and second, a corrective-like approach in which animal were first raised on HFHS diet and then switched onto CTRL diet with or without FOS. In both cases, the consequences onto gut microbiota, hedonic and motivational aspect of food reward together with brain expression of genes involved homeostatic and non-homeostatic control of feeding.

We found that prebiotics act in synergy with the diet supplied to operate change in microbiota composition, tropism for palatable food, and hypothalamic and MCL response. Using targeted metagenomics approach, we could only identify selected changes in the gut–microbiota ecosystem, especially modifying the contents of *Bifidobacterium* spp., *A. muciniphila*- and *Lactobacillus* spp., but only in animals that were either raised or had been exposed chronically to HFHS diet (**Figures 1I,J**). In the same line we found that, while FOS addition to CTRL diet increased both the drive for palatable diet (**Figures 2C–E**) and hypothalamic expression of orexigenic neuropeptides NPY and AgRP (**Figure 4B**), prebiotic addition decreased the motivation to collect food rewards after a fast and decreased hypothalamic NPY content in HFHS fed animals (**Figures 3G,I** and **4B**).

Surprisingly enough, in our hands FOS introduction to the diet only modestly affected HFHS-induced fat mass gain (**Figure 1D**; Figure S1B in Supplementary Material) but had no significantly impact of body weight gain or body weight loss after the transition from HFS to CTRL diet (**Figures 1E–H**; Figures S1C–E in Supplementary Material). However, despite the lack of effect on body weight, we could clearly demonstrate that the timing of prebiotic supplementation had a pivotal role in both molecular and behavioral responses in food reward seeking and consumption. After a 2-months HFHS exposure, we tested the capacity of prebiotic to revert molecular and behavioral dysfunctions induced by caloric overload. The shift onto CTRL diet similarly surfeited body weight gain regardless of FOS addition, however, in contrast to the chronic preventive approach, prebiotic supplementation resulted in decreased palatable food tropism and consumption (**Figures 2F–I**) without affecting operant performance (**Figures 3K–Q**) and was associated with concomitant increase in hypothalamic orexigenic markers and NAcc expression of gene involved in DA signaling. This latter result suggests that, unlike the preventive addition of FOS, prebiotic treatment after chronic HFHS exposure helped restoring the imbalance in MCL DA signaling and reward-driven tropism and overconsumption of palatable diet. Importantly, these changes primarily affected hedonic rather than motivational aspects of food reward and had a positive impact on food choice despite increased expression of hypothalamic orexigenic neuropeptides.

It is important to note that, while 10% FOS supplementation correlated with positive change in the gut microbiota ecosystem as expected from the literature, we did not observe a clear benefit on body weight. FOS introduction did mitigate fat mass gain in animal raised onto HFHS diet (**Figure 1D**) but on overall did not significantly modify body weight. Of note, however, while a decrease in body weight could be expected from prebiotic treatment it is important to highlight that while FOS supplementation has been shown to increase

post-meal satiety and hunger, change in body weight were not always consistently observed. Indeed it when compared to other dietary fibers FOS supplementation was associated with body weight gain in lean rodents (28) and obese mice (49) while other report clearly show a preventive action of FOS on high-fat-mediated body weight gain (50). A genetic model of metabolic syndrome FOS supplementation was shown to drastically alleviate excessive feeding but had no impact on body weight (29). This study comes in addition with several report that clearly established the benefits of FOS onto glucose control and insulin sensitivity (51) and in that regards it is tempting to speculate that enhanced insulin sensitivity (29), while promoting a more healthy adipose development, could mitigate the overall body weight loss.

Despite overall similar body weight in within cohort, we found very different outcomes at the behavioral and molecular level when FOS supplementation was added during or after HFHS diet exposure. We first described a paradoxical action of FOS when assessed onto CTRL diet that increased the tropism for palatable diet when assessed on a two-food choice paradigm (**Figures 2B–D**) and while this result is in good agreement with the increase in hypothalamic orexigenic peptides (**Figure 4B**) it could also potentially be the consequence of anxiolytic-like properties of prebiotic (21) which might alleviate food neophobia classically observed in C57BL6 mice (29, 52) and result in faster maximization of palatable diet intake when given the choice. Importantly, however, it should be noted that our behavioral assay was designed to address how animals spontaneously prefer, or are willing to work for food reward and although alteration on reward feeding might lead to overconsumption (46), our protocol does not provide a measure on the long-term consequence onto body weight.

Chronic palatable diet exposure has been shown to promote changes in the reward system at both molecular and behavioral levels (8, 10, 46). One possible explanation for this timing effect of prebiotic action that we observed might be encapsulated in the fact that pre exposure to energy-dense food might initiate both peripheral and central adaptive changes among which some could be selectively corrected by FOS addition. Indeed, once rodent have been exposed to reinforcing stimulus such as palatable diet or drug of abuse they are typically more prone to develop addictive-like behavior (8, 46). These adaptive changes can involve one or many components of the DA system (7, 8, 46, 53) in association with alteration of the gut microbial ecosystem (54). For instance, energy-dense food exposure leads to diet-induced central inflammation (55), neuropeptide signaling alteration (29, 44), and decrease in dopamine receptor abundance (8, 10, 46), which would presumably participate in the development of addictive/compulsive eating behavior. Aside of a direct action onto the brain, energy-dense food also target the gut to control reward acquisition. Gut detection of dietary lipids have been shown to directly control DA release and action by route of the vagal nerves (56). These regulatory processes are probably part of larger integrative aspects by which the combination of diet and microbiota can influence host appetite through change in gut-derived metabolite, intestinal barrier, immune system (48).

Hence, the combination of HFHS exposure followed by prebiotic addition might overall change the microbia–gut–brain axis resulting in the fine-tuning or resetting of DA signaling and reward-driven behavior. Indeed, when FOS was added after HFHS exposure, we could observe a restoration of mesolimbic markers of DA signaling (**Figures 4A,B**) and, despite the increase in orexigenic NPY and AgRP observed the HFHS/CTRL-FOS group displayed strong reduction in food reward tropism (**Figures 2F–H**). This points at a rather dominant function of the reward system in the control of feeding in animals pre-exposed to palatable diet. Interestingly, the study from de Cossio and colleagues also described a beneficial action of prebiotic onto hyperphagia in obese animals that was independent of any changes in NPY, POMC was blunted by prebiotic addition, hypothalamic neuropeptide related (29).

Our results suggest that manipulating of the gut–brain axis can, in specific condition, exert a satietogenic effect primarily by modulating hedonic and motivational drive for food reward. This is in good agreement with the emerging concept that microbiota–gut–brain axis is a potential avenue to modulate reward and in general addictive behavior (57, 58). Notwithstanding, a great limitation of our study lies in the use of targeted metagenomics approach that only accounted for specific bacterial strain changes. It is clear that prebiotic treatment will have consequences on gut flora that extend far beyond the changes that we described here (**Figures 1I,J**) and it is formally possible that one or multiple changes in the gut ecosystem that were not addressed here might reveal potential molecular underpinning by which bacterial–host interaction alters food reward.

In conclusion, our study depicts how timely controlled prebiotic manipulation can differentially and selectively affect positive reinforcement and motivational aspects of food reward-seeking behavior and demonstrate the efficacy of the gut–microbiota–brain axis to operate molecular adaptations in neural substrates involved in both homeostatic and non-homeostatic control of body weight. However, further studies will be warrant to precisely describe the molecular underpinning of the bacterial–host interaction in the control of food reward.

ETHICS STATEMENT

All animal experiments were performed with approval of the Animal Care Committee of the University Paris Diderot-Paris 7 and according to European directives.

AUTHOR CONTRIBUTIONS

A-SD performed all the studies. JC, RD, CM, and MQ provided technical and conceptual support for behavioral and metabolic analysis and PCR analysis. AE and PC provide analysis of microbiota composition and conceptual support. FM and SL designed the study, secured the funding, and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

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REVIEW
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Hunger and Satiety Gauge Reward Sensitivity

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Many of the neurocircuits and hormones known to underlie the sensations of hunger and satiety also substantially alter the activity of the dopaminergic reward system. Much interest lies in the ways that hunger, satiety, and reward tie together, as the epidemic of obesity seems tied to the recent development and mass availability of highly palatable foods. In this review, we will first discuss the basic neurocircuitry of the midbrain and basal forebrain reward system. We will elaborate how several important mediators of hunger—the agouti-related protein neurons of the arcuate nucleus, the lateral hypothalamic nucleus, and ghrelin—enhance the sensitivity of the dopaminergic reward system. Then, we will elaborate how mediators of satiety—the nucleus tractus solitarius, pro-opiomelanocortin neurons of the arcuate nucleus, and its peripheral hormonal influences such as leptin—reduce the reward system sensitivity. We hope to provide a template by which future research may identify the ways in which highly rewarding foods bypass this balanced system to produce excessive food consumption.

Keywords: hunger, satiety, obesity, hypothalamus, reward, superstimulus, dopamine

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INTRODUCTION

In evolutionary psychology, a supernormal stimulus or "superstimulus" is some evolutionarily novel concentration of engaging characteristics, which produces a stronger response than the natural one (1). As Pinker described it, strawberry cheesecake is a superstimulus as compared to a Neolithic human diet; it overloads the senses and drives caloric overconsumption, combining the "sweet taste of ripe fruit, the creamy mouth feel of fats and oils from nuts and meat, and the coolness of fresh water" (2). The debate is ongoing as to what exactly differentiates a superstimulus from a regular one or whether it is truly maladaptive to create them or seek them out (1). Nevertheless, an important concept for neuroscience emerges from this discussion; certain systems governing the reaction to rewarding stimulus can be overloaded and their negative feedback component overridden. This hypothesis provides explanation for the panoply of excessive behaviors we cope with as a society,

Abbreviations: AgRP, agouti-related protein; Arc, arcuate nucleus of the hypothalamus; CCK, cholecystokinin; D1R, D2R, dopamine receptors; GHS1R, growth hormone secretagogue 1 receptor (ghrelin receptor); GLP1/GLP1R, glucagon-like peptide 1/receptor; LepR, leptin receptor; LH, lateral hypothalamic area; LTD, long-term depression; LTP, long-term potentiation; MC3R, MC4R, melanocortin receptors; MCH/MCH1R, melanin concentrating hormone/receptor; MSH, melanocyte-stimulating hormone (natural MCR agonist); MSN, medium spiny neuron; MTII, melanotan II (non-selective MCR agonist); NAc, nucleus accumbens; NAcc, nucleus accumbens core; NAcSh, nucleus accumbens shell; NTS, nucleus tractus solitarius; O1R, O2R, orexin receptors; POMC, pro-opiomelanocortin; PPG, preproglucagon; PVH, paraventricular hypothalamic nucleus; VTA, ventral tegmental area.

often conceptualized as "behavioral addictions": drug addiction, internet addiction, porn addiction, food addiction, and so on (3). Even physical activity in some individuals meets the criteria for behavioral addiction, demonstrating the complex nature of this phenomenon (4). It is necessary to provide biological evidence for the existence of the balanced system which these superstimuli overload.

The neurocircuitry and endocrinology underlying hunger and satiety may represent the best studied system in this regard. It clearly produces a physiological balance in certain conditions with regular stimulus, such as a laboratory mouse fed rodent chow its whole life producing a normal weight, and is dysregulated in other conditions that contain superstimuli, such as when that mouse is fed a high-fat, high-carbohydrate diet producing obesity. The signals encoding hunger and satiety alter the brain's dopaminergic reward system in a multitude of ways. In this review, we will discuss the mechanisms by which these signals, primarily hypothalamic neurocircuits and neuropeptides in combination with peripheral hormones, modulate midbrain dopaminergic activity to alter reward salience and value. By laying out this evidence, we provide a substrate for future research to examine how superstimulus foods, such as cheesecake or high-fat/high-carbohydrate chow, drive so-called hedonic feeding and produce obesity.

The relationship of the midbrain dopaminergic reward system and hypothalamic neurocircuits governing hunger and satiety, hereafter referred to as the *reward system* and *hunger system*, is ancient. The patterned expression of genes necessary to produce segmentation of the brain at the mesencephalon (midbrain) and diencephalon (hypothalamus) occurred very early in chordate evolution (5, 6). Both dopamine neuronal receptors and hypothalamic feeding-related peptides and their associated receptors are present in most vertebrates and have similar functions across taxa (7–9). Given this intimate association, it is not surprising that they share a fundamental interdependence. For example, dopamine-deficient mice stop feeding a few weeks after birth; administration of L-DOPA reverses this phenotype and restores normal growth (10). Conversely, knockout of orexin, a hypothalamic neuropeptide associated with hunger, reduces dopamine response to cocaine (11). They also produce cross-sensitization or desensitization; food-restricted, hungry mice have enhanced response and reinforcement to amphetamine or cocaine (drugs which flood the brain with dopamine), and satiety signals such as leptin reduce the drive to seek self-administration of these drugs (12, 13). As will be discussed below, hypothalamic and endocrine components of the hunger system alter the activity of the reward system. To demonstrate this, we will first present a brief overview of the neurocircuitry of the reward system. Then, we will chart the various ways the hunger system interacts with the reward system.

NEUROCIRCUITRY OF THE REWARD SYSTEM

The Ventral Tegmental Area (VTA)

The VTA and substantia nigra pars compacta (SNc) are immediately caudal to the posterior hypothalamus, brace the third ventricle, and contain the major source of dopaminergic outflow to the rest of the brain. The SNc is best known for its role in the nigrostriatal pathway regulating the dorsal striatum in movement, and the VTA for mediating salience, motivation, and reward and aversion-related learning (14, 15). Salience refers to the attention paid to the stimulus; an increase in salience means the stimulus, if identified, will be more likely to draw the organism's attention. The value of the stimulus, whether it is rewarding or aversive, refers to whether a stimulus induces behavior to acquire it or avoid it, respectively (15). Rewarding stimuli produce a positive valence when acquired and a negative valence when unable to be acquired; the converse is true with aversive stimuli (15).

The VTA dopaminergic neurons are the primary mediators of the behavioral response to a rewarding or aversive stimulus (16). They are not uniform in their activity or projection targets, and thus activation of one neuron may produce substantially different behavioral output than another. This is why studies evaluating the rewarding nature of dopamine often focus on the VTA to nucleus accumbens (NAc) projections specifically; this will be discussed below. However, much effort has been spent elucidating the ways in which local VTA dopaminergic neurons encode reward across brain regions by alteration in firing pattern, increase or decrease in action potential frequency, and projection target. The literature is incomplete on this topic, but discussion of some of these mechanisms sets the stage for further discussion of how the hunger system interfaces with the VTA dopaminergic neurons. For example, *in vivo* recording of the VTA during a conditioned place preference task suggests that one subset of dopaminergic neurons exhibits phasic activation in response to reward-related cues or reward consumption; another exhibits phasic inhibition in response to aversive stimulus or the absence of reward consumption after a reward cue (17). Tonic activation of dopaminergic neurons can produce the opposite effect of phasic activity on the same target and will decrease reward consumption (18). Thus, a given projection target receives either increased or decreased dopamine input dependent on the valence of the reward. Furthermore, dopaminergic neurons vary in their projection targets; the VTA's projections are heterogeneous. Dependent on the projection target, an increase in dopamine outflow produces either rewarding or aversive responses (14, 19). As has been well-established, VTA projections to the NAc core (NAcc) and NAc shell (NAcSh) increase dopamine release in response to a rewarding stimulus and induce goal-direct behavior to acquire and consume it (14). Conversely, VTA dopaminergic neurons projecting to the medial prefrontal cortex are activated in response to an aversive stimulus and produce aversive behaviors (19). However, even within the same target, dopaminergic activation can code both types of behaviors; VTA dopaminergic projections to the lateral portion of the NAcSh are activated in response to both rewarding and aversive stimulus (19).

The VTA also possesses neurons releasing the classic neurotransmitters glutamate and GABA. The function of these glutamatergic and GABAergic neurons is less well-known, but recent evidence indicates they also participate in valence-related responses. VTA glutamatergic projections to the lateral habenula

(LHb) play a significant role in encoding aversive learning (20). VTA glutamatergic projections to the NAcSh act in concert with the dopaminergic projections to produce reward-mediated behavior (14). Finally, VTA GABAergic neurons projecting to the LHb appear to inhibit this area to enhance positive valence responses (21). Recent analysis has identified that some VTA neurons corelease glutamate and dopamine—it is as yet unknown whether this occurs at the same synapse or at separate synaptic targets (14). Further research is needed to fully evaluate how the classic fast-acting neurotransmitters coordinate with dopaminergic neurons to produce the full suite of valence-related behaviors and alter future learned responses.

The NAc

The NAc is part of the ventral striatum and extended amygdala in the basal forebrain, and it mediates much of the motivated behavior produced in response to VTA dopaminergic outflow after sensation of a rewarding stimulus. Many components of the hunger system act here as well as in the VTA to alter the responsiveness to rewarding stimulus; thus, some description of its components and basic activity follows. The NAc is divided into a medial shell (NAcSh) and lateral core (NAcc). Self-administration of cocaine into the NAcSh is highly rewarding and rapidly produces cue-responsiveness with locomotor sensitization to anticipation of the drug (22–24). Self-administration of cocaine into the NAcc, however, is not reinforcing (22). Phasic activity of VTA dopaminergic projections to the NAcc instead responds to risk and prediction error in response to reward presentation (22, 24, 25). Thus, a basic paradigm can be constructed, where the NAcc responds to the salience, availability, and risk of acquiring the reward to produce motivation to pursue it, and the NAcSh responds to the positive valence of the reward acquisition, learns the cues which associate with the reward, and enhances the future salience of those cues. Interestingly, if dopamine is depleted in the NAc but reward acquisition is low effort, rats will still take the reward; however, if it requires high effort, rats will choose less effort-requiring behaviors (26). Thus, the level of dopamine in the NAc may provide a rough proxy for the amount of motivation an animal has to ignore risk and effort costs of acquiring a reward.

Both the shell and the core are inhibitory on all downstream targets; the vast majority of neurons are the GABAergic medium spiny neurons (MSNs). These are divided by receptor profile. There are D1R-MSNs, possessing excitatory D1R-like dopamine receptors (D1R and D5R), and D2R-MSNs, possessing inhibitory D2R-like dopamine receptors (D2R, D3R, and D4R). A significant minority express both receptor subtypes (27). The projection fields of the NAcSh and NAcc are wide and differ from each other in several important respects for their mediation of behavior. The NAcSh densely projects to the ventromedial ventral pallidum, lateral hypothalamic area (LH), and lateral preoptic area, whereas the NAcc projects to the dorsolateral ventral pallidum, subthalamic nucleus, and substantia nigra pars reticulata (22). The NAcSh shares significant reciprocal connections with feeding-related areas of the hypothalamus, whereas the NAcc primarily interacts with the basal ganglia. Thus, the NAcSh responds more to signals from the hunger system than the NAcc and will feature more prominently in this discussion.

HUNGER NEUROCIRCUITS SELECT FOR INCREASED REWARD SYSTEM ACTIVITY IN THE PRESENCE OF FOOD

There are numerous and dense interconnections between the hypothalamic nuclei, VTA, and NAc, and a wealth of neuropeptide and neurocircuit data exists to support the powerful influence of the several hypothalamic nuclei and their specific neuronal subtypes on the reward system. The interaction is complex and dynamic, depending upon both the availability of food and the endocrine manipulation of the system, as will be discussed later. A summary of the major hunger and satiety neurocircuits influencing the reward system is shown in **Figure 1**.

Arcuate Nucleus

The arcuate nucleus of the hypothalamus (Arc) sits adjacent to the third ventricle immediately ventral to the paraventricular hypothalamic nucleus (PVH). These two nuclei share the distinction of integrating central nervous system (CNS) input into the hypothalamic–pituitary axis and are the major source of the "releasing hormones," which are secreted into the hypophyseal portal veins to alter anterior pituitary production of various hormones. Thus, the Arc possesses a multitude of neuronal populations defined by their neuropeptide content, such as gonadotropin-releasing hormone neurons, growth hormone releasing hormone neurons, kisspeptin neurons, tuberoinfundibular dopamine (TIDA) neurons regulating prolactin release, somatostatin neurons, and so on. Many of these neurons alter feeding behavior, but their interaction with the dopaminergic reward system is poorly understood at this point in time (28). Thus, our discussion will focus on the agouti-related protein (AgRP)/neuropeptide Y (NPY) neurons that govern hunger and TIDA neurons role in feeding behavior. The pro-opiomelanocortin (POMC) neurons that govern satiety will be discussed later.

AgRP/NPY-Expressing Neurons

The AgRP/NPY-expressing neurons are found solely within the Arc (29). NPY acts on NPY receptors (Y1, Y2, Y4, and Y5 which are G_iPCRs) (30). AgRP is an inverse agonist of melanocortin receptors (MCRs; MC3R and MC4R, which are G_sPCRs and MAPK pathway activators) (31–35). Perhaps because of this, these neurons share with POMC neurons the same set of connections with hypothalamic and extrahypothalamic nuclei (29, 36). Thus, AgRP neurons are strong inhibitors of their downstream targets *via* GABA release, inverse agonism of MC-Rs, and NPYR-Gi activity. These neurons respond to a wide array of peripheral and central signals of energy balance, such as leptin, ghrelin, low glucose concentration, and gustatory sensation, and are activated during fasting (37–41). Surprisingly, given this role, knockout of AgRP by itself or in combination with NPY does not produce any obvious phenotype either in *ad libitum* or starvation feeding conditions—only in old age do they demonstrate slightly reduced body weight and adiposity due to increased metabolic

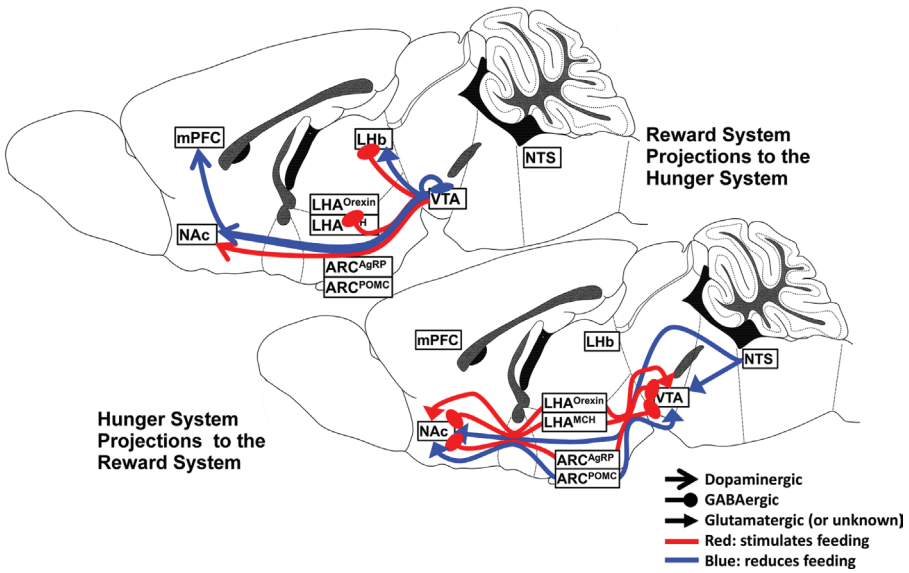


FIGURE 1 | Hunger and satiety neurocircuits interface with the midbrain–basal forebrain reward pathway.

rate (42, 43). Furthermore, neonatal destruction of the AgRP/NPY neurons has minimal effect on feeding; only adult ablation of these neurons prevents feeding behavior and leads mice in this condition to starve to death (44–46). Interestingly, several AgRP/NPY neuronal projections are not formed until a week postnatal in mice, such as to the PVH; there are many opportunities for developmental compensation to alter the neonatal AgRP/NPY ablation phenotype, which deserve further study to understand the homeostasis of feeding behavior (47). Much recent effort has been spent on understanding the acute dynamics of AgRP/NPY neuronal activity in hunger and reward.

Optogenetic stimulation produces food seeking and food consuming behaviors, with enhanced risk-taking and reduced anxiety (48–51). Their activity is aversive, as mice avoid the side of a chamber associated with their optogenetic activation (52). AgRP neurons select for food consumption; when activated, they reduce motivation to engage in other behaviors such as social interactions or drinking water when thirsty (51). Sustained AgRP neuronal activity is not necessary to produce feeding, and *in vivo* recording demonstrates that these neurons stop firing in the presence of food cues (53). Further optogenetic evidence demonstrates that a brief period of activation, prior to presentation with any food stimulus, will produce subsequent feeding, enhanced motivation to work for food, and selection for calorie dense foods (54). As strength of AgRP signal increases, there is a first-order kinetic increase in length of feeding and motivation to work which saturates (54). Stimulating AgRP projections to the PVH, bed nucleus of the stria terminalis, or LH are all individually sufficient to produce this effect (54). However, AgRP neurons also synapse on the VTA and regulate the reward system through this connection. AgRP projections to the VTA inhibit dopaminergic and glutamatergic release in the NAc and reduce the development of long-term potentiation (LTP) (55). It can be argued that AgRP neurons in the Arc reduce activity of the reward system while activating the hunger system, priming it to respond to food and not other stimuli; once food is spotted, cessation of AgRP neuronal activity releases the brakes on the reward system to enhance dopaminergic outflow. The increase in dopamine in the NAc likely increases the willingness to work for food and take risks to acquire it.

TIDA Neurons

The dopaminergic neurons of the Arc regulate the release of prolactin from the anterior pituitary. A subset of TIDA neurons appear to be functionally distinct from governing prolactin; these corelease GABA and dopamine, and deletion of prolactin receptor within this subpopulation has no effect on prolactin secretion regulation (56). A recent study demonstrated that optogenetic stimulation of these neurons produces feeding behavior independent of their stimulation of prolactin release, and inhibition of these neurons reduces body weight (57). Activation of these neurons, which are ghrelin sensitive, inhibited POMC neurons and excited AgRP/NPY neurons. While these are not considered part of the dopaminergic reward system, their role in detecting the intersection of hunger and reward is relevant to this discussion as any manipulation altering whole-brain dopamine systems (such as dopamine reuptake inhibitors such as cocaine) will alter the activity of these neurons and may subtly alter the phenotype.

The Lateral Hypothalamus

One of the major downstream targets of AgRP neurons is the LH. This was classically understood as both a hunger center and reward hot spot from early lesion and electrical stimulation studies. The LH has extensive projections divided by multiple subpopulations of neurons that express various neuropeptides as well as solely fast-acting neurotransmitter neurons (58). Many of these fast-acting projections play a role in mediating

hunger and reward. LH glutamatergic projections to the LHb prevent consumption of a conditioned reward of sucrose and has negative valence (59). Inhibition of this same projection has positive valence and induces sucrose consumption. Conversely, LH GABAergic projections appear to mediate consumption; activation of LH GABAergic neurons produces consumption regardless of the target's food value, such as ethanol, water, saccharin, sucrose, or wood (60). The projection targets of the LH GABAergic neurons may relate to different aspects of this behavior. For example, LH GABAergic projections to the PVH produce directed food consumption (61). However, activation of the LH-VTA GABAergic projections produce non-directed gnawing and licking of immediately available objects, despite the availability of a sucrose reward distant from the mouse (62). These LH-VTA GABAergic neurons inhibit both a subpopulation of dopaminergic and GABAergic neurons within the VTA. Interestingly, the reciprocal VTA-LH projection is observed to be activated in response to reward omission; the disorganized feeding behavior from LH-VTA projection may occur because of the lack of this feedback information (62). Indeed, a recent study evaluating lateral septum inhibitory influence on the LH found that one subset of LH GABAergic neurons are activated during food approach and another during food consumption, indicating that a temporal sequence of GABAergic subpopulation activation occurs to produce successful food consumption (63).

Orexin Neurons

Another reciprocal functional relationship between LH neuronal subtypes can be found in orexin (hypocretin) and melanin-concentrating hormone (MCH) neuropeptide-expressing neurons. Orexin, as the name suggests, is an orexigenic or food consumption-inducing neuropeptide released primarily by glutamatergic neurons in the medial and dorsal portions of the LH. Orexin A and B are co-released from the same neurons and bind to their receptors O1R and O2R, potently inducing arousal *via* G_s signaling. Orexin release dramatically increases in release in the human amygdala upon waking up or during arousing positive stimuli such as laughing or talking (64). Orexinergic neurons are activated in response to learned reward cues, but not novel objects; for example, activating them can reinstate extinguished drug-seeking behavior (65). Indeed, O2R protein levels in the NAc are elevated for up to 60 days after discontinuation of repeated cocaine administration (66). They are depolarized in response to low glucose, and directly activate VTA dopaminergic neurons which project to both the medial and lateral portions of the NAcSh (67). Orexin receptors also exist within both of these subdivisions; injection of Orexin A into the medial NAcSh induces a sucrose pleasure-associated facial response, whereas injection into the lateral NAcSh induces a sucrose seeking reaction (68). O1R activation increases NMDA glutamate receptor activity, a sign of LTP formation, whereas blockade of O1R in the VTA reduces the rate of self-administration of cocaine or chocolate (11, 69). Thus, orexinergic neurons clearly enhance the seeking and acquisition of a learned reward, partially *via* activation of the dopaminergic pathway as well as potentially enhancing the reinforcing quality of those rewards.

MCH Neurons

Melanin-concentrating hormone neurons are released from the anatomically adjacent dorsolateral LH and have a complex reciprocal relationship with orexin neurons. They linked with induction of sleep and directly inhibit orexin neurons *via* the MCH receptor (MCH1R) G_i protein coupled signaling (70). Their interaction with feeding behavior is complex. Knockdown of MCH1R produces hyperphagia, hyperactivity with increased foraging behaviors; however, these mice have a baseline lower body weight than controls, gain less weight on a high-fat diet, and exhibit an increased metabolic rate (71, 72). Elevation of glucose concentration from fasting levels to fed-state levels inhibits orexin neurons and depolarizes MCH neurons. It appears that MCHergic activity appears to play a role in placing a brake on orexin-induced seeking and consuming behaviors after food is acquired (73). However, injection of MCH into the lateral ventricle increases food consumption, without producing long-term weight gain (74). It appears that MCH neurons integrate olfactory, taste, and gut sensory input about the nutritional value of food and project to the VTA, NAc, and dorsal striatum in order to enhance the rewarding value of nutritionally valuable foods (75, 76). To this end, optogenetic stimulation of MCH neurons increases dopamine levels in the striatum only when paired with active consumption of an artificial sweetener; without stimulation, only caloric foods like sucrose induce this dopamine release (76). Pairing these stimuli produced a future preference for the artificial sweetener over sucrose—opposite of the control mouse preference. As further evidence of this, ablation of MCH neurons prevented the natural spike of dopamine in the striatum after the consumption of sucrose—though, interestingly, it did not ablate preference for sweet flavor over water, indicating that other mechanisms are at play (76). Thus, MCH neuronal activity increases when olfaction, taste, and gut nutrient sensors indicate that the food under active consumption is calorically valuable; it enhances the rewarding value of food by increasing VTA dopaminergic activity in the NAc.

The pattern of MCH1R activation within the NAc is similarly complex and deserves some further discussion. These receptors are coexpressed with D1R and D2R on opioid-producing MSNs. Activation of MCH1R here induces feeding and causes a depressed phenotype on the forced swim test, implying reduced locomotor drive (77, 78). Given that MCH1R is inhibitory, it is unsurprising that MSNs have decreased membrane excitability and reduced AMPA glutamatergic receptor currents; furthermore, antagonism of MCH1R reduces cocaine self-administration or cue-induced reinstatement of cocaine seeking behavior. However, on MSNs which coexpress D1R, D2R, and MCH1R, there is a unique synergy which enhances their firing activity and activates a phosphorylation cascade known to increase NMDA receptor activation (79). Some of this response is opioid dependent, as blockade of any of the three opioid receptors in the NAcSh prevents MCH-induced facial pleasure expression in response to oral gavage of sucrose (80). It appears that MCH1R activity within the NAc may shift the reward system response to enhance immediate food consumption and learning the nutrient value of food and dampen the seeking function of the reward system.

Hunger Enhances Sensitivity to Reward

The above discussion details how several neuronal populations—AgRP/NPY neurons, LH GABAergic neurons, orexin neurons, and MCH neurons—each alter the activity of the reward system in a distinct way as part of their contribution to the sensation of hunger. AgRP neurons reduce reward system sensitivity and inhibit its function until food is detected. The food cue silences their activity, disinhibiting the VTA and NAc to produce a large increase in dopamine release. This increases both the salience and value of the food cue. A subset of LH GABAergic neurons act in concert with orexin neurons to respond to these cues and produce food-seeking behavior, enhancing VTA dopaminergic release into the NAc. Other LH GABAergic neurons act with MCH neurons to also enhance the rewarding value of food and increase learning of nutritionally valuable food-related cues, producing food consumption. Once this process begins, satiety neurocircuits begin to act in order to limit overconsumption, as will be detailed below.

SATIETY NEUROCIRCUITS DECREASE THE ACTIVITY OF THE REWARD SYSTEM

The neurocircuitry of satiety is not as well-known as that of hunger. The general paradigm appears to be as follows: peripheral signals of positive energy balance, primarily hormonal, travel to the brain to inhibit the activity of hunger-producing neurocircuits. However, three populations of neurons within the CNS, defined by their neuropeptide content, are activated by these signals and directly influence the reward system. These are the POMC neurons of the Arc, the POMC neurons of the nucleus tractus solitarius (NTS), and the preproglucagon (PPG) neurons of the NTS. The NTS neurons integrate peripheral satiety signals, such as leptin, cholecystokinin (CCK), glucagon-like peptide 1 (GLP1), and gut distention, to induce rapid satiety. The role of POMC neurons is more complex, but appears to reduce the immediate value of the food reward while maintaining future responsiveness to that same reward.

PPG Neurons in NTS

The NTS has multiple functions both related to primary routing of taste information as well as being the routing point for significant amounts of autonomic and peripheral hormonal information entering the brain. It is situated in the dorsal motor vagal nerve complex and receives the vagal sensory afferents. These afferents are activated by GLP1, CCK, and leptin. The first two are released from stomach and intestinal cells and leptin from adipocytes in response to increased nutrient availability. They all synergistically increase the activity of the GLP1 producing neurons (termed PPG, or PPG neurons) in the NTS (81, 82).

The PPG NTS neurons have a wide projection field and synapse directly onto the VTA and NAc (83). Intestinally released GLP1 does not appear to cross the blood–brain barrier (BBB), and GLP1 receptor (GLP1R) signaling in the midbrain and basal forebrain requires NTS-mediated release of GLP1 (84). Activating GLP1R decreases highly palatable food intake and produces long-term weight loss; conversely, inhibiting them increases food consumption (83). Both changes occur due to changes in meal size, not frequency, indicating their role in terminating meal consumption. GLP1R activity in the VTA reduces LTP formation on dopaminergic neuron (85). GLP1 administration significantly increases dopamine transporter expression, increasing dopamine clearance from the synapse (86). GLP1R activity in the reward system may act to place the brakes on dopaminergic response to nutrient contents in the gut and prevent excessive learning of a food reward—or any reward, given the wide applicability of the reward system pathway. Consistent with this prediction, GLP1R knockout mice exhibit enhanced reward learning on the conditioned place preference test and increased cocaine-induced locomotion—receptor agonism had the inverse effect (87, 88). Thus, the PPG NTS neurons clearly mediate a dopamine-dampening function in the reward system and use this as a mechanism to reduce the reward salience and value of food or other objects.

POMC Neurons in the NTS and the Arcuate Nucleus

The role of POMC neurons is more complex, and much effort has been expended to explore their role in satiety. POMC is a precursor polypeptide which is cleaved by prohormone convertase 1 or 2 into α-, β-, or γ-melanocyte-stimulating hormone (MSH), corticotropin, and β-endorphin (89). These neuropeptides act *via* the MCR family, the best studied of which in regard to satiety are MC3R and MC4R. Deletion of MC4R produces obesity and hyperphagia; furthermore, the most common syndromic cause of obesity, Prader–Willi, occurs in part due to reduced cleavage of POMC (90, 91). The NTS and Arc POMC neurons both drive satiety *via* their actions on their receptors, but at different time scales. Optogenetic stimulation of the POMC NTS immediately halts feeding, but long-term activation does not produce weight loss (92). Stimulation of POMC Arc neurons has no immediate effect on feeding, but over many hours reduces feeding and produces weight loss (37). Both populations of POMC neurons project to the NAc and VTA—however, evidence is lacking on what effect direct stimulation of these projections produces (36). Both GABA-expressing and glutamate-expressing neurons have been found within the POMC neurons of the arcuate nucleus; but, the effects of fast neurotransmission from these neurons are not well explored (93). Instead, this discussion will focus on the pharmacological and receptor-related data that exist on the role of MC3R and MC4R in these regions.

MCR Signaling

The pharmacological effects of MCR activation are complex and their signaling mechanisms require some discussion before elaborating on their influence on the reward system. Multiple endogenous ligands for melanocortins exist. AgRP is an inverse agonist of MC3R and MC4R. α-, β-, and γ-MSHs and adrenocorticotropin hormone are produced from POMC by alternative cleavage and have varying affinities for each MCR type. Their downstream signaling activity is significantly altered dependent on their heterodimerization with other receptors such as dopamine receptors or ghrelin receptors. MC4R can couple with G_s, G_q, or $G_{i/o}$ protein complexes dependent upon allosteric binding-related conformational changes and thus produce increases in

intracellular calcium, increased cAMP signaling, or inhibit these same pathways (33). The two neuron-activating pathways, G_s and G_q, mediate distinct physiologic effects; knockout of PVH G_q, but not G_s, prevented the efficacy of MC4R agonism to prevent food intake (94). Conversely, the ghrelin receptor, when coupled with either MC3R or MC4R, selects for G_s signaling in both proteins in the PVH, for example, emphasizing that in conditions of increased hunger, MSH activity may select for activation (95). One of the most commonly used non-selective synthetic MCR agonists, melanotan II (MTII), also selects for G_s signaling (33, 95). AgRP, discussed above, is an inverse agonist of MC4R and reduces cAMP levels independent of the presence of any agonist (33–35). Hypothetically, AgRP activity may select for the G_i signaling conformation of MC4R, as it both antagonizes the ligand-binding site and binds to the allosteric site to cause a conformational change; it also induces receptor internalization and alters MC4R-mediated inhibition of L-type calcium channel activity (31–33, 96). While it has been demonstrated conclusively that intracerebroventricular infusion of the MC3R/MC4R agonist α-MSH suppresses feeding and infusion of AgRP suppresses feeding, the multiple signaling pathways invoked by each ligand as compared to MTII are important considerations while evaluating the pharmacological data for melanocortin influence on the reward system (97).

MC4R is found on D1R MSNs in the NAc. Application of α-MSH to *ex vivo* brain slices produces a decreased post-synaptic ratio of AMPA/NMDA glutamatergic receptor signaling and fewer excitatory post-synaptic currents—a sign of long-term depression (LTD) (98). These MC4R-linked D1R MSNs synapse onto LH GABAergic neurons; optogenetic activation of these prevents feeding (99). This reduction in feeding also occurs by MCR activity in the VTA; injection of MTII decreases sucrose consumption (100). Thus, MC4R activity may increase activity of these neurons to inhibit feeding, but lose their synaptic strength to prevent excessive inhibition of feeding behavior and simultaneously reduce their rewarding value. This coincides with evidence that administration of MC4R shRNA (knockdown) prevents the natural decrease in reward seeking induced by a chronic stress paradigm (98). Furthermore, blockade of NAc MC4R prevents chronic stress-elicited anhedonia, a known low-dopamine phenomenon (98).

However, in other conditions MCRs may play a role in reward-mediated learning and sensitivity to reward. After 2 h, intraventricular injection of MTII reduced the threshold for brain self-stimulation (101). Furthermore, mice with knockdown or inhibition of MC4R display reduced cocaine-induced reinforcement and reduced locomotor sensitization (102, 103). Injection of alpha-MSH into the posterior VTA increases the activity of MC4R-expressing dopaminergic neurons and induces ethanol self-administration (104). These neurons coexpress MC3R, and agonism of these neurons with γ-MSH increases motivation to work for sucrose reward in a dopamine-dependent fashion (105). Indeed, it appears that the level of glucose increase in response to food intake induces excitatory synaptic plasticity in a subpopulation of POMC neurons, which may enhance some of these downstream dopaminergic responses (106).

Thus, the multiple combinations of POMC cleavage products, the heterodimerization and allosteric-binding configurations of MCRs, and the differences in activity dependent on brain region all indicate that further evaluation of intracellular signaling pathways is needed. However, a possible synthesis may be as follows. MC4R signaling reduces ongoing feeding *via* its action on D1R MSNs in the NAc, with a naturally decaying signal because of the development of LTD. Simultaneously, in other regions and neuronal subpopulations, MC3R and MC4R enhance synaptic plasticity to encode future responses to that food reward—hence, knockdown of these receptors reduces reward consumption. The balance of melanocortin-mediated LTP and LTD in striatal neurons is important to halt ongoing food consumption, but appropriately encourage future responsiveness to rewarding food cues. One demonstration of this phenomena arises when hyperphagic MC4R knockout mice have induced second deletion of synapse-associated protein 90/post-synaptic density protein 95-associated protein 3 (SAPAP3) in the striatum (90). This double deletion both cures the compulsive grooming behavior of these mice and normalizes their weight. Deletion of SAPAP3 induces excessive LTD formation, and one may postulate that this helps reduce the excessive excitatory synapses formed in the VTA by unchecked MC3R signaling that in turn promote feeding behavior (107).

FOOD-RELATED HORMONES INFLUENCE DOPAMINERGIC ACTIVITY AND SENSITIVITY TO REWARD

The above discussion has focused on the interaction between neurons encoding hunger and satiety and the reward system. However, these are all interoceptive and are responsible for the integration of internal and external sensory information to produce unified behaviors. As is to be expected, the sensory input into this system is both neuronal (gut distention, pain, olfaction, taste, etc.) and endocrine. While the vast majority of endocrine pathways are altered in some way in response to variations in energy stores and the presence or absence of food in the gut, the best studied of these which appear to act directly on the reward system are the hunger hormone ghrelin and the satiety hormone leptin.

Ghrelin

Ghrelin is a peptide hormone first discovered as an endogenous ligand of the growth hormone secretagogue receptor (GHS1R), produced in the oxyntic glands of the stomach (108). Copies of ghrelin mRNA and ghrelin immunoreactivity are found in abundance in the antrum and fundus of the stomach and to a lesser degree in the duodenum, jejunum, ileum, and pancreas (109–111). Both peripheral and intracerebroventricular injections of ghrelin produce feeding in mice, and knockout of neuronal GHS1R prevents the development of diet-induced obesity; this places the CNS activity of ghrelin as a primary mediator of ghrelin's orexigenic effect (112, 113).

The mechanics governing ghrelin release and the neuronal response to ghrelin are complex and deserve some consideration. Ghrelin secretion occurs in ultradian pulses, peaking immediately

before onset of meals and declining soon after, with the greatest rise occurring overnight preceding breakfast (114). Several neurotransmitters, hormones, and metabolic signals also affect ghrelin release; acetylcholine, CCK, gastrointestinal peptide, and low glucose concentration enhance it, whereas insulin, gastrin, somatostatin, GLP-1, and increasing glucose concentration inhibit its release (115–117). Once ghrelin is produced, it can undergo post-translational modification with fatty acid linkage (octanoylation). This appears to affect ghrelin transport across the BBB; human octanoylated ghrelin and mouse des-octanoyl ghrelin are preferentially transported into the brain, whereas mouse octanoylated ghrelin (with two amino acid differences) is preferentially transported into the blood (118). The rate of BBB transport is enhanced by elevated serum triglycerides and fasting and blunted in aging, indicating physiological state modulation of this mechanism (119). There is also some evidence suggesting that a subpopulation of ghrelin-producing neurons may exist within the hypothalamus itself, which would not be affected by BBB transport and would have entirely unique, as yet undescribed, regulation (38). Finally, GHS1R-Gq-induced calcium flux (i.e., the signal strength) is attenuated by its heterodimerization with serotonin (specifically 5-HT2C), dopamine (D1R), and melanocortin (MC3R) receptors (120). Given that GHS1R possesses one of the highest levels of basal Gq activity of any GPCR, this dimerization may be important for reducing basal signaling except in the presence of the appropriate ligands for each receptor, potentiating the signal strength of the ghrelin-GHS1R active conformation or increasing GHS1R Gq-signaling *via* dedimerization after dopamine-D1R or serotonin-5-HT2C interactions (121).

After integrating the influences of pulsivity, BBB transport, and heterodimerization considered, ghrelin induces feeding behavior by depolarizing neurons expressing GHS1R. Chief among these, AgRP/NPY neurons express GHS1R-G_q-coupled signaling; this produces feeding behavior and reduced thermogenesis (112, 122). Thus, in the absence of any food cues, ghrelin actually reduces sensitivity to reward, acting *via* AgRP/NPY neurons and inhibiting the VTA and NAc as described earlier. However, GHS1R is also found in the VTA and laterodorsal VTA, a source of midbrain acetylcholine; injecting ghrelin into either of these places increases locomotor activity, food consumption, and NAc dopamine levels (123). Indeed, ghrelin enhances the rewarding value of high fat diet in *ad libitum*-fed mice (124). If no food is consumed after VTA ghrelin injection, VTA GABAergic neurons increase activity and reduce the release of dopamine into the NAc (123). These effects occur both due to ghrelin action on the VTA cell body and by alteration of pre-synaptic activity, especially the LH as orexin-deficient mice are resistant to the effect of ghrelin (40, 124–126).

These effects may be cross-sensitive for non-food rewards such as alcohol and amphetamines. In recovering alcoholics, ghrelin injection increases craving for alcohol; coincident with this, ghrelin receptor blockade attenuates both alcohol and amphetamine-induced locomotion sensitization (127–130). However, once sensitization has occurred, blockade of ghrelin transport into the brain does not appear to alter alcohol-induced locomotor activity or expression of conditioned place preference in rats (131). Thus, ghrelin may act to enhance learning of non-food rewards, but not be necessary to express this preference (132). Notably, GHS1R is found in the hippocampus; here it may enhance dopamine-induced synaptic plasticity within the hippocampus, promoting retention of the food-related reward *via* GHS1R-D1R heterodimer interactions (133).

Ghrelin action in gaging reward sensitivity can be summarized in three ways. First, by acting on AgRP/NPY neurons, it selects for food-seeking and food-consuming behavior and ignoring of other rewards. Second, by acting on the VTA, it increases the amplitude of dopamine release once cue-mediated silencing of AgRP/NPY neurons disinhibits these regions. Third, by acting on the hippocampus, it promotes dopamine-induced synaptic plasticity and future salience of the reward cue. Given the complexity and numerous levels of signal modification in the ghrelin system, future research will undoubtedly expand upon this story.

Leptin

Leptin is a signal of positive energy balance and appears to exert a dampening effect on the reward system. Leptin is a protein hormone released by white adipose tissue in pulses, highest around midnight in humans; the amplitude of each pulse directly correlates with the total amount of adipose tissue (114, 134). Glucose, insulin, glucocorticoids, TNF-alpha, and IL-6 all increase leptin release (134). Leptin acts on its receptors (leptin receptor, LepR), which are located in the CNS, particularly the hypothalamus. It can influence neuronal activity from the blood *via* action on the circumventricular organs and vagal afferents and is also translocated across the BBB *via* the action of tanycytes (135). The Arc, ventromedial and dorsomedial hypothalamus, LH, and NTS most densely express LepRs, and leptin action can be either inhibitory or excitatory on these neurons to inhibit food consumption and induce satiety (136). LepR also exists within the VTA on the dopaminergic projections to the extended central amygdala; exciting these neurons reduces food intake (137–139). This is consistent with the central amygdala's role in mediating the stress response.

Other populations of LepR expressing dopaminergic neurons have their activity inhibited by leptin and produce a general reward-dampening effect. Direct administration of leptin into the VTA and into the Arc increases the threshold for brain self-stimulation reward and decreases food intake (140). Conversely, knockdown or inhibition of LepRs within the VTA increases dopamine release onto the NAc and enhances cocaine-conditioned place preference (13). LepR depletion within the NAc core mediates the same effect (13). Interestingly, it also prevented D2R agonism from reducing cocaine reinforcement, implying that there is some synergy between LepR inhibitory action and D2R's reduction of synaptic plasticity mechanisms (13). This may occur *via* LepR activation of the signal transducer and activator of transcription 3 signaling pathway in the VTA, which reduces both feeding behavior and motivation to exercise (141). Finally, the hyperleptinemia of diet-induced obesity is also sufficient to produce the generalized reward-dampening effects described above. Mice with diet-induced obesity have reduced drive to self-administer cocaine, express less amphetamine conditioned place preference, and are less likely to express operant response

to sucrose (142, 143). These mice are demonstrated to have reduced dopamine turnover in the NAc (142, 143). Interestingly, alcohol consumption does not appear to follow this paradigm. Leptin administration significantly increased the consumption of ethanol in mice exposed to an ethanol deprivation-then-reinstatement procedure (144). High leptin levels at onset of alcohol withdrawal in humans were predictive of cravings and alcohol relapse; anti-opioid receptor drugs used to prevent relapse reduced leptin levels (145). Future research is necessary to understand how alcohol bypasses the dopaminergic reward system pathway to induce opioid release in a leptin-potentiated manner. Nevertheless, leptin clearly induces satiety by directly reducing activity of both the hunger system and the reward system, with generalizable reduction of the salience and value of both food and non-food rewards.

LIMITATIONS IN THE STUDY OF THE INTERFACE BETWEEN THE HUNGER AND REWARD SYSTEMS

The above reviewed evidence is not comprehensive, as the relationship between the hunger system and reward system is of great interest and under active research with exciting new evidence unveiled daily. Given the ancient association of these two systems and the necessity of proper balance of food consumption to sustain life, it is understandable that nearly every neuronal and endocrine system appears to alter food consumption and manipulate the reward system in some way. Furthermore, the developmental activity of these systems may differ from their adult activity, and disruptions to these systems may result in significant compensation over the developmental period. The techniques utilized to study feeding and reward behavior are also limited by the necessity of overactivation, for an extended period, a single node within the feeding system. Given the interconnected nature of these neuronal circuits within the hunger system, continuous activation of any part likely engages the other portions and produces the unified behavioral response. However, it is likely that each neuronal pathway described above does not independently induce or inhibit food or reward consumption, but instead activates a certain portion of a "reward consumption sequence." A clear example of this is how LH-VTA GABAergic activation induces gnawing and licking behaviors, but not sucrose seeking (62). The nature of this reward consumption sequence remains to be uncovered, and will likely require further molecular characterization of each of these components of the reward sequence, *in vivo* recording of their activity, and more advanced optogenetic and chemogenetic stimulation techniques to better approximate physiological activity.

CONCLUSION

The hunger system manipulates the reward system to increase the salience and value of food and enhance future response to rewarding food cues. Ghrelin and AgRP neurons prime the reward system to activate only in the presence of food cues. LH fast neurotransmitters and orexin neurons produce food-seeking paradigm in response to a learned cue. Other LH GABAergic neurons and MCH neurons increase food consumption, reducing reward seeking behavior and producing reward consuming behavior by their action on the VTA and NAc. Early satiety signals like GLP1 and CCK put the brakes on food consumption by dampening reward system activity, reducing its value. POMC neuronal activity produces a balanced synaptic plasticity to respond effectively to future food stimulus, while gradually inducing satiety. Late satiety signals like leptin dampen reward system activity generally to reduce effort for acquiring food or other rewards while in a positive energy state. A summary of these effects is presented in **Table 1**.

TABLE 1 | Hunger system activity and influence on the reward system.

	Fasting state	Food cue response	Feeding and fed state
Arcuate agouti-related protein (AgRP)/ neuropeptide Y neurons	Inhibits reward system activity	Decreased activity	Decreased activity
Lateral hypothalamic area (LH) GABAergic neurons	Unknown	Inhibit subpopulation ventral tegmental area (VTA) DA and GABAergic neurons	Inhibit different subpopulation of VTA DA and GABA neurons
LH Orexin neurons	Increase reward system sensitivity	Increase VTA DA firing	Reduced activity as glucose increases
LH melanin-concentrating hormone neurons	Inhibited in low glucose	Integrate gustatory cues, enhance nucleus accumbens (NAc) sensitivity	Activate in response to gustatory nutrient content, enhance NAc-mediated reward consumption
Nucleus tractus solitarius (NTS) preproglucagon neurons	Decreased activity	Decreased activity	Integrates cholecystokinin, leptin, glucagon-like peptide 1, gut distention to inhibit reward system
NTS pro-opiomelanocortin (POMC) neurons	Decreased activity	Decreased activity	Increase in response to gut distention, reduce feeding
Arcuate POMC neurons	Decreased activity	Decreased activity	Activated by leptin, decrease NAc sensitivity but increase VTA encoding of food cue
Ghrelin	Dual action: increases AgRP neuronal activity and increases intrinsic reward system sensitivity	Increases reward system sensitivity	Reduces after food consumption
Leptin	Decreased activity	Decreased activity	Increases with insulin activity and decreases reward system activity

With this paradigm in mind, we can return to the superstimulus concept and observe how extreme rewards may bypass this balanced relationship of hunger systems enhancing food rewards to increase food consumption, and satiety systems reducing food rewards to reduce consumption. Highly palatable foods can induce food consumption in fed mice, even with adult ablation of AgRP neurons—a disruption which typically produces starvation (146). This relies on dopaminergic tone to produce the feeding response. A natural extension of this finding is this: if the reward's learned value is high enough to stimulate a large dopamine spike upon sighting, overcoming the reward-dampening influence of satiety circuits and hormones, this triggers the feeding sequence. It can be speculated that normally, AgRP neurons *via* their aversive, inhibitory action sensitize the reward system so that when it is finally released, even an intrinsically low-value food cue produces a large spike in dopaminergic activity. This is in keeping with the old saying that "hunger is the best sauce." Thus, it may not be that there are separate hedonic and homeostatic feeding mechanisms, but instead that highly palatable foods bypass the natural inhibition of the reward system by the hunger system.

AUTHOR CONTRIBUTIONS

RC prepared and wrote the manuscript. QT revised the manuscript and provided critical input for new ideas and content to be added to the manuscript.

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Activity-Based Anorexia Reduces Body Weight without Inducing a Separate Food Intake Microstructure or Activity Phenotype in Female Rats—Mediation via an Activation of Distinct Brain Nuclei

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Anorexia nervosa (AN) is accompanied by severe somatic and psychosocial complications. However, the underlying pathogenesis is poorly understood, treatment is challenging and often hampered by high relapse. Therefore, more basic research is needed to better understand the disease. Since hyperactivity often plays a role in AN, we characterized an animal model to mimic AN using restricted feeding and hyperactivity. Female Sprague-Dawley rats were divided into four groups: no activity/*ad libitum* feeding (*ad libitum*, AL, $n = 9$), activity/*ad libitum* feeding (activity, AC, $n = 9$), no activity/restricted feeding (RF, $n = 12$) and activity/restricted feeding (activity-based anorexia, ABA, $n = 11$). During the first week all rats were fed *ad libitum*, ABA and AC had access to a running wheel for 24 h/day. From week two ABA and RF only had access to food from 9:00 to 10:30 a.m. Body weight was assessed daily, activity and food intake monitored electronically, brain activation assessed using Fos immunohistochemistry at the end of the experiment. While during the first week no body weight differences were observed ($p > 0.05$), after food restriction RF rats showed a body weight decrease: −13% vs. day eight ($p < 0.001$) and vs. AC (−22%, $p < 0.001$) and AL (−26%, $p < 0.001$) that gained body weight (+10% and +13%, respectively; $p < 0.001$). ABA showed an additional body weight loss (−9%) compared to RF ($p < 0.001$) reaching a body weight loss of −22% during the 2-week restricted feeding period ($p < 0.001$). Food intake was greatly reduced in RF (−38%) and ABA (−41%) compared to AL ($p < 0.001$). Interestingly, no difference in 1.5-h food intake microstructure was observed between RF and ABA ($p > 0.05$). Similarly, the daily physical activity was not different between AC and ABA ($p > 0.05$). The investigation of Fos expression in the brain showed neuronal activation in several brain nuclei such as the supraoptic nucleus, arcuate nucleus, locus coeruleus and nucleus of the solitary tract of ABA compared to AL rats. In conclusion,

ABA combining physical activity and restricted feeding likely represents a suited animal model for AN to study pathophysiological alterations and pharmacological treatment options. Nonetheless, cautious interpretation of the data is necessary since rats do not voluntarily reduce their body weight as observed in human AN.

Keywords: anorexia nervosa, body weight, brain-gut axis, eating disorder, Fos, psychosomatic, wheel running

INTRODUCTION

Anorexia nervosa (AN) is an eating disorder characterized by the desire to lose body weight or to maintain body weight at a lower level than normal for age and height. Moreover, patients suffer from an intense fear of gaining weight and a body image disturbance (American Psychiatric Association, 2013). AN has a high prevalence in adolescent girls and young women (Nagl et al., 2016); the lifetime prevalence for AN in European women was reported to be 0.9% (Preti et al., 2009), similar levels were reported for the United States (Hudson et al., 2007). The treatment of AN is challenging and mostly comprised of structured care and psychotherapy (Zipfel et al., 2014); however, treatment is hampered by a high relapse rate (Herzog et al., 1997; Zipfel et al., 2015). While only about half of the patients recover, one third improves but continues to have symptoms and 20% remain severely chronically ill (Steinhausen, 2002). Lastly, AN has a considerable weighted mortality rate (deaths per 1000 person-years) of 5.1 (Arcelus et al., 2011). It is to note that although AN is clinically well characterized, the pathogenesis underlying the disease is still not well established. Moreover, no specific pharmacological treatment is available. Therefore, more research is needed to better characterize the disease and to identify possible new treatment targets.

Progress in medical research is often achieved by establishing an animal model of a disease that can help to investigate the underlying pathophysiology. It was already in 1967 when Routtenberg and Kuznesof observed that rodents tend to self-starvation when exposed to a time-restricted feeding schedule and given the possibility of voluntary physical activity in a running wheel (Routtenberg and Kuznesof, 1967). As hyperactivity can be observed in a considerable subset (ranging from 31 to 80%) of patients with AN (Davis et al., 1997), animal models using physical activity mimic this condition. The combination of a restricted feeding schedule and the access to physical exercise using a running wheel has been used to mimic features of human AN; the model was termed activity-based anorexia (Casper et al., 2008).

Subsequently, the model has been largely characterized and several alterations observed such as an increased brain γ-aminobutyric acid (GABA) (Aoki et al., 2012) and endocannabinoid signaling (Casteels et al., 2014), disturbances in food-anticipatory dopamine and serotonin release (Verhagen et al., 2009) along with an involvement of several food intake-regulatory hormones, e.g., ghrelin (Legrand et al., 2016) and leptin (Hillebrand et al., 2005b) and lastly, an activation of the hypothalamus-pituitary-adrenal axis (Burden et al., 1993), changes that might play a role in human AN as well. These alterations are likely to be involved in several changes observed: besides a reduction in food intake and body weight also an intestinal barrier dysfunction (Jésus et al., 2014), a disruption of neural development in the hippocampus (Chowdhury et al., 2014) and an impairment of memory function (Paulukat et al., 2016), increased anxiety (Kinzig and Hargrave, 2010) and the development of stress ulcers (Doerries et al., 1991), features also observed (Kline, 1979; Ghadirian et al., 1993; Swinbourne and Touyz, 2007; Huber et al., 2015; Kjaersdam Telleus et al., 2015) or suspected in patients with AN. Taken together, activity-based anorexia—despite the major limitation of being an animal model merely mimicking features of a disease—is likely a suited tool to study aspects of the pathogenesis of human AN.

The aim of the present study was first to establish the model of activity-based anorexia in our laboratory investigating food intake, running wheel activity and body weight in female rats. Only female rats were used due to the higher prevalence of anorexia in females compared to males (Steinhausen and Jensen, 2015). Next, we investigated the food intake microstructure underlying the reduction in food intake in this animal model using an automated food intake monitoring system recently established for the use in rats (Teuffel et al., 2015). To further characterize possible underlying alterations in brain activity we used the neuronal expression marker Fos and performed a brain mapping in rats subjected to activity-based anorexia.

MATERIALS AND METHODS

Animals

Female Sprague-Dawley rats (Harlan-Winkelmann Co., Borchen, Germany) weighing 150–180 g upon their arrival were housed in groups under conditions of controlled illumination (12:12 h light/dark cycle, lights on/off: 06:00 a.m./06:00 p.m.) and temperature (21–23°C). Rats were fed with standard rat chow (ssniff Spezialdiäten GmbH, Soest, Germany) and tap water *ad libitum* unless otherwise specified. This study was carried out in accordance with the recommendation of the institutional guidelines; the protocol was approved by the state authority for animal research (#G 0117/14).

Activity-Based Anorexia

After an initial acclimatization period of 7 days, rats (total $n = 44$) were randomly assigned to one of four groups: (a) *ad libitum* group: no extra activity + *ad libitum* feeding schedule, (b) activity group: voluntary activity in a running wheel + *ad libitum* feeding schedule, (c) restricted feeding group: no extra activity + restricted feeding schedule, and (d) activity-based anorexia group: voluntary activity in a running wheel + restricted feeding schedule.

During the first week of the experiment, all rats were fed *ad libitum* and separated into single housing cages which were placed adjacent to each other to provide sight, acoustic and odor contact. Rats of the activity and activity-based anorexia group had access to a running wheel inside the cage for 24 h/day, while the sedentary groups (*ad libitum* and restricted feeding group) were housed without running wheel under otherwise identical conditions. All cages contained environmental enrichment and bedding material. Rats were acclimated to their new cages for 1 week and handled daily to become accustomed to the interaction with the investigator. This included daily removal of the rat from the cage to measure body weight. The daily routine was performed between 08:00 and 09:00 a.m.

Food restriction conditions started on day eight of the experiment. Rats of the restricted feeding as well as activity-based anorexia group received food from 09:00 to 10:30 a.m. (the 90-min feeding period during the light phase was based on Luyten et al., 2009; Wu et al., 2014), while the other two groups (*ad libitum* and activity group) continued to have access to food for 24 h/day. Body weight, food intake and activity were monitored over a period of 21 days. The experiment was discontinued and animals euthanized when the body weight loss exceeded 25%.

MEASUREMENTS

Monitoring of Body Weight

Rats were weighed daily between 08:00 and 09:00 a.m. Body weight and body weight changes were calculated for the whole 21-day experimental period (1 week of *ad libitum* food intake and 2 weeks of restricted feeding).

Monitoring of Food Intake and Food Intake Microstructure

The microstructural analysis of feeding behavior was conducted using the BioDAQ episodic food intake monitoring system for rats (BioDAQ, Research Diets, Inc., New Brunswick, NJ, USA) which allows the continuous monitoring of solid chow food intake in undisturbed rats as recently reported (Teuffel et al., 2015). The system contains a food hopper placed on an electronic microbalance, both are mounted on a regular rat single housing cage. Food intake parameters are measured continuously and can be extracted from the software (BioDAQ Monitoring Software 2.3.07); periods of interest can be chosen freely afterwards for data analysis. Every interaction of the rat with the food hopper is registered as a "bout." A meal is defined as food intake of at least 0.01 g, when feeding bouts occur after an interval of $\geq$15 min this is considered a new meal. Meal parameters extracted from the software include bout size, meal size, bout frequency, meal frequency, meal duration, time spent in meals and eating rate. The food intake microstructure was analyzed starting at 4 days after food restriction over a period of 4 days (expressed as mean value of 4 days/animal).

Monitoring of Physical Activity

Physical activity in the running wheel was assessed electronically using the software provided by the manufacturer (Campden Instruments Ltd., Loughborough, UK) and expressed as wheel rotations per day as described before (Wu et al., 2014). Here, the activity system was combined with the cages for automated food intake monitoring. Pilot studies did not indicate any deleterious interference between the two measurements (data not shown).

The estimation of energy consumption was based on an earlier study that determined oxygen consumption of rats running at a constant speed (Shepherd and Gollnick, 1976). A respiratory exchange ratio of 1.0 was assumed based on carbohydrates as largest component in the standard rat chow used (58% of calories from carbohydrates, manufacturer's information).

c-Fos Immunohistochemistry

At the end of the observation period, brain activation was assessed using c-Fos immunohistochemistry in *ad libitum* and activity-based anorexia rats ($n = 3$/group). In order to avoid signals from overfeeding and great distention of the stomach, food intake was restricted to 1.5 g in this last 1.5-h feeding period in the activity-based anorexia group. Directly after this feeding period, animals were perfused and brains processed for Fos immunohistochemistry as described before (Wang et al., 2011). Briefly, rats were deeply anesthetized by an intraperitoneal injection of 100 mg/kg ketamine (Ketanest^{TM}, Curamed, Karlsruhe, Germany) and 10 mg/kg xylazine (Rompun^{TM}2%, Bayer, Leverkusen, Germany). Transcardial perfusion was performed as described before (Stengel et al., 2009). After thoracotomy a cannula was inserted into the ascending aorta *via* the left heart ventricle. Perfusion consisted of a 1-min flush with sodium chloride (0.9%) followed by 500 ml of fixative (4% paraformaldehyde and 14% saturated picric acid in 0.1 M phosphate buffer, pH adjusted to 7.4). Afterwards, brains were removed and postfixed overnight in the same fixative at 4°C followed by a cryoprotection in 10% sucrose for 24 h. Lastly, brains were snap-frozen in dry ice-cooled 2-methylbutane (Carl Roth GmbH, Karlsruhe, Germany) and then stored at −80°C until further processing.

Rat brains from the two groups were processed in parallel to ensure similar conditions. Whole brains were cut into coronal sections (25 μm) from prefrontal forebrain to the caudal medulla using a cryostat (CryoStar NX70, Thermo Fisher Scientific, Waltham, MA, USA). Every third brain section was rinsed in phosphate-buffered saline (PBS) for 3 × 15 min. All incubations were performed using the free-floating technique at room temperature (except for the incubation with the primary antibody at 4°C) and followed by a 3 × 15 min washing step in PBS. The sections were first treated with 0.3% H_20_2 in PBS for 30 min to block endogenous peroxidase activity. After rinsing the sections, nonspecific binding was blocked by 2% normal goat serum (NGS, Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) for another 30 min. Sections were washed again and incubated in rabbit polyclonal anti-cFos (1:20,000, Catalog No. ABE457, Merck Millipore, Darmstadt, Germany) as primary antibody (2 h at room temperature followed by overnight at 4°C). Sections were rinsed again and incubated with biotinylated secondary goat anti-rabbit IgG (1:1000, Catalog No. 111-065-144, Jackson ImmunoResearch) for 2 h. After rinsing, this was followed by the incubation with the avidin-biotin-peroxidase complex (ABC, 1:500, Vector Laboratories,

Burlingame, CA, USA) in 0.3% Triton-PBS for 1 h. Staining was visualized with diaminobenzidine tetrahydrochloride (DAB, Sigma-Aldrich, Darmstadt, Germany) and nickel ammonium sulfate (Fisher Scientific, Waltham, MA, USA). The color development was frequently checked with a light microscope and stopped after about 10 min. After staining, sections were mounted, air-dried, completely dehydrated through a gradient of ethanol, cleared in xylene and cover-slipped with Entellan™ new (Merck Millipore).

In a separate experiment, specificity of the cFos antibody was assessed by pre-absorption with synthetic SGFNADYEASSSRC (amino acids 4–17 of rat c-Fos, JPT Peptide Technologies GmbH, Berlin, Germany). The peptide (5 μg/ml) was incubated with the anti-c-Fos antibody diluted at 1:20,000 (Merck Millipore, antigen:antibody ratio of 100:1) for 2 h at room temperature followed by 22 h at 4°C. The solution was centrifuged for 15 min at 13,000 × g and the supernatant used for immunostaining as described above.

Immunoreactivity of brain sections was examined using a light microscope (Axiophot, Zeiss, Jena, Germany) and images were acquired using a connected camera (AxioCam HRc, Zeiss). The density of Fos positive cells in each brain section was determined semi-quantitatively using a 10x objective and described as −, no; +, low (∼1–10 cells); ++, medium (∼10–20 cells); and +++, high (>20 Fos positive cells in a 100 μm × 100 μm area of an ocular grid with a 10x objective) density of expression. Coordinates of the brain nuclei were identified according to the rat brain atlas (Paxinos and Watson, 2007). The investigator was blinded to the experimental group. The average density of Fos immunoreactive cells derived from the total number of sections analyzed for each nucleus was determined for each animal and used to calculate the mean density of expression per group.

Statistical Analysis

Distribution of the data was determined by the Kolmogorov-Smirnov test. Data are expressed as mean ± SEM and analyzed by one-way analysis of variance (ANOVA) followed by Tukey *post-hoc* test or two-way or three-way analysis of variance followed by the Holm-Sidak method. Differences were considered significant when $p < 0.05$ (SigmaStat 3.1., Systat Software, San Jose, CA, USA).

RESULTS

Activity-Based Anorexia Rats Show the Greatest Reduction in Body Weight

During the first week of the experiment (access to the running wheel in single housing cages for activity and activity-based anorexia group; regular single housing conditions for *ad libitum* and restricted feeding group) no body weight differences were observed between the four groups (**Figure 1**). After the start of food restriction, rats of the restricted feeding group as well as activity-based anorexia rats showed a body weight decrease, while the *ad libitum* and activity groups continued to gain body weight (**Figure 1**). At the end of the 14-day food restriction period, rats of the restricted feeding group showed a body weight decrease of −13% vs. day eight ($p < 0.001$) and vs. AC (−22%, $p < 0.001$) and AL (−26%, $p < 0.001$) that gained body weight (+10% and +13%, respectively; $p < 0.001$; **Figure 1**). Activity-based anorexia rats showed an additional body weight loss of −9% compared to rats of the restricted feeding group ($p < 0.001$; **Figure 1**) reaching an average body weight loss of −22% during the 14-day observation period. Three-way ANOVA showed a significant influence of time [$F_{(13, 504)} = 5.6$, $p < 0.001$], activity [$F_{(1, 504)} = 436.1$, $p < 0.001$] and feeding regimen [$F_{(1, 504)} = 9806.3$, $p < 0.001$] as well as an interaction of these three factors [$F_{(13, 504)} = 2.0$, $p = 0.02$].

It is to note that three out of 14 rats subjected to activity-based anorexia failed to lose body weight (or even gained body weight during the experimental period, data not shown) and were therefore excluded from further analyses (final $n = 11$).

Activity-Based Anorexia Rats Show a Similar Reduction in Food Intake As Observed in the Restricted Feeding Group

The food intake observed in the two *ad libitum* fed groups (*ad libitum* and activity group) did not differ from each other and was fairly stable over the 21-day observation period (**Figure 2**). While during the first 3 days of the habituation period—although during this time also fed *ad libitum*—the food intake was lower in the activity as well as activity-based anorexia group compared to the *ad libitum* and the restricted feeding groups ($p < 0.001$) giving rise to more time spent for physical activity and less for food intake, food intake was similar on days 6 and 7 (before food restriction) in all four groups (**Figure 2**). After food restriction to 1.5 h per day in the restricted feeding and activity-based anorexia group, food intake significantly dropped by −88% in these groups on the first day compared to the *ad libitum* group ($p < 0.001$) and slowly increased afterwards to reach the same level as observed in the *ad libitum* fed groups on the last day of the observation period (**Figure 2**). Overall, rats of the restricted feeding and activity-based anorexia group ate less (−38 and −41%, respectively) compared to the *ad libitum* group during the 14-day food restriction period (**Figure 2**). Three-way ANOVA indicated a significant impact of time [$F_{(20, 776)} = 24.5$, $p < 0.001$], activity [$F_{(1, 776)} = 68.3$, $p < 0.001$] and feeding regimen [$F_{(1, 776)} = 626.5$, $p < 0.001$] as well as an interaction of these three factors [$F_{(20, 776)} = 1.8$, $p = 0.02$]. Food intake during 1.5 h did not differ between the restricted feeding and activity-based anorexia group, while 24-h food intake did not differ between the *ad libitum* and activity group ($p > 0.05$; **Figure 3**).

Activity-Based Anorexia Rats Show a Similar Physical Activity As Observed in the Activity Group

Physical activity assessed using a running wheel slightly increased during the first week from ∼1500 to ∼2000 wheel rotations/day (**Figure 4**). During the food restriction period, physical activity more prominently increased reaching ∼3500 wheel rotations/day in both, the activity and activity-based anorexia groups (**Figure 4**), corresponding to ∼1300 m/day. No daily differences were observed either in the 1.5-h (data not

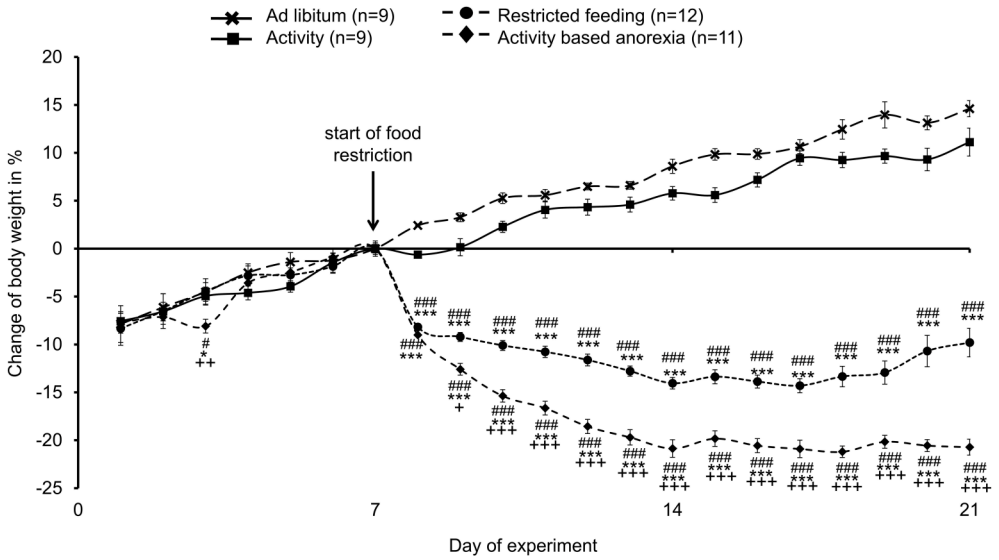


FIGURE 1 | Activity-based anorexia rats show the greatest body weight loss compared to all other groups. Animals had access to a running wheel for 24 h/day (activity and activity-based anorexia group) or were housed without wheel access under otherwise similar conditions (*ad libitum* and restricted feeding group). On day eight food intake was restricted to 1.5 h/day in the restricted feeding and activity-based anorexia group, while the activity and *ad libitum* group retained access to food for 24 h/day. Body weight changes are expressed in % changes from the day of food restriction. Data are expressed as mean ± SEM. $^{*}p < 0.05$ and $^{***}p < 0.001$ vs. *ad libitum* group; $^{\#}p < 0.05$ and $^{\#\#\#}p < 0.001$ vs. activity group; $+p < 0.05$, $++p<0.01$ and $+++p < 0.001$ vs. restricted feeding group.

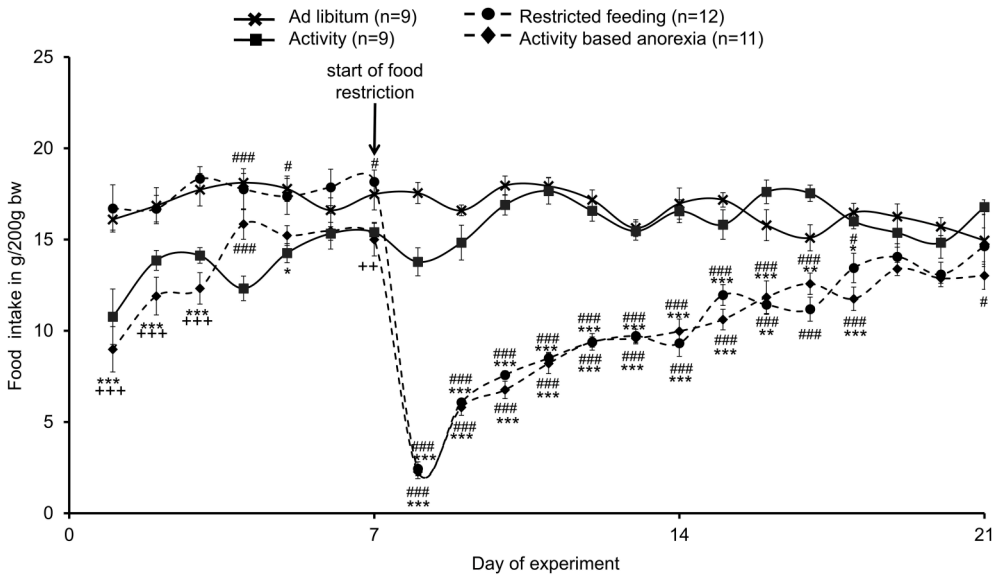


FIGURE 2 | Activity-based anorexia and restricted feeding group show a similar reduction in food intake over the whole observation period. Animals had access to a running wheel for 24 h/day (activity and activity-based anorexia group) or were housed without wheel access under otherwise similar conditions (*ad libitum* and restricted feeding group). On day eight food intake was restricted to 1.5 h/day in the restricted feeding and activity-based anorexia group, while the activity and *ad libitum* group retained access to food for 24 h/day. Food intake is calculated as g/200 g body weight; all data are expressed as mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ vs. *ad libitum* group; $^{\#}p < 0.05$ and $^{\#\#\#}p < 0.001$ vs. activity group; $++p < 0.01$ and $+++p < 0.001$ vs. restricted feeding group.

shown) or 24-h wheel rotations between the activity and activity-based anorexia group ($p > 0.05$; **Figure 4**).

The daily energy expenditure including calculated resting energy expenditure and energy expenditure while running was ∼35 kcal/200 g body weight at the beginning of the observation period (data not shown). This value slightly increased during the food restriction period in both the activity and activity-based anorexia group to ∼38 kcal/200 g body weight (data not

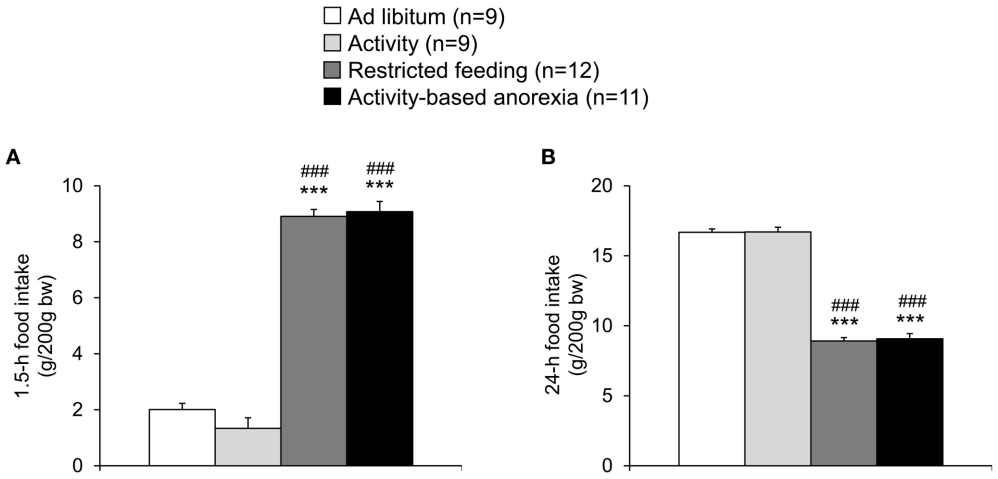


FIGURE 3 | Activity-based anorexia and restricted feeding group show a similar reduction in 1.5-h and 24-h food intake. Animals had access to a running wheel for 24 h/day (activity and activity-based anorexia group) or were housed without wheel access under otherwise similar conditions (*ad libitum* and restricted feeding group). On day eight food intake was restricted to 1.5 h/day in the restricted feeding and activity-based anorexia group, while the activity and *ad libitum* group retained access to food for 24 h/day. Food intake was monitored continuously using an automated food intake monitoring device and 1.5-h **(A)** as well as 24-h **(B)** food intake analyzed over a period of 4 days starting 4 days after food restriction (when a relatively stable food intake was observed). Data are expressed as mean ± SEM. $^{***}p < 0.001$ vs. *ad libitum* group and $^{###}p < 0.001$ vs. activity group.



FIGURE 4 | Activity-based anorexia and activity group fed *ad libitum* show similar levels of physical activity. Animals had access to a running wheel for 24 h/day. On day eight food intake was restricted to 1.5 h/day in the activity-based anorexia group, while the activity group retained access to food for 24 h/day. Physical activity was monitored by measuring the wheel rotations per day. Data are expressed as mean ± SEM. $p > 0.05$.

shown). Two-way ANOVA indicated a significant impact of time [$F_{(20,\ 377)} = 3.6$, $p < 0.001$] and feeding regimen [$F_{(1,\ 377)} = 4.3$, $p = 0.04$].

Caloric deficit was calculated by subtracting energy expenditure from calculated caloric intake. All four groups showed a caloric surplus during the first 7 days of the experimental period (ranging from 9.6 to 21.4 kcal/day on day 7, data not shown). While this surplus remained visible in the *ad libitum* fed groups over the remaining 14-day observation period (14.2 ± 4.6 kcal in the *ad libitum* and 17.1 ± 2.3 kcal in the activity group), in the restricted feeding groups a caloric deficit was observed from the start of the food restriction (greatest levels on day 8: restricted feeding: −30.5 ± 1.4 kcal, activity-based anorexia: −29.6 ± 0.9 kcal) with a progressive decrease of caloric deficit reaching a surplus again on day 21 (restricted feeding: 10.4 ± 3.6 kcal, activity-based anorexia: 2.0 ± 0.9 kcal, data not

shown). Three-way ANOVA indicated a significant impact of time [$F_{(20, 771)} = 22.3$, $p < 0.001$], activity [$F_{(1, 771)} = 125.8$, $p < 0.001$] and feeding regimen [$F_{(1, 771)} = 666.7$, $p < 0.001$] as well as an interaction of these three factors [($F_{(20, 771)} = 2.0$, $p < 0.01$].

Activity-Based Anorexia Rats Show a Similar Food Intake Microstructure Compared to the Restricted Feeding Group

After analysis of overall daily 24-h (in the *ad libitum* fed groups) and 1.5-h (in the restricted feeding groups) food intake, the underlying food intake microstructure was assessed using an automated food intake monitoring device. When analyzing the 1.5-h food intake microstructure no difference was observed between both restricted feeding groups (restricted feeding and activity-based anorexia, $p > 0.05$; **Figures 5A–G**). Similarly, the two *ad libitum* fed groups did not show a difference except for the bout size which was smaller in the activity compared to the *ad libitum* group ($p < 0.01$; **Figure 5A**). The two restricted feeding groups showed significantly higher levels for several parameters of the food intake microstructure such as meal size ($p < 0.001$; **Figure 5B**), bout frequency ($p < 0.001$; **Figure 5C**), meal duration ($p < 0.001$; **Figure 5E**), time spent in meals ($p < 0.001$; **Figure 5F**) and eating rate ($p < 0.001$; **Figure 5G**), while for meal frequency the difference was only observed when compared to the activity group ($p < 0.05$; **Figure 5D**).

Also when analyzing the 24-h food intake microstructure, no difference was observed between the two *ad libitum* fed groups (*ad libitum* and activity) or the two restricted feeding groups (restricted feeding and activity-based anorexia, $p > 0.05$; **Figures 6A–G**). While both restricted feeding groups showed higher levels in bout size ($p < 0.001$; **Figure 6A**), meal size ($p < 0.001$; **Figure 6B**), meal duration ($p < 0.001$; **Figure 6E**) and eating rate ($p < 0.001$; **Figure 6G**), values were lower for—as expected based on the feeding schedule—bout frequency ($p < 0.001$; **Figure 6C**), meal frequency ($p < 0.001$; **Figure 6D**) and time spent in meals ($p < 0.001$; **Figure 6F**).

Activity-Based Anorexia Robustly Activates Several Brain Nuclei in Different Areas of the Brain

To investigate neuronal activation of brain areas under conditions of activity-based anorexia we performed immunohistochemistry for the activity marker Fos. After pre-absorption of the Fos antibody with a synthetic Fos fragment no immunostaining was observed (data not shown) indicating the specificity of the antibody.

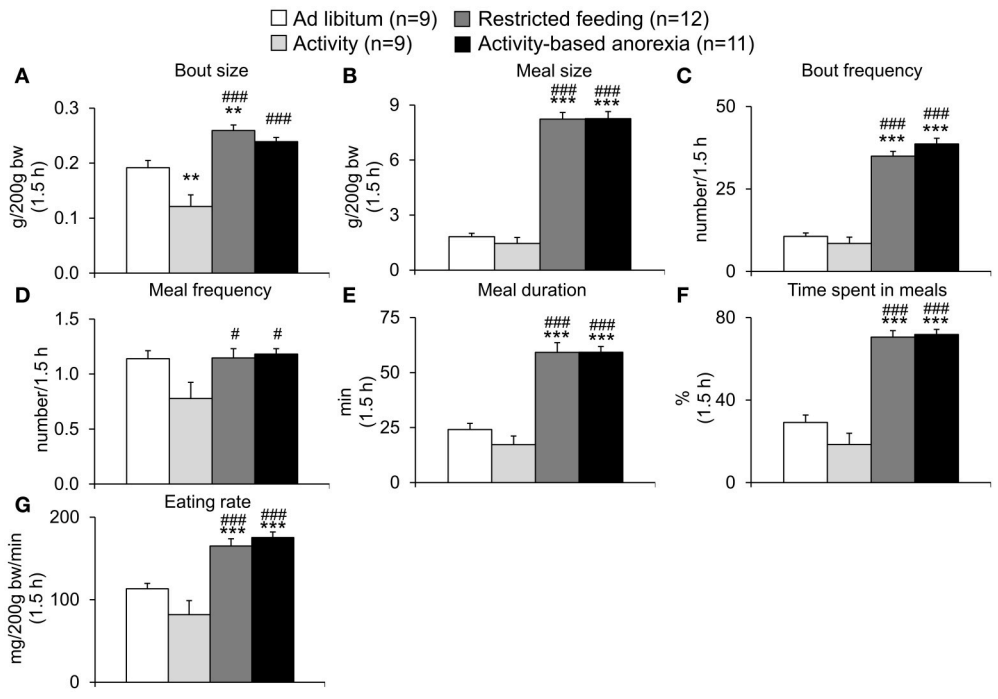


FIGURE 5 | Activity-based anorexia and restricted feeding group show similar changes in the 1.5-h food intake microstructure. Animals had access to a running wheel for 24 h/day (activity and activity-based anorexia group) or were housed without wheel access under otherwise similar conditions (*ad libitum* and restricted feeding group). On day eight food intake was restricted to 1.5 h/day (from 9:00 to 10:30 a.m.) in the restricted feeding and activity-based anorexia group, while the activity and *ad libitum* group retained access to food for 24 h/day. Food intake was monitored continuously using an automated food intake monitoring device and parameters of the food intake microstructure during the 1.5-h restricted feeding period, namely bout size **(A)**, meal size **(B)**, bout frequency **(C)**, meal frequency **(D)**, meal duration **(E)**, time spent in meals **(F)**, and eating rate **(G)** analyzed over a period of 4 days starting 4 days after food restriction (when a relatively stable food intake was observed). Data are expressed as mean ± SEM. $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. *ad libitum* group; $^{\#}p < 0.05$ and $^{\#\#\#}p < 0.001$ vs. activity group.

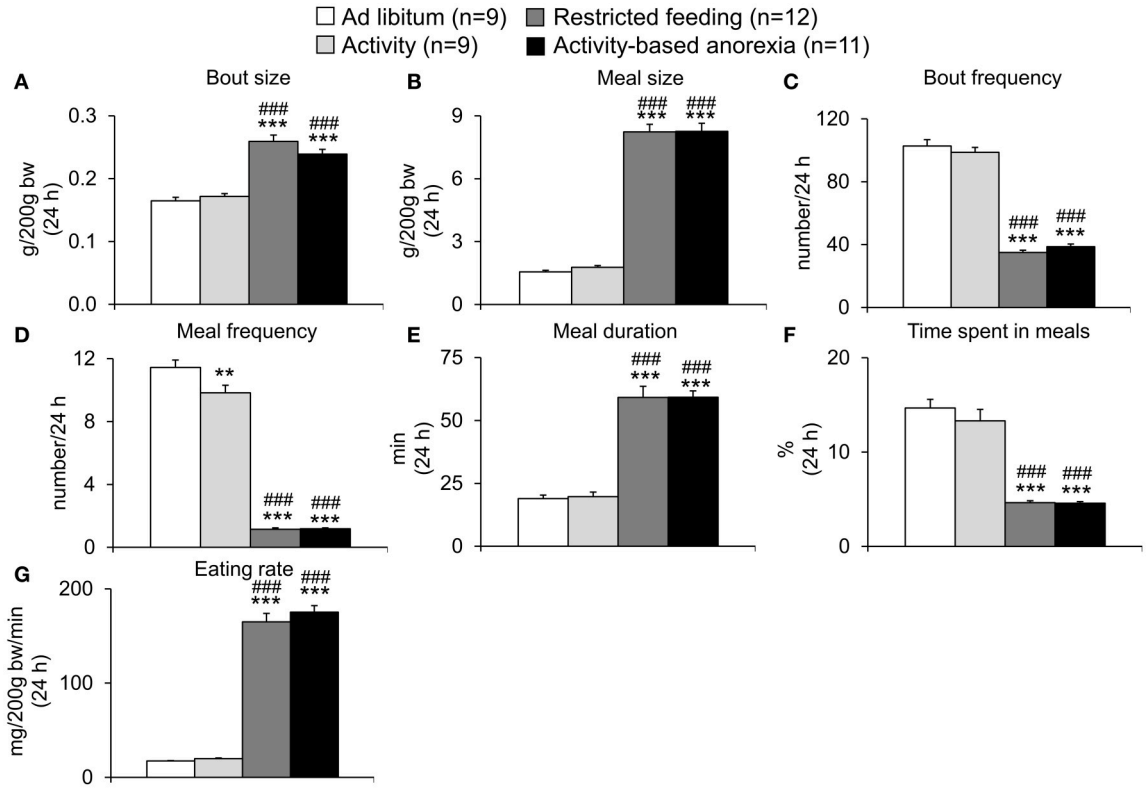


FIGURE 6 | Activity-based anorexia and restricted feeding group show similar changes in the 24-h food intake microstructure. Animals had access to a running wheel for 24 h/day (activity and activity-based anorexia group) or were housed without wheel access under otherwise similar conditions (*ad libitum* and restricted feeding group). On day eight food intake was restricted to 1.5 h/day (from 9:00 to 10:30 a.m.) in the restricted feeding and activity-based anorexia group, while the activity and *ad libitum* group retained access to food for 24 h/day. Food intake was monitored continuously using an automated food intake monitoring device and parameters of the 24-h food intake microstructure, namely bout size **(A)**, meal size **(B)**, bout frequency **(C)**, meal frequency **(D)**, meal duration **(E)**, time spent in meals **(F)**, and eating rate **(G)** analyzed over a period of 4 days starting 4 days after food restriction (when a relatively stable food intake was observed). Data are expressed as mean ± SEM. $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. *ad libitum* group and $^{\#\#\#}p < 0.001$ vs. activity group.

Overall, activity-based anorexia rats showed higher Fos activation levels compared to *ad libitum* fed rats (**Table 1**). In the forebrain, higher activity was observed in the piriform cortex, cingulate cortex, somatomotor cortex, lateral septal nucleus, caudate putamen and hippocampus (**Figures 7A,B**) of activity-based anorexia rats compared to the *ad libitum* group, while no activation was observed in the amygdala (**Table 1**). In the thalamus higher Fos activation was observed in the paraventricular thalamic nucleus of activity-based anorexia rats compared to the *ad libitum* group, while in the lateral habenula similar numbers of Fos positive cells were detected using semiquantitative assessment (**Table 1**). Evaluation of hypothalamic nuclei showed more Fos signals in the suprachiasmatic nucleus, supraoptic nucleus (**Figures 7C,D**), anterior hypothalamic area, both magno- and parvocellular parts of the paraventricular nucleus (**Figures 7E,F**), lateral hypothalamic area (**Figures 7G,H**), dorsomedial hypothalamic nucleus (**Figures 7I,J**) and the medial part of the arcuate nucleus (**Figures 7K,L**) of activity-based anorexia rats compared to the *ad libitum* group, while similar levels were observed in the ventromedial hypothalamic nucleus (**Table 1**). In the midbrain similar levels were detected in the Edinger-Westphal nucleus (**Figures 8A,B**), while higher activation was observed in the dorsal raphe nuclei (**Figures 8C,D**) and locus coeruleus (**Figures 8E,F**) of activity-based anorexia compared to the *ad libitum* fed rats (**Table 1**). Lastly, also in the medulla a higher activation was observed in activity-based anorexia rats compared to the *ad libitum* group, namely in the raphe pallidus nucleus (**Figures 9A,B**), area postrema, rostral part of the nucleus of the solitary tract (**Figures 9C,D**) and the dorsal motor nucleus of the vagus nerve (**Table 1**).

DISCUSSION

In the present study we first established the activity-based anorexia model combining voluntary physical activity in a running wheel and a time-restricted feeding protocol and showed a body weight loss of −22% following 2 weeks of restricted feeding for 1.5 h per day and 24-h access to a running wheel. This body weight loss was greater (−9%) than that observed in the restricted feeding group, while rats of the *ad libitum* and activity group gained body weight. These data indicate the importance

TABLE 1 | Localization of Fos positive neurons in brains of rats fed *ad libitum* or under conditions of activity-based anorexia.

Area	Brain structure	*Ad libitum*	Activity based anorexia
Forebrain	Piriform cortex	++	+++
	Cingulate cortex	+	++
	Somatomotor cortex	+	+++
	Lateral septal nucleus	+ − ++	+++
	Caudate putamen	++	++ − +++
	Amygdala (central, medial and basolateral)	−	−
	Hippocampus	+ − ++	+++
Thalamus	Paraventricular thalamic nucleus, anterior part	+	++
	Lateral habenula	+ − ++	+ − ++
Hypothalamus	Suprachiasmatic nucleus	++	++ − +++
	Supraoptic nucleus	−	+++
	Anterior hypothalamic area	+	++
	Paraventricular nucleus	+	++
	Lateral hypothalamic area	++	+++
	Ventromedial hypothalamic nucleus	++	++
	Dorsomedial hypothalamic nucleus	−	++
	Arcuate nucleus, medial	+	+++
Midbrain	Edinger-Westphal nucleus	++	++
	Dorsal raphe nuclei	+	++
	Locus coeruleus	−	+
Medulla	Raphe pallidus nucleus	+	++
	Area postrema	−	+ − ++
	Nucleus of the solitary tract, rostral	−	+ − ++
	Dorsal motor nucleus of the vagus nerve	−	− − +

Data are expressed as mean derived from three rats/group. It is to note that rats in the activity-based anorexia group received a fixed amount of 1.5 g during the feeding period in order to avoid signals derived from overfilling of the stomach and/or nausea. Fos expression is expressed semi-quantitatively as +, low (∼1–10 cells); ++, medium (∼10–20 cells); and +++, high (>20 Fos positive cells in a 100 μm × 100 μm area of an ocular grid with a 10x objective) density of expression; −, no cells.

of food restriction for the present model. To gain further insight into the underlying changes, we next analyzed the food intake microstructure which greatly differed from the microstructure observed in the *ad libitum* fed groups (*ad libitum* and activity group) but was very similar to the one observed in the restricted feeding group. The increase in 1.5-h food intake observed in the two groups kept on the restricted feeding schedule was based on a larger meal size and an increase in eating rate. During these 1.5 h, rats spent 71% of the time in meals (eating and interacting with the food hopper). It is important to note that despite this great drive to eat, rats still exercised during this 1.5-h period.

Interestingly, daily activity was similar between the activity and activity-based anorexia group; however, daily activity increased over time in both groups. These data are in line with previous data from mice where daily activity also increased over time; however, in mice daily activity decreased during the last days of the 2.5-week observation period (Jésus et al., 2014). Whether this represents a species difference (rats vs. mice), sex difference (female vs. male) or is related to the slower weight loss in rats (−20% reached at day 15 vs. 11 in mice) warrants further investigation. At the same time we did not observe a decrease in food intake (which rather plateaued) which is different from the human situation where food intake decreases while activity increases with the progression of the disease (Davis et al., 1994). The relatively short observation period of 3 weeks likely contributes to/explains this difference. Taken together, the combination of both food restriction and activity is key in order to exert the pronounced weight loss observed in the activity-based anorexia group.

It is to note that in the present study three out of 14 rats did not develop activity-based anorexia and were therefore excluded from further analyses. This finding is in line with previous studies reporting that 20–30% of rats are not interested in running (Mondon et al., 1985) and do not develop activity-based anorexia (Carrera et al., 2014). These data well match the dropout rate described here (3 out of 14 = 21%). Interestingly, this finding also parallels human data where up to 80% of anorexic subjects display hyperactivity, whereas 20% do not (Davis et al., 1997). Whether a difference in leptin levels, hypothesized to play a role in the semi-starvation-induced hyperactivity in rats before (Exner et al., 2000), contributes to these differences will have to be further investigated. On the other hand, all rats of the restricted feeding group (12/12) show a reduction in body weight indicating that this effect cannot be eluded. However, the fact that rats of the activity-based anorexia group show a greater body weight loss than the restricted feeding group highlights the importance of the physical activity.

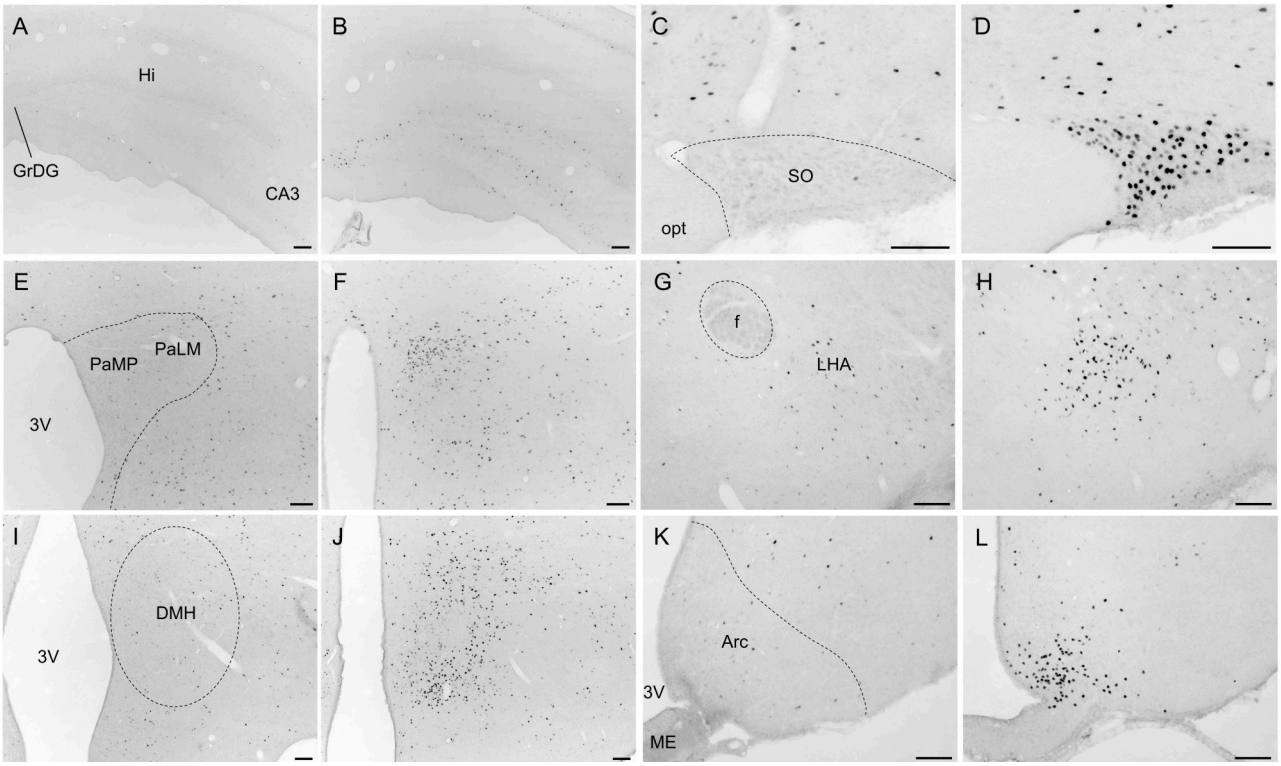


FIGURE 7 | Representative microphotographs of forebrain and hypothalamic structures in rats under *ad libitum* or activity-based anorexia conditions. Following the 21-day observation period rats received food for 1.5 h (activity-based anorexia) or were fed *ad libitum* and were transcardially perfused directly after this 1.5-h feeding period. It is to note that rats in the activity-based anorexia group received a fixed amount of 1.5 g during this feeding period in order to avoid signals derived from overfilling of the stomach and/or nausea. Brains were processed for Fos immunohistochemistry. Signals were observed in the hippocampus **(B)**, supraoptic nucleus **(D)**, magno- und parvocellular parts of the paraventricular nucleus **(F)**, lateral hypothalamic area **(H)**, dorsomedial hypothalamic nucleus **(J)** and medial part of the arcuate nucleus **(L)** of activity-based anorexia rats, while in the respective nuclei of the *ad libitum* group no **(C,I)** or few **(A,E,G,K)** signals were detected. The scale bars indicate 100 μm. Abbreviations: 3V, third ventricle; Arc, arcuate nucleus; CA3, field CA3 of the hippocampus; DMH, dorsomedial hypothalamic nucleus; f, fornix; GrDG, granular layer of the dentate gyrus; Hi, hippocampus; LHA, lateral hypothalamic area; ME, median eminence; opt, optic tract; PaLM, lateral magnocellular part of the paraventricular nucleus of the hypothalamus; PaMP, medial parvocellular part of the paraventricular nucleus of the hypothalamus; SO, supraoptic nucleus.

Despite the fact that the data mentioned above give rise to the use of the activity-based anorexia model as a suited tool to study pathophysiological alterations of AN, several limitations should be kept in mind. Although restriction of food and increased physical activity, two mean features of AN (Treasure et al., 2015) are used in this model, several other aspects such as genetic susceptibility (Clarke et al., 2012) or psychosocial and interpersonal factors (Zipfel et al., 2015) are not respected. Moreover, rats do not voluntarily reduce their body weight in contrast to human anorexic subjects. Whenever the rats' access to food is increased again, they start to regain body weight (Dixon et al., 2003; Ratnovsky and Neuman, 2011). Furthermore, the changes induced here are rather acute or subacute, while human AN is a chronic disease. Interestingly, after the initial sharp decline of food intake rats of the activity-based anorexia and restricted feeding group show a gradual increase of food intake reaching similar levels of daily food intake as observed in the *ad libitum* fed groups. Whether different dietary patterns as observed in human anorexia (Huse and Lucas, 1984; Elran-Barak et al., 2014) or a change of dietary patterns over time occurs in these rats as well will have to be further determined, preferably in a study with a longer monitoring time. Taken together, cautious interpretation of the data obtained in this model is necessary.

Several other anorexia models have been developed encompassing genetically engineered mouse models that share similarities with changes observed in AN; however, none of these reflect the multiple hormonal changes observed in AN (Méquinion et al., 2015). Lastly, other models use access to low caloric food or expose rats to various kinds of stressors (Méquinion et al., 2015). However, it is important to note that so far activity-based anorexia is considered the best animal model (Gutierrez, 2013) as it recapitulates two main features, physical activity and reduced food intake, of human AN.

To further characterize the activity-based anorexia rats we also investigated the activation of brain nuclei using the activity marker Fos (Sagar et al., 1988) and performed a whole brain mapping for activity-based anorexia and *ad libitum* fed rats. Neuronal activation was observed in brain areas involved in

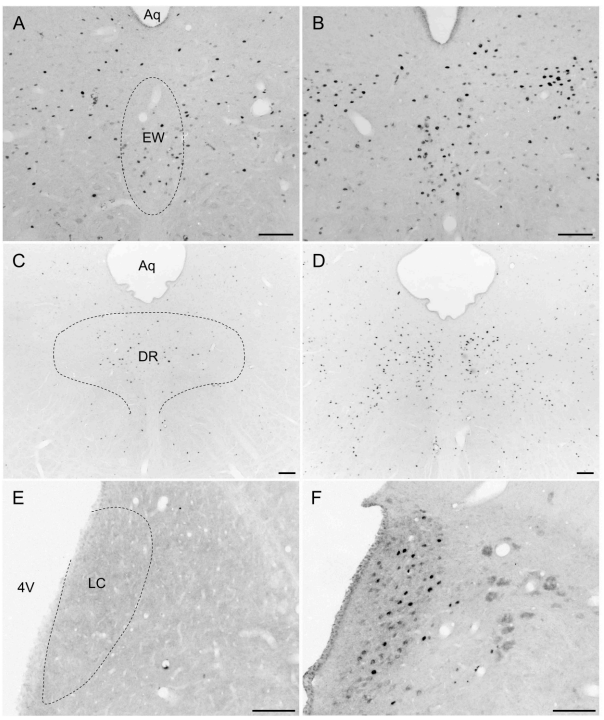


FIGURE 8 | Representative microphotographs of midbrain structures in rats under *ad libitum* or activity-based anorexia conditions. Following the 21-day observation period rats received food for 1.5 h (activity-based anorexia) or were fed *ad libitum* and were transcardially perfused directly after this 1.5-h feeding period. It is to note that rats in the activity-based anorexia group received a fixed amount of 1.5 g during this feeding period in order to avoid signals derived from overfilling of the stomach and/or nausea. Brains were processed for Fos immunohistochemistry. Signals were observed in the Edinger-Westphal nucleus **(B)**, dorsal raphe nuclei **(D)** and locus coeruleus **(F)** of the activity-based anorexia group, while in the respective nuclei of the *ad libitum* group no **(E)**, or few **(C)** signals were detected. It is to note that signals of similar density were observed in the Edinger-Westphal nucleus **(A)** of the *ad libitum* group. The scale bars indicate 100 μm. Abbreviations: 4V, fourth ventricle; Aq, aqueduct; DR, dorsal raphe nuclei; EW, Edinger-Westphal nucleus; LC, locus coeruleus.

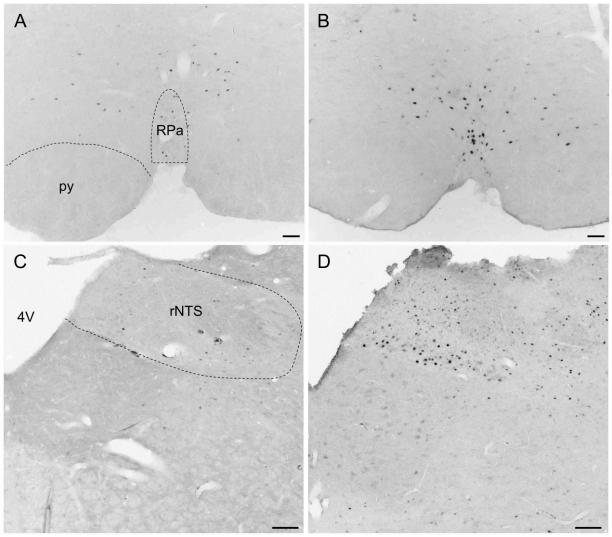


FIGURE 9 | Representative microphotographs of medulla structures in rats under *ad libitum* or activity-based anorexia conditions. Following the 21-day observation period rats received food for 1.5 h (activity-based anorexia) or were fed *ad libitum* and were transcardially perfused directly after this 1.5-h feeding period. It is to note that rats in the activity-based anorexia group received a fixed amount of 1.5 g during this feeding period to avoid signals derived from overfilling of the stomach and/or nausea. Signals were observed in the raphe pallidus nucleus **(B)** and the rostral part of the nucleus of the solitary tract **(D)** of the activity-based anorexia group, while in the respective nuclei of the *ad libitum* group no **(C)** or few **(A)** Fos positive neurons were detected. The scale bars indicate 100 μm. Abbreviations: 4V, fourth ventricle; py, pyramidal tract; rNTS, rostral part of the nucleus of the solitary tract; RPa, raphe pallidus nucleus.

the regulation of several functions such as motor activity, stress response, food intake and thermogenesis.

Analyzing brain areas involved in olfaction and the processing of olfactory stimuli (Roullet et al., 2005) an increased activation of neurons was observed in the piriform cortex, while in the lateral habenula similar Fos expression was observed in activity-based anorexia and *ad libitum* fed rats. This activation is likely associated with the increased interaction with food (as reflected in the increased number of bouts in activity-based anorexia rats compared to the *ad libitum* group) as well as the stimulated food intake during the 1.5-h feeding period. In line with this assumption, key areas of food intake regulation were activated as well, namely the lateral septal nucleus (Mitra et al., 2015), lateral hypothalamic area (Bernardis and Bellinger, 1993), the dorsomedial hypothalamic nucleus and the medial part of the Arc, both expressing the potent orexigenic transmitter neuropeptide Y (Wang et al., 2002; Bi et al., 2012) and lastly also the nucleus of the solitary tract (Stengel and Taché, 2011). Further corroborating the involvement of these nuclei in the orexigenic drive under conditions of activity-based anorexia, a previous study reported a robust upregulation of orexigenic agouti-related peptide and neuropeptide Y, whereas the anorexigenic transmitters pro-opiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART) were reduced in the Arc of activity-based anorexia rats compared to sedentary food-restricted controls (de Rijke et al., 2005). Moreover, in the lateral hypothalamic area melanin-concentrating hormone mRNA expression was increased in activity-based anorexia rats (de Rijke et al., 2005). Associated with the orexigenic response, also brain nuclei involved in the regulation of gastrointestinal motility were activated, namely the lateral hypothalamic area (Gong et al., 2013), nucleus of the solitary tract and the dorsal motor nucleus of the vagus nerve (Stengel and Taché, 2011). This pronounced activation likely underlies the robust orexigenic response of activity-based anorexia rats observed during the 1.5-h feeding period. It is to note that—although food intake was restricted to 1.5 g in the last feeding period before brain processing for Fos immunohistochemistry to avoid unspecific gastric distention and nauseating signals—a moderate activation of the area postrema, known to be involved in the mediation of nausea (Horn, 2014), has been observed in the activity-based anorexia but not in the *ad libitum* fed group. Therefore, nauseating signals—at least to a certain extent—might play a

role in this model as well and may modulate/limit food intake displayed during the restricted feeding period.

Besides the restriction of food intake, the stimulation of activity contributes to the weight loss observed in activity-based anorexia rats. Respective nuclei activated under these conditions and therefore likely implicated in the stimulation of activity encompass the somatomotor cortex (Elias et al., 2008) and caudate putamen (David et al., 2005). Interestingly, especially the dorsomedial hypothalamic nucleus has been implicated in the mediation of food-anticipatory activity under fixed-feeding conditions (Verhagen et al., 2011) as also observed in the present study. This activation likely also involves the suprachiasmatic nucleus working in a modulatory manner as part of an intrahypothalamic system (Acosta-Galvan et al., 2011). Moreover, the dorsomedial hypothalamic nucleus was implicated in the food-entrainable-related preprandial rise of body temperature, an effect that vanished after lesion of the nucleus (Gooley et al., 2006). This thermogenic response might also contribute to the observed decrease in body weight. However, it is important to note that activity-based anorexia was associated with a hypothermic response before (Hillebrand et al., 2005a) and an increase in ambient temperature was reported to reduce physical activity (Gutierrez et al., 2008). Future studies should further investigate these—likely very dynamic—changes of body temperature in activity-based anorexia rats.

Also stress mediated *via* the hypothalamic-pituitary-adrenal gland axis might play a role in the reduction of food intake and stimulation of physical activity. In the present study we observed a robust activation of lateral parvocellular neurons of the hypothalamic paraventricular nucleus of activity-based anorexia compared to *ad libitum* fed rats. This region is known for its predominant expression of corticotropin-releasing factor (CRF). Moreover, CRF mRNA expression was also reported to rise in the dorsomedial hypothalamic nucleus in rats with access to a running wheel (Kawaguchi et al., 2005). Interestingly, intracerebroventricular injection of the CRF antagonist, alpha-helical CRF attenuated the wheel-induced reduction of food intake and body weight (Kawaguchi et al., 2005) giving rise to a role of stimulated CRF signaling in activity-based anorexia. Lastly, this likely contributes to the increased circulating levels of corticosterone in rats (Burden et al., 1993) and cortisol in human anorexic subjects (Casper et al., 1979).

Lastly, also psychological parameters such as anxiety (Swinbourne and Touyz, 2007) and depressiveness (Debska et al., 2011) are often altered under conditions of AN. In the present study we observed an increased activation of the dorsal raphe nuclei and the raphe pallidus nucleus under conditions of activity-based anorexia, serotonergic nuclei that might play a role in the pathogenesis of depression (Michelsen et al., 2008). Interestingly, intraperitoneal injections of the serotonin agonist fenfluramine accelerated weight loss under conditions of activity-based anorexia compared to pair-fed controls giving rise to a mechanism other than reduced food intake (Atchley and Eckel, 2005). While the amygdala was not activated in the present study, the noradrenergic locus coeruleus showed a moderate activation in activity-based anorexia rats possibly leading to increased arousal (Aston-Jones and Waterhouse, 2016). Ascending projections might be involved in the observed activation of the paraventricular thalamic nucleus, the cingulate cortex and the hippocampus, brain structures involved in the processing of emotions and memory (Rolls, 2015). Interestingly, also the supraoptic nucleus as well as some magnocellular neurons of the paraventricular nucleus of the hypothalamus, two brain nuclei prominently expressing oxytocin, showed a robust activation in activity-based anorexia rats. It is important to note that oxytocin has been—besides its well-defined role during pregnancy—implicated in social memory, aggression and anxiety (Caldwell et al., 2016). Whether there is a direct link between anxiety or depressiveness and physical activity as suggested in humans (Holtkamp et al., 2004) warrants further investigation.

In summary, the activity-based anorexia model combines voluntary physical activity in a running wheel and time-restricted feeding to greatly reduce body weight. Interestingly, the food intake microstructure observed in activity-based anorexia rats did not differ from the one observed in the restricted feeding group arguing against a specific feeding phenotype. Also physical activity did not differ from the respective control group. Activity-based anorexia rats displayed an activation of distinct brain nuclei involved in the mediation of food intake, physical activity, thermoregulation as well as depression/anxiety and stress. Although these animal data have to be interpreted with caution, current data point toward the usefulness of the model to better understand pathophysiological alterations also occurring in AN.

AUTHOR CONTRIBUTIONS

SS performed the experiments and drafted the manuscript. PP performed the experiments and analyzed the data. MGS and PK performed the experiments and reviewed the manuscript. TH wrote and reviewed the manuscript. MR gave critical input throughout the study and reviewed the manuscript. AS planned the experiments, analyzed the data and wrote the manuscript.

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REVIEW
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Hypothalamic Integration of Metabolic, Endocrine, and Circadian Signals in Fish: Involvement in the Control of Food Intake

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The regulation of food intake in fish is a complex process carried out through several different mechanisms in the central nervous system (CNS) with hypothalamus being the main regulatory center. As in mammals, a complex hypothalamic circuit including two populations of neurons: one co-expressing neuropeptide Y (NPY) and Agouti-related peptide (AgRP) and the second one population co-expressing pro-opiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART) is involved in the integration of information relating to food intake control. The production and release of these peptides control food intake, and the production results from the integration of information of different nature such as levels of nutrients and hormones as well as circadian signals. The present review summarizes the knowledge and recent findings about the presence and functioning of these mechanisms in fish and their differences vs. the known mammalian model.

Keywords: food intake, fish, hypothalamus, review, nutrient sensing, circadian rhythm, leptin, ghrelin

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INTRODUCTION

Food intake is regulated through positive and negative loops acting at different locations and at different times (Langhans and Scharrer, 1992; Langhans, 1999). The positive loop (starting of food intake) results from the relationship among prior experience with nutrient availability, status of the animal and sensory qualities of the food. The negative loop relates to metabolic and gastrointestinal inputs displaying changes prior and after absorption (Langhans, 1999). Three regulatory levels have been suggested following this model: (i) short-term regulatory factors: those influenced by the size of a single meal, (ii) mid-term regulatory factors: those operating through several days; and (iii) long-term regulatory factors: those operating through longer time periods (weeks, months, and years) reflecting energy balance of the animal. These levels of regulation create a feedback loop that continuously modulate the immediate signal input from sense organs and internal sensors, which together with prior learning integrate information from the energy reserves of the body. The control determining the feeding behavior and food intake is elicited by the central nervous system (CNS) through various pathways, and hypothalamus is the main center involved in such regulation (Schwartz et al., 2000; Berthoud, 2002).

In fish is difficult to establish which factors act in the mid- or long-term, since most available data come from short-term studies. The basic mechanisms involved in regulation of food intake in fish appear to be similar to those of mammals with differences implying the existence of specific mechanisms in fish (Kulczykowska and Sánchez-Vázquez, 2010; Hoskins and Volkoff, 2012; Volkoff, 2016). In the following sections, we review the existing knowledge about hypothalamic integration in fish of metabolic, endocrine, and circadian information to elicit a coordinated feeding response, as summarized in **Figure 1**.

HYPOTHALAMIC NEUROPEPTIDES INVOLVED IN THE CONTROL OF FOOD INTAKE

The integration of information involved in the control of food intake takes place in mammals through a circuit mainly localized in hypothalamic areas including arcuate (ARC), ventromedial, paraventricular, and lateral hypothalamus (Berthoud and Morrison, 2008; Zheng and Berthoud, 2008). These circuits include two populations of neurons (Mobbs et al., 2005; Blouet and Schwartz, 2010; Waterson and Horvath, 2015). The first population co-expresses neuropeptide Y (NPY) and agouti-related peptide (AgRP) and the second population co-expresses pro-opiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART). Moreover, these two populations of neurons inhibit each other resulting in signaling to other higher-order neurons. The production and release of these peptides control food intake, and this occurs through integration of signals of metabolic, endocrine, and circadian nature. Other peptides such as orexins are involved in food intake modulation but they are not involved in this main integrative core.

Avaliable studies in fish (mostly through asssessment of mRNA abundance) demonstrated expression of these neuropeptides in hypothalamus. Neurons expressing NPY, AgRP, CART, and POMC in fish respond to energy challenge suggesting that the main hypothalamic pathways coordinating a response to regulate energy homeostasis are conserved throughout vertebrate evolution (Volkoff et al., 2005).

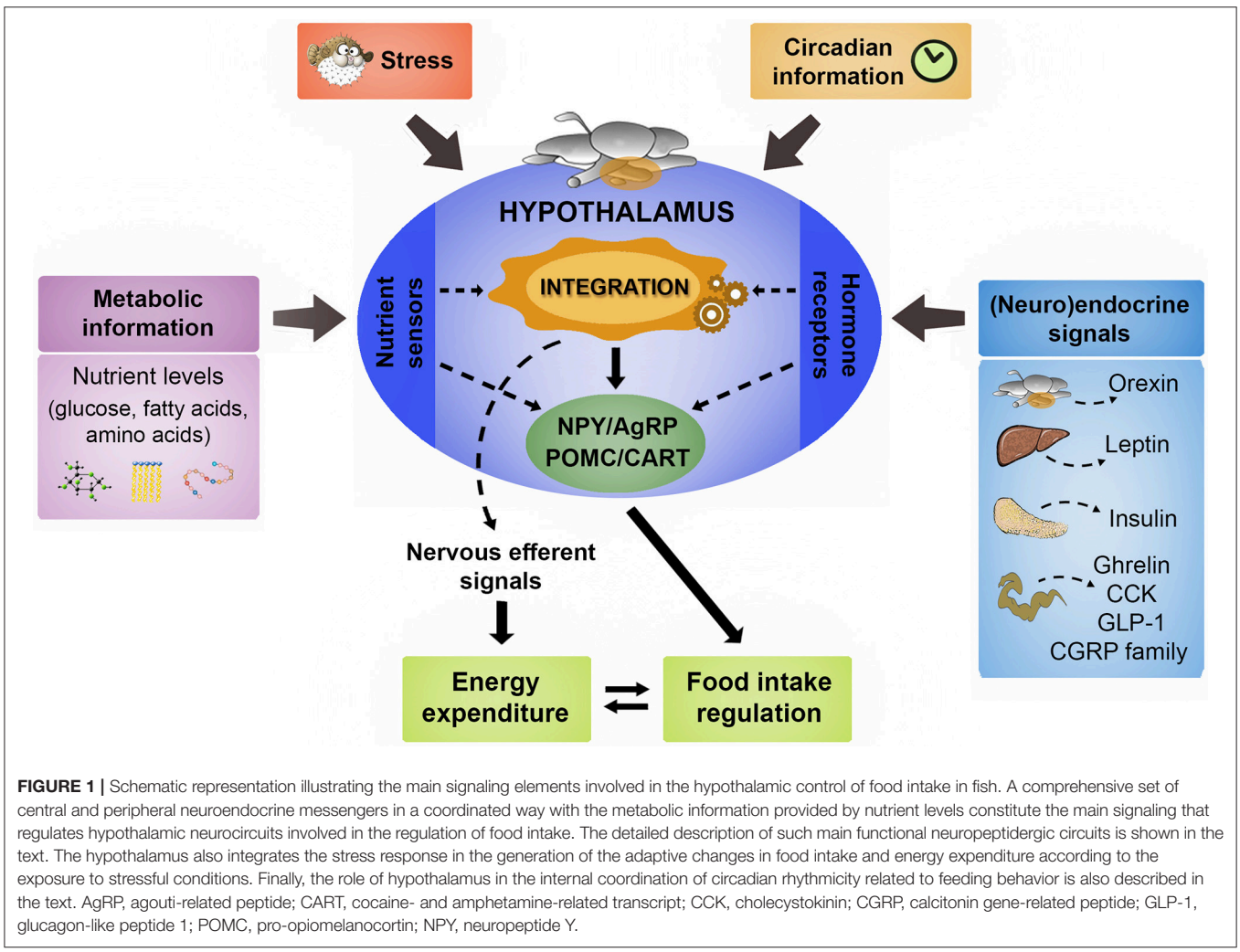


FIGURE 1 | Schematic representation illustrating the main signaling elements involved in the hypothalamic control of food intake in fish. A comprehensive set of central and peripheral neuroendocrine messengers in a coordinated way with the metabolic information provided by nutrient levels constitute the main signaling that regulates hypothalamic neurocircuits involved in the regulation of food intake. The detailed description of such main functional neuropeptidergic circuits is shown in the text. The hypothalamus also integrates the stress response in the generation of the adaptive changes in food intake and energy expenditure according to the exposure to stressful conditions. Finally, the role of hypothalamus in the internal coordination of circadian rhythmicity related to feeding behavior is also described in the text. AgRP, agouti-related peptide; CART, cocaine- and amphetamine-related transcript; CCK, cholecystokinin; CGRP, calcitonin gene-related peptide; GLP-1, glucagon-like peptide 1; POMC, pro-opiomelanocortin; NPY, neuropeptide Y.

CART/POMC

Melanocortins integrate a key system in the regulation of food intake in vertebrates. These peptides, exhibiting melanotropic (melanin stimulation) and corticotropic (corticosteroid stimulation) activity encoded in the common precursor POMC. Two discrete groups of neurons in ARC and the caudal region of the nucleus of the tractus solitarius of the medulla produce POMC, mainly processed into α-melanocyte-stimulating hormone (α-MSH) and β-endorphin. Melanocortin signaling is transduced by five different G-coupled receptors (MC1R-MC5R) but only two are conspicuously expressed in the mammalian brain, i.e., MC3R and MC4R. Central activation of these receptors appears to mediate melanocortin effects on energy balance since both MC3R knockout rat and MC4R knockout mice display severe alterations in energy homeostasis (obesity, increased food intake and linear growth in MC4R; hypophagia, increased fat mass and food efficiency but reduced lean mass in MC3R). Accordingly, central administration of MC3/4Rs agonists results in a dose-dependent reduction in food intake in mice but MC4R-deficient mice do not respond to anorectic effects of agonists, suggesting that α-MSH inhibits feeding primarily by activating MC4R (reviewed by Anderson et al., 2016).

Several studies demonstrated that melanocortin system is a key point in the regulation of energy balance in fish (Cerdá-Reverter et al., 2011). Thus, *in situ* hybridization studies demonstrated the presence of POMC neurons in hypothalamus (Cerdá-Reverter et al., 2003a) but not in the vagal lobe (homolog of the nucleus of the tractus solitarius in mammalian brain). However, more sensitive techniques (quantitative PCR) also reported POMC-A1 expression in the hindbrain (Conde-Sieira et al., 2010b). Immunohistochemical techniques further demonstrated the presence of both α-MSH and β-endorphin in the hypothalamus of several fish species, including zebrafish and tilapia (Forlano and Cone, 2007; Chabbi and Ganesh, 2016) thus suggesting that POMC is mainly processed to these peptides. However, ACTH is also present in the preoptic area of carp (Metz et al., 1994) further suggesting an alternative POMC processing in the fish brain.

Intracerebroventricular (ICV) administration of α-MSH or α-MSH agonists inhibit food intake in a dose-dependent manner in goldfish (Cerdá-Reverter et al., 2003b) and rainbow trout (Schjolden et al., 2009), whereas administration of MC4R antagonists stimulates food intake in satiated animals (Cerdá-Reverter et al., 2003b). POMC-A1 and POMC-C levels increased post-feeding in medaka (Chisada et al., 2014), rainbow trout (Gong and Björnsson, 2014), and Atlantic halibut (Gomes et al., 2015), respectively. In contrast to that expected for a negative regulator of energy balance, POMC hypothalamic levels remained unchanged after 7 progressive fasting days in goldfish (Cerdá-Reverter et al., 2003a), but POMC-A1 mRNA decreased 50% after 28 fasting days in rainbow trout (Leder and Silverstein, 2006). Surprisingly, another study demonstrated that hypothalamic POMC A1, A2 and B levels increased after 118 fasting days in the latter species (Jørgensen et al., 2016). The lack of a consistent POMC regulation by energy balance also occurred in mammalian species. Thus, sheep with about 40% of total body-weight loss did not exhibit changes in POMC mRNA levels (Henry et al., 2001). A similar situation occurred in ewes that lost about 30% body fat (Henry et al., 2000). The constrasting effects of POMC-encoded peptides could explain the paradoxical absence of POMC regulation by energy balance. Thus, central injections of β-endorphin, the C-terminal peptide of POMC, stimulate appetite in the goldfish (De Pedro et al., 1995). It is then plausible that the effects of nutritional status might regulate post-transcriptional POMC-derived peptide levels by selective regulation of prohormone convertases. Under negative energy balance situations, when animals display an enhanced feeding drive, POMC might be processed into β-endorphin. In contrast, a positive energy balance may preferentially drive the processing of POMC into production of the food intake inhibitor α-MSH.

The melanocortin activity may be also modulated by receptor signaling. Accordingly, energy balance, in the absence of an agonist regulation, could up/down regulate the neuronal receptor density in feeding-related areas of the CNS. Studies in the sea bass suggest that hypothalamic MC4R expression remains unchanged after long-term fasting precluding this regulatory pathway (Sánchez et al., 2009). Alternatively, the melanocortin system also exhibits endogenous antagonists competing by binding and activation of the melanocortin receptors. AgRP1 is consistently upregulated by fasting in the hypothalamus of all tested vertebrates including zebrafish (Song et al., 2003), goldfish (Cerdá-Reverter and Peter, 2003), Atlantic salmon (Valen et al., 2011), and sea bass (Agulleiro et al., 2014). It is therefore conceivable that AgRP1 binding to central MCRs might regulate central melanocortinergic activity whereas POMC expression remains constant as a constitutive inhibitor.

In mammals, ARC POMC neurons also produce CART but hypothalamus is not the only brain area producing this neuropeptide (Elias et al., 1998). CART was isolated form ovine hypothalamus (Spiess et al., 1981) and its expression increased in the rat *striatum* after the administration of drugs such as cocaine and amphetamine (Douglass et al., 1995). Subsequent experiments revealed that CART neurons are anatomical targets for systemic leptin to induce anorexia (Kristensen et al., 1998). In fish, CART mRNA was characterized in several species (Subhedar et al., 2014) but immunohistochemical localization of the CART peptide in brain was only studied in catfish by using antibodies against rat CART (Singru et al., 2007) or by *in situ* hybridization in zebrafish (Nishio et al., 2012; Akash et al., 2014). CART mRNA abundance decreased with food deprivation in cod (Kehoe and Volkoff, 2007), goldfish (Volkoff and Peter, 2001a), and Atlantic salmon (Murashita et al., 2009), and increased with re-feeding in channel catfish (Kobayashi et al., 2008) whereas post-prandial changes occurred in channel catfish (Peterson et al., 2012), goldfish (Volkoff and Peter, 2001a), and dourado (Volkoff et al., 2016). As many other genes, CART is duplicated in the teleost genome and four different genes (CART1-4 or CART1, 2a, 2b, 4) encoding CART peptides are reported in zebrafish (Nishio et al., 2012) or up to seven genes in Senegalese sole (Bonacic et al., 2015). All of them are expressed in zebrafish CNS but only CART2 and 4 are produced in the hypothalamus (Akash et al., 2014). Unfortunately, CART/POMC colocalization in the neurons of the tuberal hypothalamus has not been assessed in

fish yet. Both CART2 and CART4 are downregulated by fasting in ventrocaudal telencephalon (CART2) and ventral hypothalamus (CART2 and CART4) (Akash et al., 2014), as expected for a negative regulator of the energy balance and thus further suggesting their involvement in the regulation of energy balance in fish. Accordingly, ICV administration of CART peptides inhibit food intake in goldfish (Volkoff and Peter, 2000).

AgRP/NPY

Atypically, melanocortin signaling is not exclusively regulated by the binding of endogenous agonists (see above), since naturally occurring antagonists, agouti-signaling protein (ASIP) and AgRP, compete with melanocortin peptides by binding to MCRs. ASIP and AgRP molecules exhibit a cysteine-rich C-terminal domain essential for the structural and biological properties of the peptide. A basic domain and a proline-rich area precede the cysteine knot in ASIP; AgRP sequences lacks both regions but, in contrast, exhibits a processing site prior to the cysteine domain where the propeptide is cleaved (reviewed by Cerdá-Reverter et al., 2011). In mammalian species, ASIP produced in the ventral skin regulates pigment pattern and binds MC1R and MC4R with similar affinity (Cerdá-Reverter et al., 2005). In contrast, AgRP is mainly expressed in the same NPY-producing neurons in the ARC. In fact, 95% of NPY neurons co-express AgRP (Hahn et al., 1998). AgRP/NPY neurons also produce GABA (Horvath et al., 1997) and regulate the activity of hypothalamic POMC/CART neurons. In addition, the GABAergic innervation from AgRP/NPY neurons to the parabrachial nucleus in the brainstem is able to regulate feeding behavior (Wu et al., 2009). Hypothalamic AgRP sharply increases with fasting, and overexpression in transgenic mice induces overfeeding and obesity but also enhances linear growth (Ollmann et al., 1997). The selective ablation of NPY/AgRP neurons induces a rapid decrease in food intake leading to starvation (Luquet et al., 2005).

Fish exhibit two copies of AgRP named AgRP1 and AgRP2. Few studies to date have precisely localized AgRP1 mRNA in brain regions of teleost fish (Cerdá-Reverter and Peter, 2003; Song and Cone, 2007) whereas others reported AgRP-ir in fish brain (Forlano and Cone, 2007; Agulleiro et al., 2014). All studies identified AgRP1 production within the posterior region of the ventral hypothalamus of the goldfish, zebrafish and sea bass. In the zebrafish, AgRP1 and α-MSH projections strikingly match the nuclei that express MC4R (Cerdá-Reverter et al., 2003b) and MC5R mRNA (Cerdá-Reverter et al., 2003c) in the goldfish. In fact, binding studies with zebrafish receptors demonstrated that AgRP1 acts as a competitive antagonist at MC3R, MC4R, and MC5R, all of them expressed in the brain (Song and Cone, 2007). Pharmacological studies in sea bass have shown that AgRP1 works as an inverse agonist at constitutively activated MC4R (Sánchez et al., 2009). In addition, ASIP1 overexpression working at central MC4R in transgenic zebrafish model results in enhanced feeding, feed efficiency, weight and linear growth but not in obesity (Guillot et al., 2016), in a similar way to the AgRP1 transgenic zebrafish (Song and Cone, 2007). Studies in zebrafish using first developmental stages demonstrated that AgRP1 knockdown results in reduced growth suggesting that AgRP1 suppression of MC4R activity is essential for larval growth (Zhang et al., 2012). Therefore, melanocortin system seems to impose a constitutive break to feeding and growth in fish probably through constitutive activity of MC4R. AgRP1 overexpression is the soundest response to fasting reported in fish, and its hypothalamic expression is essential to counteract MC4R negative effects on energy balance by inverse agonism on the receptor thus driving fish to feeding and concomitantly enhancing fish growth both dependently and independently on feeding levels (Guillot et al., 2016). In fact, AgRP1 mRNA abundance in hypothalamus increased in food deprived zebrafish (Song et al., 2003), goldfish (Cerdá-Reverter and Peter, 2003), sea bass (Agulleiro et al., 2013), and carp (Zhong et al., 2013) but not in Atlantic salmon (Murashita et al., 2009). Post-feeding did not induce changes in AgRP1 hypothalamic mRNA abundance in medaka (Chisada et al., 2014), but the increase in food intake in the GH-transgenic carp is associated with increased values of AgRP1 mRNA (Zhong et al., 2013).

There are no studies on NPY/AgRP colocalization in hypothalamic neurons in fish brain. The NPY family of peptides consists of 36-amino-acid peptides exhibiting carboxy terminal (C-terminal) amidation (Cerdá-Reverter and Larhammar, 2000). The family comprises three different peptides, the NPY, tyrosine-tyrosine peptide, (PYY), and the pancreatic polypeptide (PP). Tetrapod species produce all three peptides, whereas non-tetrapod vertebrates have only NPY and PYY. Teleost fish synthesize two different versions of NPY and PYY but not PP (Sundström et al., 2008). Brain distribution of NPY peptides was reported in detail only in the sea bass by *in situ* hybridization (Cerdá-Reverter et al., 2000a,b), and only NPY1 expressed in the rostral hypothalamus, where the AgRP1 neurons are localized in this species. Concomitant coexpression in the rostral hypothalamus suggests that both NPY1 and AgRP1 colocalize also in the tuberal hypothalamus, ventral telencephalon and preoptic area of fish.

NPY is the most potent orexigenic factor in vertebrates (Stanley and Leibowitz, 1985) and many studies shown this effect after ICV administration in several fish (López-Patiño et al., 1999; Aldegunde and Mancebo, 2006; Kiris et al., 2007; Yokobori et al., 2012). The mRNA abundance of NPY decreased after meal in goldfish (Kehoe and Volkoff, 2007), zebrafish (Tian et al., 2015), and grass carp (Zhou et al., 2013), though responses were contradictory in other species, such as orange-spotted grouper (Tang et al., 2013), rainbow trout (Gong and Björnsson, 2014), and zebrafish (Chen et al., 2016). Food deprivation in rainbow trout also resulted in decreased mRNA abundance of NPY (Gong et al., 2016b) but also after NPY intraperitoneal (IP) injections in flounder (Li et al., 2016). Accordingly, brain expression is upregulated by fasting in several fish species (Matsuda et al., 2012b) but the number of NPY immunoreactive neurons increase in the hypothalamus and posterior tuberculum after seven days fasting in the goldfish but not in the thalamus (Yokobori et al., 2012). Peripheral leptin is also able to regulate hypothalamic NPY expression since IP injection of recombinant leptin inhibits AgRP1/NPY but upregulates POMC/CART expression in goldfish (Yan et al., 2016). In addition, NPY was suggested to link the reproductive system and the central circuitry

regulating energy balance in sea bass (Cerdá-Reverter et al., 1999).

HYPOTHALAMIC INTEGRATION OF METABOLIC INFORMATION

The detection of nutrient levels in hypothalamus modulates different neurocircuits related to food intake regulation, metabolite homeostasis, energy expenditure, and status of body reserves (Morton et al., 2006; Blouet and Schwartz, 2010). The neurons coexpressing NPY/AgRP or POMC/CART are included in these circuits, and respond with decreased or increased peptide expression, respectively, to rises in circulating levels of glucose, fatty acids, or amino acids (Mobbs et al., 2005; Blouet and Schwartz, 2010; Efeyan et al., 2015). Thus, POMC/CART neurons depolarize while NPY/AgRP hyperpolarize in response to the increase in nutrient levels (Levin et al., 2004; Fioramonti et al., 2007).

The sensing of a particular nutrient may involve the direct binding of the sensed molecule to the sensor, or occur by an indirect mechanism relying on the detection of a related molecule that reflects nutrient abundance (Efeyan et al., 2015). Different organisms detect extracellular and intracellular levels of sugars, amino acids, and fatty acids. We provide a picture of the current knowledge of the systems already characterized in fish, i.e., those involved in the sensing of glucose and fatty acids (Soengas, 2014).

As for amino acids, in mammals the increase in the levels of branched-chain amino acids (BCAA) such as leucine result in decreased food intake. This effect is mediated by activation of central amino acid sensing through changes in target of rapamycin (mTOR) and/or AMP-activated protein-kinase (AMPK), and BCAA metabolism (Heeley and Blouet, 2016; Morrison et al., 2016). Furthermore, the deficiency in essential amino acids elicit an increase in food intake through central amino acids sensors mediated by general control non-depressable 2 (GCN2) and eukaryotic initiation factor 2α (eiF2α) (Fromentin et al., 2012; Maurin et al., 2014). Considering that most fish are carnivorous (strongly dependent on dietary protein/amino acid), the presence of amino acid sensors in central areas involved in the regulation of food intake like hypothalamus is likely. However, no studies are available in fish about the presence and functioning in central areas of amino acid sensors. The only available information relates to the effect of different protein levels and/or composition on food intake with contradictory responses observed (Figueiredo-Silva et al., 2012b; Wacyk et al., 2012; Tan et al., 2016).

A summary of the main findings achieved as well as the hypothetical pathways involved in nutrient sensing in fish hypothalamus is shown in **Figure 2**.

Glucosensors and Control of Food Intake

Glucosensing Mechanisms

Hypothalamic POMC/CART neurons increase and NPY/AgRP neurons decrease their firing rate in response to increased levels of glucose in mammals (Levin et al., 2004; Marty et al., 2007). This process is glucosensing. The best characterized glucosensing mechanism is that dependent on glucokinase (GK), and is similar to that existing in β-cells of endocrine pancreas (Blouet and Schwartz, 2010; Efeyan et al., 2015). Glucose is transported into the cell by glucose facilitative carrier type 2 (GLUT2) and then phosphorylated by GK. Once phosphorylated, glucose is metabolized through glycolysis then increasing intracellular ATP/ADP ratio leading to the closure of the ATP-dependent inward rectified potassium channel (K^+_{ATP}), membrane depolarization, calcium entry through L-type voltage-dependent calcium channel, and increased neuronal activity (Marty et al., 2007; De Backer et al., 2016). However, since not all glucosensing neurons rely on this mechanism (Fioramonti et al., 2004; De Backer et al., 2016), evidence for the existence of alternative glucosensing mechanisms obtained in several studies. Thus, high glucose concentrations stimulate the expression of liver X receptor (LXR) (Mitro et al., 2007) resulting in an inhibition of gluconeogenesis (Anthonisen et al., 2010; Archer et al., 2014). The stimulation by glucose of sweet taste receptors (similar to those in lingual taste cells) depending on a heterodimer of type 1 taste receptor subunits (T1Rs) formed by T1R2 + T1R3 and the G protein α-gustducin activates an intracellular signaling cascade (Ren et al., 2009). The expression of sodium/glucose co-transporter 1 (SGLT-1) increases in response to enhanced glucose levels (Díez-Sampedro et al., 2003; González et al., 2009; Herrera Moro Chao et al., 2016). Finally, another mechanism relies on mitochondrial production of reactive oxygen species leading to increased expression of uncoupling protein 2 in response to increased glucose levels (Blouet and Schwartz, 2010). In addition, several of these systems appear to be inter-connected. Thus, for instance, T1R3 and α-gustducin are necessary for increased SGLT-1 induction by dietary carbohydrates (Wauson et al., 2013).

In fish, recent studies demonstrated the presence and functioning of components of a GK-dependent glucosensing mechanism in brain areas of different species (Polakof et al., 2011d, 2012; Soengas, 2014). The response in rainbow trout hypothalamus after IP, ICV or dietary treatments inducing changes in the levels of glucose is shown in **Table 1**. Moreover, recent studies (Otero-Rodiño et al., 2015, 2016) provided evidence in rainbow trout for the presence and response to changes in circulating levels of glucose of glucosensing mechanisms in hypothalamus dependent on mitochondrial activity, LXR, and sweet taste receptor. No other studies attempted to elucidate the presence of glucosensing mechanisms in fish though glucose-sensing properties have been recently described in medaka hypothalamus (Hasebe et al., 2016).

Glucose and Food Intake Control

The detection of changes in glucose levels induces several regulatory responses allowing the animal to control glycemia, and one of these responses is food intake regulation (Marty et al., 2007). Thus, hypo- and hyperglycemia are known to increase and decrease food intake in mammals (Baird et al., 1997; Sanders et al., 2006) and birds (Seino and Miki, 2003), respectively.

Several studies carried out in fish suggest that changes in glucose levels may also modulate food intake response (Polakof et al., 2011d; Soengas, 2014), as summarized in **Table 1**. In

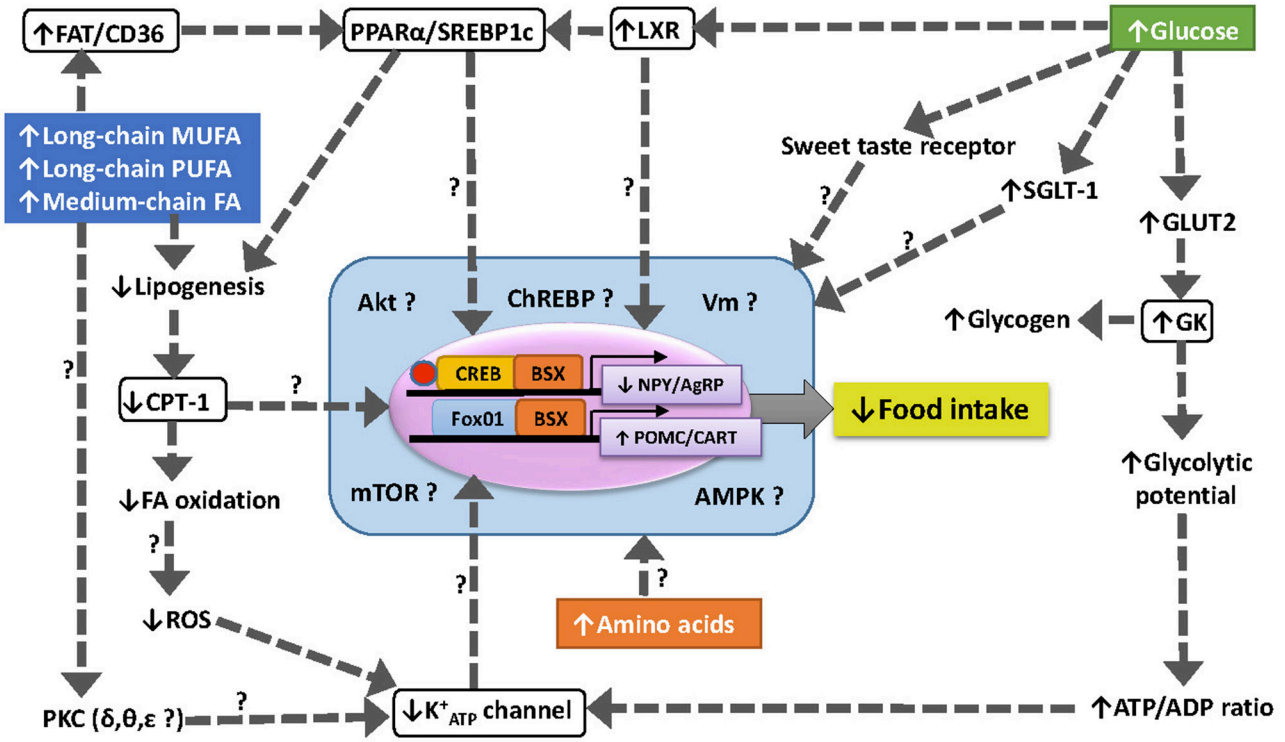


FIGURE 2 | Schematic drawing with a hypothetical model of food intake regulation through different nutrient sensing mechanisms in fish hypothalamus. The increase in levels of specific fatty acids is sensed through several mechanisms including (1) inhibition of CPT-1 to import FA-CoA into the mitochondria for oxidation, (2) increased binding to FAT/CD36 resulting in the modulation of PPARα and SREBP1c, (3) the translocation and activation of specific isoforms of PKC, and (4) the enhanced ROS production in mitochondria by electron leakage. The increase in levels of glucose is sensed through increased GK activity, activation of LXR and modulation o sweet taste receptors. The integration of different sensing mechanisms result in changes in the expression of neuropeptides controlling food intake (NPY/AgRP and CART/POMC) through unknown mechanisms.↑, increase; ↓, decrease; ?, unknown; AgRP, agouti-related peptide; Akt, protein kinase B; AMPK, AMP-activated protein kinase; BSX, brain homeobox transcription factor; CART, cocaine- and amphetamine-related transcript; ChREBP, carbohydrate-responsive element-binding protein; CPT-1, carnitine palmitoyl transferase type 1; CREB, cAMP response-element binding protein FA, fatty acid; FAT/CD36, fatty acid translocase; FoxO1, forkhead box protein O1; K^+_{ATP}, inward rectifier ATP-dependent K^+ channel; GK, glucokinase (hexokinase IV); GLUT2, facilitative glucose carrier type 2; LXR, liver X receptor; mTOR, target of rapamycin; MUFA, monounsaturated fatty acid; NPY, neuropeptide Y; POMC, pro-opio melanocortin; PPARα, peroxisome proliferator-activated receptor type α; PUFA, polyunsaturated fatty acid; SGLT-1, sodium/glucose co-transporter 1; SREBP1c, sterol regulatory element-binding protein type 1c; PKC, protein kinase C; ROS, reactive oxygen species; Vm, membrane potential.

general, fish fed with high-carbohydrate diets, or with glucose levels raised through IP or ICV treatments, displayed a decreased food intake, as demonstrated studies carried out in rainbow trout, goldfish, and tilapia. However, in some studies no changes occurred in food intake, as in sea bass or gilthead sea bream. Furthermore, fish fed a diet without carbohydrates or with low glucose levels resultant of IP or ICV treatments, increased food intake (Polakof et al., 2008a,b).

The mRNA of NPY, AgRP, POMC, and CART was detected in brain of different fish species in areas analogous to those of mammals (Cerdá-Reverter and Canosa, 2009), and changes in mRNA abundance related to food intake control (Volkoff et al., 2009). The presence in the same areas of rainbow trout brain of a marker of glucosesning such as GK protein (Polakof et al., 2009) and mRNA of neuropeptides involved in food intake control suggest that both findings are related. There are however few studies in fish assessing changes in hypothalamic neuropeptide mRNA abundance under conditions of altered glucose levels (**Table 1**). In rainbow trout NPY mRNA abundance decreased in hypothalamus after glucose treatment (Conde-Sieira et al., 2010b, 2012b; Aguilar et al., 2011; Otero-Rodiño et al., 2015) or after fish were fed with a high carbohydrate diet (Narnaware and Peter, 2002; Figueiredo-Silva et al., 2012b). Moreover, increased CART mRNA levels were observed in hypothalamus after experimental rising of glucose levels in catfish (Subhedar et al., 2011) and rainbow trout (Conde-Sieira et al., 2010b, 2012b; Otero-Rodiño et al., 2015), or in rainbow trout fed with a diet enriched with carbohydrates (Figueiredo-Silva et al., 2012b). Hypothalamic POMC-A1 mRNA abundance also increased in rainbow trout after hyperglycaemic treatment (Conde-Sieira et al., 2010b; Otero-Rodiño et al., 2015). Thus, increased glucose levels elicit increased anorectic potential whereas decreased glucose levels elicit increased orexigenic potential in agreement with the changes in food intake reported in the same species (Polakof et al., 2008a,b).

TABLE 1 | Effects in different fish species of different treatments eliciting changes in glucose levels (change) on food intake (FI), the response of hypothalamic glucosensing systems (sensing), and the mRNA abundance in hypothalamus of orexigenic (NPY, AgRP) and anorexigenic (POMC, CART) neuropeptides.

Treatment	Change	Dose	Time	Species	FI	Sensing	NPY	AgRP	POMC	CART	References
GLUCOSE											
Oral	↑	1 g/mL	4 h	Rainbow trout	↓						Soengas and Aldegunde, 2004
IP	↑	500 mg/kg	3 h	Rainbow trout	▼						Ruibal et al., 2002
	↑	500 mg/kg	6 h	Rainbow trout		▲					Polakof et al., 2007a
	↑	500 mg/kg	6 h	Rainbow trout		▲	▼		▲	▲	Conde-Sieira et al., 2010b
	↑	500 mg/kg	6 h	Rainbow trout		▲					Conde-Sieira et al., 2012b
	↑	500 mg/kg	6 h	Rainbow trout		▲	≈	≈	↑	↑	Otero-Rodiño et al., 2015
	↑	500 mg/kg	2–5 days	Rainbow trout	↓	▲					Conde-Sieira et al., 2010a
	↑	500 mg/kg	1–10 days	Rainbow trout	↓	▲					Polakof et al., 2008a
ICV		100 ng/g	2 h	Catfish						▲	Subhedar et al., 2011
		4 μg/g	6 h	Rainbow trout		↑					Polakof and Soengas, 2008
In vitro	↑	2–8 mM	1 h	Rainbow trout		▲					Polakof et al., 2007b
	↑	2–8 mM	1 h	Rainbow trout		▲	▼		▲	▲	Aguilar et al., 2011
	↑	2–8 mM	1 h	Rainbow trout		↑	↓		≈	↑	Conde-Sieira et al., 2011
	↑	2–8 mM	1 h	Rainbow trout		↑	↓		≈	≈	Conde-Sieira et al., 2012a
	↑	2–8 mM	6 h	Rainbow trout		↑					Otero-Rodiño et al., 2016
Carbohydrate-enriched diet	↑	35–55% CHO	0.5 h	Goldfish	↓		↓				Narnaware and Peter, 2002
	↑	31% CHO	1–6 h	Rainbow trout			≈			↑	Figueiredo-Silva et al., 2012b
	↑	20% CHO	1–24 h	Rainbow trout		▲					Polakof et al., 2008c
	↑	20% CHO	1–10 days	Rainbow trout	↓	▲					Polakof et al., 2008b
	↑	20% CHO	1–10 days	Rainbow trout		▲					Polakof et al., 2008c
	↑	23% CHO	28 days	Rainbow trout	↓						Krogdahl et al., 2004
	↑	31% CHO	42 days	Rainbow trout	↓						Figueiredo-Silva et al., 2012b
	↑	31% CHO	42 days	Rainbow trout	↓						Figueiredo-Silva et al., 2013
	↑	27–46% CHO	48 days	Tilapia	↓						Saravanan et al., 2012
	↑	26% CHO	48 days	Tilapia	↓						Figueiredo-Silva et al., 2013
	≈	33% CHO	70 days	Gilthead sea bream	≈						Rocha et al., 2016
	↑	19% CHO	73 days	Sea bass	≈						Castro et al., 2015
	↑	30–40% CHO	84 days	Rainbow trout	↓						Suárez et al., 2002
	↑	38% CHO	126 days	Rainbow trout	↓						Kaushik et al., 1989
Carbohydrate-free diet	↓	<0.3% CHO	1–10 days	Rainbow trout	↑	↓					Polakof et al., 2008b
	↓	<0.3% CHO	1–10 days	Rainbow trout		▼					Polakof et al., 2008c
	↓	<0.1% CHO	28 days	Rainbow trout	↑						Sánchez-Muros et al., 1998
	↓	0.2% CHO	70 days	Rainbow trout	↑						Capilla et al., 2003

↑, *Increase;* ▲, *strong increase;* ↓, *decrease;* ▼, *strong decrease;* ≈, *no changes; IP, intraperitoneal; ICV, intracerebroventricular; CHO, carbohydrate.*

Fatty Acid Sensors and Control of Food Intake

Fatty Acid Sensing Mechanisms

Evidence in mammals supports that specialized neurons in hypothalamus detect changes in circulating levels of long-chain fatty acid (LCFA), but not short- (SCFA) or medium-chain fatty acid (MCFA) contributing to neural control of energy homeostasis (Migrenne et al., 2007; Gao et al., 2013; Duca and Yue, 2014; Efeyan et al., 2015). The most accepted mechanism is of metabolic nature. Thus, increased LCFA levels in plasma induced an increase in malonyl-CoA levels and subsequent inhibition of carnitinepalmitoyltransferase 1 (CPT-1) to import FA-CoA into the mitochondria for oxidation (López et al., 2005, 2007). Other fatty acid sensing mechanisms are present in hypothalamus. Thus, the increased binding to fatty acid translocase (FAT/CD36) induced by increased levels of LCFA results in the modulation of transcription factors like sterol regulatory element-binding protein type 1c (SREBP1c), and peroxisome proliferator-activated receptor type α (PPARα) (Le Foll et al., 2009). The translocation and activation of specific isoforms of protein kinase C (PKC) in response to enhanced LCFA levels results in PI3K inhibition (Benoit et al., 2009; Blouet and Schwartz, 2010). The enhanced production of reactive oxygen species in mitochondria in response to raised levels of LCFA results in K^+_{ATP} inhibition (Blouet and Schwartz, 2010). Finally, the activity of lipoprotein lipase related to increased levels of LCFA (Picard et al., 2013). The 18 carbon monounsaturated fatty acid oleate (C18:1 n-9) is the most studied LCFA in mammals involved in the activation of fatty acid sensing systems (López et al., 2007; Blouet and Schwartz, 2010; Duca and Yue,

2014). Fatty acid unsaturation appears to be important since the saturated fatty acid palmitate (C16:0) does not activate hypothalamic fatty acid sensing systems (Ross et al., 2010; Schwinkendorf et al., 2011; Greco et al., 2014). Moreover, the presence of more than one double bond, such as for linoleate (C18:2 n-6) or docosahexanoate (C22:6 n-3), does not activate fatty acid sensing systems in mammals (Gomez-Pinilla and Ying, 2010; Ross et al., 2010; Schwinkendorf et al., 2011; Greco et al., 2014).

In fish, lipids are main nutrients involved in metabolically sustaining relevant physiological processes (Tocher, 2003; Polakof et al., 2010). Thus, it is reasonable that lipids are involved in food intake control. In recent years, the presence and function of fatty acid sensing systems in the hypothalamus was characterized in fish (Librán-Pérez et al., 2012, 2013, 2014a,b, 2015a,b), as summarized in **Table 2**. IP (Librán-Pérez et al., 2012), ICV (Librán-Pérez et al., 2014a), and *in vitro* (Librán-Pérez et al., 2013) administration in rainbow trout of oleate or the MCFA octanoate (C8:0) induced a response in hypothalamus compatible with fatty acid sensing. This included reduced potential of lipogenesis and fatty acid oxidation, decreased potential of K^+_{ATP}, and modulation of FAT/CD36 with subsequent changes in the expression of transcription factors (Librán-Pérez et al., 2012, 2013, 2014a). This response is comparable with that of mammals with the main difference of the capacity of fish to respond to increased levels of an MCFA like octanoate (Hu et al., 2011). This could relate to the finding that body lipids in teleosts contain considerable amounts of MCFA (Davis et al., 1999; Trushenski, 2009) and that in rainbow trout there is no preferential oxidation of MCFA compared with LCFA (Figueiredo-Silva et al., 2012a), in contrast with the mammalian situation (Ooyama et al., 2009).

All vertebrate species have dietary requirements for specific polyunsaturated fatty acid (PUFA), and diets for marine fish are particularly rich in long chain PUFA (Sargent et al., 2002). The brain of marine fish displays high levels of n-3 PUFA, mainly in α-linolenate (C18:3 n-3), eicosapentanoate (C20:5 n-3), and docosahexanoate (Tocher et al., 1992; Betancor et al., 2014). Therefore, it is reasonable to hypothesize that fish hypothalamic FA sensing systems, particularly in marine species, could differ from those of mammals in the ability to sense PUFA. Accordingly, a recent study in Senegalese sole (Conde-Sieira et al., 2015) demonstrated that its fatty acid sensing systems were activated not only by oleate but also by an n-3 PUFA such as α-linolenate. However, in the same study authors showed that another PUFA such as eicosapentanoate did not alter fatty acid sensing systems suggesting that the response might be specific to certain PUFA.

If fatty acid sensing systems activated when levels of specific fatty acid rise, what happens in those systems when levels of fatty acids fall? It is not possible to decrease the levels of a particular fatty acid. The only available studies in mammals used pharmacological treatments inhibiting lipolysis to induce a general decline in levels of all circulating fatty acids, which coincided with decreased activity of fatty acid sensing systems (Oh et al., 2012, 2014). A similar experimental approach in rainbow trout resulted in an inhibition of fatty acid sensing mechanisms in hypothalamus associated with the activation of the hypothalamus-pituitary-interrenal (HPI) axis (Librán-Pérez et al., 2014b).

Fatty Acids and Control of Food Intake

Feeding fish with diets enriched in lipids result in a decrease in food intake as observed in different species (**Table 2**). A comparable lower food intake also occurred in fish containing high fat stores (Shearer et al., 1997; Silverstein et al., 1999; Johansen et al., 2002, 2003). Considering the qualitative and quantitative importance of fatty acids within the lipid pool in fish diets and tissue composition, it is not surprising that the available studies are focussed on fatty acids.

In recent studies in rainbow trout, increased levels of oleate or octanoate *in vivo,* either after IP (Librán-Pérez et al., 2012) or ICV (Librán-Pérez et al., 2014a) treatments, resulted in decreased food intake, with more potent effects for octanoate. Moreover, also in rainbow trout fed diets with different lipid composition, the fish with the highest levels of fatty acid in plasma were those in which a decreased food intake occurred (Luo et al., 2014). Therefore, the inhibition of food intake reported in fish by feeding lipids is probably due to the action of central fatty acid sensors. This is also supported by the finding that the treatment of rainbow trout with C75 (fatty acid synthase inhibitor) resulted in a reduced food intake counteracted by TOFA (acetyl-CoA carboxylase inhibitor) (Librán-Pérez et al., 2012). In Senegalese sole, a decreased food intake occurred after IP treatment with oleate as well as different types of fatty acids including saturated fatty acids like stearate, and two types of PUFA such as α-linolenate and eicosapentanoate (Conde-Sieira et al., 2015). When levels of circulating fatty acids decreased through non-specific pharmacological treatment, a sound increase in food intake occurred in rainbow trout (Librán-Pérez et al., 2014b).

In mammals, enhanced levels of LCFA resulted in a decrease in mRNA abundance of AgRP and NPY as well as an increase of CART and POMC (López et al., 2005). However, few studies assessed in fish hypothalamus mRNA abundance of neuropeptides in response to changes in levels of fatty acids in circulation (**Table 2**). In rainbow trout the treatment with oleate or octanoate either IP (Librán-Pérez et al., 2012), ICV (Librán-Pérez et al., 2014a), or *in vitro* (Librán-Pérez et al., 2013) resulted in a decrease in mRNA abundance of NPY and an increase of CART and POMC-A1. The decrease in NPY is comparable to that described in rat hypothalamus after oleate ICV treatment (Blouet and Schwartz, 2010). Changes in mRNA abundance of neuropeptides suggest an enhancement of anorexigenic potential, which is in agreement with the decrease observed in food intake of rainbow trout after oleate or octanoate treatment (Librán-Pérez et al., 2012, 2014a). It is important to emphasize that the response to octanoate is unique to fish since in mammals octanoate is not inducing any change in mRNA abundance of neuropeptides (Hu et al., 2011). In Senegalese sole, the increase in circulating levels of oleate also resulted in decreased mRNA abundance of AgRP-2 and increased values of CART-2b again favoring increased anorectic potential (Conde-Sieira et al., 2015). In the same species the IP treatment with PUFA such as α-linolenate or eicosapentanoate

TABLE 2 | Effects in different fish species of different treatments eliciting changes in fatty acid levels (change) on food intake (FI), the response in hypothalamus of fatty acid sensing systems (sensing), and the mRNA abundance in hypothalamus of orexigenic (NPY, AgRP) and anorexigenic (POMC, CART) neuropeptides.

Treatment	Change	Dose	Time	Species	FI	Sensing	NPY	AgRP	POMC	CART	References
OLEATE											
IP	↑	300 μg/kg	3 h	Senegalese sole		▲	≈	▼	≈	▲	Conde-Sieira et al., 2015
	↑	60–300 μg/kg	6 h	Rainbow trout		▲	▼		▲	↓	Librán-Pérez et al., 2012
	↑	300 μg/kg	6 h	Rainbow trout		↑	≈		≈	≈	Librán-Pérez et al., 2015a
	↑	60–300 μg/kg	6–24 h	Rainbow trout	▼						Librán-Pérez et al., 2012
	↑	300 μg/kg	6–24 h	Rainbow trout	↓						Librán-Pérez et al., 2015a
	↑	300 μg/kg	4–48 h	Senegalese sole	↓						Conde-Sieira et al., 2015
ICV	↑	10 nmol/g	2–6 h	Rainbow trout		↑	↓	↓	▲	↑	Velasco et al., 2016a
	↑	10 nmol/g	6 h	Rainbow trout		↑	▼		▲	↑	Librán-Pérez et al., 2014a
	↑	10 nmol/g	6–24 h	Rainbow trout	▼						Librán-Pérez et al., 2014a
	↑	10 nmol/g	6–48 h	Rainbow trout	▼						Velasco et al., 2016a
In vitro	↑	1–100 μM	1 h	Rainbow trout		▲	▼		▲	▲	Librán-Pérez et al., 2013
Stearate (IP)	↑	300 μg/kg	3 h	Senegalese sole		≈	≈	≈	≈	↓	Conde-Sieira et al., 2015
	↑	300 μg/kg	4–48 h	Senegalese sole	≈						Conde-Sieira et al., 2015
α-Linolenate (IP)	↑	300 μg/kg	3 h	Senegalese sole		▲	≈	▼	≈	↑	Conde-Sieira et al., 2015
	↑	300 μg/kg	4–48 h	Senegalese sole	↓						Conde-Sieira et al., 2015
Eicosapentanoate (IP)	↑	300 μg/kg	3 h	Senegalese sole		≈	≈	≈	≈	▲	Conde-Sieira et al., 2015
	↑	300 μg/kg	4–48 h	Senegalese sole	↓						Conde-Sieira et al., 2015
OCTANOATE											
IP	↑	60–300 μg/kg	6 h	Rainbow trout		▲	≈		≈	↓	Librán-Pérez et al., 2012
	↑	300 μg/kg	6 h	Rainbow trout		↑	≈		≈	≈	Librán-Pérez et al., 2015a
	↑	60–300 μg/kg	6–24 h	Rainbow trout	▼						Librán-Pérez et al., 2012
	↑	60–300 μg/kg	6–24 h	Rainbow trout	↓						Librán-Pérez et al., 2015a
ICV	↑	10 nmol/g	2–6 h	Rainbow trout		↑	↓		▲	≈	Librán-Pérez et al., 2014a
	↑	10 nmol/g	6–24 h	Rainbow trout	▼						Librán-Pérez et al., 2014a
In vitro	↑	1–100 μM	1 h	Rainbow trout		▲	≈		▲	▲	Librán-Pérez et al., 2013
Lipid- enriched diet	↑	20% Lipid	1–6 h	Rainbow trout				↓		≈	Figueiredo-Silva et al., 2012b
	↑	25–34% Lipid	1–10 days	Rainbow trout	≈						Geurden et al., 2006
	↑	21% Lipid	21 days	Rainbow trout	≈						Luo et al., 2014
	↑	19% Lipid	28 days	Rainbow trout	≈	↑	↑	≈	▲	▲	Librán-Pérez et al., 2015b
	↑	30–40% Lipid	40 days	Rainbow trout	↓						Forsman and Ruohonen, 2009
	↑	19–27% Lipid	42 days	Rainbow trout	↓						Saravanan et al., 2013
	↑	20% Lipid	42 days	Rainbow trout	↓						Figueiredo-Silva et al., 2012b
	↑	20% Lipid	42 days	Rainbow trout	↑						Figueiredo-Silva et al., 2013
	↑	21% Lipid	48 days	Tilapia	≈						Figueiredo-Silva et al., 2013
	↑	18% Lipid	50 days	Rainbow trout	↓						Peragón et al., 2000
	↑	35% Lipid	56 days	Polka-dot grouper	↓						Williams et al., 2006
	↑	34% Lipid	56 days	Atlantic salmon	↓		≈	≈	≈	↑	Hevroy et al., 2012
	↑	21% Lipid	56 days	Grass carp	↓						Li et al., 2016
	↑	30% Lipid	63–84 days	Rainbow trout	↓						Gélineau et al., 2001
	↑	21% Lipid	105 days	Rainbow trout	≈						Figueiredo-Silva et al., 2012a
SDW WAG994 (IP)	↓	60 mg/kg	6 h	Rainbow trout		↓	≈		▼	▼	Librán-Pérez et al., 2014b
	↓	60 mg/kg	24–48 h	Rainbow trout	↑						Librán-Pérez et al., 2014b
Insulin (IP; het.)	↓	2 mg/kg	6 h	Rainbow trout		↑	≈		≈	↓	Librán-Pérez et al., 2015a
	↓	2 mg/kg	6–24 h	Rainbow trout	▼						Librán-Pérez et al., 2015a
Ghrelin (ICV; hom.)	↑	2 ng/g	2–6 h	Rainbow trout		↓	↑	↑	↓	↓	Velasco et al., 2016a
	↑	2 ng/g	6–48 h	Rainbow trout	↑						Velasco et al., 2016a

↑, Increase; ▲, strong increase; ↓, decrease; ▼, strong decrease; ≈, no changes; IP, intraperitoneal; ICV, intracerebroventricular; hom., homologous hormone; het. Heterologous hormone.

(Conde-Sieira et al., 2015) increased anorectic potential based on decreased mRNA abundance of AgRP-2 (α-linolenate) and increased mRNA abundance of CART-2b (α-linolenate and eicosapentanoate). The decrease in circulating levels of fatty acid induced in rainbow trout by pharmacological treatment resulted in a fall of anorexigenic potential based on decreased mRNA abundance of POMC-A1 and CART (Librán-Pérez et al., 2014b).

Integration of Nutrient Sensing Information

The precise mechanisms connecting changes in glucose or fatty acid sensing systems and the mRNA abundance of orexigenic and anorexigenic factors are mostly unknown. Changes in neuropeptide expression have been associated with the modulation of brain homeobox transcription factor (BSX), forkhead box01 (Fox01), and phosphorylated cAMP response-element binding protein (pCREB) (Diéguez et al., 2011). The actions of these factors would result in the enhancement of CART and POMC, and the inhibition of NPY and AgRP, ultimately leading to decreased food intake (López et al., 2007; Diéguez et al., 2011). How these transcription factors relate to the activity of the different nutrient sensing systems? Several possibilities were suggested in mammals including (1) direct action of malonyl CoA or CPT1c, (2) indirect action through inhibition of CPT-1, (3) modulation by protein kinase B (Akt), AMPK, carbohydrate-responsive element-binding protein (ChREBP), or mTOR, or (4) involvement of ceramides (López et al., 2007; Diéguez et al., 2011; Gao et al., 2013).

In fish, a preliminary study in rainbow trout (Librán-Pérez et al., 2015b) showed that the cell signaling pathways that are dependent on Akt, AMPK, and mTOR activated in hypothalamus of fish fed a lipid-enriched diet. In the same species, a recent study (Velasco et al., 2016b) pointed to a possible role of ceramides in connecting hypothalamic fatty acid sensing systems and neuropeptide mRNA abundance with food intake control. Besides these studies, there is no further evidence in fish about the integration of the metabolic information of different nutrient (glucose, fatty acids, amino acids) sensing systems into a unique pathway regulating transcription factors involved in the production of neuropeptides controlling food intake. A summary of hypothetical relationships is shown in **Figure 2**.

HYPOTHALAMIC INTEGRATION OF ENDOCRINE INFORMATION

The hypothalamic neurons producing neuropeptides that control food intake in response to changes in the levels of nutrients are also responsive, through binding to appropriate receptors, to the effect of hormones (Levin et al., 2004; Blouet and Schwartz, 2010). These include hormones providing information regarding metabolic stores or energy *status* such as leptin and insulin, and gastrointestinal hormones providing information regarding absence/presence of food and its composition, including ghrelin and cholecystokinin (CCK), among others (Blouet and Schwartz, 2010). The same hormones modulate the activity of fatty acid- and glucose-sensing systems and mRNA abundance of neuropeptides related to the control of food intake in hypothalamus, as summarized in **Table 2** (fatty acid) and **Table 3** (glucose).

Insulin

Insulin and insulin receptors are present in fish hypothalamus (Gutiérrez and Plisetskaya, 1994; Leibush et al., 1996; Caruso et al., 2008). The effects of insulin treatment on food intake in fish are however contradictory. Thus, in rainbow trout IP administration of insulin either resulted in inhibition (Librán-Pérez et al., 2015a) or activation (Polakof et al., 2008a; Conde-Sieira et al., 2010b) of food intake, whereas ICV treatment did not affect food intake in catfish (Silverstein and Plisetskaya, 2000) but induced a decrease in rainbow trout (Soengas and Aldegunde, 2004). The anorectic effects in rainbow trout agree with the increased anorexigenic potential (increased CART and decreased NPY mRNA abundance) observed in hypothalamus after insulin treatment (Librán-Pérez et al., 2015a). Moreover, insulin administration in rainbow trout inhibits the glucosensing system dependent on GK (Polakof et al., 2007a, 2008a; Conde-Sieira et al., 2010b) whereas no clear effects were noted on fatty acid sensing systems (Librán-Pérez et al., 2015a).

Leptin

Multiple forms of leptin exist in fishes, with the major form, leptin A, being the most likely candidate for food intake regulation and energy signaling (Gorissen and Flik, 2014). The treatment with either fish or recombinant human leptin in fish usually results in an anorectic response (De Pedro et al., 2006; Murashita et al., 2008; Kling et al., 2009; Vivas et al., 2011; Won et al., 2012; Gong et al., 2016a). This anorexigenic action of leptin appears to be mediated by brain leptin receptors, particularly in the hypothalamus (Kurokawa et al., 2008; Tinoco et al., 2012; Angotzi et al., 2016), where leptin exerts its anorectic action through regulation of neuropeptide expression. Accordingly, the leptin receptor-deficient medaka shows increased food intake accompanied by increased NPYa and AgRP mRNA abundance, and decreased POMC1 mRNA abundance (Chisada et al., 2014). Moreover, ICV injection of leptin reduced NPY mRNA levels in hypothalamus and telencephalon of goldfish (Volkoff et al., 2003), and in the whole brain of grass carp (Li et al., 2010). Peripheral treatment with recombinant salmon leptin A1 in rainbow trout induced a hypothalamic transient reduction and elevation of NPY and POMC-A1 mRNA levels, respectively (Murashita et al., 2011). Moreover, central injection of leptin increased CART-I mRNA levels in hypothalamus of goldfish (Volkoff and Peter, 2001a), and POMC-A1, A2 and B, and CART mRNA levels in rainbow trout (Gong et al., 2016a). On the other hand, the leptin anorectic effects also related in rainbow trout to the activation of central glucosensing systems (Aguilar et al., 2010, 2011).

The action of leptin on food intake regulation depends on the time of hormone administration, indicating a circadian dependence of leptin anorexigenic effects (Vivas et al., 2011). In this sense, leptin-aI and leptin-aII mRNAs in hypothalamus

TABLE 3 | Effects in different fish species of different hormone (either homologous: hom., or heterologous: het.) treatments eliciting changes in glucose levels (change) on food intake (FI), the response of hypothalamic glucosensing systems (sensing), and the mRNA abundance in hypothalamus of orexigenic (NPY, AgRP) and anorexigenic (POMC, CART) neuropeptides.

Treatment	Change	Dose	Time	Species	FI	Sensing	NPY	AgRP	POMC	CART	References
INSULIN											
IP	↓	4 mg/kg (het.)	6 h	Rainbow trout		▼					Polakof et al., 2007a
	↓	4 mg/kg (het.)	6 h	Rainbow trout		↓	≈		≈	≈	Conde-Sieira et al., 2010b
	↓	4 mg/kg (het.)	2–5 days	Rainbow trout	↑	↓					Conde-Sieira et al., 2010a
	↓	4 mg/kg (het.)	1–10 days	Rainbow trout	↑	↓					Polakof et al., 2008a
ICV		104 ng/fish (het.)	2 h	Catfish						↑	Subhedar et al., 2011
		20 ng/g (het.)	1–24 h	Catfish	≈						Silverstein and Plisetskaya, 2000
		90 ng/g (het.)	26–50 h	Rainbow trout	↓						Soengas and Aldegunde, 2004
GLP-1											
IP	≈	50–200 ng/g (het.)	1 h	Channel catfish	≈		≈		≈		Schroeter et al., 2015
	↑	100 μg/kg (het.)	2 h	Rainbow trout		▲	↑		▲	≈	Polakof et al., 2011b
		50–300 ng/g (het.)	4 h	Coho salmon	▼						White et al., 2016
ICV	↑	20 ng/g (het.)	1 h	Rainbow trout		≈	≈		↓	≈	Polakof et al., 2011b
		5–50 ng/g (hom.)	1 h	Catfish	▼						Silverstein et al., 2001
In vitro		0.1–10 nM (het.)	1 h	Rainbow trout		↑	↓		↑	↑	Polakof et al., 2011b
LEPTIN											
IP		120 ng/g (hom.)	1–8 h	Rainbow trout	▼		▼		▲	↑	Murashita et al., 2008
	↓	0.1–3.3 μg/g (het.)	2–8 h	Goldfish	▼						De Pedro et al., 2006
	↑	1 μg/g (het.)	8 h	Goldfish	▼						Vivas et al., 2011
ICV		50 ng/g (het.)	1 h	Goldfish	▼						Volkoff and Peter, 2001a
		1–100 ng/g (het.)	1 h	Goldfish	↓						Volkoff et al., 2003
		40 ng/g (het.)	1 h	Catfish	≈						Silverstein and Plisetskaya, 2000
		10 ng/g (het.)	2 h	Catfish						↑	Subhedar et al., 2011
		0.5–5 ng/g (hom.)	2 h	Rainbow trout	▼		↑		▲	↑	Gong et al., 2016a
		50 ng/g (het.)	2–6 h	Goldfish			↓			↑	Volkoff and Peter, 2001a
	↑	50 ng/g (het.)	6 h	Rainbow trout		▲					Aguilar et al., 2010
	↑	50 ng/g (het.)	6–24 h	Rainbow trout	▼						Aguilar et al., 2010
In vitro	↑	10–50 nM (het.)	1–3 h	Rainbow trout		▲	▼		≈	≈	Aguilar et al., 2011
GHRELIN											
IP		100 ng/g (het.)	0.5 h	Cavefish	▲					≈	Penney and Volkoff, 2014
	≈	200 ng/g (hom.)	1 h	Rainbow trout	↓						Jönsson et al., 2010
	≈	50–200 ng/g (hom.)	1 h	Channel catfish	▼		≈		≈		Schroeter et al., 2015
		100 μg/kg (het.)	2 h	Rainbow trout		▲	≈		≈	≈	Polakof et al., 2011c
		16 pmol/g (hom.)	1–6 h	Goldfish			≈				Miura et al., 2006
		475 ng/g (hom.)	7 days	Brown trout	▲		≈				Tinoco et al., 2014a
ICV		1 pmol/g (hom.)	1 h	Goldfish	▲						Miura et al., 2006
	≈	20 ng/g (het.)	2 h	Rainbow trout		≈	≈		≈	≈	Polakof et al., 2011c
		1 pmol/g (hom.)	1–6 h	Goldfish			↑				Miura et al., 2006
In vitro		0.1–10 nM (het.)	1 h	Rainbow trout		↑	↑		≈	↓	Polakof et al., 2011c
CCK											
IP		100 ng/g (het.)	0.5 h	Goldfish	▼						Himick and Peter, 1994
		50 ng/g (het.)	0.5 h	Cavefish	▼						Penney and Volkoff, 2014
		50–200 ng/g (het.)	1 h	Channel catfish	≈		≈		↓		Schroeter et al., 2015
	↑	50 μg/kg (het.)	2 h	Rainbow trout		≈					Polakof et al., 2011a
		50–500 ng/g (het.)	4 h	Coho salmon	▼					↓	White et al., 2016
ICV		50 ng/g (het.)	0.5 h	Goldfish	▼						Himick and Peter, 1994
		50–100 pmol/g (het.)	1 h	Goldfish	↓				▲		Kang et al., 2010
		10 ng/g (het.)	1 h	Catfish	▼						Silverstein and Plisetskaya, 2000
	↑	10 ng/g (het.)	2 h	Rainbow trout		↑					Polakof et al., 2011a

↑, *Increase;* ▲, *strong increase;* ↓, *decrease;* ▼, *strong decrease;* ≈, *no changes; IP, intraperitoneal; ICV, intracerebroventricular.*

of goldfish show 24-h rhythms under light–dark cycle and scheduled feeding conditions (Tinoco et al., 2014b). Postprandial increases of hypothalamic and liver leptin-aI expression (Huising et al., 2006; Moen and Finn, 2013; Zhang et al., 2013) are in agreement with the anorexigenic role of this hormone in fish. As expected for an anorexigenic hormone, feeding changes and nutritional status modifies the leptin system in fish, but different responses occurred depending on the feeding regime and species. Thus, leptin genes expression or circulating leptin levels rise with food restriction or starvation, when energy stores decline, and decrease during refeeding in some species (Gorissen and Flik, 2014; Johansson and Björnsson, 2015), but not in others (Huising et al., 2006; Tinoco et al., 2012). These, and other recent studies (Chisada et al., 2014; Londraville et al., 2014; Salmerón et al., 2015; Jørgensen et al., 2016) support that leptin in fish should not be considered as a lipostat signal in contrast to mammals. In this way, zebrafish knockout for leptin suggest the involvement of leptin in glucose homeostasis but not in adipostasis (Michel et al., 2016). Nevertheless, the existence of different leptin paralogs in fish differentially involved in the regulation on energy resources in a species/tissue dependent manner might explain the variety of results.

Gastrointestinal Hormones

Ghrelin

Ghrelin is a peptide mainly synthesized in the stomach or its equivalent in stomachless fish species (Kaiya et al., 2008), although its gene expression is also detected in other peripheral locations and in brain (Kojima et al., 1994; Unniappan et al., 2002; Feng et al., 2013). Some studies point to the endocrine cells in the digestive mucosa as the peripheral primary synthesizing site of ghrelin in fish (Jönsson, 2013). Ghrelin is also widely expressed in fish brain, particularly in hypothalamus (Jönsson and Holmgren, 2012; Sánchez-Bretaño et al., 2015b). Ghrelin requires a post-translational acylation (catalyzed by ghrelin O-acyltransferase, GOAT) before binding to its receptor (Yang et al., 2008). Due to tetraploidization experienced by some fish species, two paralog genes of ghrelin receptor and four or eight receptor subtypes are present in some fish (Kaiya et al., 2013). The wide distribution of transcripts of these receptors, and particularly its presence in gastrointestinal tract, liver, brain sensory areas, and pituitary may indicate multiple targets for the regulation of energy balance by this hormone (Jönsson, 2013). Particularly, the GHS-R1a subtype (specifically involved in energy balance, Kaiya et al., 2010) exhibits a dense expression in discrete hypothalamic nucleus, such as the *lateral recessus nucleus*, in support of the orexigenic role of the ghrelinergic system (Sánchez-Bretaño et al., 2015b).

Ghrelin stimulates feeding in all mammalian species studied so far, primarily through increased release of NPY and AgRP (Patton and Mistlberger, 2013). However, this generalized orexigenic effect is not so evident in fish. Ghrelin is an orexigenic peptide in many fishes (Miura et al., 2006; Kaiya et al., 2008; Picha et al., 2009; Kang et al., 2011; Jönsson, 2013; Penney and Volkoff, 2014; Tinoco et al., 2014a). However, this peptide exerts anorexigenic actions in tilapia (Peddu et al., 2009) and both anorexigenic (Jönsson et al., 2010) and orexigenic (Velasco et al., 2016a,b) effects in rainbow trout. The orexigenic action of ghrelin in goldfish is mediated by hypothalamic activation of NPY (Miura et al., 2006) and orexin-A (Miura et al., 2007; Nisembaum et al., 2014b) mRNA via vagus nerve (Matsuda et al., 2006b), as peripheral administration of ghrelin does not modify NPY mRNA expression in goldfish hypothalamus (Nisembaum et al., 2014b). Interestingly, the expression of ghs-r1 ghrelin receptor (Sánchez-Bretaño et al., 2015b) and GOAT (Blanco et al., 2016b) in hypothalamic nuclei that also express orexin-A and NPY in goldfish reinforces the suggested mechanism for the orexigenic action of ghrelin in this teleost. On the other hand, the post-feeding decrease of circulating ghrelin levels (Unniappan et al., 2004) and the preprandial rise of circulating acyl-ghrelin and GOAT (Blanco et al., 2016a) support the role of this peptide as a meal initiator in goldfish. In agreement with this action of ghrelin, the hypothalamic mRNA expression of GH secretagogue receptors (sbGHSR-1a and sbGHSR-1b) was higher in fasted than in fed seabream (Zhang et al., 2008).

In addition to the effects of ghrelin on the feeding regulatory neurons in hypothalamus, some studies suggest that the effects of ghrelin on food intake are also mediated by gastrointestinal motility in the periphery, as in zebrafish (Olsson et al., 2008). However, this is not the rule in fish, since ghrelin does not modify intestinal motility in other teleost (Kitazawa et al., 2012). Localization studies agree with this lack of ghrelin effect on gut motility since ghrelin receptor expressing cells are not present in the muscle layer of gut (Sánchez-Bretaño et al., 2015b).

Feeding and nutritional status are the main factors involved in the regulation of ghrelinergic system in mammals, but this is controversial in fish. Different responses to food deprivation occurred depending on the species, tissue, and duration of food deprivation. Accordingly, different effects are found on ghrelin release, its expression in different central and peripheral tissues, and GOAT activity (Unniappan et al., 2004; Jönsson et al., 2007; Fox et al., 2009; Hevrøy et al., 2011; Blanco et al., 2016b).

The effect of ghrelin treatment on central nutrient sensing systems was assessed in rainbow trout, and glucosensing systems appear to be activated by ghrelin treatment (Polakof et al., 2011c), an effect opposed to that addressed in mammals (Wang et al., 2008). In agreement with the well-known effect in mammals, fatty acid sensing systems are inhibited in rainbow trout by ICV ghrelin treatment (Velasco et al., 2016a,b). These changes agree with the increased mRNA abundance of AgRP/NPY and decreased mRNA abundance of POMC-A1/CART observed simultaneously (Velasco et al., 2016a,b).

CCK

CCK is a member of the CCK-gastrin family that exerts a key role in digestive physiology of vertebrates, including fish (Olsson and Holmgren, 2011). Partial and complete mRNA sequences of CCK are available for a number of fish species, and CCK-like-ir is present in gut and nervous system of several teleost species (Jönsson et al., 1987; Micale et al., 2012, 2014; Ji et al., 2015). CCK-like peptides are potent anorexigenic signals in fish (Himick and Peter, 1994; Volkoff et al., 2005; Rubio et al., 2008; MacDonald and Volkoff, 2009;

White et al., 2016). Moreover, these hormones are involved in many digestive functions and glucose homeostasis (Rajjo et al., 1988; Aldman and Holmgren, 1995; Einarsson et al., 1997; Polakof et al., 2011a; Tinoco et al., 2014b). CCK-8 may be involved in the seasonal changes of food intake experienced by salmonids (White et al., 2016), and starvation challenge experiments support the anorexigenic action of CCK-8 in fish. The expression levels of CCK mRNA decreased in brain and intestine after starvation in different fish species (Murashita et al., 2006; Feng et al., 2012; Ji et al., 2015), while CCK is released after feeding in intestine (Aldman and Holmgren, 1995). The mechanisms underlying the anorexigenic effect of CCK-like peptides are unknown, but the lack of effect after peripheral administration in channel catfish (Schroeter et al., 2015) suggests that anorexigenic action occur at the central level. Interactions with other feeding regulators, as the hypothalamic expression of amylin, have been described in goldfish (Thavanathan and Volkoff, 2006), but the mechanisms involved appear to be different when comparing peripheral and central administration (Hoskins and Volkoff, 2012). Accordingly, a differential distribution pattern of CCK receptors subtypes is present in goldfish with high expression of CCKAR subtype in the intestine, whereas the CCKBR subtype predominantly expressed in hypothalamus and vagal lobe (Tinoco et al., 2015). Finally, CCK-8 treatment activated glucosensing capacity in hypothalamus and hindbrain of rainbow trout (Polakof et al., 2011a).

Other Gastrointestinal Hormones

Glucagon-like peptide 1 (GLP-1) appears to be anorectic in fish (Silverstein et al., 2001; White et al., 2016). In rainbow trout GLP-1 treatment activated hypothalamic glucosensing systems (Polakof et al., 2011b) in parallel with altered mRNA abundance of several neuropeptides including increased values for CART and POMC-A1 and decreased values for NPY (Polakof et al., 2011b).

Other gastrointestinal peptides also show anorexigenic properties in fish including those peptides belonging to the calcitonin gene-related peptide family, such as the calcitonin gene-related peptide (CGRP), intermedin (or adrenomedulin) and amylin (or islet amyloid polypeptide). These peptides act through a calcitonin receptor-like receptor and show a broad distribution in brain and peripheral tissues in fish (Ogoshi et al., 2003; Martínez-Álvarez et al., 2008). CGRP mRNA is widely expressed in the central and peripheral nervous system, intermedin-ir is present in brain, pituitary and most peripheral tissues, including endocrine pancreas (López et al., 1999). Amylin is present in Brockmann bodies, thus suggesting that once produced in the periphery transported into brain where it has central actions (Westermark et al., 2002). ICV injections of these three peptides in goldfish induced a decrease in food intake (Martínez-Álvarez et al., 2009) mediated at the central level through unknown mechanisms. The presence of CGRP fibers innervating ventromedial hypothalamic nucleus (Batten and Cambre, 1989) support a direct effect on hypothalamus probably in a paracrine or autocrine manner (Martínez-Álvarez et al., 2009).

HYPOTHALAMIC INTEGRATION OF CIRCADIAN INFORMATION

Hypothalamus and Peripheral Circadian Clocks

Feeding shows a rhythmic pattern in a widespread of fish (Madrid et al., 2001), occurring periodically through the 24-h light/dark cycle, but tidal, lunar and seasonal feeding rhythms are also been described in some species (López-Olmeda and Sánchez-Vázquez, 2010). These feeding rhythms, as many other aspects of energy metabolism and homeostatic balance, are coordinated by the phylogenetically well-conserved circadian system, which generates an internal timing that allow animals to anticipate cyclic environmental and endogenous variations, and then, prepare the physiology for upcoming challenges. In the functional organization of circadian system, multiple endogenous clocks, synchronized by environmental and endogenous cues, generate an internal rhythmicity close to 24 h (Albrecht, 2012). The hypothalamus plays a key role in the circadian timekeeping system of vertebrates, acting as an integrative neuronal network. In mammals, a bilateral structure in the anterior hypothalamus, the suprachiasmatic nucleus (SCN), is an autonomously rhythmic nucleus that drives overt circadian rhythms in behavior and physiology, including food intake (Partch et al., 2014). The SCN is formed by clusters of interconnected neurons with individual molecular clock mechanisms inside each cell that function as an autonomous oscillator, and the intercelular coupling among these individual cells is critical for the coordination of endogenous rhythmicity and the outputs to downstream tissues (Evans and Gorman, 2016). Additional neuronal clocks are also present in ARC, ventromedial hypothalamus, and the dorsomedial hypothalamus, and in a variety of peripheral tissues, including liver, adipose tissue, adrenal and muscle (Dibner et al., 2010). In mammals, it is widely accepted that the internal coordination of circadian rhythmicity is established by the temporal signals provided by the master SCN to other hypothalamic nuclei and peripheral organs through a wide variety of direct and indirect pathways that form a network of oscillators. Fish hypothalamus also contains circadian oscillators (Velarde et al., 2009; Patiño et al., 2011; Vatine et al., 2011; Idda et al., 2012; Martín-Robles et al., 2012; Nisembaum et al., 2012; Vera et al., 2013), but no master clock has been yet identified. In contrast, the functional relevance of endogenous clocks in a variety of fish tissues supports the existence of a multiple network of endogenous oscillators in the brain (hypothalamus, diencephalon, pineal, retina, optic tectum, pituitary) and peripheral (liver, gut, gonads, head kidney) locations (Isorna et al., 2017). A summary of the main components in the fish circadian organization is shown in **Figure 3**.

The key molecules that establish the functional core of self-sustained circadian oscillators clocks are highly conserved from invertebrates to mammals, including fish (Partch et al., 2014), although important differences are found in fish regarding the additional number of copies of the clock genes resulting from the 3R teleost-specific genome duplication. The generation of

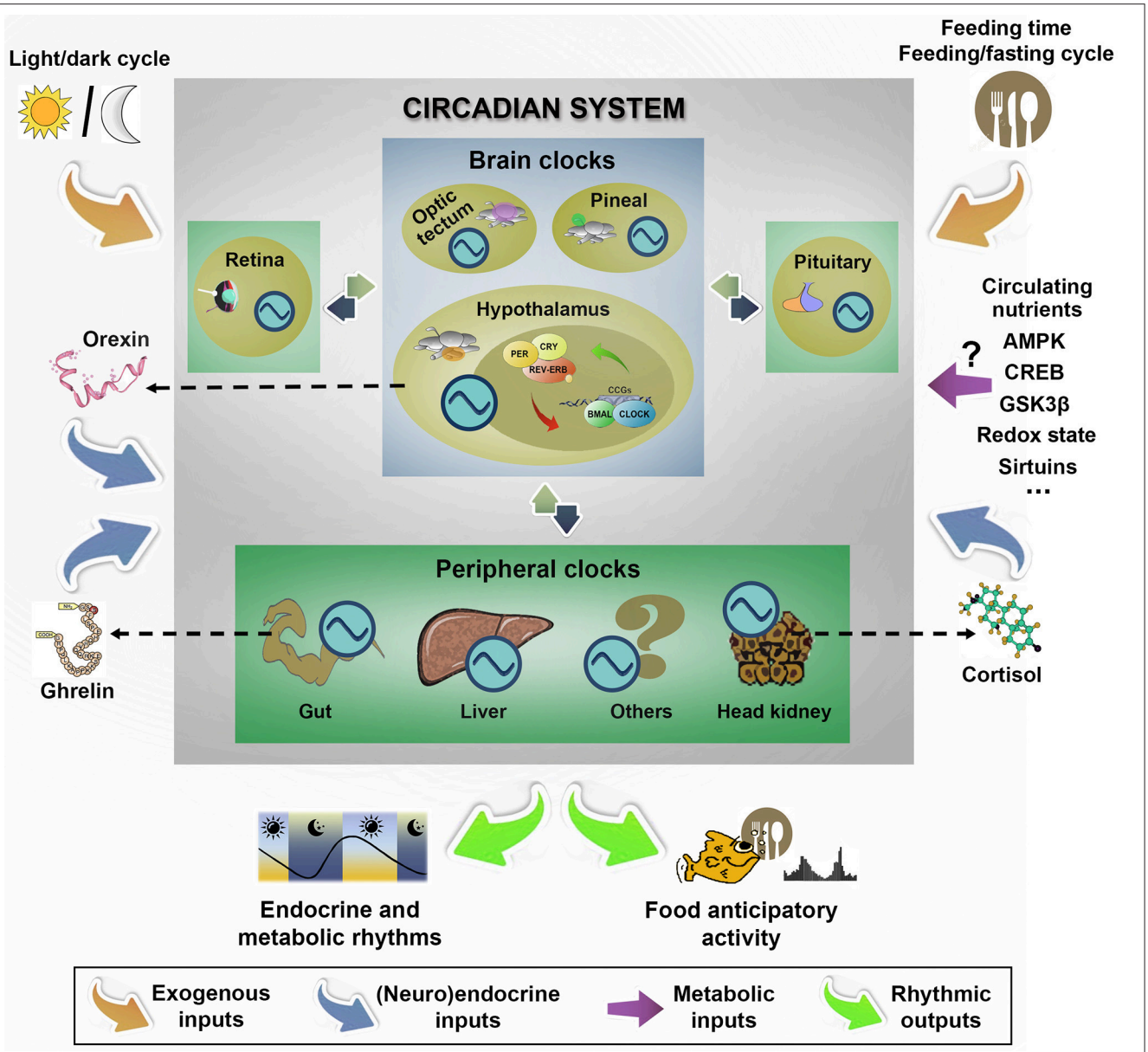


FIGURE 3 | Hallmarks of functional organization of circadian system in fish emphasizing the main external and endogenous inputs that synchronizes brain and peripheral clocks to generate rhythmic outputs, and particularly, the daily food anticipatory activity and food intake. In fish, the lack of a master clock in the functional circadian system organization shapes a more flexible model with different intercommunicated clocks located in many tissues throughout the organism. As external inputs, the light/dark cycle is one of the best characterized, but other time cues (such as feeding time and feeding/fasting cycle) are the dominant exogenous synchronizers of peripheral clocks. The possible role of circulating nutrients and some central and peripheral regulatory signals (such as orexin, ghrelin or cortisol) is discussed in the text. ?, Unknown; AMPK, AMP-activated protein kinase; CREB, cAMP response-element binding protein; GSK3β, Glycogen synthase kinase 3 beta. Clock genes: PER, CRY, REV-ERB, BMAL, CLOCK, CCG. Circadian oscillator: ∿.

cellular circadian oscillations is based on autoregulatory 24-h transcriptional/translational feedback loops of a set of clock genes (Ko and Takahashi, 2006). The proteins BMAL1, CLOCK and its analog NPAS2 act as transcriptional activators via E-box sequences driving the expression of clock controlled genes, some of them encoding other core clock protein repressors (PER and CRY). These PER and CRY proteins form complexes that are translocated into the nucleus where they shut down their own expression by removing the CLOCK(NPAS2)/BMAL1 complexes, defining a main negative loop. Some reinforcing loops are formed by the orphan nuclear receptors from the REV-ERBα-β and RORα-β families, to modulate the rhythmic transcription of *Bmal1*, *Npas* and *Clock* (Guillaumond et al., 2005; Schibler et al., 2015). Recent studies demonstrated that such oscillations in clock genes expression paralleled critical events of chromatin remodeling (Coomans et al., 2015; Masri et al., 2015).

Feeding as an Entrainment Cue for the Circadian Clocks

The light/dark cycle is the most effective signal that entrains the brain circadian clocks (Schibler et al., 2015). However, feeding related cues, including feeding time, nutritional inputs, feeding/fasting cycle and diet composition are the dominant synchronizers of peripheral clocks. Such so-called food-entrained oscillators provide animals the adaptive advantage of anticipate periodic meals within a circadian period of entrainment (Stephan, 2002; Mendoza, 2006). The food-entrained oscillators constitute the basis of the food anticipatory activity, an increase in locomotor activity occurring just before feeding time in anticipation of a predictable daily meal. This activity was evidenced in fish (López-Olmeda and Sánchez-Vázquez, 2010; Feliciano et al., 2011; Vera et al., 2013) and mammals (Mistlberger, 2009), and appears to be independent on hypothalamic SCN in mammals (Stephan, 2002) and hypothalamic clocks in fish (Velarde et al., 2009). To date, the endogenous control of the food anticipatory activity is largely unknown, but recent studies suggest that some orexigenic hormones, such as ghrelin, may be involved both in fish (Nisembaum et al., 2014b) and in mammals (Patton and Mistlberger, 2013).

The location of food-entrained oscillators is not yet certain. Neuronal feeding-synchronized clocks are suggested to reside in ventromedial and dorsomedial hypothalamus (Acosta-Galvan et al., 2011), or even in extrahypothalamic brain areas including *nucleus accumbens* and amygdala in mammals. The search and identification of feeding entrained clocks is a topical subject in fish, and both, peripheral (Nisembaum et al., 2012; Sánchez-Bretaño et al., 2015a) and brain (Feliciano et al., 2011; Vera et al., 2013; Sánchez-Bretaño et al., 2015b) are possible targets. This complex variety of locations of oscillators requires a functional organization among them to generate coordinated responses, and recent data suggest that nutritional cues may act as time givers (Dattolo et al., 2016).

Food intake is a potent phase-resetting agent for peripheral and central clocks, by entraining a number of overt rhythms in animals. The molecular mechanism underlying feeding entrainment remains largely unknown. In mammals, feeding modifies the phase of molecular oscillations in ARC and dorsomedial hypothalamic nuclei independently on the hypothalamic SCN (Feillet et al., 2008). In fish, a daily feeding schedule synchronizes rhythmicity of central (hypothalamus and optic tectum) and peripheral (gut and liver) clock genes (Feliciano et al., 2011; Nisembaum et al., 2012; Vera et al., 2013). The nature of the endogenous signals associated with feeding that may be capable of resetting peripheral clocks is not fully characterized yet, but metabolites, nutrient sensors, hormones, and possibly neuronal signals transmitted from nutrient-sensing areas to peripheral organs are good candidates.

Metabolic Signals and Circadian Clocks

The circadian system controls the expression of essential genes in numerous metabolic pathways (Ribas-Latre and Eckel-Mahan, 2016), but, in turns, it can be synchronized by its own effectors, generating a bidirectional relationship among the circadian clocks and metabolic signals (Challet, 2013).

Little is known about how specific diet components can modulate the circadian clocks, although it appears that periodic availability of circulating nutrients induces the feeding dependent resetting of peripheral and central circadian networks (Asher and Sassone-Corsi, 2015). In this context, ARC is an important target for such metabolic feedback (Uchida et al., 2016). Certain populations of neurons in ARC show rhythmicity in mammals (Ellis et al., 2008), with circadian rhythms of PER2 *in vitro* (Guilding et al., 2009). The circadian modulation of ARC neurons by circulating metabolites support the timing activation of this center for sensory metabolic information (Van den Top and Spanswick, 2006). The fact that ARC is also able to influence the functionality of the SCN (Yi et al., 2006), suggest that this ARC-SCN reciprocal interaction is essential to maintain a well-balanced metabolic circadian pattern.

Nutrient sensors evolved as time-giving signals by exerting different actions on the circadian clocks. This is the case of AMPK, regulated by glucose and fatty acids, which directly regulates CRYs proteins (Lamia et al., 2009), CREB, glycogen synthase kinase 3 beta, PPARs, and the redox state (Oosterman et al., 2015; Ribas-Latre and Eckel-Mahan, 2016). One interesting link between sensing of cellular energy status and circadian clocks is the control of clock genes expression and deacetylation of clock proteins by the sirtuins (SIRT), a (NAD^+)-dependent class III of histone deacetylases that are well characterized by their numerous effects on intracellular metabolism. Particularly, the SIRT1 and SIRT6 establish functional links between cellular metabolism and circadian clocks physiology in mammals (Masri et al., 2014; Orozco-Solis et al., 2015). The activity of SIRT1 fluctuates through the feeding/fasting cycle (Cakir et al., 2009) and is involved in the cyclic control of cofactors and peptides of circadian clocks by deacetylating BMAL1 and PER2 in liver (Nakahata et al., 2008) and activates the hypothalamic SCN pacemaker in mice (Chang and Guarente, 2013). The high expression of this enzyme in the ventromedial hypothalamus support the role of this brain area on monitoring metabolic signals through SIRT1, at least in mice. Interestingly, these ventromedial neurons expressing SIRT1 project POMC neurons in the ARC, and innervate the outer shell of SCN, providing a basis to integrate and communicate metabolic signals in the hypothalamus (Orozco-Solis et al., 2015). SIRT6 appears to be involved in the cyclic synthesis of lipids and carbohydrates (Masri et al., 2014), which may reflect differential responses to distinct nutritional intakes. Whether these molecular sensors modulate brain and peripheral clocks in fish remains to be elucidated. Preliminary studies in rainbow trout assessed changes in mRNA abundance of SIRT-1 in parallel with the functioning of fatty acid sensing systems (Velasco et al., 2016a,b). The clearly adaptive significance of circadian clocks entrainment by nutrient status is supported in fishes by the feeding-related rhythms driven by the circadian system in digestive enzymes (Vera et al., 2007; Montoya et al., 2010; Nisembaum et al., 2014b). Furthermore, changes described in parameters related to glucose, fatty acid, and amino acid metabolism in brain (Polakof et al., 2007c) and peripheral tissues (Polakof et al., 2007d;

Hernández-Pérez et al., 2015) also support the involvement of nutrient status.

Endocrine Signals in the Entrainment of Circadian Clocks

It is widely accepted that daily endocrine rhythms are outputs of the circadian system, but recent reports suggest that several hormones may act as inputs in the timing signalization of hypothalamic and peripheral clocks (Challet, 2015; Coomans et al., 2015).

Ghrelin may prove to be an endocrine signal that participates in the integration of gastrointestinal signals by the central clocks. The rhythmic expression of ghrelin transcript in hypothalamus, pituitary and gut (Sánchez-Bretaño et al., 2015b), and the periprandial variations in plasma of goldfish (Blanco et al., 2016a), and in hypothalamus of tilapia, zebrafish and goldfish (Peddu et al., 2009; Blanco et al., 2016b) indicate that ghrelin may work as an output of food-entrained oscillators. Ghrelin receptors are widely expressed in fish brain (Chen et al., 2008; Sánchez-Bretaño et al., 2015b), including those hypothalamic areas involved in the integration of food intake control, as well as the preoptic region and the anterior periventricular nucleus (Kaiya et al., 2010; Sánchez-Bretaño et al., 2015b). The expression of clock genes in these hypothalamic areas (Mazurais et al., 2000; Sánchez-Bretaño et al., 2015c) and the induction of *per* genes by ghrelin (Nisembaum et al., 2014b) support the role of ghrelin as an input of food-entrained clocks. *In vivo* (Nisembaum et al., 2014b) and *in vitro* (Sánchez-Bretaño et al., 2016b) studies carried out in goldfish reinforce the role of ghrelin in the feeding and metabolism-related signaling in hypothalamic and liver clocks. Furthermore, some reports indicate that this hormone may also participate in the regulation of food anticipatory activity in goldfish, as it modifies locomotor activity (Matsuda et al., 2006a), and this locomotor activity is blocked by a ghrelin antagonist (Nisembaum et al., 2014b).

The orexin/hypocretin system is involved in the coordination of rhythmic daily functions, such as feeding and energy balance in fish (Matsuda et al., 2012a). Taking in mind the orexigenic role of this regulator and its stimulatory effect on daily activity rhythms in the absence of other inputs (Nisembaum et al., 2014a), the possible role of orexin system in the physiology of certain brain areas involved in food intake and circadian regulation is intriguing. Orexin fibers found in some central oscillators, such as the pineal and the SCN, and other brain regions related to the regulation of activity/rest cycles (Wong et al., 2011; López et al., 2014). As a rule, orexins exert excitatory effects on various brain nuclei with the exception of a direct hyperpolarisation of clock cells in SCN during the night (Belle et al., 2014).

The orexin system is under the control of the molecular clock, as orexin neurons projecting into the pineal show circadian daily rhythmicity driven by the clock (Appelbaum et al., 2010), and daily variations in the hypothalamic expression of orexin in goldfish related to clock genes oscillations (Hoskins and Volkoff, 2012). Nevertheless, recent studies suggest that this peptidergic system also acts as an input to the clockworks. Particularly, this peptide seems to play an important role in food intake anticipation in fish, as its central administration up-regulates *per* genes in hypothalamus (central) and foregut (peripheral) clocks (Nisembaum et al., 2014a). The induction of *per* genes by orexin, as produced ghrelin, questions the existence of synergy between both orexigenic regulators in their actions on the food-entrained clocks.

Glucocorticoids display strong daily rhythms in vertebrates including fish. Cortisol daily rhythms are clear outputs of the circadian system, which are synchronized by the feeding-fasting cycle and feeding time in fish (Isorna et al., 2017). However, little is known about the possible role of glucocorticoids as inputs to the circadian system in fish. Recent reports in goldfish show that cortisol induces *per1a* and *per1b* expression, and represses the positive elements (*bmal1a* and *clock*) in liver clockwork (Sánchez-Bretaño et al., 2016a). Interestingly, such actions of cortisol on the liver, one of the most sensitive peripheral clockwork, link the entrainment of food-entrained clocks by both, metabolic signals and hormones.

HYPOTHALAMIC INTEGRATION OF OTHER FACTORS

Stress

The endocrine stress response in fish is mediated by both the hypothalamic-sympathetic chromaffin cell and the HPI axes. The activation of both systems restores homeostasis by mobilizing fuel to make energy available to cope with increased metabolic demand (Wenderlar Bonga, 1997; Mommsen et al., 1999). A disruption of the feeding behavior is a common feature of the behavioral response to stress in fish (Bernier and Peter, 2001; Bernier, 2006). The activation of HPI in response to stress involves the synthesis of corticotrophin relasing factor (CRF) in the neurons of the preotic area of the CNS that, in turn, stimulates release of adrenocorticotropic hormone (ACTH) from the corticotrophic cells in the adenohypophysis. ACTH binds to the MC2R in the surface of the interrenal cells of the head kidney to stimulate glucocorticoid release into the blood (Wenderlar Bonga, 1997; Mommsen et al., 1999). Cortisol, the main glucocorticoid in fish mediates many effects of stressors on metabolic and behavioral processes (Barton, 2002; Bernier, 2006; Aluru and Vijayan, 2009).

In the fish brain, CRF is abundantly expressed within the dorsal telencephalic structures but also in the preoptic area and tuberal hypothalamus of zebrafish (Alderman and Bernier, 2007). ICV administration induces a dose-dependent reduction in food intake levels in goldfish (De Pedro et al., 1993; Bernier, 2006) that is reverted by the injection of receptor antagonist (De Pedro et al., 1997; Bernier and Peter, 2001). Goldfish treated with glucocorticoid antagonists or inhibitors of cortisol synthesis, display increased CRF mRNA brain levels and reduced feeding, which again can be reverted by CRF receptor antagonist (Bernier and Peter, 2001). It is suggested that CRF is a major physiological transductor of stress effects on food intake in fish (Bernier, 2006). In addition, CRF seems also to regulate social effects since its increased expression is responsible of the reduced feeding levels in subordinate fish (Doyon et al., 2003).

Recent studies demonstrated that MC4R is able to bind ACTH in the presence of the melanocortin receptor accessory protein 2 in zebrafish. In addition, ACTH administration inhibits food intake in zebrafish but only in animals carrying a functional copy of MC4R (Agulleiro et al., 2013). Together with the ACTH production in carp brain (Metz et al., 1994) this suggests that ACTH can also transduce stress information into feeding circuitry downstream of the melanocortin system. In addition, MC4R is highly expressed in the magnocellular neurons of the preoptic area where CRF is synthesized thus suggesting that central melanocortins could modulate the activity of the CRF neurons (Cerdá-Reverter et al., 2003a; Sánchez et al., 2009). Stress conditions not only regulate CRF and melanocortinergic pathways in fish as the activity of many other central systems vary with stressful conditions and/or increased glucocorticoid signaling. Changes in the activity of central monoaminergic systems including serotoninergic and dopaminergic systems occurred after stressful conditions. These central monoaminergic systems can play a key role in the integration of information during the exposition to the stressor but also in organizing the coordinated stress response (Lanfumey et al., 2008). Studies in rainbow trout demonstrated that the severity of the stressors were able to produce a rated stress response, but serotonin central levels were not accordingly gradated suggesting that some other systems must be integrating the magnitude of the stress response (Gesto et al., 2015). Chronic treatment with fluoxetine (inhibitor of serotonin recapture) is able to reduce whole body cortisol levels and display anxiolytic effects in zebrafish (Egan et al., 2009) but, on the contrary, acute stress induced a rapid increase of serotoninergic and dopaminergic activity in the forebrain of rainbow trout (Gesto et al., 2013). Accordingly, both dopamine (Leal et al., 2009) and serotonin (Ortega et al., 2013) inhibit food intake in fish supporting both dopaminergic and serotoninergic pathways as neuroanatomical substrates for the integration of stress effects on behavioral pathways regulating food intake.

The reduced food intake observed in response to stress in fish could also relate to changes in the ability of nutrient sensing systems to modulate food intake. In rainbow trout chronic stress induced a readjustment in the activity of hypothalamic glucosensing mechanisms (Conde-Sieira et al., 2010a; Otero-Rodiño et al., 2015). The readjustment resulted in an inability of the fish to compensate with changes in food intake those of circulating glucose levels as observed in non-stressed fish. The response of hypothalamic mRNA abundance of CART, POMC, and NPY to glucose changed under stress conditions (Conde-Sieira et al., 2010a; Otero-Rodiño et al., 2015). CRF might be involved in the mechanism through which stress influences food intake control (Evans et al., 2004; McCrimmon et al., 2006). Accordingly, CRF treatment of rainbow trout hypothalamus *in vitro* readjusted glucosensing mechanisms (Conde-Sieira et al., 2011) in a way similar to that observed under stress conditions (Conde-Sieira et al., 2010a).

Orexins

The orexins/hypocretins are neuropeptides belonging to the incretin gene family of peptides and are involved in many physiological processes in fish, including feeding (Hoskins and Volkoff, 2012), locomotion/rest cycles (Volkoff, 2012; Nakamachi et al., 2014; Nisembaum et al., 2014a) and reproduction (Wong et al., 2011). The cDNA sequences encoding for preproorexin in fish contains orexin A and orexin B, with a high degree of homology with other orexins in vertebrates (Kaslin et al., 2004). Orexins are present in numerous fish species with a broad distribution. Thus, orexin ir-cell bodies, preproorexin mRNA, and orexin proteins are abundant in hypothalamus and preoptic area of most species so far studied (Volkoff, 2015), areas associated with the control of food intake in fish.

Orexins stimulate food intake in all fishes investigated so far. Earlier studies demonstrated this effect in goldfish (Volkoff et al., 1999; Facciolo et al., 2011; Matsuda et al., 2012a), and zebrafish (Novak et al., 2005; Yokobori et al., 2012). Currently, it is generally accepted that orexins are potent orexigenic regulators in fish (Hoskins and Volkoff, 2012; Matsuda et al., 2012a; Penney and Volkoff, 2014). The stimulation of appetite by orexin injections in many of the species studied is consistent with increases in preproorexin brain mRNA expression levels displayed after different periods of food deprivation (Novak et al., 2005; Abott and Volkoff, 2011; Volkoff, 2014). In goldfish, the presence of orexin-like-ir in the hypothalamic *nucleus recessus lateralis* is induced by fasting condition (Nakamachi et al., 2006). These stimulations of the hypothalamic orexin system by fasting suggest that this peptide play a role in long-term regulation of feeding in fish. Thus, short-term periprandial changes in the expression of orexin occurred in fishes. Orexin expression in the brain increases 1 h before the scheduled mealtime in Mexican blind cavefish (Wall and Volkoff, 2013); and hypothalamic preproorexin expression peaks around mealtime, and decreases after feeding in Atlantic cod (Xu and Volkoff, 2007) and orange-spotted grouper (Yan et al., 2011). These results suggest that orexin may be a signal preceding food supply under scheduled feeding conditions. Recently, it has been shown that orexin-A enhances locomotor activity in goldfish (Nakamachi et al., 2014), and synchronizes locomotor activity in goldfish maintained under 24 L and fasting conditions (Nisembaum et al., 2014a), which allowed authors to suggest that orexin might mediate the locomotor activity entrainment by food schedule in this teleost.

Orexin system is a good target to investigate possible interactions among food intake regulators. Central administration of orexin increases the expression of hypothalamic NPY in goldfish (Volkoff and Peter, 2001b; Nisembaum et al., 2014a), and in the orange spotted grouper (Yan et al., 2011). Desensitizing the orexin system by treatment with high doses of orexin A results in a decrease in NPY-induced feeding (Volkoff and Peter, 2001b), while the blocking of NPY receptors reduces feeding induced by orexin A (Kojima et al., 2009). In addition, ICV co-administration of orexin-A and NPY results in a synergistic orexigenic effect in goldfish (Volkoff and Peter, 2001b; Volkoff et al., 2003). Thus, a functional interdependence may exist between orexin-A and NPY in the stimulation of food intake. On the other hand, ICV orexin-A treatment stimulates ghrelin mRNA 1 h post-injection in goldfish foregut. This agrees with the increase of diencephalic ghrelin induced by orexin-A in this teleost (Miura et al., 2007), and demonstrates that the interaction between these two peptides takes place not only into the hypothalamus, but also suggests a brain-gut communication (Unniappan et al., 2004; Nakamachi

et al., 2006). Neuroanatomical evidences support the interactions above described among orexin-A and other appetite-regulating peptides, as NPY and ghrelin (Volkoff et al., 2003; Miura et al., 2007). Recent studies suggested a role of tyrosine hydroxylase in the regulation of food intake and locomotor activity in vertebrates (Wall and Volkoff, 2013). The increased mRNA expression levels of this enzyme induced by fasting or orexin in cavefish may suggest the involvement of catecholamines in the actions of orexin (Abott and Volkoff, 2011).

Melanin-Concentrating Hormone (MCH)

Melanin-concentrating hormone (MCH) characterized in chum salmon as a circulating-cyclic heptadecapeptide involved in color change (Kawauchi et al., 1983). MCH is mainly produced within the tuberal hypothalamus, stored in the pituitary neural lobe, and released under adaptation to a light-colored background (Cerdá-Reverter and Canosa, 2009). MCH is a potent orexigenic factor inducing weight gain in mammals (Qu et al., 1996). In fish, however, contradictory results were obtained, such as in goldfish (Matsuda et al., 2006c). Data reported in barfin flounder showed increased hypothalamic MCH expression after food deprivation (Takahasi et al., 2004) and enhanced somatic growth after white background adaptation (Yamanome et al., 2005). Transgenic medaka overexpressing MCH exhibit lightened body color, but development, growth, feeding behavior and reproduction do not remarkably differ from non-transgenic siblings (Kinoshita et al., 2001).

CONCLUSIONS

We aimed with this review to show readers the existing information about the way in which fish hypothalamus integrates information of metabolic, endocrine, and circadian nature to elicit a coordinated food intake response. Compared with the mammalian models, the available studies in fish are more limited resulting in the lack of knowledge in several important aspects of hypothalamic integration. Thus, we have almost no idea about how mechanisms governing food intake operate in the long-term since the available information in fish refers to mechanisms operating at short-term periods. The absence of knowledge is also relevant in terms of characterizing the hypothalamic specific nuclei involved in the process of integration of information as well as in the relationship among cells in those nuclei. In this way, almost all available studies in fish assessed the whole hypothalamus, and this may result in differences in the amount and type of neurons assessed. The large diversity of fish species is not sufficiently covered in the available studies, which in some cases (metabolic control) rely on a handful of species. The possible presence and functioning of amino acid sensing systems in fish, as well as the elucidation of signaling pathways linking activity of sensors with the effectors controlling homeostasis are also open questions demanding further research.

Even in the short-term mechanisms comparable to those known in mammals regarding the existence of neurons involved, presence of nutrient sensing systems and modulatory effects of hormones, the specific responses obtained are not identical to those observed in mammals. In some cases, these differences may relate to the limited available studies in fish precluding us to formulate clear hypothesis. Moreover, other factors might be involved, and we can only speculate about some of them. For instance, the activation of fatty acid sensing systems in mammalian hypothalamus relates to the thermogenic capacity of brown adipose tissue (Contreras et al., 2016), i.e., one of the mechanisms involved in thermoregulation that is not present in fish. Accordingly, the reduced energy expenditure in fish may relate to differential food intake regulation as suggested in a recent review (Van de Pol et al., 2017). A second reason might relate to the gene duplication present in actinopterygians resulting in multiple isoforms of neuropeptides, transporters, enzymes, etc. Accordingly, different subfunctions or different functions may attribute to different isoforms in fish, and this might explain some of the differential effects of hormones when comparing fish and mammals, but this has been evaluated only in a few cases. A third reason may relate to the fact that since fish live in a great variety of habitats they also display many species-specific adaptations such as those related to feeding behavior. Fish eating habits (carnivores, omnivores or herbivores) may be responsible of specific differences in gastrointestinal morphology and hormone functions, and this could be responsible (at least in part) of the differences observed when comparing responses among fish species. Finally, many fish species, including several of the models studied such as rainbow trout, are carnivorous, which is in contrast with the mammalian models studied so far (almost all of them omnivorus/herbivorous species). Accordingly, several of the differences in food intake regulation might relate to this fact. However, this is probably not so simple since the scarce data available in carnivorous mammals (Batchelor et al., 2011) also display differential responses compared with carnivorous fish.

The research carried out in the field in recent years provided evidence for several of the mechanisms involved, though certainly more research is needed, especially to ascertain the interactions among different regulatory mechanisms as well as to establish what are the intracellular common mechanisms through which the information is integrated to elicit a food intake response. Ongoing research in our groups, as well as in many others, will provide responses in the near future.

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All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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The Neuroendocrine Regulation of Food Intake in Fish: A Review of Current Knowledge

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Fish are the most diversified group of vertebrates and, although progress has been made in the past years, only relatively few fish species have been examined to date, with regards to the endocrine regulation of feeding in fish. In fish, as in mammals, feeding behavior is ultimately regulated by central effectors within feeding centers of the brain, which receive and process information from endocrine signals from both brain and peripheral tissues. Although basic endocrine mechanisms regulating feeding appear to be conserved among vertebrates, major physiological differences between fish and mammals and the diversity of fish, in particular in regard to feeding habits, digestive tract anatomy and physiology, suggest the existence of fish- and species-specific regulating mechanisms. This review provides an overview of hormones known to regulate food intake in fish, emphasizing on major hormones and the main fish groups studied to date.

Keywords: fish, hormones, feeding, appetite, diversity, brain, intestine

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INTRODUCTION

Feeding is a complex behavior consisting of food ingestion itself as well as foraging or appetitive behaviors (which reflect motivation to consume food; Keen-Rhinehart et al., 2013; Woods and Begg, 2016). Feeding is ultimately regulated by central feeding centers of the brain, which receive and process information from endocrine signals from both brain and periphery. These signals consist of hormones that increase (e.g., orexin; neuropeptide Y-NPY) or inhibit, (e.g., cocaine and amphetamine regulated transcript-CART; proopiomelanocortin-POMC) feeding. Feeding centers are also influenced by metabolic and neural peripheral signals providing information on meal ingestion and nutritional status (Volkoff, 2006; Volkoff et al., 2009a,b; Rui, 2013; Sobrino Crespo et al., 2014).

Fish are the most diversified group of vertebrates, with 33,200 species identified to date (FishBase, 2016), the bony fish (teleosts) containing more than half of all vertebrate species (Nelson, 2006). However, only relatively few fish species have been examined to date, with regards to their physiology, in particular feeding. The large numbers of fish species, habitats, feeding habits and digestive tract anatomy and physiology, as well as the number of extrinsic and intrinsic factors affecting feeding behavior and physiology (Volkoff et al., 2009a; Hoskins and Volkoff, 2012) most probably result in complex species-specific feeding regulating mechanisms in fish, with a number of hormones and tissues involved.

Research on the endocrine regulation of feeding in fish has progressed in recent years. New fish appetite-regulating hormones and species other than traditional models (such as goldfish, salmon and zebrafish) are gradually being examined. In addition, traditional techniques such as brain lesions and injections and biochemical purification of peptides, although still useful and being used,

have been complemented by new approaches such as gene expression studies, quantitative PCR, genomics (microarrays, RNA-seq), proteomics and metabolomics, transgenesis, gene knockout and silencing, and *in vitro* (cell and tissue culture, perifusion) studies.

The field of fish feeding endocrine physiology is evolving very rapidly and up-to-date reviews are often lacking. One of the first reviews on the endocrine regulation of feeding by R.E. Peter in 1979 (Peter, 1979) mostly focused on growth and growth hormone (GH) but predicted regions of the brain that might be responsible for feeding regulation in fish. In 1986, Matty's review described early data on the effects of GH, thyroid hormones, insulin, and gonadal steroids on feeding (Matty, 1986). Ten years later, Le Bail and Boeuf's review formulated hypotheses on mammalian hormones (e.g., leptin) that might putatively regulate feeding in fish (Le Bail and Boeuf, 1997). In the early twenty-first century, a number of reviews report recent advances on the field and include an increasing number of hormones (e.g., NPY, orexins, CART), some more comparative (Lin et al., 2000; de Pedro and Björnsson, 2001; Volkoff et al., 2005; Gorissen et al., 2006; Volkoff, 2011; Hoskins and Volkoff, 2012), some more focused on a single species (e.g., goldfish Matsuda, 2009; Matsuda et al., 2011a) or a particular group of fish (e.g., elasmobranchs Demski, 2012), some focused on growth (Won and Borski, 2013), and some on aquaculture and behavior (Papoutsoglou, 2012).

The purpose of this review is to provide an up-to-date, brief overview of the hormones regulating food intake in fish, emphasizing on recent studies, major brain hormones and the main fish groups studied thus far.

OVERVIEW OF REGULATION OF FOOD INTAKE

In fish, as in mammals (Sobrino Crespo et al., 2014), feeding behavior is regulated by specific regions in the brain, the so-called feeding centers. Early pioneer studies using stimulation and lesion experiments in teleosts (reviewed in Peter, 1979) and elasmobranchs (reviewed in Demski, 2012) seemed to indicate that the hypothalamic area was involved in feeding and that the brain control of feeding in fish might use mechanisms similar to those in mammals. However, whereas in mammals, the feeding centers appear to be restricted to the hypothalamus, evidence indicates that they might be more widespread in fish brains (Cerda-Reverter and Canosa, 2009).

Feeding centers are under the influence of hormones produced by the brain and the periphery. Neurohormones secreted by the brain, in particular the hypothalamic area, regulate energy balance by inhibiting (anorexigenic factors) or stimulating (orexigenic factors) feeding. Peripheral chemical (e.g., glucose) or endocrine (e.g., gastrointestinal hormones) factors released in the blood cross the blood brain barrier and have a direct action on feeding centers. Peripheral sensory information (mechanical or endocrine) carried by the vagus nerve can also affect feeding centers, via innervation from the brainstem (Volkoff, 2011).

HORMONES INVOLVED IN FOOD INTAKE

The list of hormones regulating feeding in vertebrates is long and increasingly so. Here, focus will be placed on major hormones and newly examined appetite-regulating factors (but not on their receptors), and the phylogeny of the fish species examined to date. **Table 1**, **Figure 1** summarize the hormones that have been examined in fish and their possible effects on feeding.

Major Appetite Regulating Factors

Central Orexigenic Factors

Agouti-related protein (or peptide, AgRP)

AgRP is a peptide released by hypothalamic NPY/AgRP neurons and is an endogenous antagonist of the melanocortin receptors MC3R and MC4R. AgRP plays a crucial role in the regulation of energy balance, as it increases food intake, by antagonizing the effects of the anorexigenic POMC product, α-melanocyte-stimulating hormone (α-MSH) (Sohn, 2015; Takeuchi, 2016).

In fish, AgRP has been identified in several species, including teleosts (e.g., goldfish *Carassius auratus* Cerdá-Reverter and Peter, 2003 and zebrafish *Danio rerio* Song et al., 2003, Atlantic salmon *Salmo salar* Murashita et al., 2009a, and seabass *Dicentrarchus labrax* Agulleiro et al., 2014, pufferfish *Takifugu rubripes* Klovins et al., 2004; Kurokawa et al., 2006), who have two genes products (AgRP1 and AgRP2; Cérda-Reverter et al., 2011) and Holocephali (Chimaeriforme, elephant fish *Callorhinchus milii* Västermark and Schioth, 2011).

AgRP appears to act as an orexigenic factor in Cypriniformes, as fasting increases hypothalamic *AgRP* expression in goldfish (Cerdá-Reverter and Peter, 2003), zebrafish (Song et al., 2003), and Ya fish *Schizothorax prenanti* (Wei et al., 2013). In addition, transgenic zebrafish overexpressing AgRP exhibit obesity, increased growth and adipocyte hypertrophy (Song and Cone, 2007). GH-transgenic common carp *Cyprinus carpio*, which display increased food intake, have higher hypothalamic AgRP1 mRNA expression levels than non-transgenic fish, further suggesting an orexigenic action (Zhong et al., 2013). However, this is contradicted by another study in carp showing that brain AgRP mRNA expression decreases after fasting and increases after re-feeding (Wan et al., 2012). In seabass (Perciforme), long-term fasting increases hypothalamic expression of AgRP1 but decreases that of AgRP2 (Agulleiro et al., 2014), suggesting an isoform-specific orexigenic action.

Within Salmoniformes, there is conflicting data with regards to the actions of AgRP. In Arctic charr *Salvelinus alpinus*, non-feeding fish have higher brain AgRP expression levels than feeding fish (Striberny et al., 2015) and transgenic coho salmon *Oncorhynchus kisutch*, which display increased feeding, have higher brain AgRP1 levels of mRNA than wild-type fish (Kim et al., 2015), suggesting an orexigenic role for AgRP. However, in Atlantic salmon, AgRP-1 brain mRNA levels decrease after fasting (Murashita et al., 2009a) and increase after feeding (Valen et al., 2011), rather pointing to an anorexigenic role.

Galanin

Galanin is a peptide expressed in both central nervous system and GIT, that regulates diverse physiological functions in

TABLE 1 | List of major hormones (in alphabetical order) potentially involved in the regulation of feeding in fish (by order, family and species studied).

Hormone	Order	Family	Species	Major references	Effect on feeding
AgRP	Cypriniforme	Cyprinidae	Common carp (*Cyprinus carpio*)	Zhong et al., 2013	+
			Common carp (*Cyprinus carpio*)	Wan et al., 2012	– ?
			Goldfish (*Carassius auratus)*	Cerdá-Reverter and Peter, 2003	+
			Ya fish (*Schizothorax prenanti*)	Wei et al., 2013	+
			Zebrafish (*Danio rerio)*	Song et al., 2003; Song and Cone, 2007	+
	Salmoniforme	Salmonidae	Atlantic salmon (*Salmo salar*)	Murashita et al., 2009a; Valen et al., 2011	–
			Arctic charr (*Salvelinus alpinus*)	Striberny et al., 2015	+
			Coho salmon (*Oncorhynchus kisutch)*	Kim et al., 2015	+
	Perciforme	Moronidae	Sea bass (*Dicentrarchus labrax*)	Agulleiro et al., 2014	+
Amylin	Cypriniforme	Cyprinidae	Goldfish (*Carassius auratus)*	Thavanathan and Volkoff, 2006	–
Apelin	Characiforme	Characidae	Blind cavefish (*Astyanax mexicanus)*	Penney and Volkoff, 2014	+
		Serrasalmidae	Red-bellied piranha (*Pygocentrus nattereri)*	Volkoff, 2014a	+
	Cyprinoforme	Cyprinidae	*Cyprinus carpio* and *Capoetta trutta*	Köprücü and Algül, 2015	?
			Goldfish (*Carassius auratus*)	Volkoff and Wyatt, 2009; Wong et al., 2013; Volkoff, 2014a; Zhang et al., 2016b	+
			Ya fish (*Schizothorax prenanti*)	Lin et al., 2014a	+
	Perciforme	Labridae	Cunner (*Tautogolabrus adspersus*)	Hayes and Volkoff, 2014	– ?
AVT (arginine vasotocin)	Salmoniforme	Salmonidae	Rainbow trout (*Oncorhynchus mykiss)*	Gesto et al., 2014	–
CART	Beloniformes	Adrianichthyidae	Medaka (*Oryzias latipes*)	Murashita and Kurokawa, 2011	–
	Characiforme	Characidae	Blind cavefish (*Astyanax mexicanus*)	Penney and Volkoff, 2014	–
			Dourado (*Salminus brasiliensis*)	Volkoff et al., 2016	0
		Serrasalmidae	Pacu (*Piaractus mesopotamicus*)	Volkoff et al., 2017	–
			Pirapitinga (*Piaractus brachypomus*)	Volkoff, 2015a	?
			Red-bellied piranha (*Pygocentrus nattereri*)	Volkoff, 2014a	–
	Cypriniforme	Cyprinidae	Common carp (*Cyprinus carpio*)	Wan et al., 2012	–
			Goldfish (*Carassius auratus*)	Abbott and Volkoff, 2011; Volkoff, 2012, 2014b; Zhang et al., 2016b	–
			Grass carp (*Ctenopharyngodon idellus*)	Zhou et al., 2013; Liu et al., 2014	–
			Zebrafish (*Danio rerio*)	Mukherjee et al., 2012; Nishio et al., 2012; Akash et al., 2014; Manuel et al., 2014, 2015; Libran-Perez et al., 2014; Woods et al., 2014; Guillot et al., 2016	–
	Gadiforme	Gadidae	Atlantic cod (*Gadus morhua*)	Kehoe and Volkoff, 2007	– ?
	Perciforme	Labridae	Cunner (Tautogolabrus adspersus)	Babichuk and Volkoff, 2013	–
	Pleuronectiformes	Pleuronectidae	Atlantic halibut (*Hippoglossus hippoglossus*)	Gomes et al., 2014	0
			Winter flounder (*Pseudopleuronectes americanus*)	MacDonald and Volkoff, 2009a	0
		Soleidae	Senegalese sole (*Solea senegalensis*)	Bonacic et al., 2015	–
	Rajiforme (elasmobranch)	Rajidae	Winter skate (*Raja ocellata*)	MacDonald and Volkoff, 2009b	0
	Salmoniforme	Salmonidae	Atlantic salmon (*Salmo salar*)	Murashita et al., 2009a; Burt et al., 2013; Kousoulaki et al., 2013	–
			Arctic Charr (*Salvelinus alpinus*)	Striberny et al., 2015	0
			Rainbow trout (*Oncorhynchus mykiss*)	Figueiredo-Silva et al., 2012; MacDonald et al., 2014	–

(Continued)

TABLE 1 | Continued

Hormone	Order	Family	Species	Major references	Effect on feeding
	Siluriforme	Clariidae	African sharptoothcatfish (*Clarias gariepinus*)	Subhedar et al., 2011	–
			Walking catfish (*Clarias batrachus*)	Barsagade et al., 2010	–
		Ictaluridae	Channel catfish (Ictalurus punctatus)	Kobayashi et al., 2008; Peterson et al., 2012	–
CCK	Characiforme	Characidae	Blind cavefish (Astyanax fasciatus mexicanus)	Wall and Volkoff, 2013	–
			Dourado (*Salminus brasiliensis)*	Pereira et al., 2015; Volkoff et al., 2016	–
			Thin dogfish (*Oligosarcus hepsetus)*	Vieira-Lopes et al., 2013	–
		Serrasalmidae	Pacu (*Piaractus mesopotamicus*)	Volkoff et al., 2017	–
			Pirapitinga (*Piaractus brachypomus)*	Volkoff, 2015a	–
			Red-bellied piranha (*Pygocentrus nattereri*)	Volkoff, 2014a	–
	Cypriniforme	Cyprinidae	Blunt snout bream (*Megalobrama amblycephala*)	Ping et al., 2013; Ji et al., 2015	–
			Common carp (*Cyprinus carpio*)	Zhong et al., 2013	–
			Goldfish (*Carassius auratus*)	Kang et al., 2010, 2011; Tinoco et al., 2015	–
			Grass carp (*Ctenopharyngodon idella*) juveniles	Liu et al., 2013	–
			Zebrafish (*Danio rerio*)	Koven and Schulte, 2012; Tian et al., 2015	–
	Gadiforme	Gadidae	Atlantic cod larvae (*Gadus morhua*)	Tillner et al., 2013	–
	Perciforme	Carangidae	Yellowtail (*Seriola quinqueradiata*)	Furutani et al., 2013; Hosomi et al., 2014	–
		Cichlidae	*Astatotilapia burtoni*	Grone et al., 2012	–
		Labridae	Cunner (*Tautogolabrus adspersus*)	Babichuk and Volkoff, 2013; Hayes and Volkoff, 2014	–
		Moronidae	Sea bass (*Dicentrarchus labrax*) larvae	Tillner et al., 2014	–
		Sciaenidae	Yellow croaker *(Larimichthys crocea*) larvae	Cai et al., 2015	–
		Sparidae	White sea bream (*Diplodus sargus)*	Micale et al., 2012	–
	Pleuronectiforme	Pleuronectidae	Atlantic halibut (*Hippoglossus hippoglossus*)	Kamisaka et al., 2001	–
			Olive flounder (*Paralichthys olivaceus*)	Kurokawa et al., 2000	–
			Winter flounder (*Pseudopleuronectes americanus*)	MacDonald and Volkoff, 2009a	–
	Salmoniforme	Salmonidae	Atlantic salmon (*Salmo salar*)	Valen et al., 2011	–
	Siluriforme	Ictaluridae	Channel catfish (*Ictalurus punctatus*)	Peterson et al., 2012	–
CRF/UCN system	Acipenseriformes	Acipenseridae	Siberian sturgeon (*Acipenser baerii*)	Zhang et al., 2016c	–
	Cypriniforme	Cyprinidae	Goldfish (*Carassius auratus*)	De Pedro et al., 1993	–
			Ya fish (*Schizothorax prenanti*)	Wang et al., 2014	–
	Salmoniforme	Salmonidae	Rainbow trout (*Oncorhynchus mykiss*)	Bernier and Craig, 2005; Ortega et al., 2013	–
Endocannabinoid system	Cypriniforme	Cyprinidae	Goldfish (*Carassius auratus*)	Cottone et al., 2013	+ ?
			Goldfish (*Carassius auratus)*	Cottone et al., 2009	+
	Perciforme	Sparidae	Sea bream (*Sparus aurata)*	Piccinetti et al., 2010	+
	Pleuronectiforme	Soleidae	Sole (*Solea solea*)	Palermo et al., 2013	?
Galanin	Cypriniforme	Cyprinidae	Goldfish (*Carassius auratus*)	de Pedro et al., 1995; Volkoff and Peter, 2001b; Unniappan et al., 2004	+
			Tench (*Tinca tinca*)	Guijarro et al., 1999	+
			Zebrafish (*Danio rerio*)	Li et al., 2013	+

(Continued)

TABLE 1 | Continued

Hormone	Order	Family	Species	Major references	Effect on feeding
Ghrelin	Anguilliformes	Anguillidae	Japanese eel (*Anguilla japonica*)	Lee et al., 2015	?
	Characiforme	Serrasalmidae	Pirapitinga (*Piaractus brachypomus*)	Volkoff, 2015a	?
			Red-bellied piranha (*Pygocentrus nattereri*)	Volkoff, 2015b	+
	Cypriniforme	Cyprinidae	Bunnei (*Barbus sharpeyi*)	Mabudi et al., 2011	?
			Gibel carp (*Carassius auratus gibelio*)	Zhou et al., 2016	+
			Goldfish (*Carassius auratus*)	Unniappan et al., 2002; Kang et al., 2011; Nisembaum et al., 2014; Blanco et al., 2016a,b	+
			Grass carp (*Ctenopharyngodon idella*)	Liu et al., 2014	+
			Zebrafish (*Danio rerio*)	Koven and Schulte, 2012	+
	Lepidosireniforme (dipnoid)	Protopteridae	West African lungfish (*Protopterus annectens*)	Kaiya et al., 2014	?
	Perciforme	Cichlidae	Tilapia (*Oreochromis mossambicus*)	Schwandt et al., 2010; Upton and Riley, 2013	+
		Scombridae	Pacific bluefin tuna (*Thunnus orientalis*)	Suda et al., 2012	?
	Pleuronectiforme	Pleuronectidae	Atlantic halibut (*Hippoglossus hippoglossus*)	Einarsdottir et al., 2011; Gomes et al., 2014	+
		Scophthalmidae	Juvenile turbot (*Scophthalmus maximus*)	Song et al., 2015	+
	Salmoniforme	Salmonidae	Atlantic salmon (*Salmo salar*)	Hevrøy et al., 2011; Vikesa et al., 2015	?
			Brown trout (*Salmo trutta*)	Tinoco et al., 2014a	+ ?
			Coho salmon (*Oncorhynchus kisutch)*	Kim et al., 2015	+
			Rainbow trout (*Oncorhynchus mykiss*)	Velasco et al., 2016	?
			Rainbow trout (*Oncorhynchus mykiss*)	Jönsson et al., 2010	–
	Siluriforme	Bagridae	Yellow catfish (*Pelteobagrus fulvidraco)*	Zhang et al., 2016a	+
		Ictaluridae	Channel catfish (*Ictalurus punctatus*)	Peterson et al., 2012	0
GnRH	Cypriniforme	Cyprinidae	Goldfish (*Carassius auratus*)	Hoskins et al., 2008; Matsuda et al., 2008	–
			Ya fish (*Schizothorax prenanti*)	Wang et al., 2014	–
			Zebrafish (*Danio rerio*)	Nishiguchi et al., 2012	–
	Gadiforme	Gadidae	Atlantic cod (*Gadus morhua*)	Tuziak and Volkoff, 2013a	0
	Pleuronectiforme	Pleuronectidae	Winter flounder (*Pseudopleuronectes americanus*)	Tuziak and Volkoff, 2013b	–
Kisspeptin	Cypriniforme	Cyprinidae	Goldfish (*Carassius auratus*)	Mawhinney, 2007	0
	Perciforme	Moronidae	Sea bass (*Dicentrarchus labrax*)	Escobar et al., 2016	+ ?
Leptin	Beloniforme	Adrianichthyidae	Medaka (*Oryzias latipes*)	Chisada et al., 2014	–
	Characiforme	Serrasalmidae	Pacu (*Piaractus mesopotamicus*)	Volkoff et al., 2017	0 ?
			Pirapitinga (*Piaractus brachypomus*)	Volkoff, 2015a	?
			Red-bellied piranha (*Pygocentrus nattereri*)	Volkoff, 2015b	0 ?
	Cypriniforme	Cyprinidae	Blunt snout bream *Megalobrama amblycephala*	Xu et al., 2016	–
			Common carp (Cyprinus carpio)	Bernier et al., 2012	–
			Goldfish (*Carassius auratus*)	Volkoff et al., 2003; Vivas et al., 2011; Tinoco et al., 2012, 2014b; Yan et al., 2016	–
			Grass carp (*Ctenopharyngodon idellus)*	Lu et al., 2015; Li et al., 2010	–
			Grass carp (*Ctenopharyngodon idellus*)	Li A. et al., 2016	?
			Topmouth culter (*Culter alburnus*)	Wang et al., 2013	?
			White-clouds minnow (Tanichthys albonubes)	Chen et al., 2016b	–
			Zebrafish (*Danio rerio*)	Tian et al., 2015; Cui et al., 2016; Michel et al., 2016	–

(Continued)

TABLE 1 | Continued

Hormone	Order	Family	Species	Major references	Effect on feeding
	Gadiforme	Lotidae	Burbot (*Lota lota*)	Nieminen et al., 2003	+ ?
	Gymnotiforme	Sternopygidae	Electric fish (*Eigenmannia virescens*)	Sinnett and Markham, 2015	?
	Perciforme	Carangidae	Golden pompano (*Trachinotus blochii*)	Wu et al., 2016	?
		Centrarchidae	Green sunfish (*Lepomis cyanellus*)	Johnson et al., 2000	+ ?
		Cichlidae	Tilapia (*Oreochromis mossambicus*)	Baltzegar et al., 2014; Douros et al., 2014	?
			Nile tilapia (*Oreochromis niloticus*)	Shpilman et al., 2014	?
		Labridae	Orange-spotted grouper (*Epinephelus coioides*)	Huang et al., 2014	?
		Labridae	Orange-spotted grouper (*Epinephelus coioides*)	Zhang et al., 2013	?
		Moronidae	European sea bass (*Dicentrarchus labrax*)	Gambardella et al., 2012	?
			Striped bass (*Morone saxatilis*)	Won et al., 2012	–
		Percichthyidae	Mandarin fish (*Siniperca chuatsi*)	Yuan et al., 2016	?
			Murray cod (*Maccullochella peelii peelii*)	Ettore et al., 2012 Varricchio et al., 2012	?
		Scombridae	Mackerel (*Scomber japonicus*)	Ohga et al., 2015	?
	Pleuronectiformes	Paralichthyidae	Fine flounder (*Paralichthys adspersus*)	Fuentes et al., 2012	+
	Salmoniforme	Salmonidae	Atlantic salmon (*Salmo salar*)	Rønnestad et al., 2010; Murashita et al., 2011; Trombley et al., 2012, 2014; Kullgren et al., 2013; Moen and Finn, 2013	?
			Arctic charr (*Salvelinus alpinus*)	Froiland et al., 2012; Jørgensen et al., 2013	?
			Rainbow trout (*Oncorhynchus mykiss*)	Gong et al., 2016	–
			Rainbow trout (*Oncorhynchus mykiss*)	Varricchio et al., 2012; Francis et al., 2014; MacDonald et al., 2014; Johansson and Björnsson, 2015; Salmeron et al., 2015; Johansson et al., 2016; Pfundt et al., 2016	?
	Siluriforme	Bagridae	Yellow catfish (*Pelteobagrus fulvidraco*)	Gong et al., 2013; Song et al., 2015; Zheng et al., 2015	?
MCH	Beloniforme	Adrianichthyidae	Medaka (*Oryzias latipes*)	Qu et al., 1996	0
	Carcharhiniforme	Sphyrnidae	Scalloped hammerhead shark (*Sphyrna lewini*)	Mizusawa et al., 2012	0
	Characiforme	Serrasalmidae	Red-bellied piranha (*Pygocentrus nattereri*)	Pérez Sirkin et al., 2013	?
	Cypriniforme	Cyprinidae	Goldfish (*Carassius auratus*)	Shimakura et al., 2006; Matsuda et al., 2007a	–
			Ya fish (*Schizothorax prenanti*)	Wang et al., 2016	+
	Gadiforme	Gadidae	Atlantic cod (*Gadus morhua*)	Tuziak and Volkoff, 2013a; Tuziak et al., 2014	+
	Pleuronectiforme	Pleuronectidae	Barfin flounder (*Verasper moseri*)	Takahashi et al., 2004; Amiya et al., 2008	+
			Olive flounder (*Paralichthys olivaceus*)	Kang and Kim, 2013b	+
			Starry flounder (*Platichthys stellatus*)	Kang and Kim, 2013a	+
			Winter flounder (*Pseudopleuronectes americanus*)	Tuziak and Volkoff, 2012	+
Nesfatin-1	Cypriniforme	Cyprinidae	Goldfish (*Carassius auratus*)	Gonzalez et al., 2010; Kerbel and Unniappan, 2012; Blanco et al., 2016a; Sundarrajan et al., 2016	–
			Zebrafish (*Danio rerio*)	Hatef et al., 2015	–
			Ya fish (*Schizothorax prenanti*)	Lin et al., 2014b	–
	Salmoniforme	Salmonidae	Rainbow trout (*Oncorhynchus mykiss*)	Caldwell et al., 2014	0
Neuromedin S	Cypriniforme	Cyprinidae	Zebrafish (*Danio rerio*)	Chen et al., 2016a	+ ?
Neuropepide B	Perciforme	Cichlidae	Nile tilapia (*Oreochromis niloticus*)	Yang et al., 2014	+ ?

(Continued)

TABLE 1 | Continued

Hormone	Order	Family	Species	Major references	Effect on feeding
NMU	Cypriniforme	Cyprinidae	Common carp (*Cyprinus carpio*)	Kono et al., 2012	?
	Cypriniforme	Cyprinidae	Goldfish (*Carassius auratus*)	Maruyama et al., 2008, 2009	–
	Perciforme	Labridae	Orange-spotted grouper (*Epinephelus coioides*)	Li et al., 2015	–
NPY	Characiforme	Characidae	Dourado (*Salminus brasiliensis*)	Pereira et al., 2015	+ ?
	Chimaeriformes	Callorhinchidae	Elephant fish (*Callorhinchus milii*)	Larsson et al., 2009	+ ?
	Cypriniforme	Cyprinidae	Blunt snout bream (*Megalobrama amblycephala*)	Xu et al., 2016	+
			Goldfish (*Carassius auratus*)	Lopez-Patino et al., 1999; de Pedro et al., 2000; Narnaware et al., 2000; Hoskins and Volkoff, 2012	+
			Grass carp (*Ctenopharyngodon idellus*)	He et al., 2013; Zhou et al., 2013; Jin et al., 2015	+
			Jian carp (*Cyprinus carpio* var. Jian)	Tang et al., 2014	+
			Ya fish (*Schizothorax prenanti*)	Wei et al., 2014	+
			Zebrafish (*Danio rerio*)	Yokobori et al., 2012; Dalmolin et al., 2015	+
	Gadiforme	Gadidae	Atlantic cod (*Gadus morhua*)	Kehoe and Volkoff, 2007; Tuziak et al., 2014	+
			Atlantic cod (*Gadus morhua*)	Kortner et al., 2011	?
	Gonorynchiforme actinopterygii	Chanidae	Milkfish (*Chanos chanos*)	Lin et al., 2016	?
	Perciforme	Carangidae	Yellowtail (*Seriola quinqueradiata*)	Hosomi et al., 2014	+
		Cichlidae	*Astatotilapia burtoni*	Grone et al., 2012	+
		Labridae	Cunner (*Tautogolabrus adspersus*)	Babichuk and Volkoff, 2013	+
			Orange-spotted grouper (*Epinephelus coioides*)	Tang et al., 2013	+
		Moronidae	Sea bass (*Dicentrarchus labrax*)	Leal et al., 2013	+
		Percichthyidae	Mandarin fish (*Siniperca chuatsi*)	Sun et al., 2014	?
		Rachycentridae	Cobia (*Rachycentron canadum*)	Van Nguyen et al., 2013	+
		Osphronemidae	Snakeskin gourami (*Trichogaster pectoralis*)	Boonanuntanasarn et al., 2012	+
	Pleuronectiforme	Pleuronectidae	Olive flounder (*Paralichthys olivaceus*)	Wang et al., 2015	+
		Paralichthyidae	Brazilian flounder (*Paralichthys orbignyanus*)	Campos et al., 2012	+
	Salmoniforme	Salmonidae	Atlantic salmon (*Salmo salar*)	Silverstein et al., 1998; Valen et al., 2011; Figueiredo-Silva et al., 2012	+
	Salmoniforme	Salmonidae	Chinook salmon (*Oncorhynchus tshawytscha*)	Silverstein et al., 1998	+
		Salmonidae	Rainbow trout (*Oncorhynchus mykiss*)	Aldegunde and Mancebo, 2006	+
	Siluriformes	Ictaluridae	Channel catfish (*Ictalurus punctatus*)	Silverstein and Plysetskaya, 2000; Peterson et al., 2012; Schroeter et al., 2015	+
	Tetraodontiformes	Tetraodontidae	Tiger Puffer (*Takifugu rubripes*)	Kamijo et al., 2011	+
Obestatin	Cypriniforme	Cyprinidae	Grass carp (*Ctenopharyngodon idellus*)	Yuan et al., 2015	– ?
Octadecaneuropeptide (ODN)	Cypriniforme	Cyprinidae	Goldfish (*Carassius auratus*)	Matsuda et al., 2007b, 2010, 2011b	–
Orexin	Characiforme	Characidae	Blind cavefish (*Astyanax fasciatus mexicanus*)	Wall and Volkoff, 2013; Penney and Volkoff, 2014	+
			Dourado (*Salminus brasiliensis*)	Volkoff et al., 2016	+
		Serrasalmidae	Pacu (*Piaractus mesopotamicus*)	Volkoff et al., 2017	+
			Pirapitinga (*Piaractus brachypomus*)	Volkoff, 2015a	?

(Continued)

TABLE 1 | Continued

Hormone	Order	Family	Species	Major references	Effect on feeding
			Red-bellied piranha (*Pygocentrus nattereri*)	Pérez Sirkin et al., 2013; Suzuki and Yamamoto, 2013; Volkoff, 2014a	+
	Cypriniforme	Cyprinidae	Goldfish (*Carassius auratus*)	Abbott and Volkoff, 2011; Facciolo et al., 2011; Hoskins and Volkoff, 2012; Crudo et al., 2013; Volkoff, 2013, 2014b; Nisembaum et al., 2014; D'angelo et al., 2016	+
			Zebrafish (*Danio rerio*)	Panula, 2010; Yokobori et al., 2011; Elbaz et al., 2012; Pavlidis et al., 2015; Sterling et al., 2015	+
	Gadiforme	Gadidae	Cod (*Gadus morhua*)	Tuziak et al., 2014; Le et al., 2016	+
	Perciforme	Cichlidae	*Astatotilapia burtoni*	Grone et al., 2012	+
			Cichlasoma dimerus	Pérez Sirkin et al., 2013	?
			Nile tilapia (*Oreochromis niloticus*)	Chen et al., 2011	+
		Labridae	Cunner (*Tautogolabrus adspersus*)	Babichuk and Volkoff, 2013; Hayes and Volkoff, 2014	+
			Orange-spotted grouper (*Epinephelus coioides*)	Yan et al., 2011	+
			Ornate wrasse (*Thalassoma pavo*)	Facciolo et al., 2009	+
	Pleuronectiforme	Pleuronectidae	Winter flounder (*Pseudopleuronectes americanus*)	Buckley et al., 2010	+
			Barfin Flounder (*Verasper moseri*)	Amiya et al., 2012	+
	Polypteriformes actinopterygian	Polypteridae	*Polypterus senegalus* and *Erpetoichthys calabaricus*	López et al., 2014	?
	Rajiformes	Rajidae	Winter skate (*Leucoraja ocellata*)	MacDonald and Volkoff, 2010	+
	Salmoniforme	Salmonidae	Rainbow trout (*Oncorhynchus mykiss*)	Varricchio et al., 2015	?
PACAP	Cypriniforme	Cyprinidae	Goldfish (*Carasisus auratus*)	Matsuda et al., 2005; Matsuda and Maruyama, 2007	–
			Grass carp (*Ctenopharyngodon idellus*)	Zhou et al., 2013	– ?
	Gadiforme	Gadidae	Cod (*Gadus morhua*)	Xu and Volkoff, 2009	– ?
POMC/α-MSH	Cypriniforme	Cyprinidae	Goldfish (*Carasisus auratus*)	Cerdá-Reverter et al., 2003; Kang et al., 2010; Kojima et al., 2010; Yan et al., 2016	–
			Zebrafish (*Danio rerio*)	Shanshan et al., 2016	–
			Zebrafish (*Danio rerio*)	Dalmolin et al., 2015	+
	Pleuronectiforme	Pleuronectidae	Barfin flounder (*Verasper moseri*)	Takahashi et al., 2005	0
			Halibut (*Hippoglossus hippoglossus)*	Gomes et al., 2015	?
			Olive flounder (*Paralichthys olivaceus*)	Kang and Kim, 2015	0
	Salmoniforme	Salmonidae	Atlantic salmon (*Salmo salar*)	Valen et al., 2011	–
			Coho salmon (*Oncorhynchus kisutch*)	Kim et al., 2015	0
			Coho salmon (*Oncorhynchus kisutch*)	Leder and Silverstein, 2006; White et al., 2016	–
PrRP	Cypriniforme	Cyprinidae	Goldfish (*Carassius auratus*)	Kelly and Peter, 2006	–
	Perciforme	Gobidae	Mudskipper (*Periophtalmus modestus*)	Sakamoto et al., 2002; Tachibana and Sakamoto, 2014	+ ?
PYY	Acipenseriformes	Acipenseridae	Siberian sturgeon (*Acipenser baerii*)	Chen et al., 2015	–
	Characiforme	Characidae	Blind cavefish (*Astyanax fasciatus mexicanus*)	Wall and Volkoff, 2013	–
		Serrasalmidae	Red-bellied piranha (*Pygocentrus nattereri*)	Volkoff, 2014a	–
	Clupeiformes	Engraulidae	Tapertail anchovy (*Coilia nasus)*	Yang et al., 2016	+ ?

(Continued)

TABLE 1 | Continued

Hormone	Order	Family	Species	Major references	Effect on feeding
	Cypriniforme	Cyprinidae	Goldfish (*Carassius auratus*)	Gonzalez and Unniappan, 2010	–
			Grass carp (*Ctenopharyngodon idellus*)	Chen et al., 2013, 2014	–
	Perciforme	Carangidae	Yellowtail (*Seriola quinqueradiata*)	Murashita et al., 2006, 2007	+ ?
	Salmoniforme	Salmonidae	Atlantic salmon (*Salmo salar*)	Murashita et al., 2009a; Valen et al., 2011; Kousoulaki et al., 2013	0
	Siluriforme	Ictaluridae	Channel catfish (*Ictalurus punctatus*)	Schroeter et al., 2015	0 ?
Secretoneurin	Cypriniforme	Cyprinidae	Goldfish (*Carassius auratus*)	Trudeau et al., 2012; Mikwar et al., 2016	+
Spexin	Cypriniforme	Cyprinidae	Goldfish (*Carassius auratus*)	Wong et al., 2013	–
	Perciforme	Labridae	Orange-spotted grouper (*Epinephelus coioides*)	Li S. et al., 2016	–
Thyroid axis	Acipenseriformes	Acipenseridae	Amur sturgeon (*Acipenser schrenckii*)	Li et al., 2012	?
	Cypriniforme	Cyprinidae	Goldfish (*Carassius auratus*)	Sinha et al., 2012	+
			Goldfish (*Carassius auratus*)	Abbott and Volkoff, 2011; Goodyear, 2012	+ ?
	Siluriforme	Ictaluridae	Channel catfish (*Ictalurus punctatus*)	Pohlenz et al., 2013	+

The effects on feeding are described as stimulatory (+), inhibitory (–), not detected (0) or unknown/uncertain (?).

mammals, including arousal/sleep, feeding, energy metabolism, and reproduction (Merchenthaler, 2010). Galanin and its receptors have been identified in a number of fish species (see review in Mensah et al., 2010). Central injections of galanin stimulate feeding in Cypriniformes (both goldfish de Pedro et al., 1995; Volkoff and Peter, 2001b, and tench, *Tinca tinca* Guijarro et al., 1999). In goldfish, brain galanin mRNA expression is not affected by fasting but increases post-prandially in unfed fish (Unniappan et al., 2004) and in zebrafish, fasting up-regulates brain mRNA expression of galanin receptors (Li et al., 2013). These data suggest that the galanin system is involved in the regulation of feeding in Cypriniformes, and perhaps other fish.

Melanin concentrating hormone (MCH)

Melanin concentrating hormone is a peptide originally isolated from the pituitary of chum salmon (*Oncorhynchus keta*) as a hormone involved in body color change (Kawauchi et al., 1983). MCH was later isolated in mammals and shown to stimulate feeding (Qu et al., 1996). In fish, the role of MCH as an appetite regulator is still unclear.

In Cypriniformes, early immunoreativity (ir) studies in goldfish showed the presence of MCH in neuron populations related to the regulation of feeding and of sleep and arousal (Huesa et al., 2005). In goldfish, central injections of MCH decrease feeding but have no effect on locomotor activity (Shimakura et al., 2006), anti-MCH serum treatments increase feeding (Matsuda et al., 2007a), and the number of certain hypothalamic neuronal cell bodies containing MCH-ir decreases in fasted fish (Matsuda et al., 2007a), altogether suggesting an anorexigenic role for MCH in this species. However, in Ya fish, MCH hypothalamic mRNA expression is higher in fasted compared to fed fish, suggesting an orexigenic role (Wang et al., 2016). Data on Gadiformes and Pleuronectiformes also seem to suggest an appetite-stimulating role for MCH: MCH brain mRNA levels increase during fasting in both Atlantic cod *Gadus morhua* (Tuziak and Volkoff, 2013a) and winter flounder *Pseudopleuronectes americanus* (Tuziak and Volkoff, 2012), and in cod fed diets with relatively high amounts of plant (camelina) material (Tuziak et al., 2014). In starry (*Platichthys stellatus*; Kang and Kim, 2013b), olive (*Paralichthys olivaceus*; Kang and Kim, 2013a) and Barfin (*Verasper moseri;* Takahashi et al., 2004) flounders, fish placed in light backgrounds have enhanced appetite and growth, which is concomitant with increased expression levels of MCH mRNA and/or numbers of MCH neurons in the brain. However, in medaka *Oryzias latipes*, transgenic fish overexpressing MCH have normal growth and feeding behavior (Qu et al., 1996) and in the scalloped hammerhead shark *Sphyrna lewini*, hypothalamic MCH mRNA levels are not affected by fasting (Mizusawa et al., 2012), suggesting little or no role of MCH in feeding regulation of Beloniformes and sharks.

Neuronal relationship between MCH- and NPY-containing neurons have been shown in goldfish (Matsuda et al., 2009) and MCH treatment increases orexin mRNA expression and decreases NPY mRNA expression in cultured goldfish forebrain slices (Matsuda et al., 2009), suggesting an interaction of MCH with appetite regulators in goldfish. Similarly, in red-bellied piranha *Pygocentrus nattereri*, orexin and MCH co-localize in pituitary and brain (Suzuki et al., 2007), and in Barfin flounder, close contacts are seen between orexin- and MCH-ir cell bodies and fibers in the hypothalamus, suggesting an interaction between the two systems and a possible role for MCH in the modulation of locomotion and feeding (Amiya et al., 2008).

Neuropeptide Y (NPY)

Neuropeptide Y (NPY) belongs to the NPY family of peptides, which also includes, peptide YY and pancreatic polypeptide (PP)

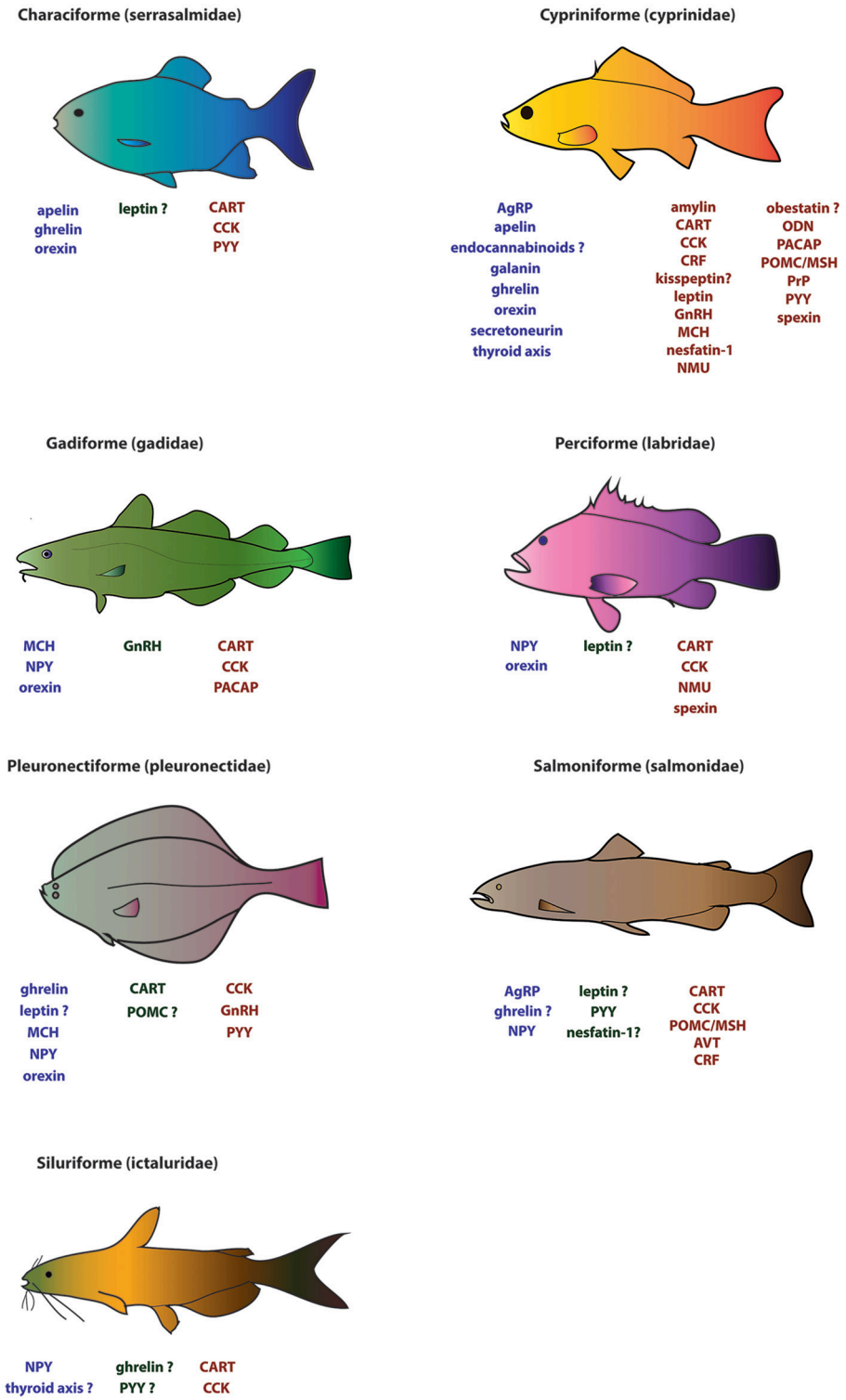


FIGURE 1 | Major appetite regulators known for seven of the most studied representative fish families (serrasalmidae, cyprinidae, gadidae, labridae, pleuronectidae, salmonidae, and ictaluridae). Factors in blue (far left) and in red (far right) under the fish diagrams represent putative orexigenic and anorexigenic factors, respectively. Factors in green (middle) represent factors with no established effect on feeding. A "?" indicates uncertainty with regards to the role of a given factor in regulating feeding.

(Holzer et al., 2012). Originally isolated from mammalian brain extracts (Tatemoto et al., 1982), NPY is one of the most abundant neuropeptides within the brain and has a major regulatory role in energy homeostasis and food intake (Loh et al., 2015).

Although reports for NPY-like ir in fish brain and other tissues appear in the 1980's (e.g., Osborne et al., 1985; Danger et al., 1990), the first fish NPY cDNAs were reported in goldfish and the electric ray *Torpedo marmorata* (elasmobranch, Torpediniformes; Blomqvist et al., 1992). One of the first studies showing the role of NPY in regulating in fish was that of Silverstein et al., showing by *in situ* hybridization (ISH) that, in chinook salmon (*Oncorhynchus tshawytscha*) and coho salmon, NPY-like mRNA signal areas were greater in fasted than fed fish (Silverstein et al., 1998). The first *in vivo* injection studies were performed in goldfish (Lopez-Patino et al., 1999; de Pedro et al., 2000; Narnaware et al., 2000) and channel catfish *Ictalurus punctatus* (Silverstein and Plysetskaya, 2000). Since then, NPY has been one of the most studied appetite-regulating hormones in fish. It has been cloned and/or shown to regulate feeding in several groups, including Characiformes (Pereira et al., 2015), Cypriniformes [(e.g., goldfish, zebrafish (Yokobori et al., 2012), blunt snout bream *Megalobrama amblycephala* (Xu et al., 2016), grass carp *Ctenopharyngodon idellus* (Jin et al., 2015), Jian carp (*Cyprinus carpio*) (Tang et al., 2014), Ya fish (Wei et al., 2014)], Gadiformes (Atlantic cod Kortner et al., 2011; Tuziak et al., 2014); Gonorynchiformes (milkfish *Chanos chanos,* Lin et al., 2016); Perciformes (yellowtail *Seriola quinqueradiata* Hosomi et al., 2014, *Astatotilapia burtoni* Grone et al., 2012, cunner *Tautogolabrus adspersus* Babichuk and Volkoff, 2013, orange-spotted grouper *Epinephelus coioides* Tang et al., 2013, sea bass Leal et al., 2013, mandarin fish, *Siniperca chuatsi* Sun et al., 2014, cobia *Rachycentron canadum* Van Nguyen et al., 2013, gourami *Trichogaster pectoralis* Boonanuntanasarn et al., 2012); Pleuronectiformes (olive flounder Wang et al., 2015, winter flounder MacDonald and Volkoff, 2009a, Brazilian flounder *Paralichthys orbignyanus* Campos et al., 2012), Salmoniformes (e.g., rainbow trout *Oncorhynchus mykiss* Aldegunde and Mancebo, 2006, Atlantic salmon Valen et al., 2011; Kim et al., 2015), Siluriformes (channel catfish, Peterson et al., 2012; Schroeter et al., 2015); Tetraodontiformes (tiger puffer *Takifugu rubripes* Kamijo et al., 2011) as well as elasmobranchs [(e.g., winter skate *Leucoraja ocellata*, Rajiforme (MacDonald and Volkoff, 2009b) and spotted catshark (*Scyliorhinus canicula*, Carcharhiniforme) Mulley et al., 2014)] and holocephalans (elephant fish Chimaeriformes; Larsson et al., 2009). The majority of these studies indicate that NPY has a widespread distribution and is present in both brain and intestinal tract, that it acts as an orexigenic factor and that its expression is affected by feeding and fasting.

Orexin

Orexins (also called hypocretins) are neuropeptides originally isolated in rats (Sakurai, 2014), that have since been identified in several fish species. The first direct evidence of an orexigenic action of orexins was shown via intracerebroventricular (ICV) injections in goldfish (Volkoff et al., 1999). As in mammals (Tsujino and Sakurai, 2009; Sakurai, 2014), orexins increase not only appetite and feeding behavior but also locomotor activity and reward-seeking/foraging behavior in fish (Panula, 2010).

In both goldfish (Volkoff et al., 1999; Nakamachi et al., 2006; Facciolo et al., 2011) and zebrafish (*Danio rerio*) (Yokobori et al., 2011) (Cypriniformes), and cavefish (*Astyanax mexicanus*) (Characiforme) (Penney and Volkoff, 2014), orexin injections increase searching/feeding behaviors. In orange-spotted grouper (Perciforme), intraperitoneal (IP) orexin injections increase hypothalamic mRNA expression levels of NPY, a major appetite stimulator (Yan et al., 2011), further suggesting an orexigenic role. However, in ornate wrasse (*Thalassoma pavo)* (Perciforme), orexin IP injections induce increases in locomotion but decreases in feeding (Facciolo et al., 2009), suggesting that the major role of orexin might be induction of hyperactivity rather than increasing food ingestion. Indeed, in goldfish, hypothalamic orexin mRNA expression levels peak when fish are active prior to a scheduled meal (Hoskins and Volkoff, 2012) and in zebrafish, increased locomotor activity is associated with increased activity of hypothalamic orexin neurons (Naumann et al., 2010) and larvae overexpressing orexin are hyperactive (Woods et al., 2014). Similarly, orexin expression decreases post-feeding in Characiformes [cavefish (Wall and Volkoff, 2013), dourado (*Salminus brasiliensis*) (Volkoff et al., 2016) and pacu (*Piaractus mesopotamicus*) (Volkoff et al., 2017)] and is higher at mealtime in orange-spotted grouper (Yan et al., 2011) and tilapia (Chen et al., 2011) (Perciformes), as well as Atlantic cod (Gadiforme) (Xu and Volkoff, 2007). In cod, orexin levels are also higher during daylight hours, when animals are active (Hoskins and Volkoff, 2012).

Fasting increases orexin brain mRNA expression in Cypriniformes (goldfish Abbott and Volkoff, 2011 and zebrafish Yokobori et al., 2011), Characiformes (cavefish Wall and Volkoff, 2013, dourado Volkoff et al., 2016, pacu Volkoff et al., 2017, and red-bellied piranha Volkoff, 2014a), and Pleuronectiformes (winter flounder Buckley et al., 2010 and Barfin flounder Amiya et al., 2012). In the mouth-brooding *Astatotilapia burtoni* (Perciforme), brain orexin mRNA levels increase in non-feeding females carrying eggs (Grone et al., 2012). In Atlantic cod (Gadiforme), orexin brain expression levels are higher in fish fed low rations than in fish fed high rations (Xu and Volkoff, 2007) or in fish fed the 30% camelina (plant) meal diet compared to fish fed a control (fish) diet (Tuziak et al., 2014), suggesting an effect of food quality and quantity on orexin expression. However, torpid cunner (Peciforme, labridae) undergoing a long-term fasting have low brain and gut orexin expression levels (Babichuk and Volkoff, 2013; Hayes and Volkoff, 2014), but this decrease might be due to a toprpor-induced general metabolic shutdown.

Anatomical studies provide further evidence for a role of orexin in nutrient digestion/abrorption and growth. In several fish species, e.g., pirapitinga (*Piaractus brachypomus*) (Characiforme) (Volkoff, 2015a), cunner (Perciforme) (Hayes and Volkoff, 2014) and rainbow trout (Salmoniforme) (Varricchio et al., 2015), orexin mRNA/protein expression is high in the gastrointestinal tract, suggesting a role of the orexin system in regulating feeding and digestive processes. Among Perciformes, in Japanese sea perch (*Lateolabrax japonicus*),

orexin-like ir is present in pituitary GH-containing cells, suggesting a control of growth by the orexin system (Suzuki et al., 2007) and in *Cichlasoma dimerus*, orexin-ir fibers are present in both hypothalamus and in pituitary, suggesting a neuroendocrine control of pituitary secretions (Pérez Sirkin et al., 2013).

In addition to teleosts, orexin has been examined in the primitive bony fish birchir *Polypterus senegalus* and rope fish *Erpetoichthys calabaricus* (Chondrosteans, Polypteriformes) for which the brain orexin ir patterns are similar to that of other fish examined (López et al., 2014) and in the Chondrichthyan winter skate (Rajiforme), in which fasting increases hypothalamic orexin expression (MacDonald and Volkoff, 2010).

Overall, it appears that in all fish species studied to date, orexin is related to both food intake and appetitive/searching behavior and perhaps to growth.

Anorexigenic Factors

CART

CART is a peptide which transcript expression is regulated by administration of cocaine or amphetamine in rodents (Vicentic and Jones, 2007; Subhedar et al., 2014) and amphetamine in goldfish (Volkoff, 2013). CART acts as an anorexigenic factor in mammals (Larsen and Hunter, 2006), and was first identified and shown to be anorexigenic in goldfish (Volkoff and Peter, 2000, 2001a).

Two CART isoforms have been identified in goldfish (Volkoff and Peter, 2001a) and common carp (Wan et al., 2012), and 4 in zebrafish (Akash et al., 2014) whereas, to date, only one form has been isolated for grass carp (Zhou et al., 2013; Liu et al., 2014), Characiformes [pirapitinga (serrasalmidae) (Volkoff, 2015a), pacu (serrasasalmidae) (Volkoff et al., 2017) and dourado (characidae) (Volkoff et al., 2016), red bellied piranha (serrasalmidae) (Volkoff, 2014a)], Salmoniformes [Atlantic salmon (Murashita et al., 2009a), rainbow trout (Figueiredo-Silva et al., 2012), Arctic charr (Striberny et al., 2015) and lake trout (*Salvelinus namaycush*) (Volkoff et al., 2007)], Siluriformes (channel catfish Kobayashi et al., 2008), Gadiformes (Atlantic cod Kehoe and Volkoff, 2007), Perciformes (cunner Babichuk and Volkoff, 2013), winter flounder (MacDonald and Volkoff, 2009a) and Atlantic halibut (*Hippoglossus hippoglossus*) (Gomes et al., 2015) (Pleuronectiformes), venomous toadfish *Thalassophryne nattereri* (Batrachoidiforme) (Magalhaes et al., 2006), rainbow smelt (*Osmerus mordax*) (Osmeriforme), pufferfishes (*Takifugu rubripes* and *Tetraodon nigroviridis*, Tetraodontiforme) and stickleback *Gasterosteus aculeatus* (Gasterosteiforme) (cited in Murashita et al., 2009a). However, six forms of CART have been identified in the medaka (Beloniforme) (Murashita and Kurokawa, 2011) and seven forms in Senegalese sole *Solea senegalensis* (Pleuronectiforme), the highest number of CART genes reported to date in a vertebrate species (Bonacic et al., 2015). The only elasmobranch CART identified to date is that of winter skate (Rajiforme) (MacDonald and Volkoff, 2009b).

CART injections induce a decrease in food intake and an increase in locomotion in goldfish (Volkoff and Peter, 2000) and enhance responsiveness to sensory stimuli in zebrafish larvae (Woods et al., 2014), suggesting that CART is involved in feeding/searching behaviors in cyprinids.

Fasting/food restriction decreases CART brain expression in Cypriniformes (goldfish Volkoff and Peter, 2001a, zebrafish Nishio et al., 2012; Guillot et al., 2016 and common carp, Wan et al., 2012), most Characiformes (red-bellied piranha Volkoff, 2014a, and pacu Volkoff et al., 2017), most Salmoniformes (Atlantic salmon, Murashita et al., 2009a; Kousoulaki et al., 2013, rainbow trout Figueiredo-Silva et al., 2012), Atlantic cod (Kehoe and Volkoff, 2007), cunner (Perciforme) (Babichuk and Volkoff, 2013), medaka (CART3) (Murashita and Kurokawa, 2011), and Siluriformes (channel catfish Kobayashi et al., 2008, African sharptooth catfish *Clarias gariepinus* Subhedar et al., 2011), suggesting an anorexigenic role for CART in teleost fish. Postprandial increases in CART brain expression have been shown in Senegalese sole (CART1a, CART 2a and CART4) (Bonacic et al., 2015), pacu (Volkoff et al., 2017), dourado (Volkoff et al., 2016), channel catfish (Peterson et al., 2012) but not in cod (Kehoe and Volkoff, 2007).

However, in Arctic charr, CART hypothalamic expression is similar throughout the seasonal feeding cycles (Striberny et al., 2015) and fasting does not affect CART expression in either dourado (Volkoff et al., 2016), winter flounder (MacDonald and Volkoff, 2009a) or Atlantic halibut larvae (Gomes et al., 2015), and in lake trout, fish exposed to the pesticide tebufenozide and control fish have similar food intakes, despite higher CART mRNA brain expression levels in exposed fish (Volkoff et al., 2007). In winter skate, 2 weeks of fasting have no effects on brain CART expression (MacDonald and Volkoff, 2009b), suggesting that CART might not have a major feeding-regulating role in elasmobranchs.

CART expression does not appear to be affected by diet, as in both cod fed a camelina (plant) diet (Tuziak et al., 2014) or rotifers or zooplankton (Katan et al., 2016) and pacu fed soybean concentrate (Volkoff et al., 2017), similar CART brain expression are seen between experimental and control fish.

Overall, there is a large interspecific variation in the number of forms and responses to fasting in the CART system in fish, although most studies tend to show that CART is mostly a central factor that might act as an appetite inhibitor.

Pro-opiomelanocortin (POMC) family of peptides

Proopiomelanocortin (POMC) is a common precursor that is processed post-translationally to generate melanocortin peptides [α-, β-, and γ-melanocyte-stimulating hormone (α-, β-, γ-MSH)], adrenocorticotropic hormone (ACTH) and other hormones that include β-endorphin (β-END) and β-lipotropic hormone (β-LPH) (Adan et al., 2006; Takahashi, 2016). POMC is mainly produced in the vertebrate pituitary, but is also found in brain, in particular the arcuate nucleus (ARC) of the hypothalamus. Receptors for melanocortin peptides include five subtypes (MC1R- MC5R) (Takahashi, 2016). In mammals, POMC and α-MSH have been shown to be involved in the regulation of appetite and energy homeostasis: POMC neurons suppress appetite by releasing α-MSH, which is an agonist at the anorectic melanocortin-4 receptor (MC4R) (Adan et al., 2006; Cone, 2006; Sohn, 2015).

Teleost fish lack γ-MSH and the POMC gene encodes an extra MSH (δ-MSH) in elasmobranchs (Cérda-Reverter et al., 2011). Fish POMC was first identified in Salmoniformes (Kawauchi, 1983; Kitahara et al., 1988) and Cypriniformes (Arends et al., 1998), followed by the identification of several forms in other fish species. As in other vertebrates, fish POMC is mainly expressed in the pituitary gland, but also within the lateral tuberal nucleus, which is equivalent to the mammalian ARC (Cérda-Reverter et al., 2011). POMC, α-MSH and the MC4R have been shown to regulate feeding in a few fish species.

In goldfish, fasting does not seem to affect hypothalamic POMC mRNA expression levels (Cerdá-Reverter et al., 2003), but ICV administration of [Nle4, d-Phe7]- α-MSH, a melanocortin agonist, inhibits food intake (Cerdá-Reverter et al., 2003), suggesting the melanocortin system participates in central regulation of food intake in Cypriniformes (Cerdá-Reverter et al., 2003). In addition, ICV injections of a MSH (MC4R) receptor agonist (melanotan II) suppress hypothalamic NPY expression (Kojima et al., 2010), and hypothalamic α-MSH-containing neurons are in close contact to NPY-containing nerve fibers, suggesting that the anorexigenic actions of the melanocortin system are mediated in part by an inhibition of the NPY system. In zebrafish larvae, although early ISH studies could not detect fasting-induced changes in hypothalamic POMC transcript levels (Song et al., 2003), more recent qPCR studies indicate that POMCa expression decreases in starved fish (Shanshan et al., 2016). In addition, GH-transgenic zebrafish, who have increased feeding, display down-regulation of POMC (Dalmolin et al., 2015), consistent with an anorexigenic role for POMC-derived peptides in Cypriniformes.

Similarly, in salmonids, POMC/α-MSH appears to have an anorexigenic role. In coho salmon, IP injections of α-MSH decrease food intake (White et al., 2016), in rainbow trout, fasting induces a decrease in hypothalamic expression of POMC-A1 (but not POMC-A2 or POMC-B) (Leder and Silverstein, 2006), and in Atlantic salmon, expression of both POMC-A1 and POMC-B increase after feeding (Valen et al., 2011). Interestingly, α-MSH treatment does not affect feeding of GH-transgenic coho salmon (White et al., 2016), despite similar hypothalamic POMC and MC4R mRNA expression levels compared to non-transgenic fish (Kim et al., 2015), suggesting that the actions of α-MSH might be inhibited by high expression levels of GH and/or AgRP.

In both olive (Kang and Kim, 2015) and Barfin flounder (Takahashi et al., 2005) (Pleuronectiformes), pituitary POMC-C (isoforms 1, 2, and 3) mRNAs are not affected by fasting, suggesting pituitary POMC might not directly related to appetite regulation. However, in fasted halibut larvae, whole brain POMC-C mRNA expression is higher in unfed fish 30 min after re-feeding compared to continuously fed fish (Gomes et al., 2015), suggesting a short-term regulation of appetite. Given the small number of studies available, and the variation in experimental protocols (adults vs. larvae, pituitary vs. brain, long-term vs. short-term feeding), conclusions are difficult to drawn regarding the role of POMC in flatfish.

Major Peripheral Factors

Ghrelin

Originally discovered in rat stomach as an endogenous ligand to the GH secretagogue-receptor (Kojima et al., 1999) ghrelin is the only known orexigenic factor in the GIT of mammals (Higgins et al., 2007). In the 2000's, a ghrelin-like peptide which stimulated GH release was first described in Nile tilapia (*Oreochromis mossambicus*; Shepherd et al., 2000) and a ghrelin-ir peptide was first detected in burbot (*Lota lota*) plasma (Mustonen et al., 2002). Using goldfish as a model, Unniappan et al. provided the first fish ghrelin cDNA sequence and the first evidence of an orexigenic role for ghrelin in fish, as central injections of ghrelin stimulated food intake (Unniappan et al., 2002). Subsequent studies on several fish species reported sequences for ghrelin and confirmed its role as an appetite stimulator in fish (see Jönsson, 2013 for a review), including other Cypriniformes [e.g., goldfish (Kang et al., 2011; Nisembaum et al., 2014; Blanco et al., 2016a); gibel carp (*Carassius auratus gibelio*) (Zhou et al., 2016); *Schizothorax davidi* (Zhou et al., 2014)], Characiformes (red-bellied piranha Volkoff, 2015b), Perciformes (Nile tilapia Schwandt et al., 2010), for which fasting-induced and periprandial changes in expression/protein levels occur. In Salmoniformes, there is contradictory evidence. In rainbow trout, central ghrelin injections and long-term peripheral treatment both decrease food intake compared to controls (Jönsson et al., 2010) and in Atlantic salmon, ghrelin plasma levels are lower in fasted fish compared with fed fish (Hevrøy et al., 2011) and show no clear periprandial changes (Vikesa et al., 2015), suggesting that ghrelin might have little effect or an inhibitory effect on feeding of in salmonids. In contrast, in brown trout (*Salmo truta*), ghrelin treatment increases foraging activity (Tinoco et al., 2014a). In rainbow trout, ICV ghrelin injections induce changes in parameters related to hepatic lipid metabolism (Velasco et al., 2016), suggesting a role of ghrelin in metabolism and nutrient storage. In yellow catfish (*Pelteobagrus fulvidraco*) (Siluriforme), although fasting increases ghrelin expression (Zhang et al., 2016a), no periprandial differences in plasma or stomach ghrelin expression are observed (Peterson et al., 2012).

It thus seems that the role of ghrelin in the regulation of feeding and metabolism of fish is still unclear, and might be species- and form-specific, so that further studies on more species are required.

Anorexigenic Factors

Cholecystokinin (CCK)

In mammals, CCK inhibits food intake and induces the release of digestive enzymes from intestine/pancreas and gallbladder (Boguszewski et al., 2010; Dockray, 2012).

In fish, CCK was first shown to have a role in digestion, as, for example, it stimulated contraction of the gallbadder in coho (Vigna and Gorbman, 1977) and Atlantic (Aldman and Holmgren, 1987) salmon, as well as bluegill (*Lepomis macrochirus*), killifish (*Fundulus heteroclitus*), and the holostean bowfin (*Amia calva*) (Rajjo et al., 1988), stimulated lipase secretion in the stomachless killifish (Honkanen et al., 1988) and inhibited gastric secretion in Atlantic cod (Holstein, 1982). The first direct evidence of the actions of CCK on feeding was

provided by injections in goldfish (Himick and Peter, 1994), followed by cloning of goldfish CCK cDNA (Peyon et al., 1998) and the demonstration of periprandial variations in CCK mRNA expression levels (Peyon et al., 1999). Subsequently, a number of studies have characterized CCK in several fish, including other Cypriniformes (e.g., common carp Zhong et al., 2013; zebrafish Koven and Schulte, 2012; Tian et al., 2015; grass carp; blunt snout bream Ping et al., 2013; Ji et al., 2015), Characiformes (e.g., cavefish Wall and Volkoff, 2013, dourado Pereira et al., 2015; Volkoff et al., 2016, thin dogfish *Oligosarcus hepsetus* Vieira-Lopes et al., 2013, pirapitinga Volkoff, 2015a, red-bellied piranha Volkoff, 2014a, pacu Volkoff et al., 2017), Salmoniformes (e.g., Atlantic salmon Valen et al., 2011), Gadiformes (Atlantic cod Tillner et al., 2013), Perciformes [e.g., yellowtail (Furutani et al., 2013; Hosomi et al., 2014); *Astatotilapia burtoni* (Grone et al., 2012); cunner (Babichuk and Volkoff, 2013; Hayes and Volkoff, 2014); sea bass (Tillner et al., 2014); yellow croaker (*Larimichthys crocea*) (Cai et al., 2015); white sea bream, *Diplodus sargus* (Micale et al., 2012, 2014)], Pleuronectiformes (e.g., winter flounder (MacDonald and Volkoff, 2009a), Atlantic halibut Kamisaka et al., 2001, olive flounder Kurokawa et al., 2000) and Siluriformes (channel catfish Peterson et al., 2012).

Overall, in all fish species studied to date, CCK appears to have similar roles in feeding and digestive processes to its role in mammals, i.e., it acts as a satiety/appetite-inhibiting factor and induces the release of digestive enzymes from the GIT.

Leptin

Leptin, a peptide originally cloned in obese *ob/ob* mice (Zhang et al., 1994), is secreted in mammals mainly by white adipose tissue, and its blood levels are proportional to body fat content (Park and Ahima, 2015). Leptin is a multifunctional hormone in both mammals (Park and Ahima, 2015) and fish (see review by Gorissen and Flik, 2014) and is involved in the regulation of not only food intake and body weight, but also reproduction, development and stress responses.

First hints of a role of leptin in fish were provided by reports of a decrease in feeding in goldfish ICV-injected with human leptin (Volkoff et al., 2003). The first fish leptin was identified in the pufferfish genome in 2005 by synteny studies (Kurokawa et al., 2005), followed by isolation of zebrafish, medaka, and carp leptins (Huising et al., 2006b). Since then, leptins have been identified in several fish species and shown to have multiple physiological functions (reviewed in Copeland et al., 2011; Angotzi et al., 2013; Londraville et al., 2014). As opposed to mammals who have a single leptin gene, several fish species have several leptin gene paralogs (e.g., lepA and lepB). Also in contrast to mammals, where subcutaneous fat is the main source of leptin, fish leptin is expressed in several tissues including liver and intestine, which is consistent with the fact that fish generally store lipids in intra-abdominal regions and liver (Birsoy et al., 2013).

Most studies on fish leptin have been conducted in Cypriniformes, in particular goldfish and zebrafish, and Salmoniformes. In goldfish, leptin injections decrease feeding and locomotor behavior (Volkoff et al., 2003; de Pedro et al., 2006; Vivas et al., 2011; Tinoco et al., 2012) in part by stimulating anorexigenic sytems (e.g., CART, CCK, and POMC) and inhibiting orexigenic ones (e.g., orexin, NPY, AgRP) (Volkoff et al., 2003; Yan et al., 2016). Similarly, in rainbow trout (Salmoniforme), central leptin administration suppresses food intake and increases the hypothalamic expressions of CART and POMC (Gong et al., 2016). Leptin treatment also inhibits feeding in grass carp (Li et al., 2010) (Cypriniforme) and increases energy expenditure in zebrafish larvae (Renquist et al., 2013). In Atlantic salmon (Salmoniforme), chronic IP treatment with leptin induces a decrease in growth rates (Murashita et al., 2011), and in hybrid striped bass (*Morone saxatilis* × *Morone chrysops*) (Perciforme), leptin treatment increases hepatic IGF-1 mRNA expression (Won et al., 2016), suggesting that leptin affects metabolism and growth.

Hepatic/gut/brain leptin increases in expressions are seen post-prandially in goldfish (Tinoco et al., 2012, 2014b), common carp (Huising et al., 2006a) and zebrafish (Tian et al., 2015) (Cypriniformes) as well as pacu (Volkoff et al., 2017) (Characiforme). However, in rainbow trout plasma leptin levels decrease post-feeding (Johansson and Björnsson, 2015).

There is a great variability in results with regards to fasting-induced changes in the leptin system. In goldfish, no significant differences in either brain or liver leptin expressions are seen between control, overfed and fasting fish, suggesting nutritional status does not affect the leptin system in goldfish (Tinoco et al., 2012). Similarly, leptin expression is not affected by fasting in the liver of common carp (Huising et al., 2006a) (Cyrpiniforme) and Nile tilapia (Shpilman et al., 2014) (Perciforme) or in the brains of red-bellied piranha (Volkoff, 2015b) and pacu (Volkoff et al., 2017) (Characiformes). However, fasting/food restriction increases hepatic leptin expression in white-clouds mountain minnow (*Tanichthys albonubes*, Cypriniforme; Chen et al., 2016b), in most Perciformes examined (orange-spotted grouper Zhang et al., 2013, mandarin fish Yuan et al., 2016, and mackerel *Scomber japonicus* Ohga et al., 2015, European sea bass Gambardella et al., 2012), in Arctic charr (Jørgensen et al., 2013) and Atlantic salmon (Rønnestad et al., 2010; Trombley et al., 2012; Moen and Finn, 2013) (Salmoniformes). In contrast, decreases in leptin expression are seen in liver of zebrafish (lepA) (Gorissen et al., 2009) and striped bass (*Morone saxatilis*) (lepB, perciforme) (Won et al., 2012) and intestine of red-bellied piranha (Volkoff, 2015b), and in blunt snout bream (Cypriniforme), higher feeding rates are associated with increased leptin pituitary expression (Xu et al., 2016). Whereas plasma leptin levels increase following fasting in rainbow trout (Salmeron et al., 2015; Johansson et al., 2016; Pfundt et al., 2016), Atlantic salmon (Trombley et al., 2012) and fine flounder *Paralichthys adspersus* (Pleuronectiforme) (Fuentes et al., 2012, 2013), they have been shown to decrease in earlier studies in fasted burbot (*Lota lota*) (Gadiforme) (Nieminen et al., 2003) and green sunfish (*Lepomis cyanellus)* (Perciforme) (Johnson et al., 2000).

In fish, leptin has been linked to metabolism. For example, in zebrafish, knocking down lepA decreases metabolic rate (Dalman et al., 2013) and in golden pompano, *Trachinotus blochii* (Perciforme), lepA gene polymorphisms are associated with different body weights, heights and lengths (Wu et al., 2016). Whereas in mammals, leptin acts as an adipostat and

its plasma levels are proportional to the amount of body fat, there is little evidence for such a role in fish. In topmouth culter *Culter alburnus* (Cyprinoforme), leptin mRNA expression is lower in wild populations, who have more muscle fat content than cultured fish (Wang et al., 2013), in grass carp, fish fed high fat diets have higher leptin expression (Li A. et al., 2016) than control fish, and in medaka, leptin receptor null-mutants have higher food intake and larger deposits of visceral fat than that of wild-type fish (Chisada et al., 2014), suggesting a correlation between leptin levels and fat. However, results from other studies seem to contradict this hypothesis: leptin receptor null adult zebrafish do not exhibit increased feeding or adiposity (Michel et al., 2016); In rainbow trout, leptin levels are higher in lean fish than fat fish (Salmeron et al., 2015; Johansson et al., 2016; Pfundt et al., 2016), and in Arctic charr, neither hepatic leptin expression nor plasma leptin levels correlate with fish adiposity (Froiland et al., 2012; Jørgensen et al., 2013); In murray cod *Maccullochella peelii peelii* (Perciforme), fish fed different experimental diets containing fish oil with or without vegetable oil have similar leptin levels (Ettore et al., 2012; Varricchio et al., 2012); In yellow catfish (Siluriforme), IP injections of human leptin reduce hepatic lipid content and the activities of lipogenic enzymes (Song et al., 2015) but Zn deficiency, which tends to increase hepatic and muscle lipid contents, does not affect leptin mRNA levels (Zheng et al., 2015).

Zebrafish lacking a functional leptin receptor have alterations in insulin and glucose levels, suggesting a role of leptin in the control of glucose homeostasis (Michel et al., 2016), which is consistent with data showing that leptin gene expression is induced by glucose in grass carp (Lu et al., 2015) and that leptin injections increase plasma glucose levels in Nile tilapia (Baltzegar et al., 2014).

Interestingly, in the Gymnotiforme *Eigenmannia virescens,* intramuscular injections of leptin increase electric organ discharges (EOD) amplitude in food-deprived but not well-fed fish, suggesting that leptin mediates EOD responses to metabolic stress in electric fish (Sinnett and Markham, 2015).

Overall, there seems to be a great species-specific variability in the functions of leptin with regards to the regulation of feeding and metabolism in fish, perhaps due to different lipid metabolism and storage areas among fish species.

Peptide YY

Peptide YY consists of two forms, PYYa and PYYb (previously called PY) (Wahlestedt and Reis, 1993; Cerdá-Reverter and Larhammar, 2000; Sundström et al., 2013) and is a brain-gut peptide that acts as an anorexigenic signal in mammals (Blevins et al., 2008; Karra et al., 2009; Zhang et al., 2012). Interestingly, one of the first studies showing an effect of PYY on feeding in mammals used fish PYY (Balasubramaniam et al., 1992). PYY was first shown to be present in the gastrointestinal tract of fish by immunochemical methods in the 1980's (daddy sculpin *Cottus scorpius* and Baltic sea cod *Gadus morhua callarias* El-Salhy, 1984) and first cloned and detected in the brain by ISH in an Agnatha, the river lamprey (*Lampetra fluviatilis*; Söderberg et al., 1994). The first indirect evidence of a role for PYY in feeding in fish was provided in sea bass, in which PYY transcripts were detected in brain areas regulating feeding (Cerdá-Reverter et al., 2000) and the first direct evidence of an anorexigenic role for PYY in fish was provided by IP injections of goldfish PYY in goldfish (Gonzalez and Unniappan, 2010). Peripheral injections of species-specific PYY also decrease food intake in another cyprinid, the grass carp (Chen et al., 2013) and in Siberian sturgeon *Acipenser baerii* (Acipenseriformes) (Chen et al., 2015). However, in channel catfish (Siluriformes), human PYY injections do not affect food intake or plasma glucose levels or hypothalamic POMC expression (Schroeter et al., 2015), suggesting perhaps that species-specific PYYs are needed to elicit an effect on feeding.

Fasting induces decreases in brain PYY expression in both goldfish (Gonzalez and Unniappan, 2010) and Ya fish (Yuan et al., 2014) (Cypriniformes) and in PPY intestinal expression in red-bellied piranha (Characiforme,) (Volkoff, 2014a), suggesting a role in satiety. However, fasting does not affect brain PYY expression in either cavefish (Characiforme) (Wall and Volkoff, 2013) or red-bellied piranha (Volkoff, 2014a), either brain or gut PYY mRNA expression in Atlantic salmon (Salmoniforme) (Murashita et al., 2009b), and induces increases in PYY gut expression in both yellowtail (Perciformes) (Murashita et al., 2006, 2007) and Japanese grenadier anchovy *Coilia nasus* (Clupeiformes) (Yang et al., 2016).

PYY mRNA expression increases post-feeding in the brain of goldfish (Gonzalez and Unniappan, 2010) and Ya fish (Yuan et al., 2014), cave fish (Wall and Volkoff, 2013) and Siberian stur*geon* (Chen et al., 2015), in the intestine of grass carp (Chen et al., 2014) and in whole larval Atlantic halibut (Pleuronectiformes) (Gomes et al., 2015). However, in Atlantic salmon, brain PYY expression shows no periprandial changes (Valen et al., 2011; Kousoulaki et al., 2013), perhaps suggesting that PYY does not play a major role as a short-term satiety factor in salmonids.

Overall, it appears that in most fish examined to date, PYY might acts as an anorectic/satiety peptide, although this does not seem to hold true for all fish species (e.g., salmon, yellowtail, or catfish).

Other Hormones and Systems

Hypothalamus-Pituitary-Thyroid Axis (HPT Axis)

The hypothalamic-pituitary-thyroid (HPT) axis regulates levels of thyroid hormones, which are essential for a number of biological functions, including food intake and energy expenditure. Hormones produced by the axis consist of thyrotropin releasing hormone (TRH), thyroid stimulating hormone (TSH) and thyroid hormones (triiodothyronine T_3 and thyroxine T_4) secreted by the hypothalamus, the pituitary and the thyroid gland, respectively (Fekete and Lechan, 2014).

In goldfish (Cypriniforme), ICV injections of TRH increase feeding and locomotor behaviors and the hypothalamic mRNA expressions of both orexin and CART (Abbott and Volkoff, 2011), and IP injections of T_4 increase food intake and locomotion (Goodyear, 2012), suggesting an orexigenic role. Fasting increases TRH hypothalamic mRNA levels (Abbott and Volkoff, 2011), further suggesting that the HPT axis regulates feeding in goldfish. In Amur sturgeon, *Acipenser schrenckii* (Acipenseriforme), lower serum levels of thyroid hormones are

seen in fish placed in high-density groups who display low feeding rates (Li et al., 2012). However, decreases in plasma levels of thyroid hormones are seen in fasted goldfish [T_3] (Sinha et al., 2012) and in fasted channel catfish [T_4 and T_3] (Gaylord et al., 2001), suggesting that food deprivation might decrease the activity of the HPT at the level of thyroid hormone synthesis and secretion, similar to what is observed in mammals (Boelen et al., 2008). A decrease in circulating thyroid hormones might inhibit the thyroid hormone negative feedback action on hypothalamic cells and contribute to the increase in hypothalamic TRH expression levels seen in goldfish. Overall, these data suggest that, in fish, TRH and thyroid hormones might affect feeding and metabolism and that nutritional status might affect the HPT axis.

Reproductive Hypothalamus-Pituitary-Gonad (HPG) Axis

Gonadotropin releasing hormone (GnRH)

GnRH is a hypothalamic hormone that stimulates the release of pituitary gonadotropins, which in turn stimulate the release of gonadal steroids. Three major forms of GnRH are present in fish, GnRH 1, 2, and 3 (Roch et al., 2014). GnRH appears to act as an anorexigenic hormone, as in goldfish, ICV injections with GnRH2 not only stimulate spawning (Hoskins et al., 2008) but also decrease food intake (Hoskins et al., 2008; Matsuda et al., 2008) and hypothalamic orexin mRNA expression (Hoskins et al., 2008). Similarly, in zebrafish, ICV injections of GnRH2 decrease food intake (Nishiguchi et al., 2012). In addition, in goldfish, treatment with orexin stimulate feeding, inhibit spawning behavior, and decrease brain GnRH2 expression, suggesting a coordinated control of feeding and reproduction by the orexin and GnRH systems (Hoskins et al., 2008).

In winter flounder, fasting reduces both brain GnRH2 and GnRH3, but not GnRH1, mRNA expression levels (Tuziak and Volkoff, 2013b) and in zebrafish, GnRH2 brain mRNA levels increase in overfed fish (Nishiguchi et al., 2012). However, in Atlantic cod, neither GnRH2 nor GnRH3 brain transcripts are influenced by food deprivation (Tuziak and Volkoff, 2013a), suggesting that the role of GnRHs in the regulation of feeding might be species- and form-specific.

RFamides

RFamide peptides, first isolated in invertebrate species in the late 1970's and later found in vertebrates, act as neurotransmitters and neuromodulators. In vertebrates, the RFamide peptide family consists of PRL-releasing peptides (PrRP), PQRFamide peptides (neuropeptide FF, NPFF), pyroglutamylated RFamide peptide (QRFP)/26RFamides, LPXRFamide peptides (gonadotropin-inhibitory hormone, GnIH, in lower vertebrates, RFamide-related peptide-3, RFRP-3, in mammals) and kisspeptins (Tsutsui and Ubuka, 2013; Osugi et al., 2016). RFamides have been shown to regulate several physiological functions in vertebrates, including feeding (Bechtold and Luckman, 2007; Quillet et al., 2016). A number of RFamides have been identified in fish, although most have been examined for their role in reproduction and are not yet well characterized with regards to their potential role as feeding regulators.

In goldfish, IP or ICV administration of PrRP decrease food intake, and hypothalamic PrRP mRNA expression increases post-prandially and after food deprivation, suggesting an anorexigenic role for PrRP in goldfish (Kelly and Peter, 2006). In line with this hypothesis, in the euryhaline fish mudskipper (*Periophthalmus modestus*, Perciforme, gobidae), freshwater fish have lower food intake/growth rates than saltwater fish and higher brain and intestine PrRP mRNA expressions, suggesting that PrRP is involved in the regulation of feeding and energy homeostasis in this species (Sakamoto et al., 2002; Tachibana and Sakamoto, 2014).

Two neuropeptide FF receptor 1 (NPFFR1) genes have been identified in carp and shown to display variations in expression associated with growth-related traits (Peng et al., 2016). As NPFF1 is receptor for neuropeptide FF (NPFF) and the LPXRFamide peptide RFamide-related peptide (RFRP), which are involved in control of feeding behavior in both invertebrates and vertebrates, these data suggest that NPFFR1s might be related to the regulation of growth and body weight in common carp (Peng et al., 2016). Similarly, in seabass, LPXRFamide-ir cells and/or fibers are present in feeding, gustatory, sensory, and behavioral centers of the brain, suggesting that it could be involved in the regulation of foraging/feeding behavior (Paullada-Salmerón et al., 2016).

In goldfish, hypothalamic expression of 26RFa increases in fasted animals (Liu et al., 2009) and IP injections of human RFRP-3 decrease food intake (Mawhinney, 2007), indicating that these neuropeptides might regulate food intake and energy balance in cyprinid fish.

In sea bass, food-restricted male fish display an increase in both kisspeptin and kisspeptin receptor expressions in both pituitary and hypothalamus (Escobar et al., 2016), suggesting the kisspeptin system is affected by nutritional status. However, in goldfish, IP injections of mammalian kisspeptin appear to have no effect on feeding (Mawhinney, 2007).

CRF and the Hypothalamus-Pituitary-Interrenal (HPI) Axis

The major endocrine components of the hypothalamic–pituitary–adrenal (HPA) axis (or interrenal, HPI in lower vertebrates) are hypothalamic corticotropin-releasing factor (CRF, or corticotropin-releasing hormone, CRH), pituitary adrenocorticotropin (ACTH) and glucocorticoids (e.g., cortisol, corticosterone) from the adrenal/interrenal gland. CRF mediates the release of ACTH, which in turn stimulates the release of steroids by the adrenal/interrenal gland (Smith and Vale, 2006). The HPI axis regulates numerous physiological functions, including metabolic functions (e.g., blood glucose levels during fasting), food intake, reproduction, growth, and immunity. Urocortins (UCN) 1 (also termed urotensin 1 in fishes), 2, and 3 belong to a recently discovered family of CRF-related peptides, which functions are still not well characterized (Majzoub, 2006).

The role of the HPI axis in the regulation of feeding of fish has been examined in several fish species. In goldfish, ICV injections of CRF decrease feeding (De Pedro et al., 1993) and increase locomotor activity (Matsuda et al., 2013). In Ya fish, fasting decreases CRF brain expression levels (Wang et al., 2014) and

goldfish exposed to the toxin fluoxetine have low food intake and increased brain expression of CRF (Mennigen et al., 2010), further suggesting an anorexigenic role for CRF in cyprinids. In goldfish, feeding fish with a diet containing low cortisol levels or implanting fish with cortisol-containing pellets result in higher food intake and CRF mRNA levels, compared to controls (Bernier et al., 2004). These results and others suggest that stress, cortisol and CRF can modulate food intake in Cypriniformes (Bernier et al., 2004).

In rainbow trout, CRF and urotensin 1 are anorexigenic, as ICV injections of either peptides inhibit feeding (Ortega et al., 2013). In addition, hypoxia stress suppresses appetite and increases forebrain CRF and urotensin mRNA levels, suggesting that, in Salmoniformes, CRF-related peptides might mediate the hypoxia-induced reduction in food intake (Bernier and Craig, 2005).

In Siberian sturgeon, IP injections of urocortin 3 inhibit feeding, and UCN3 brain mRNA expression levels increase post-feeding and decrease during fasting, suggesting that UCN3 acts as a satiety/anorexigenic factor in fish (Zhang et al., 2016c).

For more extensive reviews on the regulation of feeding by the HPI, please refer to previously published works, including (Bernier and Peter, 2001; Bernier, 2006; Flik et al., 2006; Lowry and Moore, 2006; Backström and Winberg, 2013).

"Novel" Appetite-Regulating Peptides

Amylin

Amylin (or islet amyloid polypeptide, IAPP), a hormone co-secreted with insulin from pancreatic β-cells, inhibits feeding in mammals (Riediger et al., 2003). In fish, the role of amylin in feeding has only been examined in goldfish. In this species, IP or ICV amylin treatments decrease food intake whereas ICV injections of an amylin receptor antagonist (AC 187) stimulate feeding (Thavanathan and Volkoff, 2006), suggesting an anorexigenic role for amylin in fish.

Apelin

Apelin is a peptide first identified in bovine stomach as a ligand for the orphan receptor APJ, with close identity to the angiotensin II (Ang II) receptor (Tatemoto et al., 1998; Habata et al., 1999) and subsequently shown to be involved in multiple physiological processes (see O'Carroll et al., 2013, for review) including feeding and metabolism in mammals: for example, apelin injections decrease food intake (O'Shea et al., 2003), and in adipocytes, apelin expression is inhibited by fasting (Boucher et al., 2005) and its secretion is regulated by insulin (Boucher et al., 2005).

In fish, apelin appear to be orexigenic: apelin injections increase food intake in goldfish (Volkoff and Wyatt, 2009) and cavefish (Penney and Volkoff, 2014). Fasting induces increases in brain apelin mRNA expression in Ya-fish (Lin et al., 2014a) and red-bellied piranha (Volkoff, 2014a). Moreover, in goldfish, the obesogen factor tributyltin (TBT) stimulates food intake and also increases brain apelin expression (Zhang et al., 2016b). In cavefish, IP injections of apelin increase orexin brain expression, and CCK injections induce a decrease in brain apelin expression (Penney and Volkoff, 2014), an indication that apelin interacts with other appetite regulators. Similarly, brain injections of the anorexigenic factor spexin reduce apelin brain expression (Wong et al., 2013) and *in vitro* treatment of brain fragments with apelin increase expressions of orexigenic peptides—i.e., orexin—and decrease CART expression (Volkoff, 2014b). Overall, the data suggest an orexigenic role for apelin in Cypriniformes. In cunner (Perciforme), summer fasting decreases intestinal apelin mRNA levels (Hayes and Volkoff, 2014), suggesting that GIT apelin might not be involved in the regulation of feeding. In common carp- but not in trout barb *Capoeta trutta*-, there is a negative correlation between apelin levels and body weight (Köprücü and Algül, 2015), suggesting that apelin might not be involved in metabolic processes leading to weight gain in some species.

Arginine Vasotocin

Arginine vasotocin (AVT) is the mammalian homolog of arginine vasopressin (AVP), and has been shown to have diverse and complex roles in fish physiology, including regulation of metabolic processes, stress responses and several behaviors (Balment et al., 2006). In rainbow trout, AVT treatments decrease feeding, and increase plasma levels of cortisol and glucose, brain serotonergic activity, and hypothalamic levels of POMC and CART, suggesting it acts as an anorexigenic factor in fish (Gesto et al., 2014).

Endocanabinoid System

In mammals, the endocannabinoid system (ECS), which consists of cannabinoid receptors (CB1 and CB2) and endogenous cannabinoids, is involved in the regulation of several physiological functions, including feeding and energy balance (Pagotto et al., 2006).

In goldfish, CB1 and CB2 are both expressed in brain, where CB1 co-localizes with NPY (Cottone et al., 2013). Treatment with low doses of the endocannabinoid receptor agonist anandamide (AEA) increases food intake (Valenti et al., 2005), and food deprivation increases CB1 and AEA brain mRNA levels (Cottone et al., 2009), suggesting the involvement of the ECS in the control of energy intake in Cypriniforme. Similarly, in sea bream *Sparus aurata* (Perciforme), AEA administered via water increases food intake and NPY brain mRNA levels (Piccinetti et al., 2010). In common sole, *Solea solea* (Pleuronectiforme), feeding fish with dietary nucleotides reduce CB1 brain transcript levels, suggesting that feeding and diets modulate the ECS (Palermo et al., 2013).

Nesfatin-1

Nesfatin-1, discovered in 2006 in mammals, is a peptide secreted from hypothalamic nuclei related to appetite regulation, from the precursor non-esterified fatty acid/nucleobinding 2 (NUCB2), and has been shown to reduce feeding and water intake in mammals (Ayada et al., 2015). In fish, the role of nesfatin-1 as an appetite regulator has been examined in Cypriniformes and Salmoniformes.

In goldfish, nesfatin-1 has been shown to be involved in the regulation of feeding and metabolism: nesfatin-1-like and ghrelin-like ir co-localize in both enteroendocrine and hypothalamic cells; IP or ICV injections of nesfatin-1 inhibit both food intake and brain expressions of ghrelin and NUCB2; and fasting increases both hepatic and hypothalamic NUCB2

mRNA levels (Gonzalez et al., 2010; Kerbel and Unniappan, 2012). In addition, NUCB2 mRNA levels increase in liver and hypothalamus in fish fed fat-enriched diets and decrease in gut after long-term feeding with a high-protein diet, suggesting that macronutrients regulate the expression of NUCB2/nesfatin-1 (Blanco et al., 2016a). In zebrafish, two isoforms of NUCB2 (NUCB2A and NUCB2B) exist, and both mRNAs decrease in the brain post-prandially and after food deprivation, suggesting an anorexigenic role for nesfatin-1 (Hatef et al., 2015). In Yafish, NUCB2A mRNA levels increase post-prandially in both hypothalamus and intestine, and fasting induces a decrease in NUCB2A mRNA levels in the hypothalamus, but an increase in the hepatopancreas, suggesting anorexigenic and metabolic roles (Lin et al., 2014b). However, in rainbow trout (*Oncorhynchus mykiss*), plasma nesfatin-1 levels are similar between fed and fasted females (Caldwell et al., 2014).

Neuropeptide B, Neuromedin S, and Neuromedin U

Neuropepide B (NPB), and neuromedins S (NMS) and U (NMU) are newly discovered mammalian short peptides that have been shown to affect feeding in fish.

NPB has been characterized in Nile tilapia (Perciforme), where it is expressed in brain and spinal cord. In this species, fasting increases NPB brain mRNA expression, and IP injections of NPB increase brain mRNA expression of NPY and CCK and inhibit pituitary GH expression, suggesting NPB is involved in feeding and growth in fish (Yang et al., 2014).

In both zebrafish (Chen et al., 2016a) and orange-spotted grouper (Li et al., 2015), an NMS-related protein (NMS-RP) has been identified that appears to act as an orexigenic factor. In both species, IP administration of species-specific NMS-RP increases both NPY and orexin expressions, and hypothalamic levels of NMS mRNA increase after food deprivation.

NMU has been characterized in Cypriniformes (carp, goldfish) and Perciformes (orange-spotted grouper). In both common carp (Kono et al., 2012) and goldfish (Maruyama et al., 2008), several forms of NMU (3–5) have been isolated and their mRNA expressions shown to decrease upon fasting, suggesting a role in feeding and metabolism (Kono et al., 2012). Similarly, in orange-spotted grouper, hypothalamic NMU mRNA levels decrease in fasted fish and increase post-feeding (Li et al., 2015), suggesting an anorexigenic role. In goldfish, central injections of NMU inhibit feeding and locomotor behaviors (Maruyama et al., 2008) and increase brain CRF mRNA expression levels (Maruyama et al., 2009) and in grouper, IP injections of NMU down-regulate hypothalamic NPY expression (Li et al., 2015), suggesting that the anorexigenic actions of NMU are mediated by the CRF system and an inhibition of the NPY system.

Obestatin

Obestatin, a gastrointestinal peptide discovered in 2005, is derived from the same precursor as ghrelin and inhibits food intake in mammals (Cowan et al., 2016). In grass carp, although IP injections of an obestatin-like peptide alone do not affect food intake or the expression levels of NPY, CART, or POMC, when co-injected with ghrelin, it blocks ghrelin-induced stimulation of appetite and up-regulation of expressions of NPY and NPY receptors (Yuan et al., 2015), suggesting that obestatin might inhibit the ghrelin system in Cypriniformes.

Octadecaneuropeptide

The octadecaneuropeptide (ODN) is a peptide belonging to the family of endozepines and is generated through the cleavage of diazepam-binding inhibitor (DBI) in the mammalian central nervous system (CNS) (Tonon et al., 2006). ODN acts as an inverse agonist of central-type benzodiazepine receptors (CBR) and inhibits food intake in rodents (do Rego et al., 2006).

Immunocytochemical methods first showed the presence in brain and pituitary of rainbow trout (Malagon et al., 1992) and more recently in the agnathan Atlantic hagfish, *Myxine glutinosa* (Myxiniforme, myxinidae; Candiani et al., 2000). Central injections of goldfish ODN inhibit food intake (Matsuda et al., 2007b) and stimulate locomotor activity (Matsuda et al., 2011b), and increase POMC brain mRNA levels (Matsuda et al., 2010), suggesting that the anorexigenic actions of ODN are in part mediated by the melanocortin system.

Pituitary Adenylate Cyclase Activating Polypeptide (PACAP)

Originally identified in the ovine hypothalamus (Miyata et al., 1989), pituitary adenylate cyclase-activating polypeptide (PACAP) belongs to the secretin/glucagon family of peptides that also includes secretin, glucagon, glucagon-like peptides and vasoactive intestinal peptide (Sherwood et al., 2000). In rodents, central injections of PACAP decrease food intake (Morley et al., 1992).

PACAP has been cloned in several fish, including Anguilliformes European eel (*Anguilla anguilla*) (Montero et al., 1998), Cypriniformes (e.g., zebrafish Sherwood et al., 2007, goldfish Matsuda et al., 1997), Gadiformes (cod Xu and Volkoff, 2009), Pleuronectiformes (e.g., olive flounder Nam et al., 2013), Salmoniformes (e.g., Atlantic salmon Parker et al., 1993), and Siluriformes (Thai catfish *Clarias macrocephalus* McRory et al., 1995, darkbarbel catfish *Pelteobagrus vachelli* Xu et al., 2012) as well as elasmobranchs (e.g., marbled electric ray *Torpedo marmorata* Agnese et al., 2016, stingray *Dasyatis akajei* Matsuda et al., 1998). In several fish, PACAP stimulates GH secretion by pituitary cells (see review in Gahete et al., 2009), but its role in regulating feeding is still unclear. In goldfish, central or peripheral PACAP injections inhibit food intake (Matsuda et al., 2005) and locomotor activity (Matsuda et al., 2006) and these actions might be mediated in part by the stimulation of POMC and CRH pathways (Matsuda and Maruyama, 2007). Similarly, in grass carp, central NPY injections decrease brain PACAP expression (Zhou et al., 2013), suggesting an anorexigenic role for PACAP in Cypriniformes. In Atlantic cod, PACAP inhibits intestinal smooth muscle contractions (Olsson and Holmgren, 2000), and although brain expression levels are not affected by 30 days of food deprivation, they increase after during the re-feeding period (Xu and Volkoff, 2009), suggesting that PACAP is involved in the regulation of feeding and digestive processes (Xu and Volkoff, 2009).

Secretoneurin

Secretoneurin (SN) is a short peptide derived from a secretogranin-II (SgII, also called chromogranin C) precursor protein (Zhao et al., 2009). In goldfish, ICV injections of the SN increase food intake and locomotor behavior (Trudeau et al., 2012), increase mRNA levels of hypothalamic NPY and decrease hypothalamic CART. In addition, fasting increases telencephalon SgII mRNA levels (Mikwar et al., 2016), suggesting that, in fish, SN might act as an orexigenic factor.

Spexin

Spexin (SPX) is a peptide identified in 2007 in mammalian adipose tissue. SPX expression is down-regulated in obese humans and rats, and subcutaneous injections of SPX reduce food intake and increase locomotion (Walewski et al., 2014).

In goldfish, SPX appear to act as an anorexigenic factor: brain injections of SPX inhibit both basal and NPY- or orexin-induced food consumption, decrease brain expressions of orexigenic factors (NPY, AgRP, and apelin) and increase that of anorexigenic factors (CCK, CART, POMC, MCH, and CRH), and brain SPX mRNA levels increase post-prandially (Wong et al., 2013). Similarly, in the orange-spotted grouper, IP administration of SPX increases hypothalamic mRNA levels of POMC and inhibits orexin expression, suggesting an anorexigenic role (Li S. et al., 2016). However, grouper SPX hypothalamic expression increases following long-term food deprivation (Li S. et al., 2016), suggesting that spexin might be a short-term satiety factor rather than a long-term hunger signal.

CONCLUDING REMARKS

Although the basic mechanisms regulating feeding seem to be relatively conserved between mammals and fish, it must be kept in mind that major physiological differences exist between these two groups. Fish are ectotherms and thus have lower metabolic rates than mammals and more sensitive to environmental changes, their physiology changing with their fluctuating surroundings. They also have different means of energy/nutrient storage (e.g., fat storage in liver rather than subcutaneous adipose tissue), and different growth patterns (as opposed to mammals, fish continue to grow after sexual maturity), suggesting that the endocrine regulation of energy balance, feeding and growth in fish differs from that of mammals.

Comparative studies at the genome level have revealed conserved sequences for appetite regulators across mammalian and fish species, indicating potentially conserved biological functions. Whereas the genome of all vertebrates is the result of two rounds (2R) of whole genome duplication (WGD) occurring in early vertebrate evolution, additional WGDs occurred in the teleost fish ancestor (3R) and most recently in certain teleost lineages (4R, e.g., salmonidae and cyprinidae), leading to the presence of increased gene copy numbers and multiple protein isoforms with potentially different physiological functions (Glasauer and Neuhauss, 2014), making the fish model potentially more complex. One must thus keep in mind that fish feeding-regulating hormones might not always have the same function as their mammalian homologs.

Fish are an extremely diversified group, with a great variability in feeding habits and requirements as well gut morphology and digestion processes. Fish can be carnivores, herbivores, omnivores or detritivores, with different feeding habits often seen within the same family (e.g., herbivore Mbuna cichlids and carnivore Nile tilapia in cichlidae; herbivore/omnivore pacu and carnivore piranha in serrasalmidae). Different fish species not only require different compositions of food, but also different amounts of food and feeding frequencies (Moore, 1941). Diet and feeding habits is reflected in the anatomy and physiology of the gastrointestinal tract. For example, carnivores or omnivores (such as most Characiformes and Siluriformes) have stomachs, pyloric caeca, and relatively short and straight intestines, whereas herbivores or detrivores (e.g., Cypriniformes and Cyprinodontiformes) may lack both stomach and caeca and have long and convoluted intestines (Leknes, 2015). Different diets and guts translate into different digestive enzyme profiles and different methods of nutrient storage (Day et al., 2011), as seen for lipids (e.g., in muscle in "oily" fish such as salmon and herring vs. liver in "lean" fish such as cod and flatfish), which usage might also be affected by reproductive stages and modes (guarding vs. non guarding; mature vs. immature; oviparous vs. viviparous).

Given the high diversity within fish, one should thus be careful when generalizing results from one species to all fish. Comparative studies establishing similarities and differences among species should be valuable to understand mechanisms regulating feeding. However, the large number of species poses the problem of the model species to choose. To date, most studies examining the neuroendocrine regulation of fish still use "classical" model species, i.e., cyprinids and salmonids. These somewhat differ from most fishes, as they display polyploidy, and might not represent a "perfect" model, but they are easily available and maintained, as their different holding conditions, habitats and diets, are well known. However, new species, in particular commercially important aquaculture species such as Perciformes (the largest teleost order) and Pleuronectiformes have recently been examined.

The increasing number of studies and species examined often generates conflicting and sometimes contradictory results. This variability might express true differences between species, but contradictory data also occur within same species. This variability might have several reasons. First, there is a great variability in the nature and nomenclature of isoforms examined (e.g., within CART forms). Second, when comparing studies, it is sometimes difficult to compare results obtained using different protocols (e.g., different lengths of fasting) and techniques (e.g., mRNA vs. protein vs. plasma levels), in particular because changes in gene expression do not necessarily translate into different protein levels or circulating levels. Finally, fish used between studies are often of different ages (e.g., larval vs. adult), sexual maturity (immature vs. mature spawning or non-spawning) or even environmental conditions (e.g., temperatures, photoperiods), all of these factors influencing feeding.

Even in mammals, the regulation of appetite is not yet fully understood. Using a comparative approach involving multiple

fish species, perhaps choosing representative families/species from each fish group, and complementary methods might help us start drawing accurate models for the endocrine regulation of feeding in fish.

AUTHOR CONTRIBUTIONS

HV designed this review, including table and figure, researched, acquired and analyzed all the information, drafted and revised the manuscript, and approved the version to be published. To HV's knowledge, information contained in this review and studies cited within it have been appropriately checked for accuracy or integrity.

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Neuropeptide Y-Induced Orexigenic Action Is Attenuated by the Orexin Receptor Antagonist in Bullfrog Larvae

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In bullfrog larvae at the pre- and pro-metamorphic stages, feeding behavior is regulated by appetite factors such as orexigenic peptides. In fact, food intake is enhanced by intracerebroventricular (ICV) administration of neuropeptide Y (NPY) and orexin A. Using goldfish, our previous study indicated that the orexigenic action of NPY is mediated by orexin A, suggesting the functional interaction between the two. However, there is little information about whether the action of orexin A mediates the orexigenic action of NPY in bullfrog larvae. Therefore, we examined the effect of the orexin receptor antagonist, SB334867 on the orexigenic action of NPY in larvae. The stimulatory effect of ICV injection of NPY at 10 pmol/g body weight (BW) on food intake was abolished by treatment with SB334867 at 60 pmol/g BW. These results suggest that, in bullfrog larvae, there is a neuronal relationship between the NPY and orexin systems, and that the orexigenic action of NPY is mediated by the orexin A-induced orexigenic action.

Keywords: bullfrog larvae, NPY, orexin A, orexigenic action, antagonist, neuronal interaction

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INTRODUCTION

Neuropeptide Y (NPY) was first identified from porcine central nervous system. NPY is belonging NPY family of peptides including peptide YY and pancreatic polypeptide, and it is one of the most highly conserved neuropeptides in vertebrates and even in invertebrates (Tatemoto et al., 1982; Blomqvist et al., 1992). NPY occurs abundantly in not only the central, but also peripheral nervous systems, and is involved in many roles such as cardiovascular control and neuroendocrine function in mammals (Karl and Herzog, 2007). NPY stimulates feeding behavior, and it has been considered a potent orectic neuropeptide in the mammalian brain (Woods et al., 1998). Orexin is a neuropeptide that was first isolated as an orphan receptor ligand, and subsequently identified as an appetite stimulator (Sakurai et al., 1998). Orexin has two molecular forms, orexin A and B, derived from the same precursor. In addition to its orexigenic action, orexin regulates energy consumption, rhythmicity, and neuronal apoptosis (Matsuki and Sakurai, 2008; Shioda et al., 2008).

An understanding of the evolutionary process of the NPY- and orexin-regulated system of feeding behavior could provide discernment into the physiological role of these functions throughout the vertebrates. In anuran brains, the neuroanatomical distributions of NPY and orexin have been described in detail (Danger et al., 1985; Cailliez et al., 1987; Galas et al., 2001), and primary structures of frog NPY and *Xenopus* orexin have been determined (Chartrel et al., 1991; Shibahara et al., 1999). Our previous studies have indicated that, in bullfrog larvae

at the pre- and pro-metamorphic stages, intracerebroventricular (ICV) administration of NPY and orexin A enhances food intake, suggesting that these neuropeptides act as potent orexigenic factors in the larvae of anuran amphibians (Shimizu et al., 2013, 2014b). Some previous papers have also documented the regulation of feeding by NPY and orexin A in fish (Lin et al., 2000; de Pedro and Bjornsson, 2001; Volkoff and Peter, 2001; Aldegunde and Mancebo, 2006; Gorissen et al., 2006; Matsuda and Maruyama, 2007; Xu and Volkoff, 2007; Matsuda, 2009). Especially, it has been shown that NPY and orexin A act as representative appetite enhancers in goldfish (López-Pati-o et al., 1999; Volkoff et al., 1999; Narnaware et al., 2000; Miura et al., 2006, 2007; Nakamachi et al., 2006). A previous study has also provided evidence that, in goldfish, NPY-induced appetite is mediated by orexin A, suggesting that the two peptides share a functional relationship for regulation of feeding (Volkoff and Peter, 2001). However, there is little information about the relationship between NPY and orexin A in the regulation of food intake in bullfrog larvae. Therefore, to clarify whether orexin A mediates the orexigenic action of NPY, we investigated the effect of ICV administration of an orexin receptor antagonist, SB334867, on the orexigenic action of ICV-injected NPY in bullfrog larvae.

MATERIALS AND METHODS

Animals

Bullfrog (*Rana catesbeiana*) larvae weighing 5–7 g were collected from ponds in the suburbs of Toyama City, Japan. The developmental stages of the larvae were determined according to Taylor and Kollros (1946). Since the body size of obtained larvae at premetamorphic stages was very small, experiments were done using 100 larvae at prometamorphic stages (XI–XIX). As previously described (Shimizu et al., 2013), the animals were kept for 1–2 weeks under controlled light/dark conditions (12L/12D) with the water temperature maintained at 20–24°C. The larvae were fed every day at noon with a powder diet (Itosui Co., Tokyo, Japan) until used in experiments. Animal experiments were conducted in accordance with the Invasive Alien Species Act of Japan and the University of Toyama's guidelines for the care and use of alien and laboratory animals, and were done by approval of an ethics committee of the University of Toyama and permission of government authorities (No. 05000361).

Chemicals

In order to examine the effects of ICV administration of NPY and orexin A on food intake, and the effects of NPY receptor and orexin receptor antagonists on the actions of ICV-injected NPY and orexin A in prometamorphic larvae, the following chemicals were used. A NPY Y1-receptor antagonist, BIBP3226 (diphenylacetyl-D-Arg-4-hydroxybenzylamide, Bachem AG, Bubendorf, Switzerland) and a selective orexin receptor antagonist, SB334867 [*N*-(2-methyl-6-benzoxazolyl)-*N*′-1,5-naphthyridin-4-yl urea, Tocris Cookson Ltd., Bristol, UK], were purchased commercially, dissolved in dimethyl sulfoxide at 20–50 mM for storage, and diluted with 0.6% NaCl and 0.02% Na_2CO_3 solution (saline) before use. NPY (rat NPY, Peptide Institute Co., Osaka, Japan) and orexin-A (rat orexin-A, Peptide Institute) were also purchased commercially, dissolved in saline at 0.1 mM for storage at −80°C, and diluted with saline before use. 3-Aminobenzoic acid ethyl ester (MS-222) for anesthesia was obtained commercially from Sigma-Aldrich (St. Louis, MO, USA), and was prepared at concentration of 0.15%. Each animal was exposed to this solution for 2 min.

Measurement of Food Intake

Details of the methods used for measuring food consumption in the larvae have been reported elsewhere (Matsuda et al., 2010; Shimizu et al., 2013, 2014a,b). As previously described (Shimizu et al., 2014b), two types of powder diet colored green and red, respectively (containing the same components as those described above), were obtained from Itosui Co., Tokyo, Japan. First, the test prometamorphic larvae were fed the green-colored diet and kept under laboratory conditions. Then, after a 24-h fast, an adequate amount of the red-colored food was made available at 3% of BW. Test substances were ICV-administered as described below, and after 15 min, each animal was decapitated and the gastrointestinal tract was removed. In our previous study, we observed food intake in intact and ICV-injected larvae during 60 min after recovery from anesthesia. Then, we determined that food intake is measured during first 15 min. The wet weight of the red-colored gastrointestinal contents was measured after removal of intestinal juice with tissue paper, and expressed as micrograms of food taken per gram BW. The experiments were conducted around noon.

Effect of ICV Administration of BIBP3226 and SB334867 on the Orexigenic Actions of ICV-Injected NPY and Orexin A

In order to double-check the orexigenic actions of NPY and orexin A, and the antagonistic actions of BIBP3226 and SB334867 upon them (Shimizu et al., 2013, 2014b), we examined the effects of ICV administration of NPY, orexin A, BIBP3226, and SB334867 on food intake in prometamorphic larvae. Our previous studies have indicated that simultaneous injection of antagonists with peptides induces their antagonistic effects (Shimizu et al., 2013, 2014b).

We did not have an atlas for bullfrog larval brain. However, according to our previous experiments, same procedures have been done. Therefore, following sentences were mainly quoted from our previous paper (Matsuda et al., 2010; Shimizu et al., 2013, 2014b). "For ICV administration of test substances, each animal was placed in a stereotaxic apparatus under anesthesia with MS-222. A small area (∼1 mm^2 square) of the parietal skull was carefully removed using a surgical blade (No. 19, Futaba, Tokyo, Japan), and then 0.1 μl/g BW (0.5–0.7 μl) of each test substance including Evans blue dye was injected into the third ventricle of the brain using a 10-μl Hamilton syringe with a 0.1-μl scale. The gap in the parietal skull was then filled with a surgical bonding agent (Aron Alpha, Sankyo, Japan). The accuracy of the injection site and volume was confirmed after the experiment by examining whether Evans blue dye was present in the ventricle without leakage. Control larvae in each experiment

were injected with the same volume of saline in the same way as for the experimental group. Each larva that had received the ICV injection was placed individually in a small experimental tank (diameter 11 cm) containing 700 ml of tap water. After recovery from anesthesia, each larva was supplied with the red-colored food equivalent to 3% of its BW. After 15 min, the weight of the intestinal contents was measured as described above."

"In order to check the effect of ICV injection of BIBP3226 on the orexigenic action of NPY at 10 pmol/g BW, half a microliter of BIBP3226 at 100 pmol/g BW was injected into the third ventricle of the brain of larvae as described above. The ICV-injected dose of BIBP3226 had been determined in previous experiments (Shimizu et al., 2013). Larvae in the control group were given injections of the same volume of saline-diluted dimethyl sulfoxide (vehicle). Following the administration of BIBP3226 or vehicle, either NPY at 10 pmol/g BW or saline was delivered by ICV injection. The feeding experiment was performed according to the procedures described above. In order to check the effect of ICV injection of SB334867 on the central actions of orexin A, 0.5 μl of SB334867 at 60 pmol/g BW in addition to orexin A at 6 pmol/g BW was injected into the third ventricle of the brain of the larvae. The ICV-injected dose of SB334867 had been determined by reference to a previous study (Shimizu et al., 2014b). Larvae in the control group were given an injection of the same volume of dimethyl sulfoxide diluted with saline."

Effect of ICV Administration of SB334867 on the Orexigenic Action of ICV-Injected NPY

According to our previous experiments, same procedures have been done. Therefore, following sentences were mainly quoted from our previous paper (Shimizu et al., 2014b). "In order to examine the effect of ICV injection of SB334867 on the central action of NPY, 0.5 μl of SB334867 at 60 pmol/g BW in addition to NPY at 10 pmol/g BW was injected into the third ventricle of the larval brain. Larvae in the control group were given an injection of the same volume of dimethyl sulfoxide diluted with saline. The feeding experiment was performed according to the procedures described above."

Data Analysis

According to our previous experiments, same statistical analysis have been done. Therefore, following sentences were quoted from our previous paper (Kojima et al., 2009). "All results pertaining to the effect of receptor antagonists on food intake are expressed as the mean ± SEM. Statistical analysis was performed using two-way ANOVA with Bonferroni's method. Statistical significance was determined at the 5% level."

RESULTS

Effect of ICV Injection of BIBP3226 on the Orexigenic Action of ICV-Injected NPY

ICV administration of NPY at 10 pmol/g BW stimulated cumulative food intake during the 15-min observation period in comparison with ICV injection of vehicle or BIBP3226 at 100 pmol/g BW. ICV injection of NPY at 10 pmol/g BW plus BIBP3226 at 100 pmol/g BW did not affect cumulative food intake during the 15-min observation period in comparison with ICV injection of vehicle (**Figure 1**). Interaction between treatments with NPY and BIBP3226 was significant by two-way ANOVA with Bonferroni's method ($F = 5.83$ and $p = 0.023$).

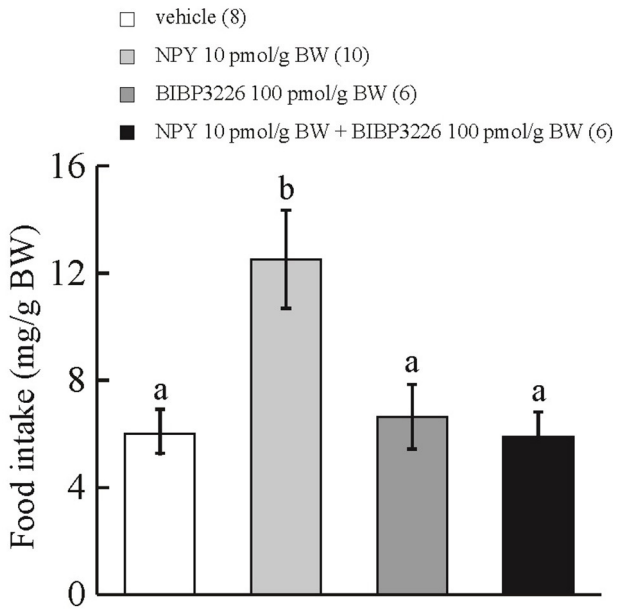


FIGURE 1 | Effect of ICV administration of a NPY Y1-receptor antagonist, BIBP3226, on the orexigenic action of NPY in prometamorphic larvae. Each column and bar represents the mean and SEM, respectively, and the numbers in parentheses in the panels indicate the number of larvae in each group. Significance of differences was evaluated by two-way ANOVA with Bonferroni's method. Different superscripts (a and b) indicate statistical significance ($P < 0.05$).

Effect of ICV Injection of SB334867 on the Orexigenic Actions of ICV-Injected Orexin A

ICV administration of orexin A at 6 pmol/g BW enhanced cumulative food intake during the 15-min observation period in comparison with ICV injection of vehicle or SB334867 at 60 pmol/g BW. ICV injection of orexin A at 6 pmol/g BW plus SB334867 at 60 pmol/g BW did not affect cumulative food intake for 15 min after feeding in comparison with ICV injection of vehicle (**Figure 2**). Interaction between treatments with SB334867 and orexin A was significant by two-way ANOVA with Bonferroni's method ($F = 8.05$ and $p = 0.009$).

Effect of ICV Administration of SB334867 on the Orexigenic Action of ICV-Injected NPY

ICV administration of NPY at 10 pmol/g BW increased cumulative food intake during the 15-min observation period

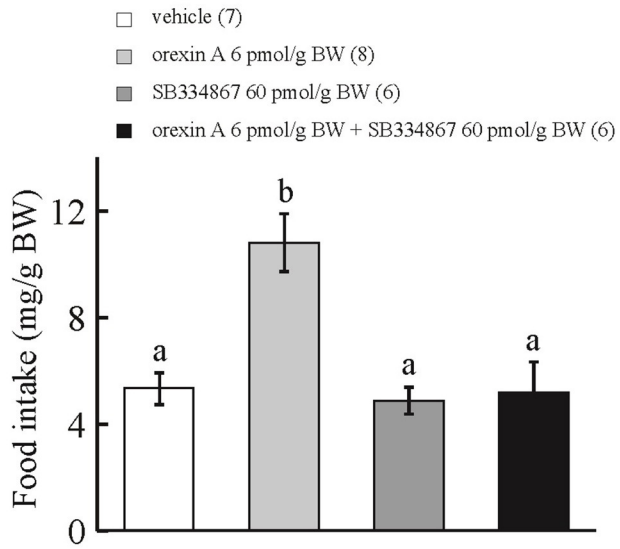


FIGURE 2 | Effect of ICV administration of an orexin receptor antagonist, SB334867, on the orexigenic action of orexin A in prometamorphic larvae. Each column and bar represents the mean and SEM, respectively, and the numbers in parentheses in the panels indicate the number of larvae in each group. Significance of differences was evaluated by two-way ANOVA with Bonferroni's method. Different superscripts (a and b) indicate statistical significance ($P < 0.05$).

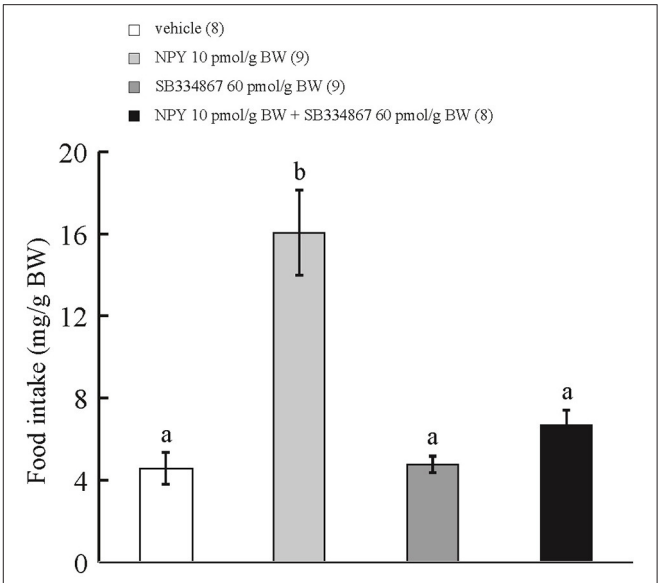


FIGURE 3 | Effect of ICV administration of SB334867 on the orexigenic action of NPY in prometamorphic larvae. Each column and bar represents the mean and SEM, respectively, and the numbers in parentheses in the panel indicate the number of larvae in each group. Significance of differences was evaluated by two-way ANOVA with Bonferroni's method. Different superscripts (a and b) indicate statistical significance ($P < 0.05$).

in comparison with ICV injection of vehicle or SB334867 at 60 pmol/g BW. ICV injection of NPY at 10 pmol/g BW plus SB334867 at 60 pmol/g BW did not affect cumulative food intake during the 15-min observation period in comparison with ICV injection of vehicle (**Figure 3**). Interaction between treatments with SB334867 and NPY was significant by two-way ANOVA with Bonferroni's method ($F = 31.9$ and $p = 0.0001$).

DISCUSSION

Larvae of anuran amphibians can feed and grow until the metamorphic climax, because as metamorphosis progresses, many body parts such as the oral and digestive organs are reconstructed (Ishizuya-Oka and Shi, 2007; Wright et al., 2011). Therefore, feeding behavior in anuran larvae seems to be regulated by appetite and satiety factors during the pre- and pro-metamorphic periods. In bullfrog larvae, ICV administration of NPY, orexin A, or ghrelin induces an increase of food consumption, whereas ICV injection of corticotropin-releasing factor attenuates food intake (Matsuda et al., 2010; Shimizu et al., 2013, 2014a,b). These findings suggest that NPY, orexin A, and ghrelin are implicated in the regulation of feeding behavior as potent orexigenic neuropeptides in the larval brain. We obtained larvae at pre- and pro-metamorphic stages. However, premetamorphic larvae were very small. In the present study, we used prometamorphic bullfrog larvae, and double-checked the orexigenic actions of NPY and orexin A, and the antagonistic effects of BIBP3226 and SB334867 upon them.

In the goldfish model, our and other previous studies have indicated that the orexigenic action of NPY is mediated by the orexin A signaling pathway (Volkoff and Peter, 2001; Kojima et al., 2009): NPY-induced action was blocked by treatment with SB334867, and it was also attenuated by treatment with an excess amount of orexin A. Using a double-immunostaining and confocal laser scanning microscopy, neuroanatomical observation has indicated that neurons with NPY- and orexin-like immunoreactivities are located in the hypothalamic region, the nucleus posterioris periventricularis, in close proximity to each other neuron (Kojima et al., 2009). Accordingly, in this species, there seems to be a functional relationship between NPY and orexin A in the regulation of feeding. In the present study, we indicated for the first time that the action of NPY is blocked by treatment with SB334867. The results suggest that the orexigenic action of NPY is mediated via the orexin receptor in bullfrog larvae as well as in goldfish. Thus, it is likely that NPY and orexin A exert orexigenic actions in bullfrog larvae. This observation also supports the role of a functional relationship between NPY and orexin A in feeding regulation among vertebrates (Yamanaka et al., 2000; Niimi et al., 2001; Sahu, 2002). Bullfrog larvae can feed and grow until the metamorphic climax, and orexigenic factors such as NPY and orexin A seem to stimulate appetite, and to regulate energy balance of larvae during the developmental stages (Shimizu et al., 2013, 2014b). The present study suggests that NPY and orexin A is mutually involved in appetite regulation during the prometamorphic stages.

In conclusion, our data indicate that, in bullfrog larvae, the orexigenic action of NPY is mediated by the orexin A-induced orexigenic action.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: KoM, TN, and NK; performed the experiments: KaM and SS; analyzed the data: KoM and KaM; contributed materials: KaM and SS; wrote the paper: KoM; revising the paper: TN and NK.

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REVIEW
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Neuropeptide Control of Feeding Behavior in Birds and Its Difference with Mammals

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Feeding is an essential behavior for animals to sustain their lives. Over the past several decades, many neuropeptides that regulate feeding behavior have been identified in vertebrates. These neuropeptides are called "feeding regulatory neuropeptides." There have been numerous studies on the role of feeding regulatory neuropeptides in vertebrates including birds. Some feeding regulatory neuropeptides show different effects on feeding behavior between birds and other vertebrates, particularly mammals. The difference is marked with orexigenic neuropeptides. For example, melanin-concentrating hormone, orexin, and motilin, which are regarded as orexigenic neuropeptides in mammals, have no effect on feeding behavior in birds. Furthermore, ghrelin and growth hormone-releasing hormone, which are also known as orexigenic neuropeptides in mammals, suppress feeding behavior in birds. Thus, it is likely that the feeding regulatory mechanism has changed during the evolution of vertebrates. This review summarizes the recent knowledge of peptidergic feeding regulatory factors in birds and discusses the difference in their action between birds and other vertebrates.

Keywords: feeding regulatory peptides, neuropeptides, feeding, central nervous system, birds

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INTRODUCTION

Domestic chickens (*Gallus gallus domesticus*) are raised worldwide for the production of meat and eggs as food for humans. Chickens have been genetically selected for efficient meat and egg production and thereby many strains have been developed. However, they are divided broadly into two strains: One is selected for meat production (meat-type chickens) and the other is for egg production (layer-type chickens) (Denbow, 1994). Meat-type strains including broilers have been selected for rapid early growth (Denbow, 1994). Nutrient and food intake during the chick stage is thought to be important for sustaining growth in meat-type chickens, thus it is essential to understanding the mechanisms underlying ingestion in these, and other, types of birds (Siegel and Wisman, 1966; Denbow, 1994).

Food intake is regulated by complex systems involving both central and peripheral sites of control, such as the gastrointestinal tract, liver, and brain. Food must enter the gastrointestinal tract, where it then must be digested and absorbed, and it is expected that this organ system has a role in regulating food intake. In most avian species, food is temporarily stored in the crop and then enters the proventriculus and gizzard. The expansion of the crop and gizzard is expected to contribute to the termination of short-term feeding (Savory, 1999). The liver is also important in regulating feeding behavior because injection of glucose into the portal vein suppresses feeding

behavior in chickens (Denbow, 1994). Intraheptic injection of other nutrients, such as lipids and amino acids, also affects feeding behavior (Denbow, 1994). Peptidergic hormones such as cholecystokinin (CCK) are also thought to be related to the regulation of feeding behavior (Denbow, 1994). These peripheral factors are sent to the central nervous system via humoral and neural pathways (Denbow, 1994; Savory, 1999). The control of the beak and the visual, taste, and smell sensations are also thought to be important in controlling feeding behavior. These sensations are sent to the central nervous system via specific pathways, such as the trigeminal sensorimotor system (for the control of grasping and mandibulation of the diet), the tectofugal and thalamofugal pathways (for visual sensation), the gustatory system, and the olfactory pathway (Kuenzel, 1989). The autonomic nervous system/parasympathetic pathway is also important in regulating feeding behavior in birds (Kuenzel, 1989).

The central nervous system plays an important role in regulating feeding behavior in birds (Kuenzel, 1989, 1994). The hypothalamus is thought to have important roles in regulating feeding in birds. Classically, the ventromedial nucleus (VMN) and lateral hypothalamic area (LHA) are respectively known as satiety and feeding centers in birds as well as mammals. In addition to these nuclei, other hypothalamic nuclei, such as the paraventricular nucleus (PVN) and infundibular nucleus [IN, an avian homolog of the arcuate nucleus (ARC)], are thought to be involved in regulating feeding behavior.

Over the past several decades, numerous bioactive molecules have been identified as affecting the activity of the neural network related to regulating feeding behavior in birds. Among them, peptidergic molecules that act in the central nervous system are sometimes called "feeding regulatory neuropeptides" and are well-studied in birds (Furuse et al., 2007). Feeding regulatory neuropeptides are conveniently divided into two categories: One suppresses feeding behavior and are called anorexigenic or anorectic neuropeptides, and the other stimulates feeding behavior and are called orexigenic neuropeptides.

The roles of feeding regulatory neuropeptides have been well-studied not only in birds but also in other vertebrates, such as mammals (Kageyama et al., 2012), amphibians (Carr et al., 2002), and teleosts (Volkoff et al., 2005). In these studies, the effects of some feeding regulatory peptides in birds are somewhat different from the effects in other vertebrates, in particular mammals (Furuse et al., 2007). This review summarizes the recent knowledge of feeding regulatory peptides in birds and discusses the difference in their action between birds and other vertebrates. Most of the studies on avian feeding regulatory peptides are well-performed using neonatal chicks (Furuse et al., 2007; Bungo et al., 2011; Honda, 2016), so previous findings on chicks are mainly described.

PERIPHERAL PEPTIDERGIC HORMONES

Several peptidergic hormones released from peripheral tissues are thought to affect feeding behavior. Among them, leptin, ghrelin, and CCK, which are mainly released from adipose tissue, stomach, and small intestine, respectively, are expected to play a key role in regulating feeding behavior in mammals (Friedman and Halaas, 1998; Miyasaka and Funakoshi, 2003; Kojima and Kangawa, 2005). These hormones have been identified in birds, but some of them produce different effects in birds compared to other vertebrates.

Leptin

Leptin is the protein product of the *obese* gene and is released from adipose tissue (Friedman and Halaas, 1998). Central or peripheral injection of leptin induces a decline in food intake, enhancement of energy expenditure, and decrease in adipose tissue and body weight in mammals (Friedman and Halaas, 1998). In addition, because lacking normal leptin or a leptin receptor induces obesity in mice (Friedman and Halaas, 1998), leptin is thought to have an important role in regulating body fat volume and body weight. The anorexigenic effect of leptin has also been observed in teleosts. For example, intracerebroventricular (ICV) or intraperitoneal (IP) injection of mouse leptin suppresses feeding behavior in goldfish, in part by modulating the orexigenic effect of neuropeptide Y (NPY) and orexin (Volkoff et al., 2003). Furthermore, leptin receptor-deficient medaka showed hyperphagia, increase in body weight, and deposition of visceral fat (Chisada et al., 2014). These results suggest that leptin is an important anorexigenic peptide in vertebrates.

The anorexigenic effect of leptin has also been observed in Asian blue quail (Lõhmus et al., 2006) and wintering white-throated sparrows (Cerasale et al., 2011). Similar to these avian species, ICV injection of human leptin suppresses feeding behavior in young chickens (Denbow et al., 2000). However, the effect seems to depend on age: ICV injection of murine leptin has no effect on feeding in neonatal chicks (Bungo et al., 1999b). Thus, the role of leptin on feeding behavior in chicks is somewhat different from that in mammals.

Taouis et al. (1998) isolated the *leptin* gene in chickens, and the amino acid sequence of chicken leptin has high homology to mammalian counterparts. However, other researchers could not find the isolated *leptin* gene in the sequenced chicken genome (Friedman-Einat et al., 1999) or other avian genomes. Recently, the *leptin* gene has been isolated in several kinds of birds (Boswell and Dunn, 2015). The chicken *leptin* gene has also been newly identified (Seroussi et al., 2016). Avian leptin exhibits ∼30% amino acid identity to its mouse and human counterparts (Boswell and Dunn, 2015). These findings suggest the necessity of reexamining the effect of leptin on feeding behavior in birds. Studies using these avian leptins are needed to clarify the actual role of leptin in regulating feeding in avian species.

Ghrelin

Ghrelin, an acylated peptide hormone released from the stomach, was originally identified as an endogenous stimulator of growth hormone (GH) release from the anterior pituitary, but later work revealed that this peptide is related to regulating feeding in mammals. In rodents, central or peripheral injection of ghrelin increases feeding behavior (Nakazato et al., 2001), demonstrating that ghrelin is one orexigenic peptide in mammals.

Ghrelin has also been identified in non-mammalian species including chickens (Kaiya et al., 2002) and stimulates GH release

(Kaiya et al., 2002). In addition, ghrelin was also demonstrated to stimulate feeding behavior in teleosts (Unniappan et al., 2002) and bullfrog larvae (Shimizu et al., 2014). Thus, ghrelin is regarded as an orexigenic peptide in both mammals and fish. In contrast, ICV injection of rat ghrelin inhibits rather than stimulates feeding behavior in neonatal chicks (Furuse et al., 2001). Moreover, chicken ghrelin suppresses feeding behavior in neonatal chicks when administered centrally (Saito et al., 2002a), demonstrating that central ghrelin is not an orexigenic peptide in chickens. ICV injection of rat ghrelin also suppresses feeding behavior in adult Japanese quail (Shousha et al., 2005a). Thus, the action of ghrelin in the feeding regulatory mechanism in the brain of birds is the opposite of that in mammals and fish.

ICV-injected ghrelin induces several behavioral changes such as vocalization and hyper activity in neonatal chicks (Saito et al., 2002b). These behaviors are also induced by ICV injection of corticotropin-releasing hormone (CRH) (Zhang et al., 2001a), and CRH itself is regarded as an anorexigenic peptide in chicks (Furuse et al., 1997b). These facts imply that the effect of central ghrelin is related to the CRH systems. Indeed, co-injection of astressin, a CRH receptor antagonist, restores the ghrelin-induced decrease in food intake in neonatal chicks (Saito et al., 2005). Thus, the anorexigenic effect of central ghrelin is likely to be mediated by the CRH system in chicks. Further studies revealed that the anorexigenic effect of ghrelin is also mediated by the serotonergic system (Zendehdel et al., 2013) and β-adrenergic system (Zendehdel and Hassanpour, 2014). In rodents, the orexigenic effect of ghrelin is thought to be mediated by NPY (Nakazato et al., 2001). In chicks, ICV injection of chicken ghrelin has no effect on the mRNA expression of NPY (Saito et al., 2005). Since ghrelin does not activate NPY neuron in the brain, ghrelin might lack its orexigenic effect in birds.

In contrast to central action, the peripheral action of ghrelin is not consistent in birds. IP injection of lower doses of rat ghrelin (0.4–0.9 nmol/100 g body weight) stimulates feeding behavior, whereas a higher dose (2.4 nmol/100 g body weight) suppresses feeding behavior in adult Japanese quail (Shousha et al., 2005a). In chicks, intravenous injection of chicken ghrelin slightly but significantly suppresses feeding behavior (Geelissen et al., 2006). On the other hand, intravenous injection of chicken ghrelin has no effect on food intake in neonatal chicks (Kaiya et al., 2007). Nevertheless, the change in plasma ghrelin concentration after food deprivation in chicks is similar to rats (Toshinai et al., 2001): 12-h food deprivation increases plasma ghrelin concentration and ghrelin content in the proventriculus in chicks (Kaiya et al., 2007). In rodents, the peripheral ghrelin signal, which is sent to the nucleus of the solitary tract via the vagus nerve, is transmitted to the hypothalamus via noradrenergic neurons, and thereafter activates NPY neurons (Date et al., 2006). It is possible that peripheral ghrelin does not affect this pathway in birds.

ANOREXIGENIC NEUROPEPTIDES

Table 1 summarizes the representative candidates for anorexigenic neuropeptides in birds. These peptides are roughly categorized as the CRH family, melanocortin, glucagon superfamily, brain-gut hormones, and others. CRH, urotensin, urocortin, and stresscopin belong to the CRH family, and they all suppress feeding behavior (Furuse et al., 1997b; Zhang et al., 2001b; Cline et al., 2009b; Ogino et al., 2014). Melanocortin is derived from the precursor protein proopiomelanocortin (POMC). Among them, adrenocorticotropin hormone (ACTH) and α-melanocyte-stimulating hormone (α-MSH) have been demonstrated to suppress feeding behavior (Deviche and Delius, 1981; Kawakami et al., 2000a). In the glucagon superfamily, glucagon, glucagon-like peptide-1 (GLP-1), GLP-2, oxyntomodulin, vasoactive intestinal peptide (VIP), pituitary-adenylate cyclase-activating polypeptide (PACAP),

TABLE 1 | Neuropeptides showing anorexigenic effects on feeding behavior in birds.

Anorexigenic neuropeptide	Change in food intake in neonatal chicks
CRH FAMILY	
Corticotropin-releasing hormone (CRH)	Decreased
Urotensin	Decreased
Urocortin	Decreased
Stresscopin	Decreased
MELANOCORTIN	
Adrenocorticotropic hormone (ACTH)	Decreased
α-melanocyte-stimulating hormone (α-MSH)	Decreased
GLUCAGON SUPERFAMILY	
Glucagon	Decreased
Glucagon-like peptide-1 (GLP-1)	Decreased
Glucagon-like peptide-1 (GLP-2)	Decreased
Oxyntomodulin	Decreased
Vasoactive intestinal peptide (VIP)	Decreased
Pituitary-adenylate cyclase-activating polypeptide (PACAP)	Decreased
GCGL	Decreased
Growth hormone-releasing hormone (GHRH)	Decreased
BRAIN-GUT PEPTIDES	
Cholecystokinin (CCK)	Decreased
Gastrin	Decreased
Gastrin-releasing peptide (GRP)	Decreased
Neuromedin B	Decreased
Neuromedin C	Decreased
OTHERS	
Calcitonin gene-related peptide	Decreased
Cocaine-amphetamine regulated transcript	Decreased
Neuromedin S	Decreased
Neuromedin U	Decreased
Neuropeptide FF	Decreased
Neuropeptide K	Decreased
Neuropeptide S	Decreased
Mesotocin	Decreased
Substance P	Decreased
Vasotocin	Decreased

and growth hormone-releasing hormone (GHRH) have been demonstrated to suppress feeding behavior (Furuse et al., 1997a; Tachibana et al., 2003a, 2015; Honda et al., 2007; Shousha et al., 2007; Cline et al., 2008b). Well-known brain-gut peptides that affect feeding behavior are CCK and bombesin-like peptides. In the bombesin-like peptides, gastrin-releasing peptide (GRP) and neuromedin B (both are homologs of bombesin) also suppress food intake after central injection (Tachibana et al., 2010b). Other peptides including vasotocin, mesotocin, neuromedin S, neuromedin U, neuropeptide FF, neuropeptide K, neuropeptide S, substance P, cocaine- and amphetamine-regulated transcript, and calcitonin gene-related peptide were reported to suppress feeding behavior (Tachibana et al., 2003b, 2004b, 2010a,c; Shousha et al., 2005b; Cline et al., 2007a,b, 2009a; Prall and Cline, 2008; Masunari et al., 2013). Most of the above are also known as anorexigenic peptides in mammalian species, although GHRH is thought to be an orexigenic neuropeptide in mammals.

CRH Family

CRH is a 41-amino acid peptide and is well-known as a neuropeptide involved in the stress response (De Souza, 1995). CRH functions as the hypothalamic signal for the hypothalamus-pituitary-adrenal (HPA) axis and stimulates ACTH release from the anterior pituitary. There are two subtypes of CRH receptor, namely CRH-R1 and CRH-R2 (Hauger et al., 2003). The CRH receptors may also bind urotensins, which were originally isolated from the urophysis of teleosts. (Vaudry et al., 2010). Urotensin I is a paralog of CRH, and urotensin II shows structural similarity to somatostatin (Vaudry et al., 2010). Further work revealed that the *urotensin II* gene exists in mammals and birds (Vaudry et al., 2010). Urocortin is also a CRH-like peptide consisting of 40-amino acids and shows similarity to urotensin I (Hauger et al., 2003). Later, two isoforms of urocortin, urocortin-2, and urocortin-3, were identified (Hauger et al., 2003). At the same time, Hsu and Hsueh (2001) identified other CRH-like peptides, stresscopin and stresscopin-related peptide. Urocortin-3 and urocortin-2 are the C-terminus fragments of stresscopin and stresscopin-related peptide, respectively. While CRH binds to both CRH-R1 and R2, urocortin-2, urocortin-3, stresscopin-related peptide, and stresscopin are selective ligands for CRH-R2 (Hsu and Hsueh, 2001; Hauger et al., 2003).

ICV injection of CRH induces a stress-like response, such as hyperactivity, vocalization, hyperthermia, and increases in corticosterone release, in neonatal chicks (Zhang et al., 2001a; Tachibana et al., 2004b). As well as a stress-like response, CRH is also expected to be related to the inhibition of feeding behavior of neonatal chicks. In fact, ICV injection of CRH suppresses food intake (Furuse et al., 1997b). Furthermore, ICV injection of mammalian urotensin-1, urocortin-1, urocortin-3, and stresscopin decreases food intake in chicks (Zhang et al., 2001b; Cline et al., 2009b; Ogino et al., 2014). Thus, the CRH family peptides are expected to be anorexigenic neuropeptides in the brain of chicks. ICV injection of CRH increases the plasma corticosterone concentration in chicks, whereas the injection of urocortin-3 has no effect (Ogino et al., 2014), suggesting that urocortin-3 binds to a different receptor than CRH and exerts its effect. Because stresscopin and urcortin-3 show high affinity to the CRH-R2 receptor (Hsu and Hsueh, 2001; Hauger et al., 2003), CRH-R2 partly contributes to the anorexigenic effect of the CRH family peptides.

CRH has been demonstrated to modify the effect of other anorexigenic neuropeptides in chicks. Indeed, the anorexigenic effects of ghrelin, GLP-1, α-MSH, VIP, PACAP, glucagon, and CCK are partly attenuated by co-injection of CRH receptor antagonist (Tachibana et al., 2004c, 2006, 2007, 2012; Saito et al., 2005; Honda et al., 2012). Thus, it is possible that CRH might be one of the downstream mediators for the anorexigenic neural pathway in the brain of neonatal chicks.

Melanocortins

Melanocortins, such as ACTH, α-MSH, β-MSH, and γ-MSH, are derived from the precursor POMC. To date, five melanocortin receptors (MC1R to MC5R) have been identified. In mammals, the melanocortin system plays an important role in regulating food intake and energy metabolism because disrupting melanocortin receptor-4 (MC4R) results in obesity, hyperphagia, and hyperglycemia in mice (Huszar et al., 1997). α-MSH is an endogenous MC4R agonist and decreases food intake in rats when injected centrally (Poggioli et al., 1986). Agouti-related protein (AGRP) is a naturally occurring antagonist for MC3R and MC4R (Fong et al., 1997), and its C-terminal fragment attenuates the anorexigenic effect of α-MSH in mammals (Rossi et al., 1998). Furthermore, the C-terminal fragment itself shows an orexigenic effect when administered centrally (Rossi et al., 1998). Thus, α-MSH and AGRP competitively regulate feeding behavior via MC4R in mammalian species.

The chicken *POMC* gene has been found to be a single copy gene and shows the same structural organization as in other vertebrates (Takeuchi et al., 1999). As in mammals, it has been demonstrated that ICV injection of α-MSH inhibits feeding behavior (Kawakami et al., 2000a), and the effect is attenuated by co-injection of AGRP in neonatal chicks (Tachibana et al., 2001a). Moreover, ICV injection of AGRP itself increases food intake in chicks and ring doves (Tachibana et al., 2001a; Strader et al., 2003). Notably, the orexigenic effect of AGRP depends on the strain of chicks: ICV injection of AGRP stimulates feeding behavior in layer chicks, whereas it has no effect on broiler chicks, which show rapid growth and heavy body weight compared with layer chicks (Tachibana et al., 2001a). Furthermore, a study using lines of White Plymouth Rock chickens reported that long-term divergent selection for low body weight shows high sensitivity to the anorexigenic effect of α-MSH, whereas sensitivity is low in the line selected for high body weight (Cline et al., 2008b). Thus, these results indicate that the melanocortin system is different between strains of chickens and is involved in regulating body weight in chicks.

In addition to alpha-MSH, the effect of some other melanocortins on feeding behavior has also been examined in birds. Although ACTH mainly exists in the anterior pituitary and functions as the pituitary signal of HPA axis, ACTH-containing neurons were found in several brain regions including the ARC of the hypothalamus (Csiffáry et al., 1990), and ACTH treatment suppresses feeding behavior in mammals (Al-Barazanji et al., 2001). The anorexigenic effect of ACTH was also observed

in birds because ICV injection of ACTH suppresses feeding behavior in domestic pigeons (Deviche and Delius, 1981) and neonatal chicks (Shipp et al., 2015). On the other hand, chicken β-MSH and γ2-MSH have no effect on feeding in birds (Saneyasu et al., 2011).

Glucagon Superfamily

The glucagon superfamily, such as glucagon, GLP-1, GLP-2, VIP, PACAP, and GHRH, are also known to have an important role in regulating feeding behavior in birds (Honda, 2016). Glucagon, GLP-1, and GLP-2 are derived from the precursor protein proglucagon (Sherwood et al., 2000). These peptides are found in the peripheral tissue, but they are likely to exist in the central nervous system because proglucagon mRNA is expressed in the medulla oblongata of neonatal chicks (Tachibana et al., 2005a). In addition, food deprivation decreases the mRNA expression of proglucagon in the medulla oblongata, suggesting that endogenous proglucagon-derived peptides are related to the regulation of feeding behavior in neonatal chicks (Tachibana et al., 2005a). Indeed, ICV injection of GLP-1 has been demonstrated to decrease food intake in neonatal chicks (Furuse et al., 1997a) and Japanese quails (Shousha et al., 2007). In addition, ICV injection of GLP-1 induces Fos expression in the VMN in young chickens (Tachibana et al., 2004a), suggesting that central GLP-1 activates neurons in the VMN, and thereby suppresses feeding behavior. In fact, direct injection of GLP-1 into the VMN decreases food intake in young chickens. Not only exogenous GLP-1, but also endogenous GLP-1 is thought to be related to the inhibition of feeding behavior because ICV injection of the GLP-1 receptor antagonist exendin (5–39) stimulates feeding behavior in neonatal layer chicks (Tachibana et al., 2001b).

In addition to GLP-1, other proglucagon-derived peptides, such as glucagon, GLP-2, and oxyntomodulin, also suppress feeding behavior in neonatal chicks (Honda, 2016). Glucagon is a 29-amino acid peptide and is known as an important regulator of glucose metabolism (Sherwood et al., 2000). In chickens, glucagon receptor mRNA is expressed in the central nervous system, and is especially highly expressed in the hypothalamus in addition to several peripheral tissues (Wang et al., 2008). Although intravascular injection of glucagon has no effect on food intake, ICV injection of glucagon decreases food intake in neonatal chicks (Honda et al., 2007), suggesting that glucagon exerts its anorexigenic effect in the brain. Honda et al. (2015) also demonstrated that ICV injection of chicken GLP-2 decreases food intake in neonatal chicks, whereas human GLP-2 has no effect. Thus, central GLP-2 is expected to function as an anorexigenic neuropeptide in birds.

Oxyntomodulin shows the same amino acid sequence as glucagon at the N-terminus region followed by a 26-amino acid extension at its C-terminus in chickens (Honda, 2016). In mammals, the C-terminus amino acid extension is shorter than that in chickens (Honda, 2016), indicating that the structure of oxyntomodulin is different in chickens and mammals. Nevertheless, ICV injection of both mammalian and chicken oxyntomodulin decreases food intake in neonatal chicks (Cline et al., 2008a; Honda et al., 2014a). However, the anorexigenic effect of oxyntomodulin seems to be weaker than that of GLP-1 and GLP-2 (Honda et al., 2015). ICV injection of chicken oxyntomodulin increases plasma glucose and corticosterone levels in neonatal chicks (Honda et al., 2014a). Similar responses have also been observed after ICV injection of glucagon (Honda et al., 2007). In addition, the similarity of the amino acid sequence suggests that glucagon and oxyntomodulin suppress feeding behavior in chicks with the same neural networks. On the other hand, injection of GLP-2 decreases the plasma glucose level and has no effect on the plasma corticosterone level (Honda et al., 2015). In addition, injection of oxyntomodulin increases Fos expression in the IN and ARC, whereas it has no effect in the VMN in neonatal chicks (Cline et al., 2008a). Because GLP-1 induces Fos expression in the VMN (Tachibana et al., 2004a), it is likely that the anorexigenic neural pathway is different between oxyntomodulin, GLP-1, and GLP-2.

VIP and PACAP are also thought to suppress feeding behavior in chicks because ICV injections of these peptides decreases food intake in neonatal chicks (Tachibana et al., 2003a; Khan et al., 2013). ICV injection of anti-chicken VIP antiserum increases food intake in neonatal chicks, suggesting that central VIP is related to the inhibition of feeding. CRH is likely to mediate the feeding-inhibitory effect of VIP and PACAP because their anorexigenic effects are attenuated by co-injection of a CRH receptor antagonist in neonatal chicks (Tachibana et al., 2004c).

A novel glucagon-like peptide named GCGL is also thought to be related to the regulation of feeding behavior because ICV injection of GCGL decreases food intake in neonatal chicks (Honda et al., 2014b). The anorexigenic effect of GCGL is also mediated by the CRH system because co-injection of CRH receptor antagonist attenuates the effect of GCGL (Honda et al., 2014b). GCGL mRNA expression in the hypothalamus is not changed by 24-h food deprivation (Honda et al., 2014b), suggesting that central GCGL might not be related to normal feeding behavior but specific feeding, such as stress-related anorexia.

GHRH is recognized as a stimulator of GH release in mammals (Sherwood et al., 2000). In addition to the GH-releasing effect, GHRH is thought to be related to the regulation of feeding behavior because central injection of GHRH stimulates feeding behavior in rats (Vaccarino et al., 1985). In chickens, GHRH-like peptide (GHRH-LP) has been identified but its amino acid sequence shows low homology to mammalian GHRH (Sherwood et al., 2000). Furthermore, GHRH-LP is less potent in stimulating GH release in chickens (Harvey, 1999). These facts implied that there might be another GHRH in chickens. In 2007, Wang et al. (2007) identified chicken GHRH, which has higher affinity to chicken GHRH receptors than GHRH-LP (Wang et al., 2010). Although the amino acid sequence of this GHRH has low similarity to mammalian GHRH, a synteny analysis indicated that the chicken *GHRH* gene is located on a conserved synteny of all vertebrate species examined, including teleosts and amphibians (Wang et al., 2007). Based on these facts, it has been demonstrated that newly found GHRH is true GHRH in chickens. It is likely that GHRH and GHRH-LP

are produced by the whole genome duplication (Wang et al., 2007).

ICV injection of synthesized chicken GHRH inhibits feeding behavior in neonatal chicks (Tachibana et al., 2015) as opposed to mammals. Notably, chicken GHRH-LP also suppresses feeding in chicks after ICV injection (Tachibana et al., 2015). Both GHRH and GHRH-LP have no effect on behavioral pattern and plasma corticosterone concentration, and it is likely that their anorexigenic effects may not be related to the induction of abnormal behavior, such as sleeping and hyperactivity, and to stress conditions (Tachibana et al., 2015). In addition, food deprivation affects mRNA expression of GHRH in the diencephalon (Tachibana et al., 2015), suggesting that endogenous GHRH in the brain is related to feeding regulation.

Brain-Gut Peptides

CCK and gastrin are well-known as gastrointestinal hormones in vertebrates. Because these peptides share the same 5-amino acid sequence at the C-terminus, they belong to the same peptide family (Miyasaka and Funakoshi, 2003). CCK is present in a variety of biologically active peptides, such as CCK58, CCK33, and CCK8 derived from the precursor peptide (Miyasaka and Funakoshi, 2003). CCK has multiple effects on the gastrointestinal system including gallbladder contraction, gut motility, gastric emptying, and the secretion of gastric acid and pancreatic enzymes (Miyasaka and Funakoshi, 2003). Additionally, numerous studies have documented the satiety-inducing role of CCK in the brain. For example, central injection of CCK suppresses feeding behavior in sheep (Della-Fera and Baile, 1980).

CCK also inhibits feeding behavior in young chickens (Denbow and Myers, 1982) and neonatal chicks (Furuse et al., 2000; Tachibana et al., 2012) when administered centrally. The anorexigenic effect of CCK depends on the length of amino acids because ICV-injected CCK33S shows a stronger effect than CCK8S (Furuse et al., 2000), and CCK4 does not affect food intake in neonatal chicks (Tachibana et al., 2012). The effect of CCK is likely mediated by CRH because a CRH receptor antagonist attenuates the anorexigenic effect of CCK (Tachibana et al., 2012). In addition to CCK, ICV injection of gastrin decreases food intake in neonatal chicks (Furuse et al., 2000), suggesting that central gastrin is also related to the inhibition of feeding behavior.

Bombesin, a 14-amino acid peptide originally isolated from the skin of frog, suppresses feeding behavior when administered centrally and peripherally in young chickens (Denbow, 1989). In mammals and birds, there are two homologs of bombesin-like peptides called neuromedin B and gastrin-releasing peptide. The fragment of gastrin-releasing peptide is called neuromedin C. ICV injections of these bombesin-like peptides decreases food intake in neonatal chicks (Tachibana et al., 2010b), suggesting that they function as anorexigenic peptides in the brain of birds.

OREXIGENIC NEUROPEPTIDES

Table 2 summarizes the representative candidates of orexigenic neuropeptides in birds. Orexigenic peptides are roughly categorized as the families of pancreatic peptide, opioid and its related peptides, Arg-Phe-NH_2 peptide (RFamide peptide), and others. In the pancreatic peptide family, NPY, pancreatic polypeptide (PP), and peptide YY (PYY) are thought to be orexigenic peptides in birds (Kuenzel et al., 1987; Ando et al., 2001). Opioid and its related peptides that stimulate feeding behavior in birds are β-endorphin, endomorphin-2, and nociception (Deviche and Schepers, 1984; Abbasnejad et al., 2005; Bungo et al., 2007). Gonadotropin-inhibiting hormone (GnIH), 26RFa, and prolactin-releasing peptide (PrRP), which are members of the RFamide peptide family, are also regarded as orexigenic peptides in birds (Tachibana et al., 2004d, 2005b; Ukena et al., 2010). In addition, AGRP, somatostatin, and galanin are also known as stimulators of feeding behavior in birds (Tachibana et al., 2001a, 2008b, 2009).

TABLE 2 | Neuropeptides showing orexigenic effects on feeding behavior in birds.

Orexigenic neuropeptide	Change in food intake in neonatal chicks
PANCREATIC PEPTIDE FAMILY	
Neuropeptide Y (NPY)	Increased
Pancreatic peptide	Increased
Peptide YY(1–36)	Increased
OPIOID AND ITS RELATED PEPTIDES	
β-endorphin	Increased
Endomorphin-2	Increased
Nociception	Increased
RFAMIDE PEPTIDES	
Gonadotropin-inhibiting hormone (GnIH)	Increased
26RFa	Increased
Prolactin-releasing peptide (PrRP)	Increased
OTHERS	
Agouti-related protein (AGRP)	Increased
Galanin	Increased
Somatostatin	Increased

In mammals, melanin-concentrating hormone (MCH), motilin, and orexin are also known as orexigenic peptides (Garthwaite, 1985; Rossi et al., 1997; Sakurai et al., 1998). However, ICV injections of these peptides have no effect on food intake in neonatal chicks (Furuse et al., 1999; Ando et al., 2000). Ghrelin and GHRH are known as orexigenic neuropeptides in mammals. However, ghrelin and GHRH inhibit rather than stimulate feeding behavior in chicks (as noted above). Thus, it is likely that the feeding-stimulating neural networks in the brain of birds are different from that in other vertebrates.

Pancreatic Peptide Family

NPY is a neuropeptide consisting of 36-amino acids and belongs to the pancreatic polypeptide family (Tatemoto et al., 1982). NPY is a potent orexigenic neuropeptide in mammals (Levine and Morley, 1984), reptiles (Morris and Crews, 1990), amphibians (Crespi et al., 2004), and teleosts (López-Patiño et al., 1999). Similarly, central injection of NPY stimulates feeding behavior in

chickens (Kuenzel et al., 1987; Chen et al., 2016), white-crowned sparrows (Richardson et al., 1995), and ring doves (Strader and Buntin, 2001). ICV injection of chicken NPY increases food intake and anti-chicken NPY antibody decreases food intake in neonatal chicks (Chen et al., 2016). In addition, fasting increases NPY content in the PVN and IN of the hypothalamus in young chickens (Zhou et al., 2005). These results suggest that endogenous NPY in the brain may function as an orexigenic neuropeptide in chicks. Furthermore, the NPY content in the hypothalamus of embryos and the mRNA expression level of NPY in the hypothalamic nuclei in chickens are different between layers and broilers (Zhou et al., 2006; Chen et al., 2007). It is therefore possible that NPY contributes to the difference in food intake and growth rate in chickens.

NPY-containing neurons in the IN of the hypothalamus are co-localized with the insulin receptor in neonatal chicks (Shiraishi et al., 2011). In addition, NPY mRNA in the brainstem is downregulated by insulin (Shiraishi et al., 2008). These findings suggest that the activity of the NPY neuron is regulated by insulin. In addition, other neuropeptides regulate the mRNA expression of NPY. For example, ICV injection of GnIH upregulates NPY mRNA expression in the hypothalamus (McConn et al., 2014). As well as the potent orexigenic effect, the existence of neural networks with other feeding regulatory peptides implies that NPY plays a key role in regulating feeding behavior in chicks.

PP is also expected to possess an orexigenic effect in chickens because ICV injection of avian PP increases food intake in young chickens (Kuenzel et al., 1987). A similar effect was also reported in neonatal chicks: ICV injection of human or rat PP increases food intake (Ando et al., 2001). Because centrally-injected PP also stimulates feeding behavior in mice (Asakawa et al., 1999), it is likely that the role of PP in the brain is conserved between chicks and rodents.

PYY belongs to the pancreatic family and is released from the gastrointestinal tract. There are two major forms of PYY, namely PYY(1-36) and PYY(3-36). ICV injection of PYY(1-36) stimulates feeding behavior in rats (Clark et al., 1987). In contrast, IP injection of PYY(3-36) inhibits feeding behavior (Batterham et al., 2002). PYY(3-36) binds to the NPY Y2 receptor, which is highly expressed on NPY neurons in the ARC of the hypothalamus and suppresses the activity of NPY neurons (Batterham et al., 2002). Intra-ARC injection of PYY(3–36) suppresses feeding behavior (Batterham et al., 2002), demonstrating that PYY(3–36) is an anorexigenic neuropeptide as a peripheral factor in mammals. In neonatal chicks, ICV injection of mammalian PYY(1–36) increases food intake as it does in rats (Ando et al., 2001). Recently, Aoki et al. (2016) identified cDNA of chicken PYY precursor and found that intravenous injection of PYY(3–36) decreases food intake in neonatal chicks. This result indicates that peripheral PYY(3–36) functions as an anorexigenic neuropeptide in chickens.

Opioid and Its Related Peptides

In mammals, the opioid system in the central nervous system was reported to stimulate feeding behavior (Kuenzel, 1994). Similarly, ICV injection of β-endorphin, an endogenous opioid, stimulates feeding behavior in pigeons (Deviche and Schepers, 1984), young chickens (McCormack and Denbow, 1988), and white-crowned sparrows (Maney and Wingfield, 1998). These results indicate that the central opioid system functions as an orexigenic neuropeptide in birds.

Among opioid receptors, δ- and κ-receptors are thought to be related to the orexigenic effect of opioids because ICV injection of δ-receptor agonists ([D-Ala^2, D-Leu^3]-enkephalin and [D-Pen^{2,5}]-enkephalin) and κ-receptor agonists (U-50488H and U-62066) increase food intake in chicks (Bungo et al., 2004). On the other hand, a μ-receptor agonist, [D-Ala^2, N-MePhe4, Gly^5-ol]-enkephalin, decreases food intake in neonatal chicks probably because of the sleep-like behavior induced by this agonist (Bungo et al., 2004). McCormack and Denbow (1987) reported that intramuscular injection of naloxone, an opioid μ-receptor antagonist, suppresses feeding behavior in young chickens. Furthermore, ICV injection of naloxone suppresses feeding behavior in white-crowned sparrows (Maney and Wingfield, 1998). Bungo et al. (2005) demonstrated that ICV injection of the μ-receptor antagonist, β-funaltrexamine, decreases food intake in neonatal chicks. Based on the studies using antagonists for the μ-receptor, this receptor is also expected to be related to the orexigenic effect of opioid in birds. In fact, the μ-receptor is demonstrated to mediate the orexigenic effect of other orexigenic peptides, such as NPY (Dodo et al., 2005), GnIH (Tachibana et al., 2008a), galanin (Tachibana et al., 2008b), and somatostatin (Tachibana et al., 2009). The opioid system is likely a downstream mediator for the orexigenic neural networks in the brain of birds.

Nociceptin (orphanin FQ), a 17-amino acid peptide, is an endogenous ligand of the nociceptin receptor, which shows structural similarity to the opioid receptor. Nociceptin is thought to have an orexigenic effect in mammals (Stratford et al., 1997). The orexigenic effect of nociceptin is also observed in chickens: ICV injection of nociceptin increases food intake and feeding time (Abbasnejad et al., 2005). Thus, the role of the opioid system in feeding behavior seems to be conserved between birds and mammals.

RFamide Peptides

GnIH is a dodecapeptide possessing a C-terminal sequence, Arg-Phe-NH_2 (RFamide peptide) (Tsutsui et al., 2000), and suppress gonadotropin release in birds and other vertebrates (Tsutsui, 2009; Tsutsui et al., 2010). Because GnIH inhibits gonadotropin release in quail and other birds, this peptide is an important factor in regulating avian reproduction (Tsutsui, 2009; Tsutsui et al., 2010). The distribution of GnIH and its receptor in the brain (Bentley et al., 2003; Ubuka et al., 2003; Yin et al., 2005) indicates that GnIH is not only related to the reproduction but also to behavioral and autonomic mechanisms (Tsutsui, 2009; Tsutsui et al., 2010). It has been demonstrated that food restriction decreases the release of gonadotropin and sex steroids in domestic hens (Richard-Yris et al., 1987). This finding suggests that gonadotropin and sex steroids are related to the regulation of energy homeostasis, including feeding behavior in avian species. In fact, ICV injection of quail and chicken GnIH and its related peptides increases food intake in neonatal chicks (Tachibana et al., 2005b; McConn et al., 2014). A similar effect of GnIH is also observed in Pekin duck (Fraley et al., 2013). ICV injection

of anti-GnIH antiserum decreases deprivation-induced feeding in neonatal chicks (Tachibana et al., 2005b). Food deprivation for 48-h induces Fos expression in GnIH-immunoreactive neurons in the PVN of the hypothalamus of Pekin duck (Fraley et al., 2013). Moreover, GnIH mRNA expression increases in the hypothalamus of neonatal chicks by fasting (McConn et al., 2016). These results suggest that endogenous GnIH in the brain is related to the regulation of feeding behavior in birds. It is likely that the orexigenic effect of GnIH is mediated by the opioid and NPY systems in neonatal chicks (Tachibana et al., 2008a; McConn et al., 2014).

26RFa was originally isolated from frog brains (Chartrel et al., 2003). This peptide was named based on its features: It consists of a 26-amino acid residue and possesses an RFamide sequence at its C-terminus. 26RFa is also found in humans and rats, and its mRNA is distributed in the LHA and VMN of the hypothalamus of rats (Chartrel et al., 2003). ICV injection of 26RFa stimulates feeding behavior in mice (Chartrel et al., 2003), suggesting that this peptide functions as an orexigenic neuropeptide in mammals. 26RFa was also identified in birds including quail and chickens (Ukena et al., 2010). The 26RFa mRNA is expressed in the diencephalon that includes the hypothalamus, and 26RFa-containing perikarya were found in the anterior hypothalamic nucleus in quail and chicks (Ukena et al., 2010). In addition, the mRNA of GPR103, a receptor for 26RFa, is distributed more in the diencephalon than other brain regions (Ukena et al., 2010). These findings suggest that 26RFa is involved in regulating feeding behavior in birds. Indeed, ICV injection of 26RFa increases food intake in neonatal broiler chicks, although it has no effect in layer chicks (Ukena et al., 2010).

PrRP was first isolated from the hypothalamus as a specific prolactin-releasing factor for mammalian pituitary cells (Hinuma et al., 1998). However, subsequent studies revealed that the peptide has less effect on prolactin release in mammals (Maruyama et al., 1999). On the other hand, ICV injection of PrRP has been demonstrated to suppress feeding behavior in rats (Lawrence et al., 2000). Concurrently with the discovery of PrRP, Carassius Arg-Phe-NH_2 peptide (C-RFa), an ortholog of PrRP, was isolated from Japanese crucian carp (Fujimoto et al., 1998). Central and peripheral injection of fish PrRP inhibits feeding behavior in goldfish (Kelly and Peter, 2006), suggesting that PrRP functions as an anorexigenic peptide in mammals and teleosts. However, in neonatal chicks ICV injection of mammalian PrRP31 stimulates rather than inhibits feeding behavior (Tachibana et al., 2004d). After the discovery of chicken PrRP, the orexigenic effect of PrRP was confirmed: ICV injection of chicken PrRP increases food intake in neonatal chicks (Tachibana et al., 2011). These results suggest that PrRP might be an orexigenic neuropeptide in birds, unlike in mammals and teleosts. Wang et al. (2012) identified new chicken PrRP and showed that there are two types of PrRP encoded by separate genes. They also demonstrated that the chicken PrRP we previously identified (Tachibana et al., 2011; Tachibana and Sakamoto, 2014) is an ortholog of C-RFa, and newly identified chicken PrRP is an ortholog of mammalian PrRP. These two types of PrRP are thought to be produced by whole genome duplication (Wang et al., 2012; Tachibana and Sakamoto, 2014). Since newly identified chicken PrRP also increases food intake in neonatal chicks when administered centrally (Tachibana and Sakamoto, 2014), the role of PrRPs on feeding behavior is expected to have changed during the evolution of vertebrates.

Somatostatin

Somatostatin is well-known as a hypothalamic inhibitor of GH release from the anterior pituitary (Brazeau et al., 1973). This neuropeptide also affects feeding behavior in mammals because ICV injection of somatostatin suppresses feeding behavior in chicks (Vijayan and McCann, 1977). However, the effect of somatostatin on feeding behavior depends on the experimental condition (Feifel and Vaccarino, 1990). Somatostatin also has an inhibitory effect on GH release in chickens (Harvey and Scanes, 1987). Although somatostatin affects feeding behavior in chicks as well as mammals, it consistently stimulates feeding behavior in neonatal chicks after ICV injection (Tachibana et al., 2009). The injection of coristatin, a neuropeptide structurally related to somatostatin, also stimulates feeding behavior in neonatal chicks (Tachibana et al., 2009).

As noted above, it is likely that the effects of GH-related peptides including ghrelin and GHRH are different between chicks and mammals. In mammals, peptides that stimulate GH release usually show an orexigenic effect, whereas peptides that inhibit GH release partly have an anorexigenic effect. In chicks, on the other hand, GHRH and ghrelin possess an anorexigenic effect, whereas somatostatin possesses an orexigenic effect. Because most of these studies were performed by injecting exogenous peptides, further studies on the effect of endogenous GH-related peptides should show the actual relationship between these peptides and feeding behavior in chicks.

Galanin

Galanin is a neuropeptide consisting of 29-amino acids and is distributed in the brain and digestive tract. Central injection of galanin stimulates feeding behavior in rats (Kyrkouli et al., 1986) and goldfish (de Pedro et al., 1995). Similarly, ICV injection of mammalian galanin increases food intake in neonatal chicks (Tachibana et al., 2008b). These results suggest that the orexigenic effect of galanin is conserved in vertebrates.

CONCLUSION

As noted above, several kinds of peptidergic molecules are related to the regulation of feeding behavior in birds, and some neuropeptides show different effects from that in other vertebrates. Notably, non-peptidergic feeding regulatory factors, such as norepinephrine, 5-hydroxytryptamine, and histamine, show a similar effect in vertebrates when administered centrally (Denbow et al., 1982; Bungo et al., 1999a; Kawakami et al., 2000b). Why avian species have acquired distinct effects in response to feeding regulatory peptides compared to mammals is still unknown. The most marked trait in birds is adaptation for flying. For the purpose of flying, birds possess a toothless beak, feathers and wings, a unique digestive tract and respiratory system, and a high metabolic rate. Their

short rectum and lightweight skeleton are also adaptations for flying. Flying requires more energy but overeating disturbs flying. This might be a partial reason why the effect of orexigenic neuropeptides in birds is different than in other vertebrates.

However, most of the studies on feeding regulation in birds have used exogenous peptide treatments. Studies on the roles of endogenous feeding regulatory peptides are needed to understand the true physiological regulation of feeding behavior in birds. In addition, it should be noted that most of the studies used neonatal and young chickens. It is possible that the roles of neuropeptides in regulating feeding behavior vary with age. Studies using adult birds should clarify the actual difference in neuropeptide control of feeding behavior between vertebrates. Clarifying the feeding regulatory mechanism should provide information on the evolution of feeding regulation in vertebrates and would be beneficial information for poultry production.

AUTHOR CONTRIBUTIONS

TT wrote this review together with co-author KT, Waseda University, Japan. In addition, TT integrated KT's sentences and edited this review as the corresponding author.

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REVIEW
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GnIH Control of Feeding and Reproductive Behaviors

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In 2000, Tsutsui and colleagues discovered a neuropeptide gonadotropin-inhibitory hormone (GnIH) that inhibits gonadotropin release in birds. Subsequently, extensive studies during the last 15 years have demonstrated that GnIH is a key neurohormone that regulates reproduction in vertebrates, acting in the brain and on the pituitary to modulate reproduction and reproductive behavior. On the other hand, deprivation of food and other metabolic challenges inhibit the reproductive axis as well as sexual motivation. Interestingly, recent studies have further indicated that GnIH controls feeding behavior in vertebrates, such as in birds and mammals. This review summarizes the discovery of GnIH and its conservation in vertebrates and the neuroendocrine control of feeding behavior and reproductive behavior by GnIH.

Keywords: gonadotropin-inhibitory hormone, gonadotropin-releasing hormone, gonadotropins, reproduction, reproductive behavior, feeding behavior

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INTRODUCTION

The discovery of "neurosecretion" in the first half of the last century created neuroendocrinology as a new research field in endocrinology. Scharrer proposed "neurosecretion" as a new concept and suggested that hypothalamic neurons that terminate in the neurohypophysis produce and release neurohormones in the 1920s. This new idea was not accepted by the scientific community easily and criticized strongly. In 1949, however, the concept of "neurosecretion" was established by Bargmann. Subsequently, hypothalamic neuropeptides, such as oxytocin (1) and vasopressin (2), which are secreted from the neurohypophysis, were identified. Harris (3) hypothesized from the histology of hypothalamic neurons that hypothalamic neurons terminating at the median eminence (ME) may produce and release neurohormones into the hypophysial portal system from the ME, and they may regulate anterior pituitary hormones secretion. Subsequently, this hypothesis was demonstrated by the discovery of several important neurohormones from the brain of mammals. Thyrotropin-releasing hormone was discovered by Burgus et al. (4) and Boler et al. (5), whereas gonadotropin-releasing hormone (GnRH) was discovered by Matsuo et al. (6) and Burgus et al. (7). Growth hormone-inhibiting hormone (somatostatin) was discovered by Brazeau et al. (8).

In early 1970s, the groups of Schally (6) and Guillemin (7) discovered a hypothalamic neuropeptide that was later named GnRH, which stimulated the release of luteinizing hormone (LH) as well as follicle-stimulating hormone (FSH) from the anterior pituitary. Thereafter, GnRHs have been identified in other vertebrates (9–12). It was generally accepted that GnRH is the sole hypothalamic neuropeptide that regulates gonadotropin release in vertebrates. However, in 2000, Tsutsui and colleagues discovered gonadotropin-inhibitory hormone (GnIH), a hypothalamic neuropeptide that

actively inhibits LH and FSH release in quail, which provides the demonstration of a hypothalamic neuropeptide inhibiting gonadotropin release for the first time in any vertebrate (13).

Studies conducted by Tsutsui and colleagues over 15 years showed that GnIH is conserved in vertebrates, from lampreys to humans and acts as a key neurohormone that regulates reproduction [see Ref. (14–26) for reviews]. In addition, recent studies have shown that GnIH has multiple functions other than the control of reproduction (27, 28). Besides regulating gonadotropin secretion, GnIH further regulates reproductive behavior by changing neurosteroid biosynthesis in the brain (28).

On the other hand, food deprivation inhibits the reproductive axis and sexual motivation. Interestingly, recent studies have further indicated that GnIH controls feeding behavior in vertebrates, such as in mammals and birds [for reviews, see Ref. (25, 26)]. Thus, the last 15 years of GnIH research has contributed to a better understanding of the mechanism of neuroendocrine regulation of feeding and reproductive behaviors as well as reproduction [for reviews, see Ref. (16–26, 29)].

Herein, this review summarizes the discovery of GnIH and its conservation in vertebrates and highlights our current understanding of the neuroendocrine control of feeding and reproductive behaviors by GnIH.

DISCOVERY OF GnIH AND ITS CONSERVATION IN VERTEBRATES

Discovery of GnIH

The discovery of "neurosecretion" led to create neuroendocrinology. In addition, recent discoveries of novel neuropeptides regulating reproductive physiology have expanded the horizons of this new research field in endocrinology. One of such discoveries was that of GnIH from a search for a novel neuropeptide that regulates pituitary hormones release in the avian brain (13).

Gonadotropin-inhibitory hormone is a new hypothalamic neuropeptide that possesses a C-terminal sequence Arg-Phe-NH_2 (RFamide peptide), which was isolated by high-performance liquid chromatography as well as competitive enzyme-linked immunosorbent assay in the Japanese quail brain (13). RFamide peptide was first identified in the late 1970s by Price and Greenberg who identified a peptide that has a sequence of Phe-Met-Arg-Phe-NH_2 from the ganglia of the venus clam and named FMRFamide (30). Subsequently, numerous RFamide peptides that act as neuromodulators, neurotransmitters, and peripheral hormones had been identified in invertebrates species. Importantly, immunohistochemical studies suggested that vertebrates also possess hypothalamic RFamide peptide(s) that may act on the anterior pituitary and regulate pituitary hormones secretion (31, 32). Tsutsui and colleagues made a breakthrough by discovering a novel RFamide peptide in 2000. The peptide had a sequence of Ser-Ile-Lys-Pro-Ser-Ala-Tyr-Leu-Pro-Leu-Arg-Phe-NH_2 (SIKPSAYLPLRFamide) and actively inhibited gonadotropin release from cultured quail anterior pituitary (**Tables 1** and **2**). This discovery provided the first demonstration of an inhibitory hypothalamic neuropeptide on gonadotropin release, which was not shown in any vertebrate (13). Given its biological action, this peptide was named GnIH (13) (**Figure 1**). In birds, GnIH neuronal cell bodies are located in the paraventricular nucleus (PVN) and terminals are found in the ME (13). The C-terminal of GnIH peptide is identical to LPLRFamide peptide of chicken (33), which may be a degraded C-terminal fragment of GnIH [for reviews, see Ref. (16, 21, 22)]. The GnIH precursor protein cDNA was cloned in quail (34) as well as other avian species [for reviews, see Ref. (16, 21, 22)]. The GnIH precursor protein encodes one GnIH and two GnIH-related peptides (GnIH-RP-1 and GnIH-RP-2) that possess an LPXRFamide (X = L or Q) motif at their C-terminus in all avian species investigated. Mature form of GnIH was also identified in starlings (35), zebra finches (36), as well as chicken (37) in birds. Quail GnIH-RP-2 was also identified (34) (**Tables 1** and **2**).

Gonadotropin-inhibitory hormone is considered to be a key neurohormone that inhibits avian reproduction as GnIH was shown to inhibit gonadotropin secretion in most of avian species that was studied [for reviews, see Ref. (16, 21, 22)] (**Figure 1**). To demonstrate the biological action of GnIH, Ubuka et al. (38) administered GnIH to mature male quail *in vivo* chronically. Chronic GnIH treatment decreases the concentration of plasma LH and testosterone and the expressions of common α, LHβ, and FSHβ subunit mRNAs. Furthermore, GnIH treatment induces apoptosis in testicular cells and decreases the diameter of seminiferous tubules in mature birds (38). Further, GnIH treatment also suppresses normal testicular growth and the increase in testosterone concentration in immature birds (38). Based on extensive studies, it appears that GnIH suppresses the development of the gonad and its maintenance by decreasing synthesis and release of gonadotropin in birds (**Figure 1**).

Conservation of GnIH in Vertebrates

To demonstrate GnIH conservation in other vertebrates, GnIHs were further identified in the hypothalamus of mammals and primates (39–43). The identified mammalian GnIH peptides possess C-terminal LPXRFamide (X = L or Q) as a common motif, as in avian GnIH and GnIH-RPs [for reviews, see Ref. (16, 17, 21–25)] (**Tables 1** and **2**). GnIH peptides were named LPXRFamide peptides based on the structure of their C-terminal. Mammalian GnIHs are also named RFamide-related peptide 1 and 3 (RFRP-1 and -3) (**Tables 1** and **2**). Administration of avian GnIH to Syrian hamsters centrally or peripherally inhibits LH release (40). It was shown that central administration of Siberian hamster GnIHs (RFRP-1 and -3) to Siberian hamsters also inhibits LH release (43). Centrally administered rat GnIH (RFRP-3) also inhibits LH release in rats (44) as well as GnRH-elicited LH release (45, 46). GnIH (RFRP-3) also inhibits GnRH-elicited gonadotropin synthesis and release and reduces LH pulse amplitude in ovine (47, 48) as well as bovine (49). Since human GnIH (RFRP-3) has the same structure as ovine GnIH (RFRP-3) (42), the biological action of human/ovine GnIH (RFRP-3) was investigated in the ovine pituitary. It was clearly shown that human/ovine GnIH (RFRP-3) inhibits GnRH-elicited secretion of LH and FSH (47), demonstrating that human/ovine GnIH inhibits synthesis and release of gonadotropin as well as GnRH-elicited gonadotropin secretion like avian GnIH [for reviews, see Ref. (16, 17, 21–26)] (**Figure 1**).

TABLE 1 | Molecular structure and behavioral actions of mature gonadotropin-inhibitory hormone (GnIH) peptides identified in birds and mammals.

Vertebrates	Molecular structure of mature GnIH peptides	Receptor and cell signaling of mature GnIH peptides	Behavioral actions of mature GnIH peptides
Mammals	MPHSFANLPLRFa (*human GnIH*; *RFRP-1*) VPNLPQRFa (*human GnIH*; *RFRP-3*) SGRNMEVSLVRQVLNLPQRFa (*macaque GnIH*; *RFRP-3*) SLTFEEVKDWAPKIKMNKPVVNKMPPSAANLPLRFa (*bovine GnIH*; *RFRP-1*) AMAHLPLRLGKNREDSLSRWVPNLPQRFa (*bovine GnIH*; *RFRP-3*) ANMEAGTMSHFPSLPQRFa (*rat GnIH*; *RFRP-3*) SPAPANKVPHSAANLPLRFa (*Siberian hamster GnIH*; *RFRP-1*) TLSRVPSLPQRFa (*Siberian hamster GnIH*; *RFRP-3*)	GPR147 is the primary and GPR74 is the secondary receptor for human GnIH (*human*). Activation of GPR147 suppresses cAMP production (*human*). Mammalian GnIHs (RFRPs) suppress adenylate cyclase /cAMP/ protein kinase A-dependent extracellular signal-regulated kinase phosphorylation (*mouse gonadotrope cell line*). Gonadotropin-releasing hormone (GnRH) neurons express GPR147 (*Siberian hamsters*). GnRH neurons express GPR147 mRNA (*mice*). Kiss1 neurons express GPR147 and GPR74 mRNAs (*mice*). Gonadotropes express GPR147 mRNA (*human*).	Central administration of mammalian GnIH (RFRP-3) decreases male sex behavior (*rats*). Central administration of avian GnIH reduces sexual motivation and vaginal scent marking (*female hamsters*). Central administration of mammalian GnIHs (RFRPs) induces anxiety-related behavior (*rats*). Central administration of mammalian GnIH (RFRP-3) increases food intake (*male rats, sheep*). Bilateral intraamygdalar administration of mammalian GnIH (RFRP-3) decreases food intake (*male rats*). Bilateral intraamygdalar administration of mammalian GnIH (RFRP-1) has positive rewarding–reinforcing properties (*male rats*).
Birds	SIKPSAYLPLRFa (*quail GnIH*) SSIQSLLNLPQRFa (*quail GnIH-RP-2*) SIRPSAYLPLRFa (*chicken GnIH*) SIKPFANLPLRFa (*European starling GnIH*) SIKPFSNLPLRFa (*zebra finch GnIH*)	GPR147 is the primary receptor for avian GnIH (*quail, chicken*). Activation of GPR147 suppresses cAMP production (*chicken*). GnRH1 and GnRH2 neurons express GPR147 mRNA (*starlings*). GPR147 is colocalized with luteinizing hormone β or follicle-stimulating hormone β mRNA in the pituitary (*chicken*).	Central administration of avian GnIH inhibits copulation solicitation (*female white-crowned sparrows*). Avian GnIH RNA interference reduces resting time, spontaneous production of complex vocalizations, and stimulated brief agonistic vocalizations (*white-crowned sparrows*). Central administration of avian GnIH decreases socio-sexual behavior (*male quail*). Central administration of avian GnIH, GnIH-RP-1 and GnIH-RP-2 stimulates food intake (*chicks*). Central administration of avian GnIH stimulates feeding (*adult Pekin drakes*).

Names of the investigated species or organism are shown in the parenthesis.

Tsutsui and colleagues further identified GnIH peptides in the brains of reptiles, amphibians, and fish. All of the identified or putative GnIHs also had a characteristic C-terminal LPXRFamide (X = L or Q) motif in these species, which avian and mammalian GnIHs have (50–57) (**Table 2**). Accordingly, GnIH peptides are produced in the brains of vertebrates across fish to humans [see Ref. (14–25) for reviews]. Three GnIHs, gfLPXRFa-1, -2, and -3 are encoded in goldfish GnIH precursor cDNA (52) (**Table 2**). It was shown that gfLPXRFa-1, -2, and -3 have inhibitory as well as stimulatory effects on synthesis and release of gonadotropin, which may depend on the reproductive phase (58–61). It was also shown that zfLPXRF-3, zebrafish GnIH, inhibits gonadotropin release (62).

As mentioned above, GnIH peptides were identified in gnathostomes from humans to fish. However, in the most ancient lineage of vertebrates, agnathans, the presence of GnIH peptide was not known (63). Accordingly, Tsutsui and colleagues searched for agnathan GnIH. Osugi et al. (64) cloned sea lamprey GnIH precursor cDNA. Three mature GnIH peptides were identified from the sea lamprey brain using immunoaffinity purification as well as mass spectrometry (64) (**Table 2**). The identified lamprey GnIHs have a C-terminal PQRFamide motif in common (64). Lamprey GnIH neuronal cell bodies exist in the hypothalamus and their immunoreactive fibers project to GnRH3 neurons (64). A lamprey GnIH peptide increases the expressions of lamprey GnRH3 and gonadotropin β mRNA. Lamprey GnIH may also act on GnRH3 neurons and stimulate gonadotropin β expression in the pituitary (64). Accordingly, GnIH may be a stimulatory neuropeptide in agnathans and changed its function to be an inhibitory neuropeptide during the evolution of vertebrates.

Evolutionary Origin of GnIH

Most GnIH peptides have C-terminal structure of LPXRFamide (X = L or Q), within a member of the RFamide peptide family [see Ref. (14–25) for reviews]. The neuropeptide FF (NPFF; PQRFamide peptide) group is also a member of the RFamide peptide family as NPFF peptides have a C-terminal PQRP motif [for reviews, see Ref. (14–17)]. Since the C-terminal structure of GnIH peptides is similar to that of NPFF peptides, further clarification of the NPFF peptide gene was warranted in agnathans. Tsutsui and colleagues accordingly identified the precursor cDNAs of NPFF peptides from the brains of lamprey and hagfish (65, 66). The phylogenetic analysis showed that agnathans genes encode GnIH and NPFF (65, 66). The identified agnathan NPFF peptides had the same C-terminal PQRFamide motif that also exists in agnathan GnIH peptides (65, 66). Based on these findings, Tsutsui and colleagues hypothesized that GnIH and NPFF genes derive from their ancestral gene of protochordates.

TABLE 2 | Molecular structure of gonadotropin-inhibitory hormone (GnIH) peptides in chordates.

Chordates	Peptide name	Molecular structure
Mammals	*Human RFRP-1*	MPHSFAN**LPLRFa**
	Human RFRP-3	VPN**LPQRFa**
	Macaque RFRP-3	SGRNMEVSLVRQVLN**LPQRFa**
	Bovine RFRP-1	SLTFEEVKDWAPKIKMNKPVVNKMPPSAAN**LPLRFa**
	Bovine RFRP-3	AMAHLPLRLGKNREDSLSRWVPN**LPQRFa**
	Rat RFRP-3	ANMEAGTMSHFPS**LPQRFa**
	Siberian hamster RFRP-1	SPAPANKVPHSAAN**LPLRFa**
	Siberian hamster RFRP-3	TLSRVPS**LPQRFa**
Birds	*Quail GnIH*	SIKPSAY**LPLRFa**
	Quail GnIH-RP-2	SSIQSLLN**LPQRFa**
	Chicken GnIH	SIRPSAY**LPLRFa**
	European starling GnIH	SIKPFAN**LPLRFa**
	Zebra finch GnIH	SIKPFSN**LPLRFa**
Reptiles	*Red-eared slider GnIH*	SIKPVAN**LPLRFa**
	Red-eared slider GnIH-RP-1	STPTVNKMPNSLAN**LPLRFa**
	Red-eared slider GnIH-RP-2	SSIQSLAN**LPQRFa**
Amphibians	*Bullfrog GRP/R-RFa*	SLKPAAN**LPLRFa**
	Bullfrog GRP-RP-1	SIPN**LPQRFa**
	Bullfrog GRP-RP-2	YLSGKTKVQSMAN**LPQRFa**
	Bullfrog GRP-RP-3	AQYTNHFVHSLDT**LPLRFa**
	Red-bellied newt LPXRFa-1	SVPN**LPQRFa**
	Red-bellied newt LPXRFa-2	MPHASAN**LPLRFa**
	Red-bellied newt LPXRFa-3	SIQPLAN**LPQRFa**
	Red-bellied newt LPXRFa-4	APSAGQFIQTLAN**LPQRFa**
Teleost fishes	*Goldfish LPXRFa-3*	SGTGLSAT**LPQRFa**
Agnathans	*Sea lamprey LPXRFa-1a*	SGVGQGRSSKTLFQ**PQRFa**
	Sea lamprey LPXRFa-1b	AALRSGVGQGRSSKTLFQ**PQRFa**
	Sea lamprey LPXRFa-2	SEPFWHRTR**PQRFa**
Protochordates	*Amphioxus PQRFa-1*	WDEAWR**PQRFa**
	Amphioxus PQRFa-2	GDHTKDGWR**PQRFa**
	Amphioxus PQRFa-3	GRDQGWR**PQRFa**

Only mature endogenous peptides structurally determined by high-performance liquid chromatography and mass spectrometry are shown. Characteristic C-terminal sequences for GnIH peptides, -LPXRFamide (X = L or Q) sequences, are shown in bold.

To demonstrate this hypothesis, Tsutsui and colleagues further cloned a precursor cDNA of PQRFamide peptide, which encodes three putative PQRFamide peptides in amphioxus (67). Mature forms of these three endogenous amphioxus PQRFamide peptides were identified by immunoaffinity purification as well as mass spectrometry (67) (**Table 2**). Phylogenetic analysis suggested that the amphioxus PQRFamide peptide precursor occurred before the divergence of GnIH and NPFF groups in vertebrates (67). Importantly, the conserved synteny region exists around the loci of the amphioxus PQRFamide peptide gene, as well as GnIH and NPFF gene in vertebrates (67). Namely, the amphioxus PQRFamide peptide gene is located close to the HOX cluster, whereas the GnIH and NPFF genes in vertebrates are located close to the HOXA and HOXC clusters, respectively. These results suggest that the GnIH and NPFF genes may duplicated by whole-genome duplications (67). The amphioxus PQRFamide peptide gene is therefore considered to be related to the ancestor of the GnIH and NPFF genes (67, 68). Thus, it is possible that the GnIH and NPFF genes diverged from the ancestral gene in protochordate during vertebrate evolution by whole-genome duplication.

CONTROL OF GnIH EXPRESSION AND MODE OF GnIH ACTION

Control of GnIH Expression by Environmental and Internal Factors

Studying the mechanisms controlling GnIH expression is important to understand the physiological role of GnIH. Stress inhibits reproduction in vertebrates (69). Calisi et al. (70) examined the effect of capture-handling stress on GnIH expression in house sparrows to investigate if stress changes GnIH expression. The number of GnIH-positive neurons increased in birds sampled in fall compared with birds in spring, and the numbers of GnIH-positive neurons increased in spring birds by capture-handling stress (70). These findings indicate that stress influences GnIH expression during the breeding season (70). Thus, stress may inhibit reproductive function in birds through GnIH neurons. In mammals, it was also found that acute and chronic immobilization stress both upregulate GnIH expression in the dorsomedial hypothalamic area of rats with a decrease in the activity of hypothalamic–pituitary–gonadal axis (HPG axis) (71). This

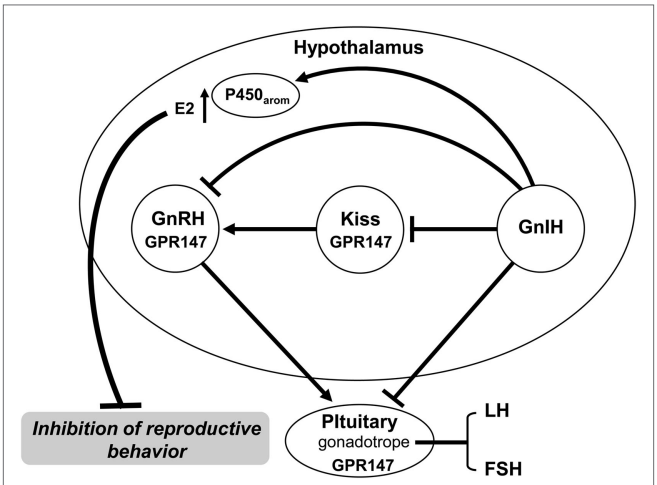


FIGURE 1 | Gonadotropin-inhibitory hormone (GnIH) control of reproduction and reproductive behavior. GnIH participates in the control of reproduction and reproductive behavior in birds and mammals. GnIH neurons project to the median eminence to inhibit gonadotropin synthesis and release *via* GnIH receptor (GPR147) expressed in gonadotropes. GnIH neurons also project to gonadotropin-releasing hormone (GnRH) neurons that express GPR147 and inhibit GnRH neuronal activity. Thus, GnIH inhibits gonadotropin synthesis and release by decreasing the activity of GnRH neurons as well as directly inhibiting pituitary gonadotrope function. GnIH neurons also project to kisspeptin (Kiss) neurons that express GPR147 in mammals. GnIH neurons further project to P450 aromatase neurons and stimulate aromatase activity to produce neuroestrogen (E2) that inhibits reproductive behavior. See the text for details.

stress-induced increase in GnIH expression is abolished by adrenalectomy (71). Immunohistochemical analysis further revealed that GnIH neurons express glucocorticoid receptor (GR) (71), suggesting that adrenal glucocorticoids directly act on GnIH neurons *via* GR to increase GnIH expression. Thus, it is considered that GnIH serves as an important stress integrator in the suppression of the reproductive axis in vertebrates.

Son et al. (72) found that GnIH neurons in the PVN express GR mRNA in quail, which suggests that glucocorticoids can directly control GnIH transcription in birds. In addition, corticosterone (CORT) treatment increases expression of GnIH precursor mRNA in the quail diencephalon (72). Furthermore, Son et al. (72) examined the transcription mechanism of GnIH gene by CORT using rHypoE-23 cells, a rat hypothalamic GnIH-expressing neuronal cell line. It was shown that rHypoE-23 cells express GR mRNA and CORT increases expression of GnIH mRNA (72). In addition, CORT stimulates the recruitment of GR to the GC response element in the promoter region of rat GnIH, supporting the idea that CORT induces GnIH expression *via* GR in GnIH neurons (72). It thus appears that stress reduces gonadotropin release by increasing GnIH expression in GnIH neurons.

It is thought that photoperiodic mammals utilize the annual changes in the nocturnal melatonin secretion to regulate reproductive activities (73). In photoperiodic birds, melatonin participates in the regulation of seasonal reproductive processes, such as gonadotropin secretion and gonadal activity (74–77), despite the dogma that seasonal changes in melatonin secretion is not used to time reproductive activities in birds (78, 79). Tsutsui and colleagues therefore investigated whether melatonin is involved in the regulation of GnIH expression in quail, a highly photoperiodic avian species (80). Melatonin is mostly produced in the pineal gland and eyes in quail (81). Ubuka et al. (80) found that pinealectomy together with orbital enucleation (Px + Ex) decreases GnIH precursor mRNA expression and GnIH peptide concentration in the quail brain. Melatonin administration increases GnIH precursor mRNA expression and GnIH peptide concentration in the quail brain (80). Importantly, a melatonin receptor subtype Mel_{1c} is expressed in GnIH neurons in the PVN (80). Chowdhury et al. (82) further demonstrated that melatonin increases GnIH release as well as GnIH expression in quail. GnIH release increases under short day (SD), when nocturnal secretion of melatonin is long (82). Importantly, GnIH release is negatively correlated with plasma LH concentration with their diurnal changes in quail (82). Based on these findings, it is considered that melatonin synthesized in the pineal gland and eyes acts directly on GnIH neurons *via* Mel_{1c} to induce GnIH expression and release in birds (24, 80, 82).

In contrast to birds, melatonin reduces GnIH expression in Syrian and Siberian hamsters, photoperiodic mammals (43, 83, 84). GnIH precursor mRNA levels as well as the number of GnIH cell bodies decrease in sexually inactive Syrian and Siberian hamsters kept under SD photoperiods, compared with sexually active animals kept in long day (LD) photoperiods. These photoperiodic changes in GnIH expression disappear in Px hamsters; however, melatonin injections to LD hamsters reduce GnIH expression to SD levels (43, 83). Seasonal GnIH expression patterns were similar in European and Turkish hamsters (85, 86) and the semi-desert rodent, Jerboa (87). Although these results suggest a role for GnIH in seasonal breeding, it is inconsistent with the seasonal reproductive control model. Hamsters may require abundant GnIH to suppress GnRH in LD, whereas high level of GnIH is unnecessary in SD hamsters of regressed reproductive axis. Ubuka et al. (43) clearly showed that GnIH administration suppresses gonadotropin secretion in LD, but stimulates it in SD, suggesting the role of GnIH to fine tune the reproductive axis according to different photoperiods. In sheep (88, 89) and rats (90), there are also reports showing that GnIH expression is controlled by season and melatonin. Accordingly, GnIH expression is modulated by melatonin in mammals, as in birds.

In addition to stress and photoperiod that are important environmental factors, social environment may also influence GnIH expression because reproductive physiology and behavior can vary between individuals even in the same natural environment. To determine this possibility, Calisi et al. (91) investigated the effect of competition for mating on GnIH. The opportunities of nesting for European starlings pairs were restricted and GnIH precursor mRNA and GnIH content in the brain were investigated. Birds that occupied nest boxes had fewer GnIH cells than birds without nest boxes. These results suggest a role of GnIH in the modulation of reproductive function in response to the social environment (91).

There is evidence that female bird presence and copulation decrease plasma T concentrations in male quail rapidly (92, 93).

Tsutsui and colleagues therefore examined the mechanism of how social stimuli change reproductive physiology and behavior. Recently, Tobari et al. (27) first found that the release of norepinephrine (NE) increases in the PVN rapidly in male quail when viewing a conspecific female. Likewise, GnIH precursor mRNA increases in the PVN associated with plasma LH decrease in male when viewing a female. Tobari et al. (27) then demonstrated a link between these two changes by showing that NE applied to diencephalic tissue blocks stimulates GnIH release *in vitro*. Tobari et al. (27) further found that GnIH neurons are innervated by noradrenergic fibers and express α2A-adrenergic receptor mRNA in male quail. Accordingly, it is considered that female presence increases NE release in the PVN that stimulates GnIH release, which suppress LH release in male quail (27).

Mode of GnIH Action on Gonadotropin Secretion

To reveal the mode of GnIH action on the secretion of gonadotropin, Tsutsui and colleagues characterized the receptor for GnIH in quail. The GnIH receptor, GPR147, identified in quail is a member of the G-protein coupled receptor (GPCR) superfamily (94), which is also named neuropeptide FF receptor 1 (NPFF1). The COS-7 cells membrane fraction transfected with GnIH receptor cDNA specifically binds GnIH and GnIH-RPs at high affinities (94). By contrast, non-amidated GnIH cannot bind the GnIH receptor. Accordingly, the C-terminal LPXRFamide (X = L or Q) motif is critical for its binding to GnIH receptor (94). GnIH receptor cDNA was also cloned in chicken (95). The GnIH receptor exists in gonadotropes in the pituitary, and GnIH acts on gonadotropes directly to reduce gonadotropin synthesis and release in birds [for reviews, see Ref. (16, 17, 21–26, 29)] (**Figure 1**). Ultrastructural studies of GnIH neurons to explore the neurosecretory nature are progressing. GnIH neurons further project to GnRH1 neurons expressing GnIH receptor in birds (35, 96–98) (**Figure 1**). Accordingly, it appears that in birds GnIH acts on gonadotropes as well as GnRH1 neurons to inhibit gonadotropin synthesis and release [see Ref. (16, 17, 21–26, 29) for reviews] (**Figure 1**).

In mammals, Hinuma et al. (99) identified a mammalian GnIH (RFRP) specific receptor that is identical to GPR147, and it was named OT7T022. In the same year, Bonini et al. (100) reported two different GPCRs for NPFF and named NPFF1 (same as GPR147) and NPFF2 (same as GPR74). As mentioned previously, NPFF has a C-terminal PQRFamide motif and is involved in pain modulation. GnIH (LPXRFamide peptide) and NPFF (PQRFamide peptide) genes may have evolved from the ancestral gene by gene duplication (64, 67, 68). GPR147 and GPR74 are thought to be paralogous (101). GnIH binds GPR147 at higher affinity, whereas NPFF binds GPR74 at high affinity (100, 102, 103). Thus, GPR147 (NPFF1, OT7T022) is considered to be the primary GnIH receptor.

To demonstrate the mode of GnIH action on the gonadotropes, Tsutsui and colleagues investigated GnIH receptor signaling pathways in LβT2 cells, a mouse gonadotrope cell line, which expresses GnIH receptor mRNA (104). In LβT2 cells, mouse GnIHs effectively reduce cAMP production and phosphorylation of extracellular signal-regulated kinase (ERK) induced by GnRH (104). Furthermore, mouse GnIHs reduce GnRH-induced LHβ expression and LH release (104). Adenylate cyclase (AC) and protein kinase A (PKA) inhibitors suppress the stimulatory effect of GnRH on gonadotropin expression (104). Thus, mouse GnIH reduces GnRH-stimulated gonadotropin secretion by specifically interfering with GnRH actions *via* a AC/cAMP/PKA-dependent ERK pathway (104).

Kisspeptin (Kiss) encoded by the *Kiss-1* gene is also a newly identified neuropeptide in mammals, following the discovery of GnIH. In mammals, Kiss possesses a C-terminal RFamide or RYamide motif. In contrast to GnIH, Kiss stimulates GnRH neurons and upregulates the HPG axis in mammals (105–108). Importantly, GnIH neurons project to GnRH1 neurons and Kiss neurons that express GnIH receptor (16, 17, 21–26, 109) (**Figure 1**). Therefore, GnIH may act on GnRH1 neurons and Kiss neurons to regulate their activities (16, 17, 21–26, 109) (**Figure 1**). Furthermore, GnIH neurons project not only to GnRH1 neurons but also to GnRH2 neurons and many other neurons in the brain, suggesting multiple actions of GnIH [for reviews, see Ref. (16, 17, 21–26)].

Because GnIH neurons express ERα and respond to E2 administration with c-Fos expression in rodents, Kriegsfeld et al. (40) suggested that GnIH is involved in estrogen feedback to GnRH neurons. Gibson et al. (110) showed that when endogenous E2 concentration is high at the time of the GnRH/LH surge the activity of GnIH neurons is low, suggesting that high E2 removes its negative feedback effect by decreasing the negative effect of RFRP-3 on GnRH neurons in female hamsters. It was further shown that the suprachiasmatic nucleus (SCN) projects to a large population of GnIH neurons, and SCN, GnRH, and GnIH neuronal activities are coordinated with ovulation (110). Molnár et al. (111) investigated the involvement of GnIH neurons in E2 feedback in mice. GnIH mRNA expression was compared in ovariectomized mice with and without E2 replacement. Subcutaneously, administered E2 for 4 days significantly suppressed GnIH mRNA expression. Salehi et al. (112) measured GnIH gene expression during the estrous cycle in the rat hypothalamus and found that GnIH mRNA expression during proestrus is lower when endogenous E2 concentration is the highest than the diestrus phase (112). Furthermore, Jørgensen et al. (113) showed that c-Fos-positive RFRP-1-ir neurons increase in diestrus when endogenous E2 concentration is lower as compared with proestrus in the female rat brain (113). Accordingly, downregulation of GnIH expression by estrogen may be the mechanism of estrogen positive feedback to GnRH/LH release at least in female rodents [see Ref. (114, 115) for reviews].

Direct Control of Reproduction by Gonadal GnIH

Accumulated findings indicate that GnIH is a key neuropeptide in the control of reproduction, by decreasing the activity of GnRH1 neurons in the hypothalamus to reduce gonadotropin synthesis and release and directly decreasing the activity of pituitary gonadotropes, resulting in the suppression of spermatogenesis and gonadal steroid secretion. In addition to the central actions of

GnIH, direct control of reproduction by gonadal GnIH is becoming clear [for reviews, see Ref. (16, 17, 21–26, 28, 29)]. GnIH and GnIH receptor are expressed in steroidogenic and germ cells in the gonads of birds and mammals, possibly acting in autocrine or paracrine mechanisms to suppress production of gonadal steroid and germ cell differentiation and maturation (116–122). There are also several reports in songbirds, showing that gonadal GnIH is directly regulated by melatonin, metabolic challenge, and stress according to season (123–125).

GnIH CONTROL OF FEEDING BEHAVIOR

Importantly, several lines of evidence indicate that GnIH not only regulates neuroendocrine functions but also behavior. Animals use photoperiod to time breeding according to maximal food availability anticipated in environments where energy availability changes according to season (73). Reproduction is temporarily inhibited when food become scarce during the breeding season (126). Reproductive function and sexual motivation are inhibited by deprivation of food and other metabolic stress (127–131). Therefore, it is considered that GnIH may control neural feeding circuits by transferring metabolic information to the HPG axis [for reviews, see Ref. (25, 26)].

In fact, intracerebroventricular (ICV) injection of GnIH stimulates food intake in chicks (132). Administrations of GnIH-RP-1 and GnIH-RP-2 also stimulate food intake in chicks (132). In further support of these findings, immunoneutralization of GnIH by central antiserum administration suppresses fasting-induced appetite, but does not modify feeding at *ad libitum* conditions in chicks (132). Similarly, ICV injection of GnIH, but not of GnIH-RP-1, stimulates feeding and suppresses plasma LH in adult Pekin ducks (133). Together, it is considered that at least GnIH is involved in the control of feeding behavior as well as reproduction in birds (**Table 1**; **Figure 2**).

To clarify the neurochemical cascade underlying GnIH actions on feeding behavior, Tachibana et al. (134) examined if GnIHs orexigenic effect occurs *via* the opioid and nitric oxide (NO) systems. According to Tachibana et al. (134), the orexigenic effect of centrally injected GnIH is attenuated by co-injection of an opioid μ-receptor antagonist β-funaltrexamine but not an opioid δ-receptor antagonist ICI-174,864 and an opioid κ-receptor antagonist nor-binaltorphimine in chicks. GnIH-induced feeding behavior is not affected by co-injection of a non-selective NO synthase inhibitor (134). More recently, McConn et al. (37) also investigated the mechanism of the orexigenic response of GnIH in chicks. ICV injection of chicken GnIH increases neuropeptide Y (NPY) mRNA but decreases pro-opiomelanocortin (POMC) mRNA in the chick hypothalamus (37). Additionally, ICV injection of chicken GnIH increases c-Fos expressed cells in the lateral hypothalamic area (LHA) (37). McConn et al. (37) further showed that in the isolated LHA, ICV administration of GnIH increases melanin-concentrating hormone (MCH) mRNA. Based on these findings, it is considered that opioid μ-receptor-positive neurons, and NPY, POMC, and MCH neurons are part of the orexigenic regulation by GnIH in birds.

In mammals, ICV administration of GnIH stimulates food intake in rats (44) and sheep (135). Additionally, food restriction activates GnIH neurons and GnIH infusion inhibits sexual motivation in hamsters (136, 137). GnIH mRNA levels are lower in male and female obese mice than wild-type animals, suggesting that GnIH stimulation of feeding circuits is reduced when energy storage is maximum (138). Furthermore, the inhibition of LH concentrations by food restriction is reduced in GnIH receptor (GPR147) knockout mice (139). Fu and van den Pol (140) reported in mouse brain slices that chicken and human GnIH inhibit POMC neurons and decrease Kiss cell excitation by opening potassium channels. Jacobi et al. (141) also reported in mice that GnIH inhibits POMC neurons' firing rate and predominantly inhibits NPY neurons' action potential activity as shown in **Figure 2**. Jacobi et al. (141) further reported that GnIH fibers have close contacts to NPY neurons.

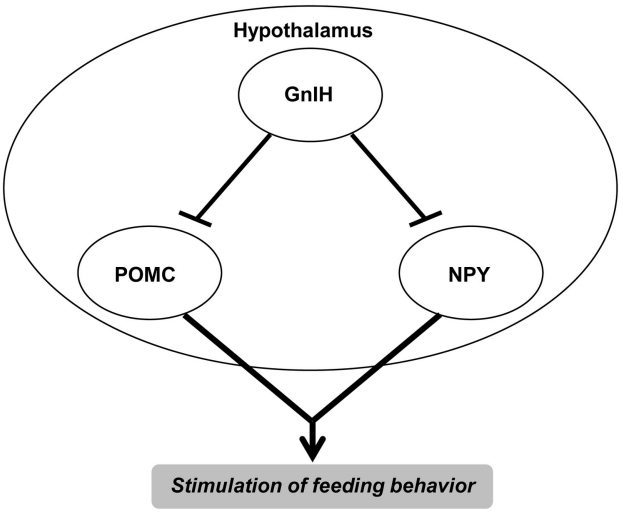


FIGURE 2 | Gonadotropin-inhibitory hormone (GnIH) control of feeding behavior. GnIH also participates in the control of feeding behavior in birds and mammals. Central administration of GnIH increases food intake in birds and mammals. GnIH neurons project to neuropeptide Y (NPY) neurons and pro-opiomelanocortin (POMC) neurons. GnIH inhibits the firing rate in POMC neurons and has a predominantly inhibitory effect on action potential activity in NPY neurons. Thus, GnIH neurons may stimulate feeding behavior by inhibiting POMC neurons and NPY neurons in the hypothalamus. Future studies are needed to further develop the concept of central mechanism of GnIH actions on the control of feeding behavior. See the text for details.

Together, these results indicate that GnIH is involved in the control of feeding behavior in birds and mammals by the similar mechanisms (**Table 1**; **Figure 2**). Future studies are needed to further develop the concept of central mechanism of GnIH actions in the regulation of feeding behavior.

GnIH CONTROL OF REPRODUCTIVE BEHAVIOR

Gonadotropin-inhibitory hormone also acts in the brain to control reproductive behaviors including sexual and aggressive behaviors (28, 142, 143) (**Table 1**; **Figure 1**). First, Bentley et al.

(142) reported that central administration of GnIH inhibits copulation solicitation of estrogen-primed female sparrows exposed to male song. There is evidence that GnRH2 enhances copulation solicitation of estrogen-primed female sparrows exposed to male song (144). GnIH neurons terminate in the close proximity of GnRH2 neurons and it was also shown that GnRH2 neurons express GnIH receptor mRNA in songbirds (35). Accordingly, GnIH may inhibit copulation solicitation by inhibiting GnRH2 neuronal activity in female songbirds (142). Subsequently, Ubuka et al. (143) investigated this possibility by testing how RNA interference (RNAi) of GnIH gene affects the behavior of male and female white-crowned sparrows. It was found that GnIH RNAi reduces not only resting time, but also spontaneous production of complex vocalizations and stimulates agonistic vocalizations. In addition, it was shown that GnIH RNAi increases song production of short duration when they were exposed to novel male songs in male birds. These findings indicate that GnIH gene silencing induces arousal in birds. Ubuka et al. (143) further found that the activities of the birds are correlated negatively with GnIH mRNA expression in the PVN. In female birds, GnIH RNAi decreases GnIH neuronal fiber density in the ventral tegmental area. Further, GnRH1 and GnRH2 neurons' number, which receives appositions of GnIH neuronal fiber terminals, is correlated negatively with the activity of male birds (143). Recently, Ubuka et al. (28) have further demonstrated in male quail that GnIH inhibits aggressive behavior. It is thus becoming clear in birds that GnIH decreases sexual and aggressive behaviors [for reviews, see Ref. (24, 25, 115)] (**Table 1**; **Figure 1**).

In mammals, Johnson et al. (44) also reported that central administration of GnIH decreases male sex behavior in rats. On the other hand, there is a report showing that central administration of GnIH decreases sexual motivation and vaginal scent marking without affecting lordosis behavior in female hamsters (137). Piekarski et al. (137) showed that GnIH administration alters fos expression in the medial preoptic area (POA), the medial amygdala as well as the bed nucleus of the stria terminalis, key neural loci implicated in female sexual behavior. These findings suggest that GnIH is a modulator of proceptive sexual behavior and motivation in female animal (**Figure 1**). Accordingly, GnIH does not only control the HPG axis, but it may also modulate the neural circuitry underlying socially motivated behavior as in birds [see Ref. (26) for a review].

It is known that the interactions of neuropeptides and neurosteroids regulate brain functions [for a review, see Ref. (145)]. Recently, Ubuka et al. (28) discovered that GnIH activates cytochrome P450 aromatase (P450arom) and stimulates neuroestrogen synthesis in the quail brain (28) (**Figure 1**). Abundant GnIH immunoreactive neuronal fibers are distributed in the vicinity of P450arom immunoreactive cells in the POA (28). It was also shown that GnIH receptor is expressed in P450arom immunoreactive cells in the POA (28). Furthermore, GnIH increases neuroestrogen synthesis by stimulating P450arom activity through GnIH receptor in the POA (28) (**Figure 1**). Importantly, GnIH actions on neuroestrogen synthesis decrease aggressive behavior in birds (28) (**Figure 1**), providing a new finding that GnIH modifies neurosteroidal milieu in the brain to modulate aggressive behavior [see Ref. (146) for review]. Future studies are needed to develop the emerging concept of GnIH and other hypothalamic neuropeptides modifying the neurosteroidal milieu in the brain and the impact of its function.

CONCLUSION

The discovery of GnIH in 2000 and the studies to understand its functions have advanced reproductive neuroendocrinology. It appears that GnIH acts on the pituitary and within the brain and modulates the reproductive axis as well as reproductive behaviors. Furthermore, recent studies have demonstrated that GnIH controls feeding behavior in vertebrates, such as birds and mammals. Thus, the last 15 years of GnIH research has led to a better understanding of the neuroendocrine control mechanism of feeding and reproductive behaviors as well as reproduction.

AUTHOR CONTRIBUTIONS

All the authors listed have made substantial, direct, and intellectual contribution to the work and approved it for publication.

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MINI REVIEW
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The Neuropeptide 26RFa (QRFP) and Its Role in the Regulation of Energy Homeostasis: A Mini-Review

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This mini-review deals with the neuropeptide 26RFa (or QRFP) which is a member of the RFamide peptide family discovered simultaneously by three groups in 2003. 26RFa (or its N-extended form 43RFa) was subsequently shown to be the endogenous ligand of the human orphan receptor GPR103. In the brain, 26RFa and GPR103mRNA are primarily expressed in hypothalamic nuclei involved in the control of feeding behavior, and at the periphery, the neuropeptide and its receptor are present in abundance in the gut and the pancreatic islets, suggesting that 26RFa is involved in the regulation of energy metabolism. Indeed, 26RFa stimulates food intake when injected centrally, and its orexigenic effect is even more pronounced in obese animals. The expression of 26RFa is up-regulated in the hypothalamus of obese animals, supporting that the 26RFa/GPR103 system may play a role in the development and/or maintenance of the obese status. Recent data indicate that 26RFa is also involved in the regulation of glucose homeostasis. 26RFa reduces glucose-induced hyperglycemia, increases insulin sensitivity and insulinemia. Furthermore, an oral ingestion of glucose strongly stimulates 26RFa release by the gut, indicating that 26RFa is a novel incretin. Finally, 26RFa is able to prevent pancreatic β cell death and apoptosis. This brief overview reveals that 26RFa is a key neuropeptide in the regulation of energy metabolism. Further fields of research are suggested including the pathophysiological implication of the 26RFa/GPR103 system.

Keywords: RFamide peptide, G protein-coupled receptor, food intake, glucose homeostasis, obesity, diabetes

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DISCOVERY OF 26RFa AND ITS RECEPTOR GPR103

26RFa also referred to as QRFP (for pyroglutamilated RFamide peptide) is a 26-amino acid peptide discovered simultaneously by three different groups in 2003 including our team (Chartrel et al., 2003; Fukusumi et al., 2003; Jiang et al., 2003). These teams have developed either a bioinformatic approach or a comparative approach using frog brains as a source of neuropeptides (Chartrel et al., 2002, 2003; Fukusumi et al., 2003; Jiang et al., 2003). 26RFa and/or its N-terminal extended form, 43RFa, have been subsequently biochemically characterized in the human hypothalamus and the rat brain (Bruzzone et al., 2006; Takayasu et al., 2006), and molecular cloning has revealed that a

26RFa precursor-encoding sequence is present in human (Chartrel et al., 2003; Fukusumi et al., 2003), ox (Fukusumi et al., 2003), rat (Chartrel et al., 2003; Fukusumi et al., 2003; Jiang et al., 2003), mouse (Fukusumi et al., 2003; Jiang et al., 2003), quail (Ukena et al., 2010), chicken (Ukena et al., 2010), and goldfish (Liu et al., 2009), indicating that 26RFa is widely distributed among vertebrates.

26RFa is the cognate ligand of the human orphan receptor GPR103, also designated SP9155 or AQ27 (Chartrel et al., 2003, 2011; Jiang et al., 2003). GPR103 is a 7-transmembrane G protein-coupled receptor (GPCR) that shares significant amino acid identity (52%) with NPFF2 (Bonini et al., 2000; Lee et al., 2001), another receptor for mammalian RFamide peptides. Binding studies and functional assays indicated that the N-elongated form of 26RFa, 43RFa, binds also with a high affinity to GPR103 and has the same efficacy as 26RFa to inhibit cAMP production (Fukusumi et al., 2003; Jiang et al., 2003). In contrast, GPR103 is not activated by other mammalian RFamide peptides, such as PrRP, RFRP-1 and -3 (Dockray, 2004), indicating that GPR103 selectively recognizes 26RFa/43RFa. Conversely, 26RFa displays moderate affinity and selectivity for NPFF-2 (Gouardères et al., 2007). Data mining revealed that two orthologues of human GPR103 are present in the mouse and rat genome (Kampe et al., 2006; Takayasu et al., 2006). The two GPR103 genes from rodents exhibit between 79 and 85% homology with human GPR103 (Kampe et al., 2006; Takayasu et al., 2006). 26RFa/43RFa bind with a similar affinity the two forms of GPR103 in both mouse and rat (Kampe et al., 2006; Takayasu et al., 2006). Up to now, the occurrence of two distinct GPR103 receptors has only been reported in rodents.

26RFa AND CONTROL OF FEEDING BEHAVIOR

Neuroanatomical studies have revealed a discrete localization of 26RFa-expressing neurons in various hypothalamic nuclei including the ventromedial hypothalamic nucleus (VMH), the lateral hypothalamic area (LHA) and the arcuate nucleus (Arc) (Chartrel et al., 2003; Bruzzone et al., 2007). GPR103-containing neurons are found in the same hypothalamic structures but also in other brain nuclei, such as the piriform cortex and the nucleus of the solitary tract (Bruzzone et al., 2007). All of these nuclei mentioned above are known to be involved in the control feeding behavior raising the hypothesis that 26RFa may be implicated in the hypothalamic regulation of food intake. This is the case as intracerebroventricular (i.c.v.) administration of 26RFa in mice stimulates food consumption in a dose-dependent manner, and expression of the 26RFa precursor is up-regulated in the hypothalamus of fasted mice (Chartrel et al., 2003; Do Rego et al., 2006; Takayasu et al., 2006). 43RFa exerts a similar effect and is even more potent than 26RFa in stimulating appetite (Do Rego et al., 2006; Moriya et al., 2006; Takayasu et al., 2006). In addition, chronic administration of 43RFa for 2 weeks results in an important increase of body weight and fat mass in mice that also exhibit a hyperphagic behavior (Moriya et al., 2006). These effects of 43RFa are more pronounced when mice are fed a moderately high fat diet (Moriya et al., 2006). Finally, 26RFa mRNAs are increased in genetically obese *ob/ob* and *db/db* mice (Takayasu et al., 2006), suggesting that up-regulation of 26RFa may play an important role in the maintenance of obesity.

26RFa has also been found to stimulate food intake in rats fed a standard chow (Kampe et al., 2006; Lectez et al., 2009). Consistent with this observation, it has been recently shown that direct administration of 26RFa into the medial hypothalamus increases food consumption (Zagorácz et al., 2015), and that the concentrations of 26RFa/43RFa in the VMH are significantly increased in rats fed a standard chow (Beck and Richy, 2009). It has also been found that 26RFa still stimulates appetite when rats are fed a high fat diet (Primeaux et al., 2008), and this phenomenon is accompanied by an up-regulation of prepro26RFa and GPR103 in the VMH and the Arc (Schreiber et al., 2016). By contrast, these authors (Primeaux et al., 2008; Schreiber et al., 2016) as well as Patel et al. (2008) failed to find any effect of 26RFa or 43RFa on food consumption when rats are fed a standard chow. Interestingly, in both mice and rats, 26RFa potently stimulates food intake when the animals are deprived of food for 18 h prior to the injection of the neuropeptide (Chartrel et al., 2003; Do Rego et al., 2006; Lectez et al., 2009) strongly suggesting that starvation potentiates the orexigenic activity of 26RFa. To conclude, these data indicate that in both mice and rats, 26RFa/43RFa strongly stimulate food consumption when the animals are fed a moderate or a high fat diet (Moriya et al., 2006; Primeaux et al., 2008), and that the expression of prepro26RFa is enhanced in the hypothalamus of animals submitted to such a fat diet (Moriya et al., 2006; Primeaux et al., 2008). These data support therefore the notion that 26RFa/43RFa plays a role in the establishment and maintenance of the obese status in mammals. However, Beck and Richy (2009) have recently reported a decrease of 43RFa levels in the VMH of rats fed a high fat diet. Conversely, a single study has investigated the expression/production of 26RFa under chronic undernutrition (Galusca et al., 2012). This study has been conducted in young women suffering from anorexia nervosa in which circadian plasma 26RFa levels have been measured. The data reveal significant higher levels of circulating 26RFa in anorectic patients as compared to healthy volunteers, suggesting the occurrence of an adaptive mechanism of the organism to promote energy intake and to increase fat stores in response to chronic undernutrition (Galusca et al., 2012).

Interestingly, it has been reported that 26RFa promotes arousal in mice (Takayasu et al., 2006), raising the hypothesis that the orexigenic activity of the neuropeptide may be related to its wake-promoting effect, as previously suggested for the other orexigenic neuropeptide orexin. However, a recent paper reveals that, in the zebrafish, the overexpression of 26RFa in the hypothalamus inhibits locomotor activity and promotes sleep whereas lack of 26RFa signaling results in increased locomotor activity and decreased sleep during the day (Chen et al., 2016).

One neuronal pathway by which 26RFa/43RFa exerts its orexigenic activity in the hypothalamus has been elucidated. The investigation has focused on the neuropeptide Y (NPY)/proopiomelanocortin (POMC) system of the Arc as a high expression of the 26RFa receptor is found in this nucleus

(Sakurai et al., 1998; Fukusumi et al., 2003; Takayasu et al., 2006; Bruzzone et al., 2007). i.c.v. administration of 26RFa induces an increase in the expression and release of NPY in the Arc and, simultaneously, a decrease in POMC expression and α-MSH release (an anorexigenic POMC-derived peptide) which is associated with an increase in food consumption (Lectez et al., 2009). In addition, in this study, Lectez et al. (2009) show that the effects of 26RFa on the activity of POMC neurons is indirect as these neurons do not express the GPR103 transcript. Specific antagonists of the Y1 and Y5 NPY receptors (which are expressed by POMC neurons) totally abolish the inhibitory effect of 26RFa on POMC expression and α-MSH release, as well as 26RFa-induced food intake, indicating that 26RFa increases consumption by stimulating the release of NPY which in turn inhibits the activity of POMC neurons via the activation of the Y1 and Y5 receptors (Lectez et al., 2009, **Figure 1**). A possible involvement of the orexin system in the orexigenic hypothalamic effect of 26RFa has also been examined because the orexin system shows important similarity with the 26RFa/GPR103 system. As a matter of fact, orexins stimulate food intake and orexin-expressing neurons are localized in the LHA (Sakurai et al., 1998), like 26RFa. The orexin neurons innervate the NPY neurons of the Arc (Ciriello et al., 2003) that express the OX-R1 receptor (Bäckberg et al., 2002). Orexin-A stimulates the activity of NPY neurons (López et al., 2002) and the orexigenic activity of the neuropeptide is abolished when the animals have previously received Y1 and Y5 NPY receptor antagonists (Dube et al., 2000; Yamanaka et al., 2000). Finally, it has been recently demonstrated that orexin receptors and GPR103 can form functional heterodimers to exert their effects through activation of ERK½ (Davies et al., 2015). However, the fact that 26RFa still stimulates appetite in orexin invalidated mice (Takayasu et al., 2006) indicates that the orexin system is not recruited during 26RFa-induced food intake. Alternatively, it is not known whether the OX receptor needs to dimerise with GPR103 to initiate orexin-induced food intake or whether orexin and 26RFa may act synergically via heterodimerization of their receptors to potentiate their orexigenic activities. This interesting hypothesis deserves further investigation.

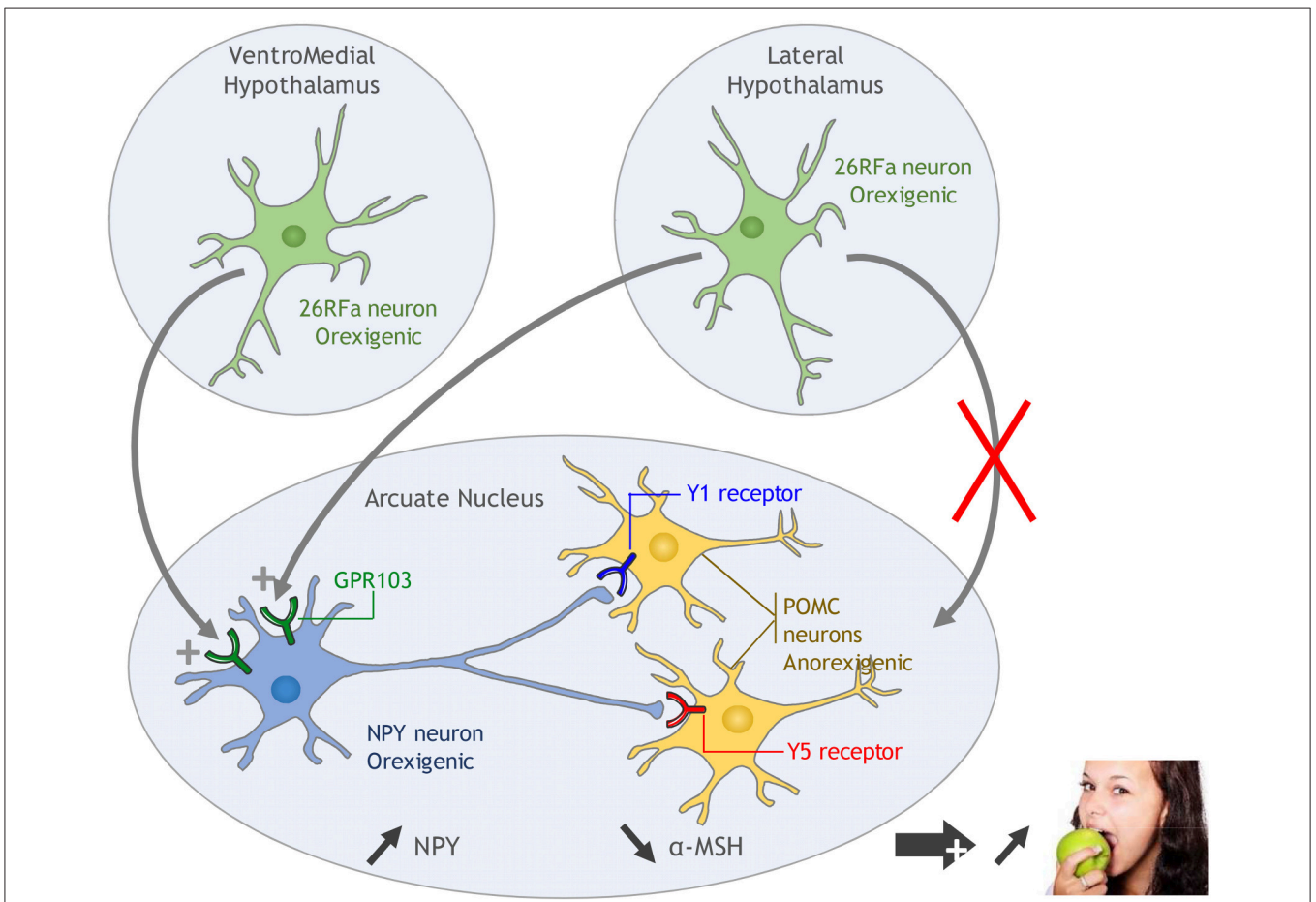


FIGURE 1 | Proposed mechanism of action of 26RFa in the hypothalamic control of food intake. 26RFa, produced by neurons of the ventromedial hypothalamic nucleus (VMH) and the lateral hypothalamic area (LHA), stimulates the activity of NPY neurons of the arcuate nucleus (Arc) via activation of GPR103. Subsequent NPY release in the Arc inhibits the activity of proopiomelanocortin (POMC) neurons via activation of the Y1 and Y5 receptors, leading to a stimulation of appetite.

In addition to its central action, accumulating data indicate that 26RFa can also regulate energy homeostasis at the periphery. It has notably been shown that, in the adipocyte cells 3T3-L1, 26RFa, and 43RFa stimulate triglyceride accumulation and fatty acid uptake, and increase the expression of genes involved in lipid uptake (Mulumba et al., 2010). Concurrently, the expression of GPR103 is enhanced in the adipose tissue of a mouse model of diet-induced obesity whereas that of prepro26RFa is decreased, and the neuropeptide inhibits lipolysis in adipocytes of these animals (Mulumba et al., 2010, 2015). It thus appears that 26RFa plays a crucial role in the central and peripheral regulation of body weight and energy homeostasis, and may be involved in the development and maintenance of obesity in vertebrates.

26RFa AND CONTROL OF GLUCOSE HOMEOSTASIS

Type 2 diabetes, which is a frequent consequence of obesity, is characterized by chronic hyperglycemia induced by impaired insulin secretion due to decreased β cell mass and function, and increased insulin resistance (Butler et al., 2003; Kahn et al., 2014). Recent studies suggest a peripheral role of hypothalamic neuropeptides controlling feeding behavior in the regulation of glucose homeostasis, leading to the new concept that hypothalamic neuropeptides may serve as a link between energy and glucose homeostasis, and identifying them therefore as potential therapeutic targets for the treatment of diabetes and obesity (Greenwood et al., 2011). With regard to these observations a potential role of 26RFa/43RFa in the regulation of glucose homeostasis has been examined. A primary study in 2007 has investigated the effect of 26RFa on insulin and glucagon secretion by rat perfused pancreas (Egido et al., 2007). This study reports that 26RFa reduces glucose, arginine and exendin-4 (a GLP-1 agonist)-induced insulin release without affecting glucagon secretion (Egido et al., 2007). In addition, these authors show that the inhibitory effect of 26RFa on exendin-4-induced insulin release is not observed in pancreas from pertussis toxin-treated rats suggesting the involvement of a pertussis toxin-sensitive G_i protein negatively coupled to the adenylyl cyclase system (Egido et al., 2007). Recently, the role and mechanism of action of 26RFa/43RFa in the regulation of glucose metabolism has been studied more thoroughly (Granata et al., 2014; Prévost et al., 2015). The two studies show that 26RFa/43RFa and GPR103 are expressed by the pancreatic islets as well as by the rodent insulin-secreting cell lines INS-1E and MIN6. Granata et al. (2014) report that 26RFa and 43RFa prevent cell death and apoptosis induced by serum starvation, cytokines and glucolipotoxicity in INS-1E β cells and in isolated human pancreatic islets. In addition, these authors indicate that 43RFa promotes, whereas 26RFa inhibits, glucose—and exendin-4-induced insulin secretion through $G\alpha_s$ and $G\alpha_{i/o}$ proteins, respectively (Granata et al., 2014). They also show that inhibition of GPR103 expression by small interfering RNA in INS-1E β cells totally blocks the insulinotropic effect of 43RFa but not the insulinostatic action of 26RFa, suggesting that the insulinotropic effect of 43RFa is mediated via activation of GPR103 whereas, conversely, the insulinostatic effect of 26RFa is mediated via another unknown receptor (Granata et al., 2014). Finally, the same study reveals that 43RFa promotes glucose uptake by β cells whereas 26RFa does not (Granata et al., 2014).

Our team has also investigated the role and mechanism of action of 26RFa in the regulation of glucose homeostasis (Prévost et al., 2015). Clinical studies performed in human revealed a positive correlation between plasma 26RFa and plasma insulin in obese, type 2 diabetic patients and healthy volunteers. In addition, measurement of plasma 26RFa during an oral glucose tolerance test shows an increase in the circulating levels of the neuropeptide during the test, indicating a link between the 26RFa system and glucose homeostasis (Prévost et al., 2015). Finally, immunohistochemical experiments describe the presence, in abundance, of 26RFa in the gut, from the stomach to the colon, suggesting that the gut is the primary source of circulating 26RFa that can be released under an oral glucose load. In mice, it was found that i.p. administration of 26RFa does not alter basal glycemia. In contrast, the neuropeptide strongly attenuates glucose-induced hyperglycemia during a glucose tolerance test, indicating that the neuropeptide exerts an antihyperglycemic effect rather than a hypoglycemic effect. In addition, it is reported that 26RFa enhances insulin sensitivity and increases insulin

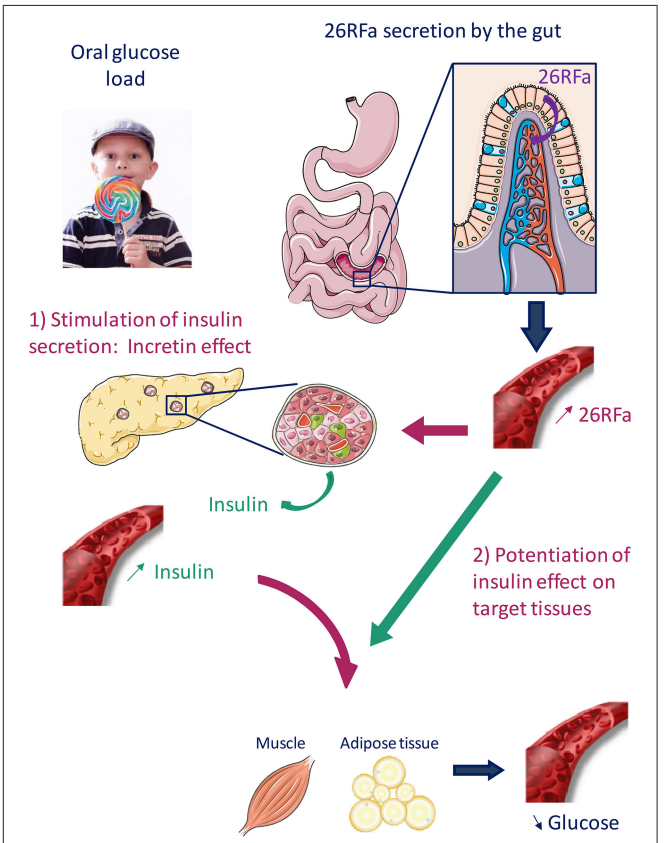


FIGURE 2 | Proposed mechanism of action of 26RFa in the control of glucose homeostasis. 26RFa is abundantly produced by the gut and released in the general circulation after an oral glucose load. 26RFa stimulates insulin release by pancreatic cells and potentiates insulin sensitivity on target tissues (muscle, adipose tissue), leading to a decrease of glycemia.

production, suggesting that the two mechanisms contribute to the antihyperglycemic effect of 26RFa. 26RFa-induced insulin production is due to a direct action of the neuropeptide on pancreatic β cells as 26RFa stimulates insulin release by the MIN6 cells that express GPR103, and as invalidation of GPR103 in these cells totally abolishes 26RFa-induced insulin secretion (Prévost et al., 2015). These observations are partially in agreement with those of Granata et al. (2014) reporting that 43RFa stimulates insulin secretion by human pancreatic islets and INS-1E β cells by activating GPR103. However, the same authors found that 26RFa exerts an opposite effect to that of 43RFa, and that the inhibiting effect of 26RFa on insulin secretion is not mediated by GPR103. They thus suggest that NPFF2, another receptor that 26RFa can bind to but with a much lower affinity than to GPR103 (Moriya et al., 2006), may be involved in the insulinostatic activity of 26RFa. In our study (Prévost et al., 2015), we show that MIN6 cells do not express NPFF2 that might explain the discrepancy between the two studies.

26RFa has also been found to increase insulin sensitivity and GPR103 is co-expressed with the insulin receptor and the glucose transporter, GLUT-4, in the muscle, liver and adipose tissue (Prévost et al., 2015). In addition, a recent study indicates that 26RFa enhances insulin's effects on glucose uptake in rat skeletal muscle cells (Allerton and Primeaux, 2015). Altogether, these observations strongly suggest a direct action of the neuropeptide on insulin target tissues. Finally, it has been observed that an oral ingestion of glucose induces an increase in plasma 26RFa levels 30 min after the glucose load, whereas this phenomenon is not observed when glucose is administrated i.v., indicating therefore that elevation of 26RFa in the blood is due to a massive release of the neuropeptide by the gut (Prévost et al., 2015). In conclusion, these studies promote evidence for an important role of 26RFa, acting as an incretin, in the regulation of glucose homeostasis (**Figure 2**). Interestingly, a genome wide identification of new genes causing type 1 diabetes with autoimmune thyroiditis has revealed a strong association of the GPR103 gene with this pathology (Tomer et al., 2015), supporting the involvement of the 26RFa/GPR103 system in the regulation of glucose metabolism.

CONCLUSION

Accumulating data obtained during the last decade reveal that the 26RFa/GPR103 system plays an important role in the regulation of food intake and glucose homeostasis. Obesity associated with type 2 diabetes is a major world-wide problem of public health as 11% of the world population is obese (Organisation Mondiale de la Santé, 2016) and 400 millions people are affected by type 2 diabetes in the world (Kahn et al., 2014). One main axis for further research would be to investigate whether dysfunction of the 26RFa/GPR103 system is associated with diabetes/obesity that could serve as a basis to develop 26RFa analogs to treat the pathology. Supporting this idea, it can be reminded that, during the last decade, the therapeutic arsenal to treat type 2 diabetes has been enlarged with the occurrence of novel classes of drugs, such as GLP-1 agonists and inhibitors of DPPIV.

Besides, an increasing body of evidence supports the existence in the hypothalamus of a glucoregulatory system that acts coordinately with pancreatic islets to regulate blood glucose levels, via both insulin-dependent and insulin-independent mechanisms, that would be responsible of 50% of glucose homeostasis. Considering this latter observation, it would be important to investigate whether the hypothalamic neuronal populations expressing 26RFa are involved in the central regulation of glucose homeostasis.

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All of the authors have contributed to the design, writing and correction of the present mini-review.

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frontiers in Endocrinology

REVIEW
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Role of Melatonin, Galanin, and RFamide Neuropeptides QRFP26 and QRFP43 in the Neuroendocrine Control of Pancreatic β-Cell Function

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Glucose homeostasis is finely regulated by a number of hormones and peptides released mainly from the brain, gastrointestinal tract, and muscle, regulating pancreatic secretion through cellular receptors and their signal transduction cascades. The endocrine function of the pancreas is controlled by islets within the exocrine pancreatic tissue that release hormones like insulin, glucagon, somatostatin, pancreatic polypeptide, and ghrelin. Moreover, both exocrine and endocrine pancreatic functions are regulated by a variety of hormonal and neural mechanisms, such as ghrelin, glucagon-like peptide, glucose-dependent insulinotropic polypeptide, or the inhibitory peptide somatostatin. In this review, we describe the role of neurohormones that have been less characterized compared to others, on the regulation of insulin secretion. In particular, we will focus on melatonin, galanin, and RFamide neuropeptides QRFP26 and QRFP43, which display either insulinotropic or insulinostatic effects. In fact, in addition to other hormones, amino acids, cytokines, and a variety of proteins, brain-derived hormones are now considered as key regulators of glucose homeostasis, representing potential therapeutic targets for the treatment of diabetes and obesity.

Keywords: neurohormones, melatonin, galanin, QRF26, QRFP43, pancreatic β-cells

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INTRODUCTION

Type 1 and type 2 diabetes (T2D) are characterized by a reduced insulin secretion from the pancreas, due to shortage of β-cells and decreased β-cell function. Because both types of diabetes eventually lead to β-cell loss, a major goal in research is to identify strategies to preserve β-cell mass and increase β-cell function (1, 2). Pancreatic exocrine and endocrine secretion is partly controlled by neuronal projections from the vagus nerve, as well as many hormones produced in peripheral tissues, including the gastrointestinal tract. These comprise the gastric peptide ghrelin, the intestinal peptides glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotropic polypeptide, somatostatin, produced by pancreatic δ-cells, or the adipose tissue-derived peptide leptin. Insulin release by β-cells is also influenced by non-hormonal signals, such as small proteins, amino acids, lipids, and cytokines. Moreover, recent studies have demonstrated that different neuropeptides are implicated in the regulation of glucose homeostasis and β-cell function, providing a physiological link between the brain and the endocrine pancreas (3, 4).

In the present review, we describe the role of neurohormones whose effects on insulin secretion and glucose homeostasis have been less well characterized compared to others. These include neuropeptides mainly displaying inhibitory functions on insulin secretion, such as the chronobiotic

hormone melatonin, produced in the pineal gland, and galanin, released by the central and peripheral nervous systems and the gastrointestinal tract. Furthermore, we illustrate the effects of the hypothalamic RFamide peptides QRF26 and QRFP43, which, in addition to regulating feeding behavior, display both insulinostatic and insulinotropic actions and also promote pancreatic β-cell survival. Overall, because of their different ability to regulate β-cell function and glucose homeostasis, these hormones may be considered as potential therapeutic agents in diabetes and metabolic diseases.

Melatonin

Melatonin is a hormone predominantly produced by the pineal gland of the mammalian brain. It is synthesized and secreted in a circadian manner at night and functions as chronobiotic agent, regulating the seasonal and circadian rhythms, such as the sleep–wake cycle. Therefore, it is a "Zeitgeber," entraining circadian rhythm and indicating the time of day to various different organs and tissues in the body (5). In addition to the pineal gland, melatonin is produced by neuroendocrine cells in the retina and peripheral tissues, such as gastrointestinal tract, pancreas, and immune cells. In fact, because of its widespread production, melatonin acts in both endocrine and paracrine/autocrine manner. Furthermore, its effects have been shown in the cardiovascular and immune system, and on the regulation of metabolic functions (6–8).

At the cellular level, melatonin signals through two inhibitory G-protein (Gi)-coupled receptors, MT1 and MT2, whose binding results in inhibition of cAMP production. These receptors are widely distributed in the brain as well as in peripheral tissues, including the pancreas (9, 10). Furthermore, melatonin binding sites in cell nuclei of rat liver hepatocytes have been demonstrated (11) and identified as retinoid-related orphan receptor, mediating the genomic effects of the hormone (12, 13). Melatonin also interacts with cytosolic proteins, including calmodulin and calreticulin, implicated in the regulation of the cytoskeleton and the control of nuclear receptors (14, 15).

Interestingly, a variant of the human melatonin receptor 1 b gene (*MTRB1*) has been associated with high plasma glucose levels, reduction of insulin response to glucose, and increased risk of T2D (16–18). However, the role of melatonin on insulin secretion has not been clearly elucidated, as both inhibitory and stimulatory actions have been reported, probably because of the pleiotropism at the level of the receptor and second messengers (10, 19). Interestingly, most studies suggest that melatonin inhibits insulin secretion from pancreatic β-cells (20–22), while there are reports showing lack of effect (23). In fact, in INS-1 pancreatic β-cells, expressing MT1 receptors, acute treatment with melatonin inhibited GLP-1-induced insulin secretion. However, prolonged pretreatment with melatonin, enhanced insulin secretion in the presence of either the cAMP activator forskolin or GLP-1. Similar findings were observed in isolated rat islets (24). In another study, Peschke et al. demonstrated that melatonin inhibits cAMP and insulin secretion in INS-1 β-cells stimulated with forskolin, in a $G\alpha_i$-dependent manner. Melatonin also inhibited insulin release in INS-1 cells treated with the inositol trisphosphate stimulator carbachol; however, in pertussis toxin (PTX)-incubated cells, the hormone increased carbachol-induced insulin release. These results suggested that in β-cells, MT1 receptor activates different signaling pathways displaying opposite effects on insulin secretion (25). Interestingly, downregulation of MT1 receptor expression in INS-1 β-cells reduced the insulinostatic effect of melatonin, indicating that, at least in rodent β-cells, the effects of the hormone are mainly mediated by this isoform of the receptor (26). Recently, rat islets and INS-1 cells were found to express MT2 (27), which is also involved in the inhibitory effect of the hormone on insulin secretion (27, 28). Of note, in isolated human pancreatic islets expressing both MT1 and MT2, melatonin promotes insulin secretion, in contrast with the effects in rodent β-cells and islets, possibly through an indirect action involving stimulation of glucagon secretion following its binding to MT1 receptors (29). In addition, melatonin has been shown to promote the secretion of glucagon in pancreatic αTC1.9 α-cells, expressing MT1 and MT2, treated with different concentrations of glucose (30). Furthermore, long-term administration of melatonin resulted in elevation of plasma glucagon concentrations in Wistar rats (WR), whereas in type 2 diabetic Goto-Kakizaki rats glucagon levels were decreased compared to untreated animals (30). Interestingly, mRNA expression for glucagon receptor, which was slightly reduced in the liver of untreated GK rats compared to WR, was upregulated by melatonin in GK rats and decreased in WR. Furthermore, MT1 and MT2 mRNA was elevated in the liver of MT1 or/and MT2 knockout (KO) mice compared to wild-type animals, suggesting that melatonin influences pancreatic glucagon secretion and displays metabolic effects in the liver.

With regard to melatonin and glucose homeostasis, it has been demonstrated that high levels of melatonin, due to blindness (31) or to exogenous administration of melatonin, result in an increase in blood glucose levels (32); moreover, glucose levels are reduced and insulin levels increased after pinealectomy (33, 34). However, most studies suggest that the pineal gland has an inhibitory effect on pancreatic β-cell function, as melatonin reduces insulin levels and glucose tolerance in animals and humans (35–38). Furthermore, elevation of insulin has been shown to inhibit the synthesis of melatonin from the pineal gland (39). Collectively, these findings suggest an antagonism between insulin and melatonin functions. This is further sustained by the fact that in man, insulin levels are elevated during the day and low at night, whereas the opposite occurs for melatonin (40); interestingly, diabetic patients show an abnormal circadian rhythm of melatonin (5). In addition, melatonin has been shown to promote the expression and release of GH and prolactin in female primates through MT1 (41), and the secretion of prolactin in humans (42–44), whereas ACTH secretion was found to be inhibited in the mouse pituitary corticotrope tumor cell line AtT20 (45). Hence, some of the actions of melatonin on glucose metabolism may be mediated by its effects on secretion of pituitary hormones.

A recent study has demonstrated that the risk variant rs10830963 of MTNR1B is an expression quantitative trait locus (eQTL), conferring increased expression of MTNR1B mRNA in human islets, which likely results in a reduction in insulin

secretion and increased risk of T2D (22). Furthermore, melatonin was found to inhibit cAMP levels and insulin secretion in INS-1 832/13 β-cells, and these effects were further enhanced in β-cells overexpressing MTNR1B (22). Of note, melatonin is a prescription drug for improving sleep and for jet lag (8); therefore, it should be carefully administered in individuals with sleep disturbances, particularly in obese patients and carriers of the MNTR1B risk allele. However, administration of melatonin has been shown to improve sleep quality independently of rs10830963 genotype, despite the negative effect on insulin secretion (22). Moreover, the reduction of insulin release at night, mediated by the high levels of melatonin, when the metabolic demands are low because of reduced food intake, may be a protective physiological mechanism to prevent nocturnal hypoglycemia (22).

Interestingly, mice with a disruption of the receptor have been shown to secrete more insulin, despite no change in glucose levels, suggesting reduced insulin sensitivity but unchanged insulin tolerance (22). In addition, melatonin treatment in a human recall-by-genotype study was found to reduce insulin secretion in all subjects and to increase glucose levels; moreover, insulin reduction was even enhanced in individuals with the risk variant (22). Collectively, these findings suggest that increased melatonin signaling in islets impairs β-cell function, resulting in hyperglycemia and increased risk of T2D.

Galanin

Galanin, a 29- to 30-amino acid neuropeptide initially discovered in porcine intestine (46), is expressed in the central and peripheral nervous systems and intestinal neuroendocrine system of many mammalian species (47–51). Galanin co-localizes and is coexpressed in neurons with a number of neurotransmitters and displays strong inhibitory effect on synaptic transmission (52–55). Because of its broad expression, galanin regulates many neuronal functions, such as memory and learning, neuropathic pain, neuroprotection, and neuroendocrine activity, representing a therapeutic potential for diseases such as Alzheimer's disease, epilepsy, and diabetes (51, 56–58). Three distinct G-protein-coupled receptors GalR1, GalR2, and GalR3 are involved in the effects of the neuropeptide. GalR1 and GalR3 are coupled to the inhibitory G-protein Gi, whereas GalR2 associates with either Gi or Gq/11, thus displaying both inhibitory or stimulatory responses (51, 59).

Galanin-positive nerve fibers have been shown in the pancreas of different species, including rat, mouse (60, 61), and humans (62–64). Furthermore, a number of studies have indicated that galanin displays strong inhibitory effects on insulin secretion. In fact, galanin administration was found to reduce insulin levels in many species (65–67). In addition, a whole-genome profile study has demonstrated that the expression levels of a number of hippocampal genes, including galanin, and from the prefrontal cortex, such as GalR2, were dysregulated in type 2 diabetic rats, further suggesting the importance of the galanin system and the complexity of insulin signaling in modulating brain functions (68). Interestingly, infusion of galanin into animals through the pancreatic artery, at a concentration similar to that released from stimulated pancreatic nerve termini, resulted in inhibition of insulin secretion (69). However, conflicting results have been reported in humans, as galanin either suppresses insulin levels (70) or has no effect (71, 72). Moreover, galanin levels were inversely correlated with plasma insulin levels in postmenopausal women, whereas in controls there was a positive correlation (73).

Galanin and galanin analogs have been shown to reduce glucose-induced insulin secretion in isolated rat and pig islets (66, 74–76). The inhibitory action on insulin secretion in rat and mouse islets was found to involve a G_{o2} protein, through the regulation of both K_{ATP} and Ca^{2+} channels (60, 77). In line with these inhibitory effects, galanin infusion increased the levels of blood glucose in dogs but not in humans (69, 78). Furthermore, glucagon levels are upregulated by galanin, suggesting a role for glucagon in mediating the effects of galanin in glucose increase (49, 69).

Of note, transgenic mice overexpressing galanin showed visceral adiposity, increased body weight, increased serum cholesterol and triglycerides, hyperinsulinemia, and impaired glucose tolerance, indicating that elevated circulating galanin levels contribute to the development of metabolic syndrome (79). The obese phenotype was observed in the absence of increased food intake, suggesting defects in energy expenditure, since these mice had reduced oxygen consumption, as well as carbon dioxide and heat production (79). Surprisingly, mice with a loss-of-function mutation in the galanin gene [galanin KO mice] showed impaired inhibition of insulin secretion after activation of autonomic nerve, suggesting that galanin may act on sympathetic nerves to inhibit insulin secretion (80). Furthermore, insulin secretion was found reduced in galanin KO mice in response to glucose and arginine, compared to wild-type mice, and β-cells showed reduced sensitivity to glucose (80). Collectively, these findings suggest that in addition to regulating energy expenditure, galanin may be involved in the regulation of normal β-cell function. Conversely, galanin infusion has no effect on glucose tolerance in humans (71, 81, 82) and does not influence the postprandial rise of plasma glucose levels (70).

Reduced levels of pancreatic galanin were found in obese, hyperinsulinemic mice (83), and galanin-expressing cells were found to be strongly reduced in islets of diabetic rats (61). Interestingly, in rat and bovine pancreatic islets, galanin-like immunoreactivity co-localized with that of insulin, suggesting that galanin may influence insulin secretion in an autocrine/paracrine manner (61, 84). Furthermore, administration of a centrally active galanin analog with high affinity for GalR1 has been recently shown to reduce insulin secretion and promote hyperglycemia, providing a further understanding on the role of GalR1 *in vivo* (85).

However, a beneficial effect for galanin in animal models of diabetes has been also reported (86), therefore, additional studies are required to shed light on the role of galanin in human metabolic disorders and diabetes. Importantly, intranasally administered galanin-like peptide (GALP), whose aminoacid sequence 9–21 is identical to that of galanin 1–13, reduces body weight, food intake, water intake, and locomotor activity in leptin-deficient *ob/ob* mice and in diet-induced obese (DIO) mice (87). The decrease in body weight was found to be stronger in hyperglycemic

compared to mormoglycemic mice, suggesting that intranasally administered GALP displays its best effect in obese mice with higher glucose levels. Interestingly, in DIO mice, the decrease in body weight after intranasal treatment with GALP was observed in spite of a reduction in locomotor activity, suggesting that GALP restrains energy intake and promotes energy expenditure (87). Other studies have demonstrated that intracerebroventricular GALP reduces food intake and stimulates energy expenditure; however, these effects did not persist over time, suggesting that the mice become insensitive to repeated treatment with GALP (88, 89). Conversely, repeated intranasal administration of GALP continued to decrease food intake and locomotor activity compared with repeated intracerebroventricular injection, suggesting that sensitivity to GALP is maintained and intranasal administration is the best way for GALP to exert its effects against obesity (87).

RFamide Neuropeptide QRF26 and QRF43

The neuropeptide QRFP26 and its N-extended form QRFP43 are members of the RFamide peptide family, discovered in 2003 by three different groups (90–92). The gene encoding the QRFP26/QRFP43 precursor is widely distributed among vertebrates, including humans, mice, rats (90–92), and other species (93–95), indicating that these neuropeptides have been highly conserved during evolution (96).

QRFP26 and QRFP43 are the cognate ligands of the former orphan receptor GPR103, also called SP9155 or AQ27, and now renamed QRFPR (90, 97). QRFPR is a G-protein-coupled receptor, with a 52% amino acid identity with neuropeptide FF receptor 2 (NPFF2), another receptor for mammalian RFamide peptides. However, whereas QRFP26 also displays low moderate affinity for NPFF2, QRFP43 only binds to QRFPR, which, in turn, is not recognized by other mammalian RFamide peptides (98). Two isoforms have been described for QRFPR (QRFPR1 and QRFPR2) in rodents, sharing high homology with the unique form of human QRFPR, and QRFP26/QRFP43 bind with similar affinity to both forms of the receptor in rodents (99, 100).

The genes for QRFP26/QRFP43 precursor and QRFPR are mainly located in the hypothalamic nuclei, as well as in other brain areas involved in the control of feeding behavior (90, 101). Accordingly, intracerebroventricular (i.c.v.) injection of both QRFP26 and QRFP43 in mice has been shown to promote food intake and to increase body weight and fat mass (90, 97, 100, 102, 103). In addition to the central distribution, QRFP26/QRFP43 and QRFPR are expressed in peripheral organs, including adipose tissue and macrophages (104–106), eye, trachea, mammary gland, and testis, endocrine glands, including the pituitary, thyroid, and parathyroid glands, coronary artery, gastrointestinal tract, bladder, and prostate (91, 92, 100, 107). Thus, because of the broad distribution of QRFPR, QRFP26/QRFP43 have been shown to regulate a variety of physiological functions, including adipogenesis, lipolysis and inflammation (104–106), blood pressure (100), bone formation (108), and hypothalamo–pituitary–gonadal activity (109, 110).

Although initially not found in mouse and rat pancreas (91, 92), expression of QRFP26/QRFP43 and QRFPR mRNA and protein was later found in human endocrine pancreas and isolated pancreatic islets (107, 111), rat INS-1E β-cells (111), and mouse insulinoma MIN6 cells (107). Moreover, in human islets, QRFPR co-localized with insulin, suggesting autocrine/paracrine action of locally produced QRFP26/QRFP43 and direct binding of the peptides with its receptor in pancreatic β-cells (111).

Interestingly, QRFPR displays sequence similarity with NPY and galanin receptors (112), and like NPY and galanin, QRFP26/QRFP43 have been shown to regulate insulin secretion. In fact, QRFP26 was found to reduce glucose-, arginine-, and exendin-4-induced insulin secretion in rat perfused pancreas, showing no effect on glucagon secretion. Since the insulinostatic action of QRFP26 was inhibited by PTX upon treatment with exendin-4, it was suggested the involvement of a pertussis-sensitive Gα inhibitory ($G\alpha_i$) protein negatively coupled to the adenylyl cyclase pathway (113). However, the authors of this study were unable to identify the receptor implicated in these effects, likely because previous reports failed to demonstrate QRFPR expression in the pancreas (91, 92).

In accordance with the findings of Egido et al. (113) QRFP26 was later found to inhibit glucose- and exendin-4-induced insulin secretion in INS-1E β-cells and human pancreatic islets, through mechanisms mediated by $G\alpha_i$ and reduction of intracellular cAMP levels (111). Of note, knocking down QRFPR in these cells did not affect the insulinostatic action of QRFP26, suggesting the involvement of a different receptor. By contrast, QRFP43 potentiated insulin secretion in β-cells and human islets treated with both glucose or exendin-4, through engagement of a Gα stimulatory protein ($G\alpha_s$) and elevation of cAMP levels (111). The insulinotropic effect of QRFP43 was suppressed when QRFPR was knocked down in INS-1E β-cells using small interfering RNA, whereas the insulinostatic effect of QRFP26a was maintained. Furthermore, QRFP43, but not QRFP26 increased glucose uptake by β-cells. At variance with the opposed effects observed on β-cell function, both peptides reduced apoptosis and cell death induced by serum starvation, inflammatory cytokines and glucolipotoxicity in β-cells and human islets, to an extent comparable to that induced by exendin-4. QRFP43-induced protection involved activation of the survival and proliferative pathways phosphatidylinositol 3-kinase/Akt and extracellular signal-related kinase 1/2 (ERK1/2), whereas only ERK1/2 was required for the survival function of QRFP26 (111). At present it is unclear why both QRFP26 and QRFP43 promote survival of β-cells, while having opposed effects on insulin secretion. The possible explanation would be that, in addition to QRFPR, these peptides bind to one or more yet unknown alternative receptors involved in their survival action.

The role of QRFP26 was recently investigated on the regulation of glucose homeostasis (107). It was demonstrated a positive association between the levels of plasma QRFP26 and plasma insulin in patients with diabetes; furthermore, QRFP26 increased in response to an oral glucose tolerance test. In mice, QRFP26 attenuated glucose-induced hyperglycemia, increased insulin sensitivity and plasma insulin concentrations but did not alter basal glycemia, suggesting antihyperglycemic action. In addition, QRFP26 promoted insulin secretion in MIN6

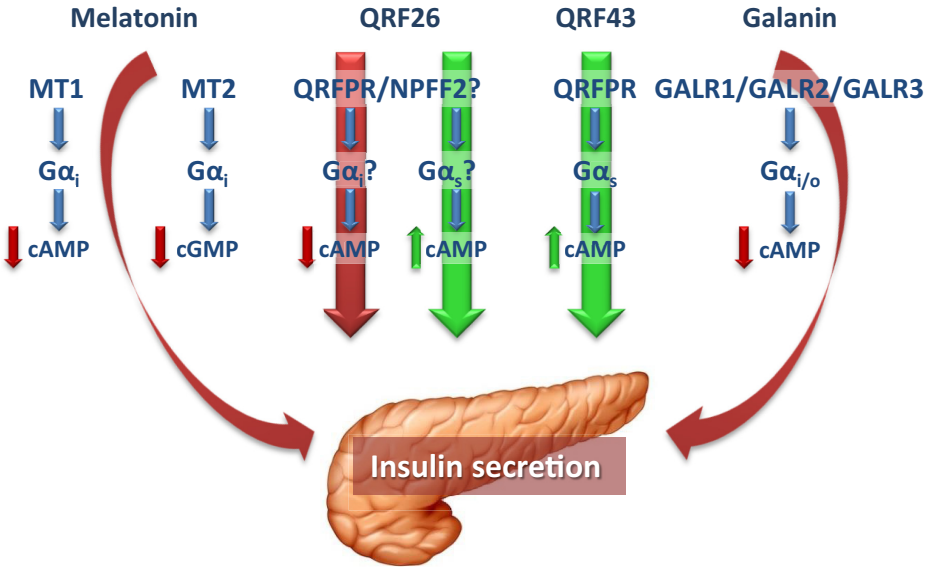


FIGURE 1 | In pancreatic β-cells, the pineal hormone melatonin reduces insulin secretion through binding to the receptor isoforms MT1 and MT2, which, by coupling to $G\alpha_i$ proteins, inhibit cAMP and cGMP, respectively. The hypothalamic peptide QRFP26 has been shown to both inhibit and promote insulin secretion in different β-cell models, through receptors that in part remain to be defined. The stimulatory and inhibitory actions of QRFP26 are likely mediated by activation of $G\alpha_s$ and $G\alpha_i$ proteins, respectively. On the other hand, QRFP43 acts as an insulinotropic neuropeptide by binding to a $G\alpha_s$-coupled QRFPR, to increase cAMP levels. Galanin, widely distributed in both central and peripheral nervous systems, displays inhibitory effects in the endocrine pancreas and, like melatonin, reduces insulin secretion in β-cells by binding to $G\alpha_i$-coupled receptors (GALR1-3). Red and green arrows indicate the inhibitory and stimulatory effects on insulin secretion, respectively. cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; GALR, galanin receptor; $G\alpha_i$, inhibitory guanine triphosphate-binding protein α-subunit; $G\alpha_s$, stimulatory guanine triphosphate-binding protein α-subunit; MT1 and MT2, melatonin receptor 1 and 2; NPFF2, neuropeptide FF receptor 2; QRFPR, QRFP receptor.

insulinoma cells, in a QRFPR-dependent manner, as inhibition of QRFPR expression using specific siRNA blocked the insulinotropic effect of the peptide. Accordingly, MIN6 showed expression for QRFPR but not for NPFF2, the other RFamide receptor that can be recognized by QRFP26. Conversely, in INS-1E β-cells the insulinostatic action of QRFP26 was independent QRFPR binding, suggesting that other receptor(s), such as NPFF2 would be involved. However, to date, the presence of NPFF2 in INS-1 β-cells or human pancreatic islets remains to be determined. Thus, the different effect of QRFP26 on insulin secretion in different β-cell types may be attributed to the different expression pattern of the receptor(s). Interestingly, in *both in vivo* and *in vitro* experiments, high concentrations of glucose induced a massive secretion of QRFP26 by the small intestine (107). Overall, at variance with the results of Granata et al. these findings indicated that QRFP26 acts as an incretin hormone to regulate glucose homeostasis.

Overall, the results from different reports indicate that QRFP26/QRFP43 regulate glucose homeostasis and β-cell function; however, further understanding is required to disentangle the discrepancies observed in the various experimental models and for elucidating the role of the receptor(s) involved in these effects. Of note, these neuropeptides increase the survival of β-cells and human pancreatic islet cells, suggesting potential therapeutic implications in diabetes.

CONCLUSION

Many important questions on the regulation of β-cell function remain unanswered, as a variety of players, and even more to be discovered, are implicated in this complex process. In addition to their central actions, it is becoming increasingly clear that, together with peripheral hormones, neuropeptides are also key regulators of glucose homeostasis and insulin secretion, displaying both direct and indirect actions in the endocrine pancreas (**Figure 1**). Thus, it is important to further understand their specific role and mechanisms, in order to increase the wide range of potential therapeutic targets for the treatment of diabetes and metabolic diseases.

AUTHOR CONTRIBUTIONS

IG, TV, DB, and GG contributed to the writing of the different topics and edited the manuscript; RG wrote the paper and supervised the work of the co-authors.

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MINI REVIEW
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Cannabinoid Receptor Signaling in Central Regulation of Feeding Behavior: A Mini-Review

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Cannabinoids are lipid messengers that modulate a variety of physiological processes and modify the generation of specific behaviors. In this regard, the cannabinoid receptor type 1 (CB_1) represents the most relevant target molecule of cannabinoids so far. One main function of central CB_1 signaling is to maintain whole body energy homeostasis. Thus, cannabinoids functionally interact with classical neurotransmitters in neural networks that control energy metabolism and feeding behavior. The promotion of CB_1 signaling can increase appetite and stimulate feeding, while blockade of CB_1 suppresses hunger and induces hypophagia. However, in order to treat overeating, pharmacological blockade of CB_1 by the inverse agonist rimonabant not only suppressed feeding but also resulted in psychiatric side effects. Therefore, research within the last decade focused on deciphering the underlying cellular and molecular mechanisms of central cannabinoid signaling that control feeding and other behaviors, with the overall aim still being the identification of specific targets to develop safe pharmacological interventions for the treatment of obesity. Today, many studies unraveled the subcellular localization of CB_1 and the function of cannabinoids in neurons and glial cells within circumscribed brain regions that represent integral parts of neural circuitries controlling feeding behavior. Here, these novel experimental findings will be summarized and recent advances in understanding the mechanisms of CB_1-dependent cannabinoid signaling being relevant for central regulation of feeding behavior will be highlighted. Finally, presumed alternative pathways of cannabinoids that are not driven by CB_1 activation but also contributing to control of feeding behavior will be introduced.

Keywords: cannabinoid receptor type 1, endocannabinoids, hypothalamus, feeding behavior, anorexia, cachexia, overeating, obesity

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INTRODUCTION

Central regulation of feeding behavior is indispensable to life, since animals and men have to consume energy in terms of food to exert essential daily functions (Gao and Horvath, 2016). In this regard, a network of neural circuitries evolved that ensures constant energy supply by providing a "pro-feeding" behavioral outcome: in times when food is plentiful, energy intake dominates energy expenditure, so that excessive energy could be stored and used when food was restricted or temporarily not available (Koch and Horvath, 2014).

Cannabinoids, such as THC interfere with central regulation of feeding behavior by acting upon G protein-coupled cannabinoid receptor type 1 (CB_1) in the brain (Williams and Kirkham, 1999).

However, the underlying molecular and cellular mechanisms of central CB_1 signaling in control of feeding and other behaviors are still far from being fully understood (Mazier et al., 2015). Moreover, better insight into the aforementioned network being responsible for central control of feeding behavior is of significant interest, since nowadays, the respective neural circuitries are of substantial clinical relevance. Most importantly, availability of food no longer represents an evolutionary pressure, since food exists in abundance in many (albeit not all) countries around the world. Moreover, energy-dense foods high in carbohydrates and rich in fat can be obtained with little or no efforts. Thus, many people are suffering from chronic overload with nutrients in today's world, which, when accompanied by overall decreased physical activity is often leading to a morbid increase in body fat mass and resulting in obesity. On the other hand, a significant number of patients is affected from a complete loss of appetite (anorexia), which may be caused by psychiatric disorders, or by cancer and infectious diseases, and make these patients suffering from chronic under-nutrition (Scarlett and Marks, 2005; Park et al., 2014). Thus, decoding of the underlying cellular and molecular mechanisms in the central nervous system (CNS) that control feeding behavior may help to develop pharmacological interventions not only for disorders related with anorexia, but also for the treatment of the ever-increasing number of obese patients worldwide (Dietrich and Horvath, 2012).

Since time immemorial, cannabis extracts are used for recreational purposes. However, it is clear today that not only the psychotropic properties but also the well-known appetite stimulating effects of the plant-derived cannabinoid THC are mediated by CB_1 activation (Silvestri and Di Marzo, 2013). CB_1 belongs to the endocannabinoid system (ECS) that further consists of endocannabinoids (eCBs) as intrinsic CB_1 ligands, and of eCB synthesizing and hydrolyzing enzymes (Piomelli, 2003). These enzymes steadily control eCB levels in a temporal and spatial fashion to guaranty functional CB_1 signaling in a region and cell type specific manner (Pertwee, 2014). Interestingly, malfunction of the central ECS is associated with overeating and obesity (Engeli, 2008; Mazier et al., 2015). Thus, the main purpose here is to summarize recent experimental findings for central control of feeding behavior in health and disease, with special focus on central CB_1 signaling. Finally, presumed alternative, non-CB_1 driven pathways by which eCBs might also contribute to feeding regulation will be introduced.

DOES CB_1 STILL LEND ITSELF AS A THERAPEUTIC TARGET IN CENTRAL FEEDING REGULATION?

CB_1 was discovered almost 30 years ago and later identified as a promising target molecule in the CNS to pharmacologically interfere with feeding behavior (Matsuda et al., 1990; Devane et al., 1992; Williams and Kirkham, 1999). Besides feeding, several other physiological functions, and behaviors being modulated by central CB_1 signaling were deciphered so far (Lutz et al., 2015), and many pharmacological, biochemical, and morphological aspects of central CB_1 signaling were characterized.

The vast majority of CB_1 is located at presynaptic terminals in order to suppress the further release of classical neurotransmitters, such as GABA or glutamate (Castillo et al., 2012). However, different localizations and functions of CB_1 were also discovered (**Figure 1**). In principle, the acute pharmacological promotion of central CB_1 signaling can evoke food intake and thus still represents a promising approach to treat anorexia (Williams and Kirkham, 1999; Aigner et al., 2011; Reuter and Martin, 2016). However, it was discovered a couple of years ago that only administration of low to moderate doses of CB_1 agonists were able to increase food intake in mice, while moderate to high doses of CB_1 agonists decreased feeding (Bellocchio et al., 2010). In this, hypophagia was induced by CB_1-mediated reduction of GABAergic transmission, while hyperphagia was stimulated by CB_1-driven suppression of glutamatergic conduction (Bellocchio et al., 2010; Busquets Garcia et al., 2016). This fundamental finding in mice might explain the contrary results of different clinical trials on the use of CB_1 agonists in order to treat anorexia in humans (Aigner et al., 2011; Reuter and Martin, 2016). Thus, further approaches are needed to carefully reconsider the beneficial effects of CB_1 agonists for the treatment of anorexia (Whiting et al., 2015). In contrast to CB_1 agonists, the overall blockade of CB_1 by rimonabant generally suppressed hunger and induced hypophagia (Colombo et al., 1998; Simiand et al., 1998), but unfortunately also resulted in psychiatric side effects in humans. To develop more specific and safe pharmacological interventions for the treatment of overeating, the recently presented molecular ultrastructure of human CB_1 may deliver new opportunities for the design of next-generation CB_1 directing pharmaceuticals as novel anti-obesity drugs (Hua et al., 2016; Shao et al., 2016). Moreover, allosteric agents directed against CB_1 such as hemopressin or pregnenolone (Heimann et al., 2007; Dodd et al., 2010, 2013; Vallee et al., 2014) may supply medications with a significantly improved side effect profile (Busquets Garcia et al., 2016). Finally, another pharmacological approach aimed at selective blockade of peripheral CB_1, which basically was shown to induce metabolic benefits independently from modification of feeding behavior (Nogueiras et al., 2008; Tam et al., 2012). Nevertheless, it is primarily the knowledge about the cell type specific functions of CB_1 signaling in different types of neurons, and, as discussed later, also in glial cells, such as astrocytes (Metna-Laurent and Marsicano, 2015), which will determine if and in how far the full therapeutic potential of CB_1 pharmacology in feeding regulation can be leveraged.

In this regard, complexity of central CB_1 signaling was further broaden by the observation that CB_1, as a G protein-coupled receptor, is not exclusively expressed at the plasma membrane but also located at the outer mitochondrial membrane (Benard et al., 2012; Hebert-Chatelain et al., 2014). By interfering with respiratory chain complex I, mitochondrial CB_1 was recently shown to promote the amnesia-inducing effects of CB_1 agonists in the hippocampus (Hebert-Chatelain et al., 2016; Harkany and Horvath, 2017). Accordingly, effects of cannabinoids on food intake are also transmitted via CB_1-induced mitochondrial adaptations, since induction of feeding by CB_1 agonists depended on the expression of mitochondrial uncoupling protein 2 and the formation of reactive oxygen

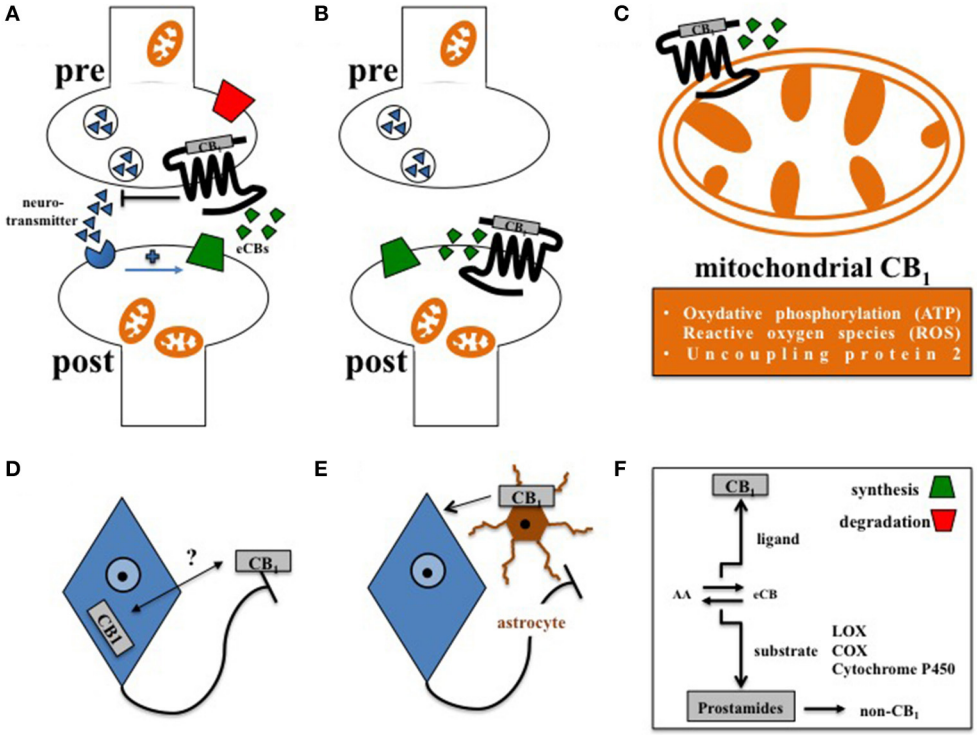


FIGURE 1 | Principles of central CB_1 signaling in control of feeding behavior. (A) Retrograde signaling of eCBs at presynaptic CB_1 impacts feeding (Bellocchio et al., 2010). **(B)** Postsynaptic CB_1 at POMC neurons affects feeding in DIO (Morello et al., 2016). **(C)** Cannabinoids interfere with mitochondrial CB_1 in hypothalamic feeding regulation (Koch et al., 2015). **(D)** Whether activity-dependent subcellular distribution of CB_1 (Thibault et al., 2013) accounts for control of food intake is still open. **(E)** Astroglial CB_1 regulates the metabolic effects of leptin in cultured astrocytes (Bosier et al., 2013), and thus might contribute to astrocyte-dependent control of feeding behavior in the hypothalamus (Kim et al., 2014). **(F)** Enzymes of eCB synthesis or degradation control eCB levels in a spatial and temporal manner (Pertwee, 2014). Moreover, eCBs not only function as CB_1 ligands, but also as substrates of specific enzymes, such as lipoxygenases (LOX), cyclooxygenases (COX), or cytochrome P450, supporting the idea that the ECS might also transmit metabolic effects independently from CB_1 signaling (non-CB_1).

species (ROS) in the hypothalamus (Koch et al., 2015; Kruger, 2016), finally pointing toward region-specific functions of mitochondrial CB_1 signaling in the brain (Harkany and Horvath, 2017). However, CB_1 driven control of ROS seems to be multifaceted, since cannabinoids reduced leptin-mediated ROS formation in cultured hypothalamic neurons by CB_1 dependent peroxisome proliferator-activated receptors (PPAR)-gamma and subsequent catalase activation (Palomba et al., 2015). Overall, about 15% of total brain CB_1 is associated with mitochondria (Benard et al., 2012; Hebert-Chatelain et al., 2014), and it appeared that CB_1 is present in mitochondria of both pre- and postsynaptic terminals (Busquets Garcia et al., 2016). However, CB_1 is most abundantly expressed at the plasma membrane of axonal shafts and presynaptic terminals (Pertwee, 2010), and significant amounts of CB_1 in the forebrain are constantly activated, internalized, and recycled at steady state (Thibault et al., 2013). Whether internalization and redistribution of CB_1 between axonal plasma membrane and somato-dendritic endosomes account for control of feeding behavior still needs to be investigated. Moreover, functional expression of CB_1 is also observed at the postsynaptic plasma membrane (Castillo et al., 2012). In the course of diet-induced obesity (DIO), orexin-A represses satiety-promoting pro-opiomelanocortin (POMC) neurons in the hypothalamic arcuate nucleus (ARC) by eCB-mediated activation of postsynaptic CB_1 on POMC neurons (Morello et al., 2016).

In addition to neurons, CB_1 is also expressed in astrocytes (Metna-Laurent and Marsicano, 2015; Oliveira Da Cruz et al., 2016), and plays an important role in neuroinflammation (Walter and Stella, 2004), and in physiological neurotransmission (Navarrete and Araque, 2010; Han et al., 2012). Interestingly, astrocyte-dependent energetic support of neurons also involves CB_1, since leptin-induced astroglial glycogen accumulation depends on CB_1 signaling in cultured astrocytes (Bosier et al., 2013). However, the relevance of astroglial CB_1 in distinct hypothalamic feeding centers has to be considered *in vivo*. Accordingly, structural analyses determined CB_1 in the immediate vicinity to astrocytes at tripartite synapses in the ARC (Morozov et al., 2017). Moreover, hypothalamic astrocytes and microglia show morphological adaptations in DIO (Baufeld et al., 2016; Argente-Arizon et al., 2017), and astrocytes, via leptin signaling, actively control hypothalamic neuronal circuits, and feeding (Kim et al., 2014). Thus, it is of significant interest to study the function of CB_1 signaling in glial cells under normal and high fat diet (HFD).

Together, studies focusing on the cell type specific expression and subcellular distribution of CB_1 delivered unique mechanistic insights into central CB_1 signaling, which provides an important prerequisite to uncover the physiological role of CB_1 in distinct homeostatic and hedonic feeding centers of the CNS.

RECENT ADVANCES IN UNDERSTANDING HOMEOSTATIC AND HEDONIC FEEDING CONTROL: WHAT IS THE RELEVANCE OF CB_1?

Homeostatic feeding centers supervise the body's energy resources and are located in the hypothalamus and caudal brainstem (Koch and Horvath, 2014), while hedonic feeding centers relevant for palatability and rewarding aspects of food are pinpointed to the mesolimbic system (Alonso-Alonso et al., 2015; Pandurangan and Hwang, 2015). Although both control systems are anatomically located in different brain areas, it becomes more likely that they are functionally closely interconnected to each other (Munzberg et al., 2016).

CB_1 obtains a conserved distribution in the CNS among different mammalian species (Herkenham et al., 1990). High CB_1 expression levels in the hippocampus or basal ganglia are attributed to cannabinoid-induced effects on memory formation and movement (Castillo et al., 2012). Low CB_1 expression levels in hypothalamic or caudal brainstem nuclei display significant functions in regulation of feeding behavior (Cardinal et al., 2012; Mazier et al., 2015). In this, distinct groups of hypothalamic neurons measure the body's energy resources by sensing circulating nutrients and detecting metabolic hormones, such as leptin, insulin, or ghrelin (Varela and Horvath, 2012; Vogt and Bruning, 2013; Muller et al., 2015). Moreover, hypothalamic neurons are directly affected by cannabinoids, since infusion of CB_1 agonists into distinct hypothalamic nuclei acutely induced feeding (Jamshidi and Taylor, 2001; Koch et al., 2015). Interestingly, hypothalamic CB_1 signaling interferes with signal transmission of metabolic hormones. While leptin suppressed feeding correlates with decreased hypothalamic eCB levels (Di Marzo et al., 2001), ghrelin triggered acute feeding accompanies with increased hypothalamic eCB levels, and depends on paraventricular nucleus (PVN) CB_1 signaling (Kola et al., 2008). However, CB_1 mediated control of feeding in the PVN is more complex than thought before, since under an experimental fasting/re-feeding paradigm, blockade of local CB_1 in the PVN increased hyperphagy in hungry mice, and enhanced the hyperphagic effect of ghrelin in fed animals (Soria-Gomez et al., 2014b). Thus, hypothalamic eCBs represent local neuromodulators that are actively involved in rapid rewiring of hypothalamic feeding circuits in accordance to the current prandial state (Pinto et al., 2004). In DIO, imbalanced hypothalamic eCB levels and defective CB_1 signaling seem to be the consequence of central leptin resistance (Silvestri and Di Marzo, 2013). In the lateral hypothalamus (LH), CB_1 is involved in physiological control of melanin-concentrating hormone and orexin-A neurons (Silvestri and Di Marzo, 2013). In DIO, eCBs in the LH promote hyperphagia by remodeling the synaptic input organization of orexin-A neurons (Alpar and Harkany, 2013; Cristino et al., 2013).

In the ARC, at least two neuronal populations with opposing effects on feeding behavior can be distinguished: the hunger promoting Agouti-related protein/neuropeptide Y (AgRP/NPY) neurons that acutely promote food intake, and POMC neurons that drive gradual onset of satiety (Varela and Horvath, 2012). Systemic blockade of CB_1 by rimonabant reduced NPY levels, indicating that AgRP/NPY neurons are controlled by local eCBs (Verty et al., 2009). AgRP/NPY neurons do not contain CB_1 (Cota et al., 2003; Horvath, 2003), but CB_1 was predominately found at GABAergic terminals innervating AgRP/NPY neurons (Morozov et al., 2017). Thus, local eCBs in the ARC might promote feeding by retrograde dis-inhibition of AgRP/NPY neurons. However, POMC neurons are also affected by cannabinoids via pre- and postsynaptic CB_1 (Hentges et al., 2005; Koch et al., 2015; Morello et al., 2016). In fed mice, CB_1 agonists rapidly converted POMC neurons from promoters of long-term satiety into acute drivers of hunger (Koch et al., 2015; Patel and Cone, 2015). In DIO, orexin-A repressed POMC neurons by constitutive eCB signaling at postsynaptic CB_1 in POMC neurons (Morello et al., 2016). Mapping of hypothalamic neuronal subtypes by single-cell RNA sequencing (Romanov et al., 2017) and molecular indexing of local ARC cell types by gene expression profiling identified novel cell types of putative relevance for regulation of distinct vegetative body functions, including feeding (Campbell et al., 2017). Thus, it would be interesting to dissect the functional relevance of CB_1 signaling in these cell types. Accordingly, glutamate-releasing neurons in the ARC that express oxytocin receptors were identified as an integral part of a rapid ARC to PVN satiety pathway (Fenselau et al., 2017). However, whether acute effects of cannabinoids on feeding might be further transmitted by this novel pathway remains elusive. Alongside, local ARC dopaminergic cells were identified that reciprocally control activity of AgRP/NPY and POMC neurons (Zhang and Van Den Pol, 2016). This finding is of substantial interest in order to study CB_1 controlled homeostatic feeding, since dopamine modulates rewarding aspects of food mainly through dopaminergic ventral tegmental area (VTA) to nucleus accumbens (NAc) projections (Volkow et al., 2011), and CB_1 signaling was shown to modulate dopaminergic signaling in the NAc and VTA to regulate hedonic aspects of feeding (Melis et al., 2007; Di Marzo et al., 2009).

Beside the VTA located in the rostral brainstem, CB_1 signaling is also interfering with the functional activity of caudal brainstem nuclei, such as parabrachial nucleus, dorsal motor nucleus of the vagus, and nucleus of the solitary tract. In this, CB_1 basically controls food preferences, such as digestion of palatable foods being rich in fat (Busquets Garcia et al., 2016). Finally, hypothalamic AgRP/NPY and POMC neurons are not only directly affected by food intake itself, but also rapidly respond to sensory detection of available food (Chen et al., 2015). It is thus likely that hypothalamic neurons not only transmit internal signals causing hunger or satiety in response to eating and internal sensing of energy resources, but also receive external information on the incentive value of food,

such as sight, smell, and taste in order to rapidly react to food stimuli and transmit motivational aspects on feeding being generated via the mesolimbic system (Seeley and Berridge, 2015). Processing of food sensations such as olfactory or gustatory signals indeed involve CB_1 signaling, since fasted mice displayed CB_1-dependent increased odor detection in the main olfactory bulb (Soria-Gomez et al., 2014a).

BESIDES CB_1: DOES THE ECS PROVIDE OTHER RELEVANT TARGET MOLECULES IN FEEDING REGULATION?

Within the ECS, it is the availability of eCBs that provides the routes and directions of CB_1 signaling in the brain. While research was long-time focusing on pharmacological modulation of CB_1 signaling by direct interaction at CB_1 in order to interfere with feeding and other behaviors, numerous evidence arose that targeting of classical enzymes involved in biosynthesis or degradation of eCBs will also allow to induce adaptations in feeding behaviors (Pertwee, 2014). For example, degradation of the eCB 2-arachidonoylglycerol (2-AG) into arachidonic acid and glycerol is basically controlled by three different serine hydrolases: while monoacylglycerol lipase (MAGL) accounts for 85% of 2-AG degradation, alpha/beta-hydrolase domain containing (ABHD) 6, and 12 are responsible for hydrolysis of 5 and 10%, respectively (Savinainen et al., 2012). Indeed, it was shown that knockdown of ABHD6 in the ventromedial hypothalamus resulted in locally elevated 2-AG levels, finally resulting in a blunted fasting-induced feeding response and in a general diminished efficacy of the mice in order to adapt to other metabolic shifts (Fisette et al., 2016).

Generally, eCBs do not resemble to classical neurotransmitters that are stored in synaptic vesicles (Piomelli, 2003). Instead, eCBs, as being arachidonic acid derivatives, are produced on demand from lipid precursors. Most eCBs display a relative short half-life, since they are attracted by both classical eCB degrading enzymes in order to terminate CB_1 signaling, and by different classes of enzymes aiming transformation of eCBs into other classes of lipidergic signaling molecules, such as prostamides (Urquhart et al., 2015). The fact that eCBs belong to the family of polyunsaturated fatty acids makes them indeed attractive substrates for enzymatic oxidation, as induced by lipoxygenases (LOX), cyclooxygenases (COX), or cytochrome P450 (Rouzer and Marnett, 2011). Numerous eCBs have been described so far and in addition to 2-AG it is arachidonoylethanolamine (AEA) representing by far the best-studied intrinsic ligand of CB_1 today. However, beside CB_1 and CB_2 as the most relevant G protein-coupled receptors of cannabinoids, it is likely that eCBs also act upon several other G protein-coupled receptors, such as GPR18, GPR55, and GPR119. These former orphan receptors are putative candidates for nomination of CB_3, however their relevance in feeding regulation has to be further investigated. Nevertheless, it appeared that GPR18 and GPR55 signaling is involved in processes of metabolic dysfunction (Liu et al., 2015; Rajaraman et al., 2016). Besides G protein-coupled receptors, eCBs such as AEA were also shown to act upon other types of receptors, such as transient receptor potential (TRP) vanilloid 1 (Pertwee, 2010). Moreover, several enzymes involved in eCB biosynthesis, such as the AEA synthesizing N-acyl phosphatidylethanolamine-specific phospholipase D (NAPE-PLD) not only give rise to the CB_1 ligand AEA, but also to structural very similar lipid messengers that do not bind and activate CB_1. In this, it was shown that oleoylethanolamine (OEA) and palmitoylethanolamine (PEA), as close related lipids of AEA, bind to PPARs (Fu et al., 2003; Lo Verme et al., 2005; Gaetani et al., 2010), which are well-known to contribute in control of glucose, lipid, and energy metabolism (Grygiel-Gorniak, 2014). Thus, the overall metabolic role of the enzymes in the ECS, beside CB_1, may deliver future targets for therapeutic interventions in control of feeding behavior. Indeed, targeted lipidomics of different brain regions derived from mice either deficient for CB_1, the AEA degrading enzyme FAAH or the aforementioned 2-AG degrading MAGL revealed that AEA and 2-AG hydrolyzing enzymes, when compared to CB_1, link the ECS to a broader lipid signaling network in contrasting ways, which again may open an avenue in altering neurotransmission and behaviors independently of CB_1 signaling (Leishman et al., 2016a). This assumption is further supported by another lipidomic analysis. In this, mice deficient for NAPE-PLD not only displayed a shift in the concentration of AEA, but also shifted several other lipids, not binding to CB_1, such as OEA and PEA, that as mentioned before signal upon different metabolic relevant targets, such as PPARs (Leishman et al., 2016b).

OUTLOOK

Actually, there has been significant increase of knowledge about central CB_1 signaling in control of feeding behavior. Despite the significant setback that occurred in the past on clinical use of CB_1 inverse agonists in order to treat overeating, there still is strong confidence in the field that the recent discoveries on central CB_1 signaling soon will leverage the therapeutic potential of CB_1.

AUTHOR CONTRIBUTIONS

MK designed this review, including **Figure 1**.

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REVIEW
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Central Control of Feeding Behavior by the Secretin, PACAP, and Glucagon Family of Peptides

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Constituting a group of structurally related brain-gut peptides, secretin (SCT), pituitary adenylate cyclase-activating peptide (PACAP), and glucagon (GCG) family of peptide hormones exert their functions *via* interactions with the class B1 G protein-coupled receptors. In recent years, the roles of these peptides in neuroendocrine control of feeding behavior have been a specific area of research focus for development of potential therapeutic drug targets to combat obesity and metabolic disorders. As a result, some members in the family and their analogs have already been utilized as therapeutic agents in clinical application. This review aims to provide an overview of the current understanding on the important role of SCT, PACAP, and GCG family of peptides in central control of feeding behavior.

Keywords: secretin, PACAP, and glucagon family peptides, hypothalamus, feeding behavior, energy homeostasis, metabolic diseases

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INTRODUCTION

Secretin (SCT), pituitary adenylate cyclase-activating peptide (PACAP), and glucagon (GCG) family or SCT family, a group of short peptides that were classified based on their structural homology and named after the first hormone discovered, include SCT, PACAP, vasoactive intestinal peptide (VIP), GCG, glucagon-like peptide-1 (GLP-1), GCG-like peptide-2 (GLP-2), glucose-dependent insulinotropic polypeptide (GIP), growth hormone–releasing hormone (GHRH), and peptide histidine isoleucine (PHI) or peptide histidine methionine (1). While sharing some biological functions, each of these hormones possesses distinct physiologic actions such as the involvement of SCT in water homeostasis (2). Similar to their ligands, receptors mediating functions of the peptides are structurally related and are grouped in the class B G protein-coupled receptors (3, 4). Recent pharmacological interests for these receptors have paved way for novel therapeutic strategies for intervention of pathological conditions (5–8). For instance, liraglutide and exenatide (GLP-1 receptor agonists) are being used for treating diabetes (5).

Feeding is a complex behavior involving the integration of homeostatic systems that sense energy balance with hedonic (reward) behavior (9). Areas in the central nervous system (CNS) that appear to be important for regulation of feeding behavior are distributed across regions of forebrain and caudal brain stem (10, 11). Of particular significance is the hypothalamic neural circuitry, long known to be involved in the control of energy homeostasis in response to various endocrine, nutritional, metabolic, and thermal signals. The role of hypothalamus in energy homeostasis regulation has been discussed in detail in several review articles (10, 12–16). Arcuate nucleus (ARC), paraventricular nucleus (PVN), ventromedial hypothalamus (VMH), dorsomedial hypothalamus (DMH), and lateral hypothalamus (LH) are hypothalamic regions with reciprocal

connections that are known to be involved in regulation of food intake and energy homeostasis. In the ARC above the median eminence, neurons expressing neuropeptide Y (NPY) and agouti gene-related protein (AgRP) stimulate food intake and are found medially, while pro-opiomelanocortin (POMC) (precursor of α-melanocyte-stimulating hormone; α-MSH) and cocaine- and amphetamine-regulated transcript (CART) induce anorexia and are coexpressed in the neuronal population of lateral ARC. These neurons project to the PVN which controls feeding and provides preganglionic autonomic output to the brainstem. NPY reduces energy expenditure and stimulates food intake through the Y1 and Y5 receptors in the PVN. Conversely, CART inhibits NPY-induced feeding (17–19). Also, POMC and its products such as α-MSH suppress feeding behavior. AgRP is an endogenous antagonist of α-MSH and, therefore, increases food intake and weight. Other hypothalamic peptides, such as melanin-concentrating hormone and hypocretin, are orexigenic in nature and are expressed in distinct populations of neurons in the lateral hypothalamic area (20–22). Another important site in regulation of feeding behavior is caudal brain stem. In the brain stem, neurons in the nucleus tractus solitarius (NTS) and dorsal motor nucleus of the vagus (DMV) receive and integrate inputs from vagus nerve, which is involved in sensing nutrient accumulation in the stomach and the duodenum (23). Highly interconnected with the hypothalamus, brainstem regulates responses to fasting through ascending projections to the hypothalamus (10) and short-term satiety signals through descending projections from the hypothalamus (24). Apart from the brain circuits regulating hunger and satiety, nuclei embedded within the mesolimbic reward circuitry [ventral tegmental area (VTA) and nucleus accumbens (NAc)], which are well known for their importance in reinforcing properties of drugs of abuse and natural rewards (25), as well as nuclei in the amygdale and hippocampus are involved in the rewarding effects of food (26).

The SCT family peptides and their receptors have been found to exist in various brain regions, including hypothalamus, implying their neuroactive functions (27, 28). Most of them have also been found to be important in the central regulation of energy balance (29). Therefore, in the sections below, we have reviewed in detail the role of SCT family hormones in the central control of feeding behavior.

SECRETIN

Secreted from the S-cells of the duodenum in response to acid, SCT is a 27-amino acid peptide and the first ever hormone discovered (30). SCT primarily functions in the gastrointestinal tract to stimulate bicarbonate secretion from the pancreas to neutralize acid (31). A century-long research has gone into studying the gastrointestinal functions of SCT, and only recently it has been found as a neuropeptide (2, 32–36). As we summarized before (28), SCT was found to be expressed in multiple brain sites, including PVN, supraoptic nucleus (SON), and ARC of hypothalamus, NTS of brainstem, central amygdala (CeA), hippocampus, and cerebral cortex (37, 38). SCT receptor (SCTR) expressions were also found in PVN, SON, and ARC of the hypothalamus, among other brain regions (39). In spite of contradictory evidences in the past (40–42), our laboratory has recently confirmed an anorectic effect of peripheral and central SCT in mice (39), supported by our findings that intracerebroventricular (i.c.v.) and intraperitoneal (i.p.) injections of SCT reduced food intake in wild-type mice. This observation was absent in SCTR knockout ($SCTR^{-/-}$) mice, further confirming the specificity of SCT–SCTR axis in regulating feeding behavior.

The previous review from our lab (28) has concluded that circulating SCT might not cross the blood–brain barrier (BBB) for specifically exerting its anorectic effect (38, 39), although SCT has been shown to be able to cross the BBB (43). Thus peripheral SCT, through SCTR in the intestinal vagal afferents, communicated to the brain to bring about satiety control without causing conditioned taste aversion (CTA) (39, 44). However, a very recent study has provided another piece of the puzzle on the appetite regulation by peripheral SCT. By using male oxytocin (OXT)-monomeric red fluorescent protein 1 (mRFP1) transgenic rats, the study showed that i.p. SCT (100 μg/kg), apart from inducing a reduction in food intake, stimulated mRFP1 fluorescence (OXT indicator) in the dorsal division of the parvocellular PVN (dpPVN) and increased mRFP1-positive granules in the axon terminals of dpPVN OXT neurons in the NTS. An upregulation of c-Fos expression was also observed in the NTS and OXT neurons of dpPVN. Therefore, peripheral SCT might regulate feeding behavior, at least in part, *via* an OXTergic pathway from the dpPVN to the NTS (45).

Consistently, central SCT is also able to reduce food intake. It has been shown that central SCT induced c-Fos immunoreactivities in the ARC and PVN of hypothalamus. In the ARC, POMC neurons were found to be colocalized with activated c-Fos as well as with SCTR. An increase in POMC and a reduction in AgRP transcript levels were observed in the ARC after i.c.v. and i.p. SCT (39, 46). In addition, it has been reported that central and peripheral SCT increased melanocortin-4 receptor (MC4R) mRNA in the PVN, and administration of MC4R antagonist, SHU9119, in the PVN reduced the anorectic effect of central and peripheral SCT, indicating the involvement of melanocortin system (39, 46). We previously showed that K^+-induced depolarization of hypothalamic explants released SCT endogenously through voltage-gated sodium and calcium channels, suggesting that this endogenous SCT could function as a neurotransmitter in the region (34, 35). Taken all together, SCT has been suggested as a pleiotropic regulator of feeding behavior and neuroendocrine signaling in the hypothalamus. Microinjection of SCT into the CeA has been recently shown to reduce cumulative food intake through cAMP-activated protein kinase pathway, while electrophysiological recordings indicated that SCT may exert its anorectic actions, at least in part, by modulation of spontaneous firing of CeA neurons (47).

Absence of a truly functional SCTR antagonist is partly a hindrance for studying the region-specific (hypothalamus or amygdala) role of SCT in modulating food intake behavior. Recent establishment of several animal models such as $SCT^{-/-}$ or $SCTR^{-/-}$as well as the $SCT^{fl/fl}$ for cell-specific knockout of SCT should help to reveal the relationship between SCT and

anorectic functions. Research on food intake modulations by SCT has been initiated for the past few years but still requires further efforts to test its potential in therapeutic interventions.

PITUITARY ADENYLATE CYCLASE-ACTIVATING PEPTIDE

Pituitary adenylate cyclase-activating peptide, a 38-residue peptide hormone, was first isolated from ovine hypothalamic tissue as a hypophysiotropic peptide that would activate adenylate cyclase in cultured rat pituitary cells (48) and is widely distributed in the body including brain, gonads, and adrenal gland (49, 50). Various regions of the brain like central thalamic nuclei, amygdaloid complex, and hippocampus expressed PACAP and yet the most abundant expression was found in the ARC, PVN, VMH, and SON of the hypothalamus (50, 51), which has been well documented to be involved in appetite regulation. Double labeling studies have shown PACAP to be colocalized with POMC neurons of the ARC but not POMC neurons within the brain stem (52). Receptors for PACAP (PAC1R and/or VPAC2R) were also found to be expressed in approximately half of POMC neurons (53, 54) and a significant proportion of NPY neurons (53, 54). Consistent with its distribution pattern in the brain, i.c.v. administration of PACAP was found to reduce food intake by several studies (55–57). Notably, in rat, Mizuno's group showed that PACAP induced a long-lasting reduction of food consumption (58). As we have reviewed before (28), a study showed that 100 nM PACAP increased POMC mRNA, α-MSH tissue content as well as α-MSH release in mediobasal hypothalamic explants (53, 54). Another study further revealed that central PACAP stimulated c-Fos expression in POMC neurons and increased POMC and MC4R transcripts in the ARC; PACAP-specific receptor (PAC1R) knockout mice had lower POMC transcript levels in the ARC compared to wild-type animals and pre-injection of SHU9119, MC3R/MC4R antagonist, abolished the hypophagic effects of i.c.v.-PACAP, suggesting that PACAP might act through PAC1R receptor and then melanocortin system to exert its anorectic effect (57). Using AtT20PL, a clone of the AtT20 mouse corticotroph tumor cells stably transfected with rat POMC 5′ promoter-luciferase fusion gene, PACAP and VIP were found to increase the POMC promoter activity and expression *via* a PKA-independent intracellular signaling pathway (59).

Intra-PVN injection of PACAP has been shown to reduce food intake as well as lead to significant reductions in meal size, duration, and total time spent eating (60), consistent with the loss-of-function study showing that there was a pronounced hyperphagia in PVN-lesioned animals (61), and PACAP receptor-specific study showing that the feeding behavior was primarily controlled by PAC1R and this receptor subtype was abundantly distributed in the PVN (60). Although PVN has been suggested as the predominant site of action for PACAP-mediated hypophagia, the PACAP stimulation of the VMH may serve primarily to stimulate energy expenditure (60). Direct acute injection of PACAP into the VMH, a region heavily expressing PAC1R, was able to inhibit food intake for 6 h which could be reversed by the PAC1R antagonist. Intra-VMH injection of PACAP also increased POMC mRNA in the ARC while not affecting NPY and AgRP mRNA levels (62). Previous studies have shown that PACAP is capable of modulating the activation of ionotropic glutamate receptors (63, 64). Recently, it has been shown that glutamatergic signaling *via* NMDA receptors was required for the hypophagic effects of intra-VMH PACAP (65). A novel model of binge behavior that could temporally separate homeostatic feeding from palatable food-driven (hedonic) feeding behavior (66), has shown that the microinjection of PACAP into NAc mimics the actions of GABA agonists and reduces the intake of palatable food without altering homeostatic feeding, while microinjection of PACAP into the VMH mimics the actions of AMPA by decreasing homeostatic feeding without altering hedonic feeding. Furthermore, it has been shown that transcript levels of PACAP in the VMH was regulated according to energy status, as fasting reduced and high-fat diet increased PACAP expression in VMH (67).

Recently, monosynaptic interactions of PACAP-expressing neurons in the VMH with appetite-suppressing POMC neurons of the ARC have been shown (68). Nevertheless, this study also showed, using channel rhodopsin-assisted circuit mapping, that appetite-stimulating AgRP neurons in the ARC had excitatory PACAPergic afferents originating within the PVN. Incidentally, a previous study has reported that PACAP could stimulate NPY neurons to elevate cytosolic Ca^{2+} levels (69). These exciting new findings suggested that, based on their neuroanatomical location, PACAP could stimulate both orexigenic and anorexigenic effects (70). A very recent study showed a role of PACAP/PAC1 signaling during light-regulated feeding behavior (71), adding more complexity on the appetite regulation by PACAP in the hypothalamus. Therefore, further detailed studies are required to understand how these pathways interact under various energy states and converge to modulate feeding behavior.

Apart from its anorectic role, local PVN as well as i.c.v. infusion of PACAP-38 significantly induced plasma glucose concentration, endogenous glucose production, and c-Fos immunoreactivity in the autonomic neurons in the PVN. These neurons project to preganglionic sympathetic neurons in the spinal cord and are involved in hepatic glucose production (72). Furthermore, PACAP has been shown to interact with other peptides to modulate feeding behavior. Central administration of PACAP provokes increases in hypophysiotropic neurohormones in the hypothalamus, such as vasopressin, GnRH, somatostatin, and CRF (73, 74). In chicks (75) and goldfish (76), it has been shown that anorectic effect of central PACAP was inhibited by CRH receptor antagonist, astressin, and α-helical $CRH_{(9–41)}$, respectively, and GnRH2 has been found to mediate CRH-signaling pathway in goldfish (77). PACAP mRNA was found to be colocalized with steroidogenic factor-1in the VMN and leptin signaling was required for normal PACAP expression in these cells, while blocking of endogenous central PACAP signaling attenuated leptin-stimulated hypophagia and hypothermia (67). Consistently, i.p. administration of PACAP has been found to suppress appetite with a decrease in plasma ghrelin and an increase in plasma GLP-1 and leptin (78, 79). For its integrative role in glucose and energy homeostasis, PACAP receptor subtype-specific agonists and/or antagonists are being

considered as potential therapeutic agents for metabolic disorders in addition to appetite disorders.

VASOACTIVE INTESTINAL PEPTIDE

Distributed throughout the gastrointestinal tract (80, 81) and CNS including cerebral cortex, suprachiasmatic nucleus (SCN), and PVN of the hypothalamus and thalamus (82, 83), VIP is a 28-amino acid peptide hormone known for its role in vasodilation and hypotension acting through VPAC1R and VPAC2R receptors. VPAC1R expression has been found mainly in the cerebral cortex and hippocampus, while VPAC2R was expressed in the thalamus, midbrain and in the PVN and SON magnocellular cells and SCN of the hypothalamus (83, 84). Previous studies have demonstrated that plasma VIP concentrations increased following either a carbohydrate meal or water loading (85), and short-term fasting altered the VIP levels in the hypothalamus and other brain regions, suggesting a potential for VIP in modulating appetite and food intake (86). Indeed, i.c.v. administration of VIP induced anorexia in chicken (87, 88), goldfish (89, 90), and rat (91). Disruption in food intake and metabolic rhythm occurred in VIP and VPAC2R knockout mice (92). VIP could possibly stimulate hypothalamic–pituitary–adrenal (HPA) axis as intra-PVN injection of VIP increased secretion of ACTH and corticosterone (93), possibly by activating the CRH neurons (94). Additionally, stimulation of hypothalamic explants by VIP significantly stimulated the release of α-MSH (91), suggesting VIP could also work through the activation of melanocortin system to inhibit food intake. As mentioned earlier, cell line studies with transfected rat POMC promoter-luciferase fusion gene have shown that VIP induces POMC promoter activity through a PKA-independent pathway (59). Recently, VIP knockout mice were shown to have a disrupted pattern of circadian feeding behavior resulting in a significantly reduced nocturnal/diurnal feeding along with reduced body weight and fat mass accumulation (78, 79). The study also showed that, in VIP knockout mice, the release of anorexigenic hormones, such as GLP-1, leptin, PYY, and insulin was altered in both fasting and post-prandial conditions, revealing a possibility of VIP cross-talking with other hormones to inhibit food intake. However simultaneously, orexigenic hormones were also found to be altered suggesting the role of VIP in both anorexigenic and orexigenic effects (78, 79). There were also reports indicating an absence of anorectic effects by VIP as i.c.v. injection of VIP did not influence appetite in fasted mice (57) and administration of VIP receptor antagonist in the PVN had no effect on food intake in rats (94). With such contrasting evidences, the role of VIP on appetite control remains currently unclear.

GLUCAGON

Derived from proglucagon that contains sequences for GLP-1 and GLP-2, GCG is a 29-amino acid peptide hormone secreted by pancreatic α cells in response to low blood glucose. Counteracting hypoglycemia, it antagonizes insulin action by stimulating hepatic glucose synthesis and mobilization. In contrast to its peripheral action, recent reports have shown that hypothalamic action of GCG inhibited hepatic glucose production (95, 96). Its role in energy homeostasis and metabolism has been reviewed in detail before (97–99). Also as we have reviewed before (28), peripheral GCG induced satiety in humans (100, 101) and in rats (102, 103) without causing CTA (104); GCG affected meal size rather than meal interval (105); and hepatic vagal afferents were found to mediate this effect of peripheral GCG (106). In addition, another study reported that subcutaneous injection of GCG was able to reduce appetite and through immunohistochemical analysis c-Fos expression could be detected in the NTS, area postrema (AP), and CeA but absent in the ARC, PVN, and DMH regions of the hypothalamus, suggesting the involvement of brainstem and amygdala in the appetite control by peripheral GCG (107). GCG receptor (GCGR) distribution has been found in the dorsal vagal complex (DVC) of brainstem (108). A recent study investigating the role of GCG in high-protein feeding has demonstrated that elevated circulating GCG found during high-protein feeding acted in the DVC through GCGR-dependent PKA–Erk1/2–K_{ATP} signaling cascade to contribute to the effect of high-protein feeding (109). However, relatively low levels of GCGR were also found in the hypothalamus, and it is noteworthy that circulatory GCG has been shown to suppress glucose sensing *via* LH, DMH and VMH neurons of hypothalamus, hence the possibility of hypothalamic activation in appetite control by peripheral GCG could not be excluded (110). While the mechanisms behind the anorectic effect of peripheral GCG were not fully understood, there were also reports suggesting that peripheral GCG increased food intake (111).

Central administration of GCG has been observed to induce anorexia in rats (112, 113), chicks (114, 115), and sheep (116) with much higher anorectic effect appeared after i.c.v. GCG than its peripheral effect in rats (112). Although the mechanisms underlying satiety regulation by central GCG remains a mystery, a previous review has discussed several possibilities. For example, it suggested the stimulation of hypothalamic corticotropin-releasing factor and activation of HPA axis to be involved in the anorectic effect of GCG (117). Microinjection of GCG into the LH reduced appetite and stimulated sympathetic activity (118). Although high levels of immunoreactive GCG and relatively low levels of GCGR were found in the hypothalamus (108), the role of hypothalamic neurons in appetite control by GCG was not clear, until a recent report proved that central GCG reduced appetite through hypothalamic pathway (119). Hypothalamic GCG activated GCGR to stimulate downstream PKA pathway in the hypothalamic ARC, as i.c.v. co-infusion of the GCGR antagonist des-His^1-[Glu^9] GCG amide or the PKA inhibitor H-89 negated the ability of central GCG to induce anorexia. And as the downstream factor of PKA, central GCG injection also reduced protein levels of Ca^{2+}-calmodulin-dependent protein kinase kinase β (CaMKKβ) and its downstream target phosphorylated AMP-activated protein kinase (AMPK) in the ARC. AMPK was upstream Acetyl-CoA carboxylase (ACC), and it was hence observed that the injection of a constitutively active AMPK virus in the ARC was able to recover the decrease of ACC caused by central GCG and attenuated the anorectic effects of GCG. Consistent with above findings as well as the co-localization of the GCGR

in AgRP neurons of the ARC (95), a significant reduction in the expression of AgRP was observed after central GCG injection. Diet-induced obesity abolished the anorectic effects of GCG but it was restored by molecular inhibition of CaMKKβ in the ARC *via* adenoviruses encoding dominant negative CaMKKβ. Central GCG, therefore, exerted its acute anorectic effects through PKA/AMPK/CaMKKβ-dependent pathways in the ARC and CaMKKβ mediated its obesity-induced hypothalamic resistance (119). Taken together, even though several aspects of the central GCG's role in controlling feeding behavior have been discussed above and also by other studies, further research is still required to get better understanding and shed light on the beneficial effects by coordination of central GCG with other hormones such as insulin and GLP-1.

GLUCAGON-LIKE PEPTIDE-1

Processed from the preproglucagon (PPG), GLP-1 is a 30-amino acid peptide hormone secreted from the L-cells of the intestinal epithelium in response to nutrient intake (120) and acts as an incretin along with GIP stimulating glucose-dependent insulinotropic action primarily (121, 122). As the most explored hormone for its role in feeding behavior among the SCT family of peptides, a plethora of research has brought clarity and understanding on the peripheral and central effects of endogenous and exogenous GLP-1 on food intake and glycemic control as reviewed in several reports (123–126). Acting with GLP-1 receptors (GLP-1Rs), GLP-1 altered food intake behavior through various neural substrates, including hypothalamus (ARC, PVN, and LH), hindbrain nuclei [parabrachial nucleus (PBN), area postrema (AP), medial NTS (mNTS)], ventral hippocampus (vHP), and nuclei embedded within the mesolimbic reward circuitry (VTA and NAc). Within these areas, the diverse neural circuitry involved in feeding control by GLP-1 has been recently reviewed in detail (127). Following nutrient intake and entry into the gastrointestinal tract, peripheral GLP-1, endogenously released from the L-cells, acted in a paracrine manner on the GLP-1R that was expressed on dendritic terminals of the celiac and gastric branches of the vagal afferents which innervated the intestine to exert its satiation effect. This vagal activation *via* vagal-to-NTS glutamatergic signaling relayed signals to nodose ganglion for activating the NTS neurons in the brain (128). On GLP-1 stimulation, GLP-1R on the intestinal vagal afferents also stimulated pancreatic insulin secretion *via* vago-vagal reflex (128, 129). Indeed, it was possible that peripheral GLP-1 through a vagal afferent-independent pathway crossed the BBB to directly activate the central GLP-1R of NTS. But a recent finding that after subdiaphragmatic vagal deafferentation, i.p. injection of GLP-1 did not affect the size of the first meal suggested otherwise (130). Although there was a question about whether peripheral GLP-1 was able to act directly in the brain, in the case of peripheral injections of long-acting GLP-1 analogs like liraglutide and exendin-4 (Ex-4), it was quite clear that they brought about reductions in food intake, at least partly by crossing the BBB and directly acting on the brain regions (131–133).

Central GLP-1 was a potent modulator of blood glucose utilization along with an anorectic effect (134). GLP-1 was found to be produced endogenously in the caudal nucleus of NTS and in the ventrolateral medulla while GLP-1R expression was found in the PVN, ARC, and DMH of hypothalamus as well as in the NTS, AP, and PBN of brainstem (135) revealing the functional sites of GLP-1. In the hypothalamus, i.c.v.-GLP-1 induced c-Fos expression in the PVN (134), while intra-PVN GLP-1 produced satiety (136, 137). PVN GLP-1R activation caused anorexia (138) and selective blockade of PVN GLP-1R resulted in hyperphagia and weight gain (139). GLP-1 (and/or GLP-1 analogs) primarily activated CRH and nesfatin-1 neurons (and to a lesser extent OXT neurons) in the PVN (139). Additionally, HPA axis and catecholamine release was stimulated by central GLP-1 and GLP-1R in the PVN (140). Although it has been reported that central GLP-1 induced an acute dose-dependent anorectic effect and this effect was abolished after the damage of ARC (141), there were inconsistent findings supporting and negating the anorectic actions of GLP-1 injected into the ARC (142). Thus, it warrants further research to clarify the discrepancy. Intra-LHGLP-1 induced hypophagia that was short latency and short lasting, while liraglutide reduced food intake for 24 h after its injection into LH (143). Local injections of GLP-1 into the VMH or the DMH also resulted in short latency, short-lasting (1–2 h) hypophagia while there was no effect for liraglutide in these areas (144). In spite of multiple action sites of GLP-1 in the hypothalamus, which indicated its indispensable role in regulating appetite *via* neuroendocrine pathways, some negative side effects of centrally administrated GLP-1 could not be easily ignored. For example, i.c.v. GLP-1 induced CTA effect (145) and central GLP-1R participated in LiCl-induced CTA effect as GLP-1R antagonist abolished the response. Bilateral lesions of CeA reversed the aversive behavior but not anorectic effect while bilateral lesions in the PVN was *vice versa* indicating that PVN was important for inhibition of food intake and CeA for aversive effect of GLP-1 (137, 146). However, it is still in need of more research to increase the knowledge of regional-specific effects of GLP-1 and expand the positive effect on clinical applications.

Caudal brain stem processing has been found to be sufficient for carrying out the various effects of peripheral and hindbrain GLP-1R activation (147). While all three nuclei of the DVC of the hindbrain (NTS, AP, DMV) expressed the GLP-1R, it was NTS GLP-1R expressing cells that were physiologically and pharmacologically more significant in modulating food intake behavior (127). It was unclear if NTS-PPG neurons expressed GLP-1R, while there was a report showing that they did not respond to GLP-1R ligands (148). Hindbrain GLP-1R activation has been found to suppress food intake *via* PKA-mediated suppression of AMPK activity and simultaneous activation of p44/42 MAPK in NTS neurons (149). In the same report, Ex-4, a GLP-1R agonist, activation of the same signaling cascades in GT1-7 neuronal cells and in NTS lysates supports the view that these pathways occur in GLP-1R-expressing neurons. As the mechanisms underlying the anorectic function of NTS GLP-1R activation are being studied, there were complimentary reports suggesting that intra-mNTSGLP-1 analogs mediated nausea responses and hence the anorexic effect was induced at least in part by reducing motivation to feed or by eliciting pica response and illness like

behavior (150). GLP-1 producing neurons in the NTS projected to lateral and medial PBN in rodents (151, 152) and consistently, GLP-1Rs have been found to be expressed in the lateral PBN (lPBN) (153). Activation of GLP-1R by microinjection of Ex-4, in lPBN resulted in reduced food intake and body weight along with reduced ingestion of palatable food and motivation to work for it (152, 154). Taken all together, these studies have provided another aspect of appetite control by GLP-1through brainstem pathways.

Glucagon-like peptide-1 neurons in the hindbrain projected directly to the NAc and VTA of the mesolimbic reward system (155), and both these areas expressed GLP-1R (153). GLP-1R activation in these nuclei led to reduction in reward-motivated behaviors for palatable food (156), alcohol (157), and cocaine (158). Injection of Ex-4 into the VTA or into the NAc core or shell reduced body weight, intake of regular chow, and intake of high-fat diet while not inducing pica response or CTA (155, 156, 159). Furthermore, injection of GLP-1R antagonist into mesolimbic nuclei increased the intake of regular chow, high-fat diet, liquid sucrose meal, and alcohol suggesting a role of endogenous GLP-1 in these nuclei (152, 157, 159). Interestingly, blockade of GLP-1Rs in the NAc-core increased high-fat diet intake, whereas blockade of shell GLP-1Rs did not show the effect (152), indicating a difference in actions of endogenous GLP-1 in the core and shell of NAc. Furthermore, it has been recently found that GLP-1R activation in NAc core suppresses food intake by increasing glutamatergic AMPA/Kainate signaling (160). *Ex vivo* electrophysiological studies revealed that GLP-1R activation in NAc core activates GABAergic medium spiny neurons predominantly by a presynaptic, AMPA/kainate-mediated glutamatergic mechanism and does not involve dopamine signaling. Consistently, *in vivo* intra-NAc core GLP-1R activation-induced food intake suppression and body weight reduction were attenuated by blockade of AMPA/Kainate receptors but not NMDA receptors (160).

Recently, central GLP-1 has been found to increase dopamine signaling in amygdala (161). Central activation of GLP-1R increased tyrosine hydroxylase, rate limiting enzyme for dopamine synthesis in the VTA (161, 162), which might contribute to increase in somatodendritic release of dopamine in the VTA (163). VTA dopaminergic neurons also projected to the amygdala where central Ex-4 acutely upregulated dopamine turnover and amygdale-dopamine receptor activation-induced satiety (161). Hence, novel VTA-amygdala dopamine circuit is being proposed as one of the underlying circuits involved in the anorectic effect of GLP-1 (161). In addition, a recent study has revealed that GLP-1R activation in vHP robustly reduced feeding, high-fat palatable food in particular while antagonizing GLP-1R in vHP region increased feeding (164), suggesting vHP as another target of GLP-1 controlling appetite. Surprisingly, the knockdown of GLP-1R did not alter the food intake in mice (165) and consistently body weight gain induced by high-fat feeding was also unaltered in GLP-1R knockout mice (166). These indicated that there were other compensatory factors that could make a good combination with GLP-1. Indeed for instance, GLP-1 has been identified to be one of the downstream mediators of leptin and leptin has been suggested to enhance the central GLP-1 activity (167).

The use of Ex-4 and liraglutide, two GLP-1R agonists, for treatment of diabetes produced small yet significant reductions in body weight (168–171). GLP-1 also induced weight loss after bariatric surgery (172). Dual agonism of GLP-1R/GCGR (173) and very recently triagonism with GLP-1R/GIPR/GCGR (174) have been found to have potent effects to reverse obesity in rodents. A GLP-1R agonist has also been found to have therapeutic effects on alcohol use disorders in mice (175). With these increasing therapeutic advantages, the hunt for GLP-1R agonists and combinatorial integrative effect of GLP-1 with other hormones is increasing to obtain maximum efficiency in therapeutic treatments.

GROWTH HORMONE–RELEASING HORMONE

Named after its stimulating effect of pituitary growth hormone, GHRH or growth hormone-releasing factor is a 44-amino acid hypothalamic peptide (176–179). Predominantly in the hypothalamic region, GHRH containing cell bodies are located primarily in the ARC along with the DMH and VMH (180, 181). It has been shown that i.p. injection of GHRH failed to alter food intake in rodents (182) while peripheral injection of chicken GHRH inhibited feeding behavior in chicks (183), suggesting the complexity of the peripheral GHRH in feeding regulation. The i.c.v. injection of GHRH stimulated food intake at doses that did not stimulate GH release (182, 184) while the i.c.v. injection of GHRH suppressed feeding behavior in rats at a higher dose (185), suggesting that not only peripheral and central GHRH could have different effects on appetite control but different doses of GHRH might also cause different feeding behavior.

As we reviewed before (28), the cell bodies of ARC-GHRH neurons had varied projection sites with their nerve terminals being at perifornical region, lateral preoptic area and SCN/medialpreoptic area (SCN/MPOA) (186) and mapping studies have identified SCN/MPOA as the central site of action for GHRH to induce orexigenic effects (184, 187). Orexigenic effect of GHRH has been found to be photoperiod sensitive which was consistent with the role of SCN/MPOA in controlling circadian rhythms (188). The i.c.v.-GHRH stimulated a dose-dependent increase and suppression of feeding during the light and dark phases of photocycle, respectively, in rats (189), and intra-SCN/MPOA-injected GHRH antiserum reduced dark onset feeding (190) with selective suppression of protein intake (191). Collectively, these reports suggested a role of endogenous GHRH in the regulation of circadian feeding rhythm. GHRH action was found to stimulate protein intake with no effects on carbohydrate intake (192). The observation that intra-ARC injected morphine stimulated protein intake was reversed by intra-SCN/MPOA pretreatments with GHRH antiserum (193), further confirming the significant role of GHRH in the regulation of protein intake. Additionally, Intra-PVN injection of opiate antagonist inhibited the effect of i.c.v.- (194) and SCN/MPOA-injected GHRH (192) suggesting a role of opiates in GHRH-induced feeding behavior. Transcriptional profiling of hypothalamic glucose-sensing neurons along with electrophysiological studies has revealed that hypoglycemia activated GHRH neurons (195). The electrical

patterns that controlled the hypothalamic GHRH neurons have remained elusive while recently somatostatin has been found to bring about irregular suppression of the neuronal activity of GHRH neurons (196). In summary, it is the important role in GH secretion along with its involvement in nutritional and circadian feeding behavior that has made GHRH vital in integration and coordination of diverse aspects related to metabolism, growth and nutrient regulation (190, 197).

OTHER PEPTIDES

Other peptides from SCT family also shared a certain degree of functional similarities on the feeding behavior. Derived from the PPG, GLP-2 was known to have anorectic effect (198, 199). Central but not peripheral injection of GLP-2 reduced food intake and stimulated c-Fos expression in the ARC, PVN, DMH, VMH, and LH (200). The anorectic effect by central GLP-2R

TABLE 1 | Secretin (SCT), pituitary adenylate cyclase-activating peptide (PACAP), and glucagon (GCG) family of peptides in neural regulation of appetite.

Peptide	Primary physiological function	Effect on feeding behavior	Central site of action	Central mode of action
SCT	Stimulates bicarbonate release from pancreas and water homeostasis regulation	Anorectic	• Hypothalamus: ARC and PVN • Brainstem: NTS • Amygdala: CeA	• Activates oxytoxinergic pathway from the dpPVN to the NTS • Stimulates POMC neurons in ARC • Activates melanocortin system in PVN • Modulates spontaneous firing of CeA neurons
PACAP	Strong modulator of hypothalamic magnocellular neurons	Anorectic	• Hypothalamus: ARC, PVN, NAc and ventromedial hypothalamus (VMH)	• Stimulates POMC neurons of ARC and activates melanocortin system through MC4R in PVN • Increases ARC-POMC expression by intra-VMH administration and acts through glutamatergic signaling *via* NMDA receptors in VMH • Acts through excitatory PACAPergic afferents originating within the PVN to AgRP/NPY neurons of ARC activating NPY neurons (possibly orexigenic effect) • In NAc, mimics actions of GABA and reduces hedonic but not homeostatic feeding • In VMH, mimics AMPA and reduces homeostatic but not hedonic feeding
Vasoactive intestinal peptide	Vasodilation and hypotesion	Not clear	• Hypothalamus: PVN and SCN	• Stimulates HPA axis and melanocortin system
GCG	Induces glucose release	Anorectic	• Hypothalamus: ARC, LH • Brainstem: DVC	• Acts through GCGR-dependent PKA–Erk1/2–K_{ATP}signaling cascade in DVC • Stimulates HPA axis • Acts through PKA/AMPK/CaMKKβ-dependent pathways and reduces AgRP in ARC
GCG-like peptide-1 (GLP-1)	Incretin	Anorectic	• Hypothalamus: PVN, ARC, LH, DMH, and VMH • Brainstem: mainly NTS, lPBN as well as AP, DMV • Mesolimbic reward system: VTA and NAc • Amygdala: CeA • Hippocampus: vHP	• Activates NTS via vagal to NTS glutamatergic signaling • Stimulates HPA axis in PVN • Causes CTA in CeA • Reduces motivation to feed or elicits pica response in NTS • Acts through NTS to lPBN circuit to activate lPBN leading to reduced ingestion of palatable food and motivation to work for it in lPBN • Reduces reward-motivated intake of palatable food, alcohol and cocaine in VTA and NAc (no pica response or CTA) • Acts in NAc core neurons through GLP-1R activation *via* presynaptic, AMPA/kainate-mediated glutamatergic mechanism • Acts through VTA to amygdala dopamine circuit • Reduces feeding and high-fat palatable food in vHP
GCG-like peptide-2 (GLP-2)	Intestinal function	Anorectic	• Hypothalamus: ARC	• Stimulates POMC and activates melanocortin pathway
Growth hormone–releasing hormone	Growth hormone release	Orexigenic	• Hypothalamus: ARC, DMH, SCN/MPOA	• Participates SCN/MPOA mediated circadian feeding stimulation • Stimulates protein intake • Induces opiate-involved feeding behavior
Peptide histidine isoleucine	Prolactin regulation	Anorectic	• Hypothalamus: PVN • Amygdala: CeA	• Acts through oxytocin/vasopressin system in PVN and activates PVN OT neurons by intra-CeA infusion

ARC, arcuate nucleus; PVN, paraventricular nucleus; NTS, nucleus tractus solitarius; CeA, central amygdala; POMC, pro-opiomelanocortin; dpPVN, dorsal division of the parvocellular PVN; VMH, ventromedial hypothalamus; MC4R, melanocortin-4 receptor; AgRP, agouti gene-related protein; NPY, neuropeptide Y; SCN, suprachiasmatic nucleus; HPA axis, hypothalamic–pituitary–adrenal axis; LH, lateral hypothalamus; DVC, dorsal vagal complex; AMPK, AMP-activated protein kinase; CaMKKβ, Ca^{2+}-calmodulin-dependent protein kinase kinase β; DMH, dorsomedial hypothalamus; lPBN, lateral parabrachial nucleus; AP, area postrema; DMV, dorsal motor nucleus of the vagus; VTA, ventral tegmental area; NAc, nucleus accumbens; vHP, ventral hippocampus; CTA, conditioned taste aversion; MPOA, medialpreoptic area.

activation was reversed in MC4R knockout mice suggesting the involvement of melanocortin system and specific deletion of GLP-2R in the ARC POMC neurons resulted in increased intake (199).

Unlike its incretin counterpart GLP-1, GIP alleviated obesity through increased energy expenditure but did not affect food intake (201–203). However, recent evidences have shown the modulation of hypothalamic gene expression by i.c.v.-GIP (204). The i.c.v. administration and intra-PVN or -CeA injection of PHI decreased food consumption in overnight-deprived rats (205). These peptides' roles in appetite regulation has not been well understood, hence further research is warranted for clarity in understanding the role of these peptides in neuromodulation of feeding behavior.

CONCLUSION

This review has focused on central roles of the SCT, PACAP and GCG family of peptides in regulating food intake behavior. With members of the family such as GLP-1 exhibiting significant therapeutic advantage, SCT family of peptides have been considered as an important group of hormones in neural appetite modulation. With the exception of GHRH which is an orexigenic peptide, members of the SCT family of peptides mostly exhibit anorectic roles with GLP-1 and PACAP being most-studied among all the peptides. Central sites of action, effects on specific aspect of feeding behavior control, and the central circuitry recruited to carry out these effects vary widely among the SCT family of peptides and they have been summarized in **Table 1**. More information on the site-specific endogenous actions of these peptides on food intake modulation and the neural pathways involved along with the mechanistic insights is still unclear. Of the neural circuitry involved, melanocortin system (206), stimulated mainly through activation of POMC neurons, is the most common neural pathway through which these peptides, at least partially, exert their anorectic effect. Yet, the combinatorial effect of these peptides, along with the mechanisms and the neural pathways involved in such a scenario, is important to understand if they have synergistic additive effects on food intake reduction. Importance of this research is clearly evident as a novel monomeric peptide triagonist acting on GLP-1, GIP, and GCG receptors (174) has been found to be the most effective among existing pharmacological agonists/strategies in reversing obesity in mice (207, 208).

Recent research on the neurobiology of food intake behavior has taken a leap with the advances in neuro-technology for mapping, manipulating, and monitoring molecularly defined cell types that are exponentially expanding our understanding into appetite modulating neural circuits (209). Although there is a huge gap between our current knowledge of the SCT family peptides and valuable clinical implications of these peptides for disease treatment, significance on the clinical applications of these peptides has already been evident by the significant therapeutic advantage by GLP-1R agonists in human (210, 211). In overweight or obese individuals, GLP-1R agonists have been shown to produce clinically relevant reductions in weight, body mass index, and waist circumference (212, 213). Liraglutide 3.0 mg day^{-1} has been approved for weight management in the US on December 23, 2014 (214), and in the EU on March 23, 2015 (215). Novel combinatorial hormone therapies are being researched at the pre-clinical stage for obesity treatment (216), wherein the novel unimolecular GLP-1R/GIPR/GCGR triagonist at a very low dose of 2 nmol/kg decreases cumulative food intake and reduces the body weight of HFD-mice by 18.3% and is more effective than any other available therapy (174). While the application prospect of SCT family peptides is exciting, some aversive consequences brought by injection of these peptides should not be easily ignored. Further regional and functional specific research as well as the understanding on combinational effects of SCT family peptides needs to be greatly enhanced. Future studies with the new tools that give neurobiologists opportunity to rigorously examine neural circuits in modulating feeding behavior should provide insights and novel therapeutic approaches to combat pathophysiological conditions related to appetite disorders and obesity.

AUTHOR CONTRIBUTIONS

RS contributed to manuscript preparation and manuscript definition of intellectual content. LW also contributed to manuscript preparation and followed by manuscript editing and revision. These two authors contributed equally to this work and should be listed as co-authors. BC, as the corresponding author, approved the final version of the manuscript.

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Corrigendum: Central Control of Feeding Behavior by the Secretin, PACAP, and Glucagon Family of Peptides

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In the published article, there was an error regarding the contributions. Revathi Sekar and Lei Wang should be listed as equal contributors.

The authors apologize for this error and state that this does not change the scientific conclusions of the article in any way.

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Pituitary Adenylate-Cyclase Activating Polypeptide Regulates Hunger- and Palatability-Induced Binge Eating

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While pituitary adenylate cyclase activating polypeptide (PACAP) signaling in the hypothalamic ventromedial nuclei (VMN) has been shown to regulate feeding, a challenge in unmasking a role for this peptide in obesity is that excess feeding can involve numerous mechanisms including homeostatic (hunger) and hedonic-related (palatability) drives. In these studies, we first isolated distinct feeding drives by developing a novel model of binge behavior in which homeostatic-driven feeding was temporally separated from feeding driven by food palatability. We found that stimulation of the VMN, achieved by local microinjections of AMPA, decreased standard chow consumption in food-restricted rats (e.g., homeostatic feeding); surprisingly, this manipulation failed to alter palatable food consumption in satiated rats (e.g., hedonic feeding). In contrast, inhibition of the nucleus accumbens (NAc), through local microinjections of GABA receptor agonists baclofen and muscimol, decreased hedonic feeding without altering homeostatic feeding. PACAP microinjections produced the site-specific changes in synaptic transmission needed to decrease feeding via VMN or NAc circuitry. PACAP into the NAc mimicked the actions of GABA agonists by reducing hedonic feeding without altering homeostatic feeding. In contrast, PACAP into the VMN mimicked the actions of AMPA by decreasing homeostatic feeding without affecting hedonic feeding. Slice electrophysiology recordings verified PACAP excitation of VMN neurons and inhibition of NAc neurons. These data suggest that the VMN and NAc regulate distinct circuits giving rise to unique feeding drives, but that both can be regulated by the neuropeptide PACAP to potentially curb excessive eating stemming from either drive.

Keywords: hypothalamus, accumbens, obesity, hedonic, homeostatic

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INTRODUCTION

A fundamental barrier in treating obesity is the challenge associated with isolating individual feeding drives. Understanding these could lead to the identification and development of potential new treatments based on the mechanisms underlying each unique form of caloric intake. Discrete forms of obesity, binge eating, or other eating disorders may differentially stem from pathological

changes in circuitry underlying feeding typically driven by homeostatic needs (e.g., hunger-driven feeding) or hedonic motivations for highly palatable foods (e.g., palatable-driven feeding) (Lowe and Levine, 2005; Lowe and Butryn, 2007). However, the degree to which potential anorexigenic substances can suppress distinct feeding drives has been difficult to determine because feeding in many preclinical models likely involves multiple feeding drives. This is particularly problematic with paradigms comparing the consumption of standard chow and highly-palatable food in combination with food deprivation. For example, in the limited-access binge model, subjects have *ad lib* access to standard chow in conjunction with brief access to a highly palatable food, which promotes binge eating (Corwin, 2004; Corwin and Hajnal, 2005; Czyzyk et al., 2010). While *ad lib* access to standard chow should limit hunger-driven feeding, animals show self-induced food deprivation with reduced consumption of the devalued standard chow (Corwin and Buda-Levin, 2004). The potential that a confluence of homeostatic and hedonic drives exists in this model is evident by the observation that daily caloric intake and body weight remain stable in this paradigm despite the addition of the high-caloric food (Bake et al., 2014). In the current studies, we modified this approach by restricting access to both diets in order to promote conditions whereby hunger-driven consumption of standard chow resulted in satiety prior to providing subjects access to highly palatable food. By doing this, homeostatic and hedonic drives are more clearly separated, which enabled us to examine the cellular and molecular components of each feeding drive.

Using this new model of binge eating, we first sought to characterize the cellular or regional contributions to hunger- and palatable-driven feeding. Initially, we examined the impact of VMN activation on feeding primarily driven by homeostatic or hedonic feeding drives. Although the VMN have historically been viewed as satiety centers regulating feeding behavior (King, 2006), it is unknown if the VMN-satiety signal also gates feeding stemming from other distinct drives (e.g., palatable-driven feeding). We then targeted subregions of the nucleus accumbens (NAc), which has been principally linked to hedonic drives; the degree to which the NAc regulates other motivations to eat including homeostatic-based feeding is less well studied (Baldo and Kelley, 2007; Johnson and Kenny, 2010; Baldo et al., 2013). Each of these experiments is important because human obesity can stem from either abnormal homeostatic feeding or, over consumption of highly palatable foods even in the absence of homeostatic need (Boggiano, 2016). Hence, these and future studies have the potential to identify drive-specific circuitry, a discovery that could help narrow attempts to outline the neural basis for unique forms of obesity.

An additional objective was to examine the potential for a single anorexigenic substance to modify the activity of both NAc- and VMN-related circuitry through either hunger- or palatable-feeding drives. Recently, we found that intra-VMN administration of pituitary adenylate cyclase-activating polypeptide (PACAP) markedly suppressed feeding and reduced body weight even in fasted animals via the PAC1R receptor subtype (Resch et al., 2011, 2013). Of the three PACAP receptors, PAC1R is primarily involved in the hypophagic properties of intra-VMN PACAP whereas the contribution of VPAC1 and VPAC2 are not (Resch et al., 2013). While the VMN express an abundant amount of PACAP mRNA, retrograde tracing has revealed numerous extra-hypothalamic efferents including PACAP containing projections from the medial amygdala and lateral parabrachial (Resch et al., 2013). In the NAc, similar retrograde studies show different PACAP containing efferent projections to the NAc such as the medial prefrontal cortex (unpublished data). PACAP is a highly conserved neuropeptide that is often expressed in glutamatergic neurons and has been primarily implicated in neurohormone signaling, learning and memory, and neurodegenerative responses (Pellegri et al., 1998; Zhou et al., 2002). Thus, it represents an interesting molecular candidate because prior studies have shown that this neuropeptide is capable of activating and inhibiting ionotropic glutamate receptors (Macdonald et al., 2005; Toda and Huganir, 2015). For example, PACAP's anorexic actions in VMN likely augments glutamate signaling by potentiating NMDA receptors (Resch et al., 2014b). Hence, the capacity for PACAP to produce bidirectional changes in excitatory signaling may position this poorly understood anorexigenic peptide to inhibit NAc-related circuitry and suppress palatable-driven feeding while stimulating VMN-related circuitry to restrict hunger-driven feeding.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (Harlan; Indianapolis, IN) weighing 350–400 g, were housed individually in either a BioDAQ feeding system, a computer automated data acquisition system that records food intake measurements using an algorithmic load cell technology (Research Diets, New Brunswick, NJ) or standard hanging wire cages under a 12:12 light/dark cycle. Feeding was measured via the BioDAQ system or by weighing food bins before and after experimental sessions (including spilled food). Body weights were collected daily. All animal procedures were approved by the Marquette University Institutional Animal Care and Use Committee.

Diets

We used Harlan standard chow (SC; #8604; 32% protein, 54% carbohydrate, 14% fat; 3.0 kcal/g) or a palatable western diet (WD; #D12079B; Research Diets; New Brunswick, NJ; 17% protein, 43% carbohydrate, 41% fat; 4.7 kcal/g). When indicated, standard chow was flavored with either vanilla, almond (0.05% pure vanilla extract, 0.05% imitation almond extract; The J.R. Watkins Co; Winona, MN) or vehicle (water).

Cannulation Surgery and Microinjections

Surgery

Animals were anesthetized with ketamine/xylazine/acepromazine (77:1.5:1.5 mg/ml/kg; i.p.). Twenty-six-gauge bilateral guide cannulae (Plastics One; Roanoke VA) were stereotaxically placed 2–3 mm above the ventromedial nuclei (VMN; anterior/posterior, −2.5 mm from bregma; medial/lateral, ± 0.6 mm from midline; dorsal/ventral, −6.2 mm from surface of the skull) or the nucleus accumbens (NAc;

anterior/posterior, +1.6 mm from bregma; medial/lateral, +2.2 from midline; dorsal/ventral, −4.8 mm from surface of the skull) and secured to the surface of the skull (Paxinos and Watson, 2007). Afterwards, brains were collected, immediately frozen and embedded in OCT for analysis of cannula placement. Thirty micrometers thick sections were Nissl stained and only those with correct placements were included in the studies (**Figure 5**).

Microinjections

Pituitary adenylate cyclase activating polypeptide (PACAP; 50 pmol/0.25 μl/side; California Peptide Research, Napa, CA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA; 74.5 ng/side; Tocris Bioscience, Minneapolis, MN); baclofen+muscimol (106.8 ng/5.7 ng/side; Tocris Bioscience, Minneapolis, MN) or saline (vehicle) were microinjected into the VMN (0.25 μl/side) or NAc (0.5 μl/side) over a 2 min period (using a syringe pump) in gently restrained awake animals followed by an additional minute to prevent backflow.

Restricted Feeding

At the onset of dark, animals ($n = 12$ total) were entrained (1 week/regimen) to various restricted feeding durations (2, 3, or 4 h/day in BioDAQ) using only SC. During the remaining 22, 21, or 20 h animals did not have access to food. Body weights were recorded daily. In addition to the restricted feeding groups, animals ($n = 6$/group) fed SC and WD *ad libitum* served as control groups for feeding and body weight measurements.

Two-Meal Model (M1-M2)

Rats ($n = 12$/group) were entrained to consume their daily SC intake in a 2-h period after the onset of the dark phase (Meal 1; M1). After establishing consistent feeding patterns and weight gain (40–50 kcal/2 h; body weight gain 2–3 g/day), animals were offered a short 15 min meal (Meal 2; M2) of either SC or WD ($n = 6$/group) ∼30 min following M1 for 7 days before experimentation. Food intake and body weight measurements were recorded in an additional group ($n = 6$/group) of rats that were *ad lib* fed either SC or WD as additional control groups.

In separate studies, animals were entrained to the two-meal model (M1-M2) for 5 days before undergoing VMN or NAc cannulation surgery. VMN microinjections of vehicle ($n = 9$–10/group), PACAP ($n = 7$/group), AMPA ($n = 6$/group), or baclofen+muscimol ($n = 3$/group) were separately administered ∼30 min prior to either M1 or M2. Similarly, NAc microinjections of vehicle ($n = 9$–10/group), PACAP ($n = 9$/group), baclofen+muscimol ($n = 9$/group), or AMPA ($n = 3$/group) were administered ∼30 min prior to M1 or M2.

Slice Electrophysiology

Rats were anesthetized by isoflurane inhalation and decapitated. Coronal slices (250 μm; $n = 6$–7 slices/brain region) containing the VMN and the NAc were cut using a vibrating slicer (VT1000S, Leica) at 4°C with a sucrose-based solution containing the following: 220 mM sucrose, 25 mM $NaHCO_3$, 2.5 mM KCl, 1.25 mM NaH_2PO_4, 0.5 mM $CaCl_2$, 7 mM $MgSO_4$, and 10 mM glucose. The slices were recovered in a sucrose-NaCl-based solution containing the following: 68 mM sucrose, 78 mM NaCl, 25 mM $NaHCO_3$, 2.5 mM KCl, 1.25 mM NaH_2PO_4, 2 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM glucose for 30 min at room temperature. The slices were then transferred to artificial cerebrospinal fluid (ACSF) containing the following: 125 mM NaCl, 2.5 mM KCl, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, 1.25 mM NaH_2PO_4, 25 mM $NaHCO_3$, and 10 mM glucose. The slices were maintained in ACSF for at least 1 h before electrophysiology recordings. All solutions are saturated with 95% O_2 and 5% CO_2.

Whole-cell or cell-attached recordings were made from the VMN and NAc using patch-clamp amplifier Multiclamp 700B under infrared-differential interference contrast (DIC) microscopy. The VMN is an egg-shaped region located in the mediobasal hypothalamus adjacent to the third ventricle, and the NAc is an area around the optic nerve about 200 μm from the edge of the anterior commissure. Data acquisition was performed using DigiData 1440A digitizer (Molecular Devices). Glass pipettes (4–6 MΩ) were filled with an internal solution containing (in mM): 140 potassium gluconate, 5 KCl, 10 HEPES, 2 $MgCl_2$, 0.2 EGTA, 2 MgATP, 0.3 Na_2GTP, and 10 Na_2-phosphocreatine (pH 7.4 with KOH). Signals were filtered at 2 kHz and sampled at 10 kHz. Spikes were driven by current injections from −60 to 300 pA. PACAP (100 nM) was added to the brain slices after the membrane potential was stable and a baseline measurement (control) of spontaneous activity and spike firing followed by application of PACAP to obtain within cell treatment effects. Glutamate receptor antagonist CNQX (10 μM) and $GABA_A$ receptor blocker picrotoxin (50 μM) were present throughout all physiological recordings. Recordings were performed at 32 ± 1°C using an automatic temperature controller (Warner Instrument).

Corticosterone (B) Radioimmunoassay

In a separate group of animals offered *ad lib* SC ($n = 12$) or restricted SC access (2 h/day at the onset of the dark cycle; $n = 12$) for 2 weeks, half were sacrificed at the onset of the dark cycle (prior to eating), and the remaining half sacrificed 2 h into the dark cycle or after the restrict feeding session. Plasma B was measured from trunk blood using a radioimmunoassay (MP Biomedicals, Santa Ana, CA).

Statistics

Data are presented as means ± standard error of the mean and analyzed by ANOVA (with repeated measures when appropriate) or Student's *t*-test. Fisher LSD analysis was used for *post-hoc* group comparisons using Sigma Plot 11 software (Systat Software Inc.; San Jose, CA). $p < 0.05$ = statistical significance.

RESULTS

Two-Meal Model (M1-M2) and Restricted Feeding

The two-meal model tested food consumption in satiated vs. hungry rats. After entrainment to a 2 h SC meal (M1), animals were offered a second meal (M2; 15 min) consisting of either SC or WD (**Figure 1A**). Animals consuming WD during M2 (SC-WD) consumed significantly more total daily calories than rats receiving SC (SC-SC) [**Figure 1A**; DIET $F_{(1,\ 183)} = 78.428$, $p <$

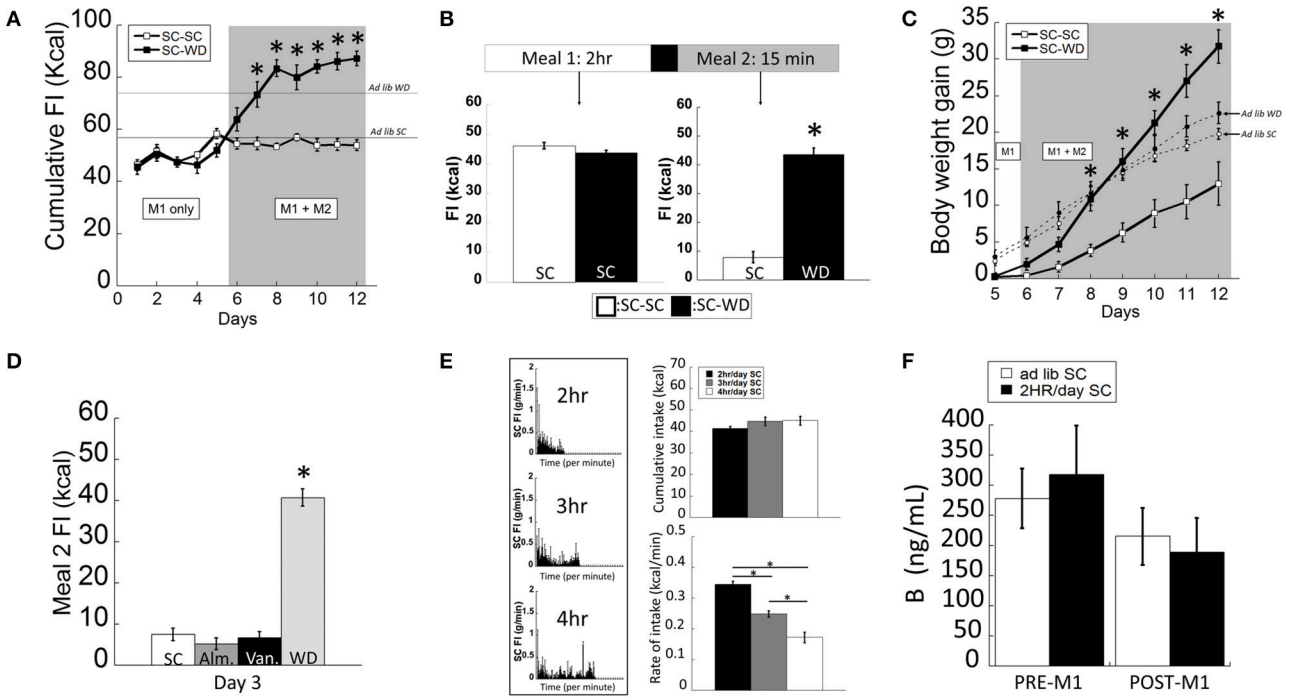


FIGURE 1 | The two-meal paradigm maintained animals on restricted SC (standard chow) intake (M1; 2 h/day) followed by access to a second meal (M2; 15 min/day) of SC (M1(SC)-M2(SC)) or a highly palatable diet (WD) shortly after M1, (M1(SC)-M2(WD)). (A) SC-WD displayed higher total food intake (FI) levels than SC-SC animals (and *ad lib* fed SC or WD animals). **(B)** By day 12, satiated rats offered WD (SC-WD) consumed more calories than animals offered SC (SC-SC). **(C)** Animals consuming WD (SC-WD) during M2 gained significantly more weight than animals offered SC (SC-SC) and gained more than *ad lib* fed SC and WD animals. **(D)** Food intake levels of SC flavored with almond (Alm.) or vanilla (Van.) during M2 did no differ compared to unflavored SC and were significantly lower than WD. **(E)** Left: SC food intake (FI) levels for 2, 3, or 4 h daily access; Upper-right: cumulative daily SC intake did not differ between 2, 3, or 4 h; Lower-right: 2 h feeding periods resulted in significantly faster feeding rates compared to 3 or 4 h access. **(F)** Plasma B (corticosterone) levels in *ad lib* and restrict fed animals before M1 and after the onset of dark and 2 h into the dark cycle (after M1). Data expressed as mean ± SEM. $^*p < 0.05$.

0.001; DIET × TIME $F_{(7, 183)} = 13.279$, $p < 0.001$]. Moreover, **Figure 1A** shows SC-WD fed animals consumed more calories than animals provided SC (58.6 ± 0.5 Kcal) and WD *ad libitum* (74.9 ± 1.4 Kcal) demonstrating that 2 h restricted feeding or SC-SC resulted in ∼25% reduction in daily caloric intake compared to a SC *ad lib* fed animals and that SC-WD animals consumed more calories than *ad lib* WD fed rats. By day 12, animals consumed as many calories from WD during the 15-min M2 as was consumed during the 2 h M1 (**Figure 1B**; $p < 0.001$) and as a result gained significantly more body weight than rats receiving SC for M2 [**Figure 1C**; DIET × TIME; $F_{(6, 150)} = 32.983$; $p < 0.001$]. Notably, this increase was significantly greater than even *ad lib* WD fed rats. A significant difference in body weight gain was evident by the third presentation of WD for M2 compared to the SC-SC group ($p < 0.001$; **Figure 1C**). In addition, SC-WD animals gained body weight faster than animals maintained on *ad lib* SC or WD (**Figure 1C**). To determine if the two-meal model was a product of novelty, we offered SC made novel by flavoring with either vanilla or almond extract (or control) during M2. There were no differences in M2 intake over 3 days access to flavored SC (**Figure 1D**; SC vs. almond $p = 0.714$; SC vs. vanilla $p = 0.902$; almond vs. vanilla $p = 0.807$), which contrasted the marked increase in palatable WD intake over the same time period (**Figure 1D**; WD vs. SC, almond or vanilla $p < 0.001$).

In order to develop a feeding paradigm that produced fully-satiated animals (i.e., minimal homeostatic-based feeding), we measured the total calories consumed during periods of 2–4 h of restricted feeding in 1 min bins (**Figure 1E**). Interestingly, total intake of SC did not differ in rats permitted 2, 3, or 4 h daily access [$F_{(2, 35)} = 0.781$; $p = 0.466$]. As would be predicted, animals provided 2 h access to SC ate at a faster rate (kcal/min) compared to rats allowed 3 or 4 h access [**Figure 1E**; $F_{(2, 35)} = 76.749$; $p < 0.001$]. Restricted feeding of SC at all durations was sufficient to produce a modest weight gain (data not shown). We and others have shown that animals entrained to 2 h of restricted feeding show normal circadian rhythmicity and low basal and normal peak levels of corticosterone (**Figure 1B**; <3 μg/dl and >20 μg/dl, respectively) suggesting that they were not chronically stressed (Krieger, 1980; Choi et al., 1998). In support, we confirmed that circulating B levels did not differ between *ad lib* and 2 h restrict fed animals (before and after their meal) during a period of peak B activity [**Figure 1F**; FEEDING REGIMEN $F_{(1, 23)} = 0.017$, $p = 0.915$; FEEDING REGIMEN × TIME $F_{(1, 23)} = 0.302$, $p = 0.589$]. Taken together, we chose the 2-h restricted

feeding to ensure a state of satiety in the shortest amount of time.

VMN Microinjections

Intra-VMN PACAP (**Figure 5A** for anatomy) administered prior to M1 produced a significant reduction in SC consumption during M1 compared to non-injected (No INJ) and vehicle injected animals (**Figure 2A**; $p < 0.001$ for both). Intra-VMN AMPA administration also significantly suppressed consumption of SC during M1 (**Figure 2A**; AMPA vs. No INJ or vehicle, $p < 0.001$; baclofen+muscimol vs. No INJ, $p = 0.148$; vs. vehicle, $p = 0.282$) indicating that PACAP and AMPA produced similar behavioral actions in the VMN. Surprisingly, there were no differences in the calories consumed during M2 of either SC or WD when PACAP was administered prior to M1 (**Figure 2A**; PACAP vs. No INJ, $p = 0.624$; vs. vehicle, $p = 0.713$) or just prior to M2 (**Figure 2B**; PACAP vs. No INJ, $p = 0.613$; vs. vehicle, $p = 0.868$). Baclofen+muscimol injections into the VMN did not alter feeding during either M1 or M2 suggesting that PACAP actions in the VMN are primarily excitatory. Every cannula placement into the VMN was confirmed at the conclusion of the study resulting in a 90% accuracy rate.

NAc Microinjections

NAc injections of PACAP (**Figure 5B** for anatomy), AMPA, or baclofen+muscimol (prior to M1) had no effect on feeding behavior during M1 [**Figure 3A**; $F_{(4, 80)} = 0.463$; $p = 0.763$]. However, intra-NAc injections of PACAP and baclofen+muscimol significantly reduced WD intake during the subsequent 15 min M2 compared to vehicle and non-injected controls (**Figure 3A**; PACAP vs. No INJ, $p < 0.001$; vs. vehicle, $p < 0.002$; baclofen+muscimol vs. No INJ or vehicle, $p < 0.001$). Similarly, PACAP and baclofen+muscimol administered just prior to M2 also suppressed WD intake (**Figure 3B**; PACAP vs.

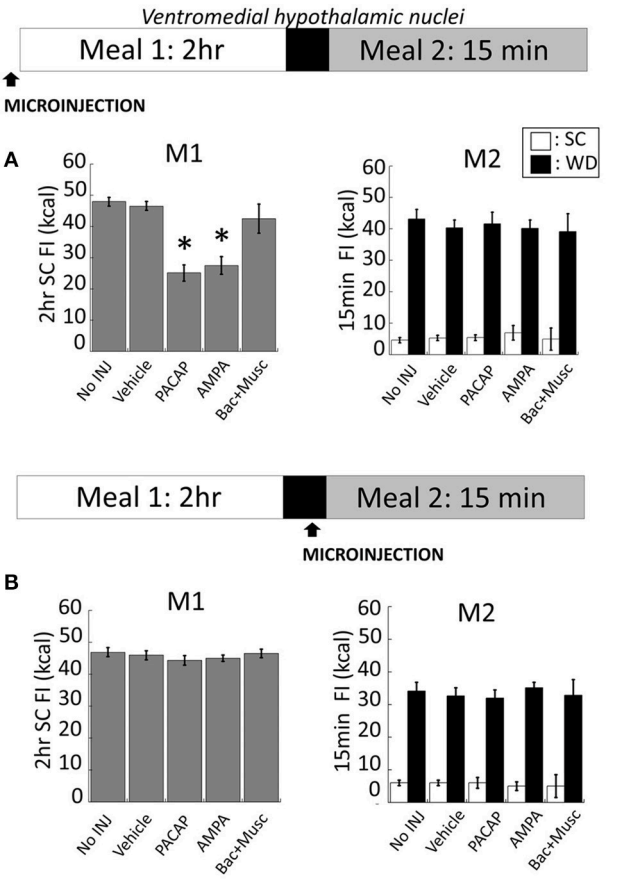


FIGURE 2 | PACAP or AMPA microinjections into the hypothalamic ventromedial nuclei (VMN) suppressed hunger-induced feeding (meal 1; M1) without affecting palatable food consumption (meal 2; M2). (A) Intra-VMN PACAP or AMPA administered prior to M1 significantly suppressed hunger-induced feeding of standard chow (SC) compared to baclofen+muscimol, vehicle (saline), and non-injected controls, whereas there were no significant treatment difference in the consumption of SC or palatable diet (WD) during M2. **(B)** No changes in SC or WD consumption following microinjections administered just prior to M2. Data expressed as mean ± SEM. *$p < 0.05$.

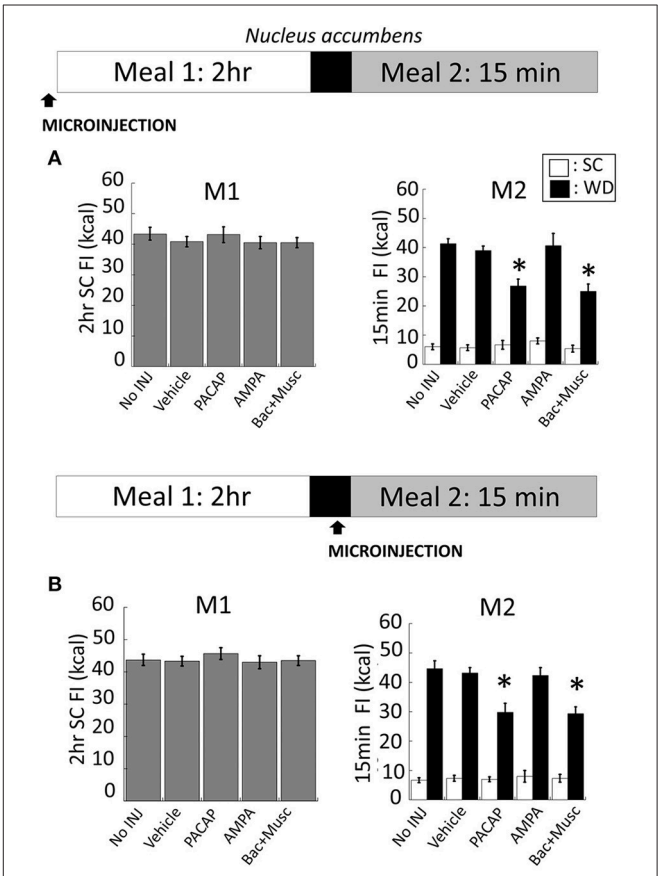


FIGURE 3 | PACAP and baclofen+muscimol microinjections into the nucleus accumbens (NAc) suppressed palatable food consumption (meal 2; M2) without effecting standard chow (SC) intake (meal 1; M1). (A) No changes to SC intake following microinjections administered prior to M1 compared to controls. However, intra-NAc PACAP or baclofen+muscimol significantly reduced WD intake compared to AMPA, vehicle and non-injected controls during M2. **(B)** Similarly, WD intake was reduced following PACAP and baclofen+muscimol administration into the NAc just prior to M2. Data expressed as mean ± SEM. *$p < 0.05$.

No INJ or vehicle, $p < 0.001$; baclofen+muscimol vs. No INJ or vehicle, $p < 0.001$). By contrast, AMPA administration into the NAc prior to either M1 or M2 had no effect on food consumption suggesting that PACAP actions in the NAc were inhibitory. Every cannula placement into the NAc was confirmed at the conclusion of the study resulting in a 90% accuracy rate.

Slice Electrophysiology

We determined whether PACAP affected action potential firing rates in VMN and NAc slices. All recordings were made in the presence of the glutamate receptor antagonist CNQX (10 μm) and the $GABA_A$ receptor blocker picrotoxin (50 μm) to block excitatory and inhibitory synaptic transmission. Cell-attached patch clamp recordings were made on VMN neurons, which displayed spontaneous action potential firing. Bath application of PACAP (100 nM) significantly increased the frequency of spontaneous action potential firing in VMN neurons [**Figure 4A**, $t_{(6)} = -4.062$, $n = 7$, $p < 0.004$, Paired *t*-test]. We next examined whether PACAP also affected action potential firing in the NAc. Since medium spiny neurons (MSNs) in NAc slices do not fire spontaneous action potentials at resting membrane potential (∼−80 mV), we made whole-cell current-clamp recordings and evoked action potential firing by injecting depolarizing current steps. Bath application of PACAP (100 nM) significantly decreased the number of spikes in responses to depolarizing current injections [**Figure 4B**, 120 pA, $t_{(5)} = 4.828$, $p < 0.005$; 180 pA, $t_{(5)} = 4.620$, $p < 0.006$; 240 pA, $t_{(5)} = 11.364$, $p < 0.001$; 300 pA, $t_{(5)} = 5.937$, $p < 0.002$, $n = 6$]. These effects were independent of excitatory and inhibitory synaptic inputs as these studies were conducted in the presence of both CNQX and picrotoxin. Thus, PACAP increased spontaneous action potential firing in the VMN whereas, it decreased evoked action potential firing in the NAc.

DISCUSSION

Obesity can stem from excessive or binge-like consumption of food generated by different homeostatic and hedonic-related drives, each of which may involve distinct circuitry in the brain. This study extends earlier findings revealing that PACAP administration into the hypothalamic VMN markedly suppressed feeding behavior (Resch et al., 2011, 2013) by determining the capacity of this novel anorexigenic peptide to regulate distinct forms of eating stemming from homeostatic and hedonic feeding drives. To do this, we developed a novel binge-eating paradigm (rapid consumption of a high volume of food within a short time period) that would better isolate distinct feeding drives. Using this paradigm, it is likely that VMN activation suppressed the consumption of standard chow (SC) in restrict-fed rats without altering palatable food intake in a satiated rat. Inhibition of the NAc produced the opposite outcome in that consumption of palatable food in a satiated rat was reduced, while SC intake was not altered. Interestingly, PACAP signaling in the VMN and NAc produced the precise changes in synaptic transmission needed to suppress each form of eating. Collectively, these data suggest that distinct feeding drives may involve at least partially non-overlapping circuitry, and that targeting PACAP signaling may be an effective strategy at reducing both homeostatic and hedonic-related feeding.

Isolation of Homeostatic- and Hedonic-Related Feeding Drives

A challenge in the study of the neurobiology of obesity is that multiple feeding drives are likely *simultaneously* activated under most experimental conditions thereby, obfuscating efforts to identify the cellular or molecular basis of discrete feeding drives (Lowe and Levine, 2005; Lowe and Butryn, 2007). Many rodent models assess consumption of a highly-palatable food combined with some degree of food deprivation, thereby demonstrating the presence of multiple feeding drives even in those designed to separate distinct drives. For example, in the limited-access binge model, rodents are provided *ad lib* access to SC and limited-access to palatable foods often high in both fat and sugar (Corwin, 2004; Corwin and Hajnal, 2005; Czyzyk et al., 2010). While *ad lib* SC intake should mitigate hunger-driven feeding during the limited access to a highly palatable diet, rats display

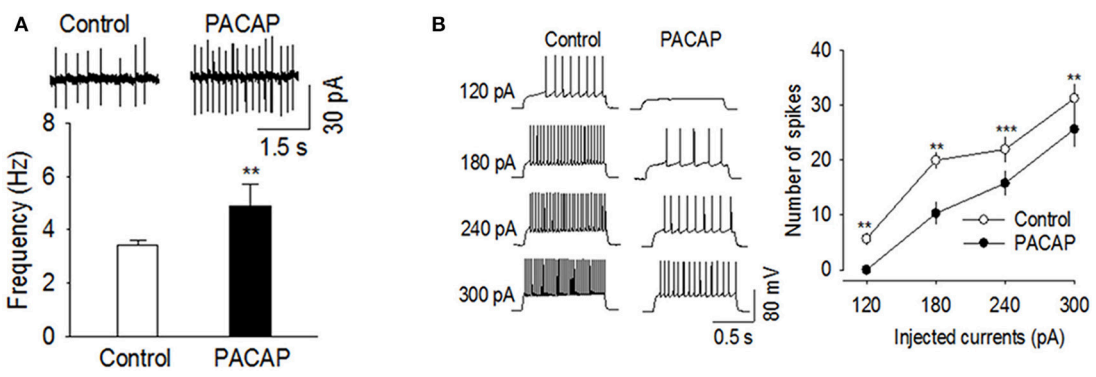


FIGURE 4 | PACAP produced opposite effects on action potential firing in the VMN and NAc. (A) PACAP significantly increased the frequency of spontaneous action potential firing in the VMN ($n = 7$, $^{**}p < 0.01$ vs. control). **(B)** PACAP decreased the number of spikes in the NAc in response to depolarizing current injections (120–300 pA, $n = 6$, $^{**}p < 0.01$ vs. control, $^{***}p < 0.001$ vs. control, Paired *t*-test). All physiological recordings were collected in the presence of CNQX and picrotoxin.

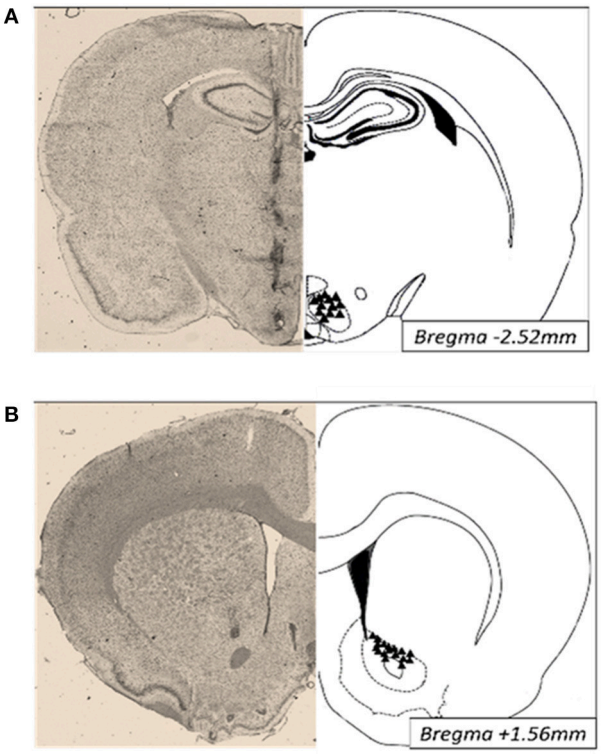


FIGURE 5 | Photomicrographs of cannula placements (triangles, right) and a representative Nissl stained section (30 μm; left) of the VMN (A) and NAc (B).

self-imposed deprivation evident by significantly decreased SC consumption. Thus, both hunger- and palatability-driven feeding drives are likely engaged during the limited access period. In the current approach, we limited the co-existence of hunger-driven and palatability-driven feeding drives by creating conditions in which restricted-feeding produces heightened hunger-driven feeding that is satiated with a low-palatable diet. It is important to note that the study of the neural mechanisms underlying feeding involving *hunger*- or *palatability*-related drives requires the manipulation of these variables to understand the specific contribution of each of these drives. There are three key design aspects used to create this desired experimental condition. First, subjects were restrict fed for 2 h; these conditions do not result in overt increases in stress hormones (Choi et al., 1998) and has previously been used by numerous others to enhance hunger-driven feeding (Hagan and Moss, 1997; Denis et al., 2015; Wei et al., 2015; Baldo et al., 2016). Confounding interactions stemming from malnourishment in these animals is extremely unlikely since rats with similar long-term caloric restriction paradigms (25% reduction) show improved life expectancy and health outcomes (Keenan et al., 1996, 2013). Second, SC was used as the low-palatable diet, which is illustrated in other studies to show diminished motivation for SC after exposure to a palatable diet (South et al., 2014). Third, the duration of the 2-h restricted feeding of SC was sufficient to produce satiety as evident by the lack of increased consumption when the access period was lengthened to 3 or 4 h. Hence, these conditions permit the study of the cellular or molecular basis of hunger-driven eating that culminates in a robust state of satiety.

In the next phase of the paradigm, palatability-driven feeding was assessed by measuring feeding in satiated rats provided additional access (15 min) to either low- or high-palatable diets. As expected, palatable food consumption significantly increased compared to the minimal consumption of SC. Remarkably, the average number of calories consumed of a highly-palatable food by satiated rats (M2) was equivalent to the number of SC calories consumed during the 2-h M1. This marked increase in the highly palatable food is unlikely to be due to novelty or stimulus-specific satiety since a similar increase was not obtained when SC was made novel with either vanilla or almond flavorings (**Figure 1D**). Hence, these conditions likely permit the study of the cellular or molecular basis of palatable-driven feeding with limited influence from hunger-driven eating.

VMN Gate Hunger but Not Palatability-Driven Feeding

Historically, the VMN were thought to be critical components of the brain's "satiety center" (Kennedy, 1950) and later described as the inhibitory counterpart to the lateral hypothalamus (promoting feeding) in the dual-center hypothesis for motivated behavior (Stellar, 1954). Recent studies continue to support the VMN as key sites in the regulation of energy homeostasis by demonstrating that specific genetic deletions in the VMN lead to obesity (Kim et al., 2009), altered fMRI activity is evident in the VMN after ingesting a glucose solution (Liu and Gold, 2003), and a positive correlation between the degree of medial hypothalamic damage and excess weight gain (Pinkney et al., 2002). However, an important outstanding question is whether the satiety signal from the VMN regulates multiple distinct feeding drives (e.g., hunger and palatability-driven feeding). Using the two-meal paradigm, we found that VMN activation achieved by local AMPA injections decreased consumption of SC in restrict-fed rats but, surprisingly, it did not alter palatability-driven feeding. While more work is needed to more thoroughly characterize this effect, these findings are consistent with the conclusion that hunger- and palatability-driven feeding involve at least partially non-overlapping circuitry.

NAc Gates Palatability but Not Hunger-Driven Feeding

The NAc has been strongly implicated in a wide-range of motivated behaviors, including palatability-driven feeding (Robbins and Everitt, 1996; Wise, 1998; Aragona et al., 2006; Baldo and Kelley, 2007). However, an open question is whether NAc-related circuitry are also involved in hunger-driven feeding, in part because many studies measure intake when both hunger- and palatability-related drives would be present. We found that local inactivation of the NAc by baclofen+muscimol reduced palatability-driven but not hunger-driven feeding. Our finding that GABA agonists into the NAc did not reduce hunger-driven consumption of SC is consistent with earlier work (Stratford and Kelley, 1997). However, we are the first to show that inhibition

of the NAc decreased hedonic-driven feeding in rats that were accustomed to binge eating a palatable meal. While it is possible that regions of the NAc or ventral striatum not impacted by our manipulations may contribute to both forms of eating, our results, at a minimum, reinforce the concept that each of these feeding drives can involve unique circuitry. Illustrating this point is the evidence that GABA agonist administration in other regions of the NAc show increased feeding behavior (Basso and Kelley, 1999). Thus, discretely mapping the anatomical underpinnings of various feeding drives could provide key insight into the etiology of eating behavior underlying distinct forms of obesity. For example, individuals displaying excess eating stemming from enhanced hunger-driven feeding vs. those that display enhanced (or the inability to suppress) palatable-driven feeding may express unique molecular and cellular pathological changes that could be targeted by more focused therapeutic intervention.

PACAP Gates Both Hunger- and Palatability-Driven Feeding

In the NAc, microinjections of PACAP did not alter homeostatic feeding but effectively reduced consumption of a highly palatable diet. Specifically, intra-NAc PACAP only altered consumption of high-fat, high-carb food in a satiated rat. The lack of an effect on homeostatic feeding is unlikely to be due to an insufficient dose or drug duration given that identical parameters were used in the VMN to block homeostatic feeding and in the NAc to block palatable feeding. Interestingly, the activation of NAc efferents, all of which are GABAergic, is linked to multiple forms of motivated behavior including palatability-driven feeding, as described above (Robbins and Everitt, 1996; Wise, 1998; Aragona et al., 2006; Baldo and Kelley, 2007). Thus, our observation that PACAP in the NAc mimicked the behavioral effects of GABA agonists suggests that PACAP likely inhibited at least some of these circuits, although as discussed below, the precise mechanism is unknown.

In the VMN, we found that microinjections of PACAP reduced homeostatic but not hedonic feeding. In support, PACAP microinjections into the VMN decreased consumption only when rats displayed a pronounced hunger drive (e.g., following a 22 h fast). Once the animal achieved a state of satiety, PACAP microinjections into the VMN did not alter the consumption of either standard chow or a highly palatable food source. Interestingly, PACAP in the VMN mimicked the actions of AMPA microinjected into this structure. Given that previous studies have established the VMN as a satiety center of the brain in which activation of this structure reliably decreases feeding, these collective results suggest that both PACAP and AMPA excited VMN efferents involved with satiety. While our experiments did not identify the type of cell impacted by PACAP, previous studies have revealed that the majority of VMN cells are glutamatergic (Bowers et al., 1998; Ovesjö et al., 2001). In support, studies have shown highly dense expression of the glutamatergic marker vGlut2 (Ziegler et al., 2002) with minimal expression of non-glutamatergic cells.

Our finding that PACAP signaling in the VMN reduces homeostatic but not hedonic feeding extends existing work establishing the hypophagic and metabolic actions of this neuropeptide. Although PACAP signaling has been implicated in feeding behavior and body weight regulation for over 20 years (Morley et al., 1992; Chance et al., 1995), only recent studies have begun to delineate its regional and mechanistic details. PACAP administration into the VMN reduces *ad lib* feeding without malaise specifically through the PAC1R receptor subtype, while also increasing thermogenesis and spontaneous locomotor activity (Resch et al., 2011). Likely as a result of both the anorexia and the increased metabolic indices, PACAP in the VMN results in dramatic body weight loss even after a single acute administration (Resch et al., 2011, 2013). Moreover, PACAP administration in the VMN increases both POMC mRNA expression in the arcuate nuclei and fasting glucose levels further illustrating a role for PACAP in the regulation of energy balance.

Given the historical roles for the NAc in generating motivated behaviors and the VMN in suppressing feeding, it would seem that a molecule acting in each structure would need to have the remarkable capability of inhibiting the NAc while activating the VMN to regulate each form of eating. While more work needs to be done to confirm these effects for PACAP, our data are consistent with this type of region-specific regulation. In the current study, we found that bath application of PACAP to VMN slices increased action potential firing and that microinjections of PACAP and AMPA produced the same behavioral effect. In the NAc, PACAP appears to produce the opposite effect in that bath application of PACAP to NAc slices decreased evoked potentials and microinjections of PACAP into the NAc mimicked the effects of baclofen+muscimol on feeding.

While the current results do not identify the molecular basis for PACAP mimicking GABA agonists in the NAc and AMPA in the VMN, previous work has shown that PACAP is able to increase or decrease the activity of glutamate ionotropic receptors, including NMDA (Shioda et al., 1997; Vaudry et al., 2009; Toda and Huganir, 2015). Lastly, previous work has also linked PACAP to other glutamatergic mechanisms, such as system x_c^- (Resch et al., 2014a; Kong et al., 2016) and activation of metabotropic glutamate receptors (Baker et al., 2002, 2003), which may display region-specific differences in expression (Gu et al., 2008). Regardless, these data show the degree to which the complexity of the glutamate network can differ across discrete brain regions yet be regulated by the same neuropeptide, potentially revealing PACAP to be a powerful regulator of caloric intake by both activating or inhibiting circuits associated with satiety (e.g., VMN) and appetitive (e.g., NAc) signals, respectively. Future studies will be needed to explore this intriguing possibility.

Collectively, these data suggest that PACAP signaling suppresses multiple feeding drives, which positions this novel anorexigenic peptide as an important target in understanding and possibly treating obesity. Toward the latter observation, identifying therapeutic targets capable of modulating multiple feeding drives may be especially important in the treatment of obesity given the widely observed propensity for tolerance to anti-obesity medications to have long-term utility (Fernstrom and Choi, 2008), an effect that could be due to compensatory changes across distinct drives. Thus, these findings may address a fundamental barrier in treating obesity by better isolating

individual feeding drives and demonstrating the potential for PACAP signaling to regulate unique forms of overeating.

AUTHOR CONTRIBUTIONS

MH, DB, QL, and SC designed research; MH, BM, MB, MF, MR, EK, YC, and YL performed research; MH, MF, MR, YC, YL, DB, QL, and SC analyzed data; MH, DB, QL, and SC wrote the paper.

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Glial Endozepines Inhibit Feeding-Related Autonomic Functions by Acting at the Brainstem Level

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Endozepines are endogenous ligands for the benzodiazepine receptors and also target a still unidentified GPCR. The endozepine octadecaneuropeptide (ODN), an endoproteolytic processing product of the diazepam-binding inhibitor (DBI) was recently shown to be involved in food intake control as an anorexigenic factor through ODN-GPCR signaling and mobilization of the melanocortinergic signaling pathway. Within the hypothalamus, the DBI gene is mainly expressed by non-neuronal cells such as ependymocytes, tanycytes, and protoplasmic astrocytes, at levels depending on the nutritional status. Administration of ODN C-terminal octapeptide (OP) in the arcuate nucleus strongly reduces food intake. Up to now, the relevance of extrahypothalamic targets for endozepine signaling-mediated anorexia has been largely ignored. We focused our study on the dorsal vagal complex located in the caudal brainstem. This structure is strongly involved in the homeostatic control of food intake and comprises structural similarities with the hypothalamus. In particular, a circumventricular organ, the area postrema (AP) and a tanycyte-like cells forming barrier between the AP and the adjacent nucleus tractus solitarius (NTS) are present. We show here that DBI is highly expressed by ependymocytes lining the fourth ventricle, tanycytes-like cells, as well as by proteoplasmic astrocytes located in the vicinity of AP/NTS interface. ODN staining observed at the electron microscopic level reveals that ODN-expressing tanycyte-like cells and protoplasmic astrocytes are sometimes found in close apposition to neuronal elements such as dendritic profiles or axon terminals. Intracerebroventricular injection of ODN or OP in the fourth ventricle triggers c-Fos activation in the dorsal vagal complex and strongly reduces food intake. We also show that, similarly to leptin, ODN inhibits the swallowing reflex when microinjected into the swallowing pattern generator located in the NTS. In conclusion, we hypothesized that ODN expressing cells located at the AP/NTS interface could release ODN and modify excitability of NTS neurocircuitries involved in food intake control.

Keywords: astrocytes, tanycytes, dorsal vagal complex, nucleus of the tractus solitarius, area postrema, food intake, swallowing, octadecaneuropeptide

INTRODUCTION

In the early eighties, the search for an endogenous ligand for the benzodiazepine binding site of the $GABA_A$ receptor led to the discovery of a precursor polypeptide named Diazepam-Binding Inhibitor (DBI; Costa et al., 1983; Guidotti et al., 1983; Corda et al., 1984). DBI and its proteolytic fragments octadecaneuropeptide (ODN; Ferrero et al., 1984) and triacontatetraneuropeptide (TTN; Slobodyansky et al., 1989) were collectively called endozepines (Tonon et al., 2013; Farzampour et al., 2015). DBI is identical to acyl-CoA-binding protein (ACBP, Knudsen, 1991), a cytosolic protein involved in fatty acid metabolism (Mogensen et al., 1987). It has been shown that astrocytes secrete DBI/ACBP through an unconventional pathway, in response to a variety of treatments (for review see Tonon et al., 2013; Farzampour et al., 2015).

Endozepines actually bind three molecular targets, the benzodiazepine binding site of the $GABA_A$ receptor, also called Central-type Benzodiazepine Receptor (CBR; Guidotti et al., 1983; Tonon et al., 1989; Bormann, 1991; Louiset et al., 1993), the Peripheral-type Benzodiazepine Receptor (PBR), also called Translocator Protein (TSPO), a cholesterol transporter at the outer mitochondrial membrane (Berkovich et al., 1990; Papadopoulos et al., 1991; Gandolfo et al., 2001), and a still unidentified ODN G-protein-coupled receptor (ODN-GPCR; Patte et al., 1995; Gandolfo et al., 1997; Leprince et al., 2001).

The DBI gene is widely expressed in the central nervous system (Alho et al., 1989; Tonon et al., 1990; Costa and Guidotti, 1991; Malagon et al., 1993), with particularly high levels of expression in areas involved in food intake control such as the ventromedial and dorsomedial hypothalamus and the lateral hypothalamic area (Alho et al., 1985; Tonon et al., 1990; Malagon et al., 1993), suggesting a role of endozepines in food intake control. Accordingly, intracerebroventricular administration of ODN, or of its C-terminal octapeptide OP, in rodents exerts a potent anorexigenic effect (de Mateos-Verchere et al., 2001; do Rego et al., 2007; Lanfray et al., 2013). Moreover, intraparenchymal unilateral injection of OP in the arcuate nucleus of the hypothalamus also reduces food intake (Lanfray et al., 2013). The anorexigenic effect of endozepines does not depend on CBR or PBR signaling, but solely on ODN-GPCR signaling, since it is blunted by co-treatment with a selective ODN-GPCR antagonist (do Rego et al., 2007; Lanfray et al., 2013). Although, DBI mRNA and immunoreactivity has been found both in neurons and astrocytes (Alho et al., 1985, 1989; Tonon et al., 1990; Bouyakdan et al., 2015), DBI is mainly expressed in non-neuronal cells, such as ependymocytes, tanycytes, and proteoplasmic astrocytes (Tong et al., 1991; Lanfray et al., 2013; Bouyakdan et al., 2015). Consistent with a role of glial endozepines as anorexigenic factors, food deprivation reduces the mRNA expression of DBI in ependymocytes bordering the third and lateral ventricle as well as in median eminence tanycytes and arcuate protoplasmic astrocytes (Compère et al., 2010; Lanfray et al., 2013). The impact of endozepine signaling on neuronal populations involved in food intake control has been evaluated, with a focus on the hypothalamic leptin-sensitive neurons. Two populations of leptin-sensitive neurons located in the arcuate nucleus of the mediobasal hypothalamus play a major role in food intake control, one of which is inhibited by leptin and expresses the orexigenic neuropeptide Y (NPY), whereas the other one is activated by leptin and expresses the proopiomelanocortin (POMC), the endoproteolytic processing of which leads to the anorexigenic alpha-melanocyte stimulating hormone (α-MSH). It has been shown that central administration of ODN or OP reduces the expression of arcuate NPY mRNA, whereas it increases the expression of POMC mRNA (Compère et al., 2003, 2005). Moreover, pharmacological blockade of the melanocortin receptors type 3/4 (MC3/4R) abolishes the effects of OP on food intake, suggesting that the melanocortin signaling is a downstream effector of ODN-GPCR signaling in food intake control (Lanfray et al., 2013).

Up to now, the relevance of extrahypothalamic targets for endozepine signaling-mediated anorexia has been largely ignored. The dorsal vagal complex (DVC) located in the caudal brainstem is strongly involved in the homeostatic control of food intake. It comprises three interconnected structures: the area postrema (AP), a circumventricular organ, the nucleus tractus solitarius (NTS) and the dorsal motor nucleus of the vagus nerve (DMNX). Similarly to the arcuate nucleus of the hypothalamus, the NTS comprises a population of POMC-expressing neurons. Several lines of evidence showed that NTS POMC neurons mediate the acute anorectic effect of melanocortin signaling. NTS POMC neurons are activated by refeeding (Appleyard et al., 2005) and cholecystokinin (Fan et al., 2004). Moreover, their selective pharmacogenetic stimulation induces a rapid and strong anorectic effect (Zhan et al., 2013). By contrast, acute optogenetic (Aponte et al., 2011) or pharmacogenetic (Zhan et al., 2013) selective stimulation of arcuate POMC-neurons does not reduce food intake. Since endozepines mobilize melanocortin signaling, the DVC appears as a probable target site for endozepine-signaling-mediated anorexia. We previously studied the particularly dense glial coverage within the DVC. Interestingly, we described leptin-sensitive tanycyte-like cells form a barrier between the AP and the NTS and named these cells vagliocytes (Pecchi et al., 2007; Dallaporta et al., 2009, 2010). In the present study, we explored DBI expression profile within the DVC, as well as the effects of 4th ventricle administration of endozepines.

We show here that DBI is highly expressed in non-neuronal cell types within the dorsal vagal complex, with a strong labeling in ependymocytes and vagliocytes, and a more scattered labeling in protoplasmic astrocytes. Intracerebroventricular injection of ODN or its C-terminal octapeptide OP in the fourth ventricle reduces food intake in starved-refed rats treated in the light phase. This anorexigenic endozepine action goes with a cellular activation specifically located within the DVC. We also show that, similarly to other anorexigenic effectors acting at the brainstem level, microinjection of ODN into the swallowing pattern generator (SwCPG) located in the NTS inhibits the swallowing reflex elicited by stimulation of the solitary tract (ST). The swallowing reflex is a motor component of the alimentary canal involved in ingestive behavior. It allows the propulsion of the alimentary bolus from the oral cavity to the stomach.

ANIMALS

Experiments were performed on adult male Wistar rats (Janvier, France) of 250–350 g body weight. The animals were housed at controlled temperature on a 12:12 h light/dark cycle (lights on at 07.00 am) with food (SAFE, AO4) and water available *ad libitum*. Experiments carried out in this study were performed in strict accordance with European Economic Community guidelines (86/609/EEC) for the care and use of laboratory animals. The experimental procedures have been approved by our local Animal Care Ethics Committee (Comité Ethique de Provence N°13; license N° #2207-2015100819154639).

SURGERY AND INTRACEREBROVENTRICULAR INJECTION OF ENDOZEPINES

Cannula implantation: Animals were anesthetized by an intraperitoneal (ip) injection of ketamine (100 mg/kg; Imalgen 1000, Merial) and xylazine (6 mg/kg; Rompun, Bayer), and placed in a digital stereotaxic apparatus (Model 502600, WPI) coupled to the neurostar software (Neurostar GmbH). A 26-gauge stainless steel cannula was implanted into the fourth ventricle at the following coordinates: 12.7 mm posterior to bregma, 0.2 mm lateral to the midline, and 7.2 mm ventral to the skull surface. Verification of coordinates was performed by injecting 10 µl of China ink through the cannula. After rapid cryofreezing, 40 µm brain sections were realized and observed under an optic microscope (Nikon Eclipse E600W). The presence of the blue colorant in the walls on the 4th ventricle attested the right placement of the cannula. The right cannula placement was also checked at posteriori for each animal by histological observation of the cannula trace. The cannula was secured to the skull with dental cement and sealed with removable obturators. The animals were sutured, placed in individual cages and allowed to recover for 7 days. During this resting period, animals were injected with physiological saline every other day for habituation. One week post-surgery, rats were administered either 7 µl (2.2 µL/minute) of physiological saline, ODN or OP (2 µg/animal) solution 2 h after lights on. The correct cannula positioning was checked for each animal at the end of experiment by cresyl violet staining of brain sections. Subgroups of rats were anesthetized as previously described and perfused with paraformadehyde 4%, 90 min after injections for c-Fos procedures.

FAST-REFED EXPERIMENTS AND FOOD INTAKE MEASUREMENTS

Rats were fasted for 20 h before being injected. Food was removed 6 h before lights off. Two hours after lights on, rat received either icv administration of ODN ($n = 10$), OP (2 µg/animal, $n = 7$) or vehicle (NaCl 0.9%, $n = 12$) as described above. The corresponding molar amounts for microinjection experiments of ODN (1912.13 g/mol) and OP (911.11 g/mol) are 1 nmol and 2.1 nmol, respectively. Forty-five minutes after treatment, a fresh supply of pre-weighed food was given and food intake was calculated as the difference between the pre-weighed and the remaining pellets measured with a precision balance (0.01 g; Denver Instrument from Bioblock) as previously described (Gaigé et al., 2014).

IMMUNOHISTOCHEMISTRY

Adult rats ($n = 15$) were deeply anesthetized with a mixture of ketamine (100 mg/kg ip; Merial, France) and xylazine (16 mg/kg ip; Bayer, France), transcardially perfused with 0.1 M sodium phosphate buffer (PBS; pH 7.4) and then, with freshly depolymerized 4% paraformaldehyde (PFA) solution in 0.1 M PBS. The brains were immediately removed, post-fixed 1 h in 4% PFA at room temperature and then cryoprotected for 24 to 48 h in 30% sucrose at 4°C. After freezing of the brains in cold-isopentane, coronal, horizontal and sagittal free-floating sections (30–40 µm thick) were cut on a cryostat (Leica CM3050, France) and rinsed in PBS. Sections were then treated with PBS containing 3% bovine serum albumin (BSA) to block non-specific binding sites and 0.3% Triton X-100. Sections were incubated overnight at 4°C with the respective primary antibody at 1/1000 (ODN: 403 2207 Tonon et al., 1990; GFAP: G3893, Sigma; vimentin: AB5733, Merck Millipore), washed in PBS and incubated for 2 h at room temperature with respective secondary antibody (1/200, Vector, CA, USA). Fluorescent images were acquired on a confocal microscope (Zeiss LSN 700) using the 488-nm band of an Ar-laser and the 543-nm band of a He/Ne-laser for excitation of FITC and TRITC, respectively. In double labeling experiments, images were sequentially acquired. All images were further processed in Adobe Photoshop 6.0; only contrast and brightness were adjusted and figures were not otherwise manipulated.

For c-Fos immunohistochemistry ($n = 6$), an anti-c-Fos rabbit antiserum (1/5000, Santa Cruz; SC-52) was used. Briefly, the free-floating sections were incubated 10 min in a solution containing 0.3% H_2O_2 in PBS 0.1 M for quenching of endogenous peroxidase activity. After 1 h in PBS containing 3% normal goat serum (NGS) and 0.3% Triton X-100, sections were incubated for 48 h at 4°C in PBS containing 3% NGS, 0.3% Triton X-100 and anti-c-Fos antibody. A biotinylated goat anti-rabbit IgG (1/400, Vector Labs) was used as secondary antibody. After incubation with the avidin-biotin complex (1/200, Vector Labs), horseradish peroxydase activity was visualized using a nickel-enhanced diaminobenzidine (DAB) as the chromogen. The reaction was closely monitored and terminated when optimum intensity was achieved (3–5 min) by washing the sections in distilled water. Three animals of each conditions and height sections per structure were analyzed. Non-specific labeling was assessed on alternate slices that were treated identically to the above but in which the primary antibody was omitted. c-Fos immunostaining photomicrographs were acquired using a 10-fold lens with a DXM 1200 Camera (Nikon) coupled to ACT-1 software. The microscope was set at a specific illumination level, as was the camera exposure time.

ELECTRON MICROSCOPY

Adult male Wistar rats (400–500 g, $n = 6$) were deeply anesthetized with a cocktail of ketamine (100 mg/kg ip; Merial, France)/xylazine (16 mg/kg ip; Bayer, France) and perfused by intraortic perfusion of 50 mL of 0.1 M PBS followed by 500 mL of 2% paraformaldehyde-1% glutaraldehyde in 0.1 M phosphate buffer. The brains were collected and immediately postfixed in the same solution for 3 h and then in 4% paraformaldehyde in 0.1 M phosphate buffer for 3 h. Coronal and sagittal serial sections of 50 μm thickness were made at the brainstem level using a Leica vibratome. The sections were treated with 1% sodium borohydride for 5 min. After thorough washes in PBS, they were successively incubated in 10% NGS for 30 min, in 10% NGS, 1% BSA and 0.1M lysine for 30 min, and then in rabbit anti-ODN antibody (#403; 1/800) overnight at 4°C. Following three washes, the sections were then transferred in a biotinylated goat anti-rabbit IgG (Vector Lab.) diluted 1/400 for 2 h at room temperature, washed thrice and incubated in the avidin biotin complex (Elite Vectastain kit, Vector Lab.) diluted 1/400 for 2 h at room temperature. Unless specified, the dilutions and washes between each of the above steps were made in PBS containing 1% NGS. The peroxidase activity was revealed with 0.003% of DAB and 0.01% H2O2. After that, the sections were post-fixed in 1% osmium tetroxide in 0.1 M phosphate buffer for 45 min, dehydrated in ethanol, and embedded in Epon. A portion of the DVC containing the interface between the AP and the NTS was then cut off under binocular. Ultrathin sections of ∼70 nm in thickness were cut with an Ultracut ultramicrotome (Leica) and counterstained with uranyl acetate (5 min) and lead citrate (5 min). The ultrathin sections were then examined with a Philips CM 10 electron microscope (Center for Microscopy and Imaging of the Jean-Roche Institute) or JEOL JEM 2010F URP22 (Pluridisciplinary Center of Electron Microscopy and Microanalysis).

REAL TIME RT-PCR

DBI mRNA expression within the brainstem and the hypothalamus was examined as previously described (Gaigé et al., 2014). Briefly, total RNA was extracted from frozen brainstem and hypothalamus ($n = 5$) using Trizol. After RNA reverse transcription, gene expression analysis by real time PCR was performed using the ABI Thermocycler 7500 fast (Applied Biosystems). The equivalent of 6.25 ng initial RNA was subjected to PCR amplification in a 10 μl final volume using specific 2.4 μM primers and SYBR Green PCR master mix (Applied Biosystems). Product formation (DBI primers: Fw TGCTCCCGCGCTTTCA; Rev CTGAGTCTTGAGGCGCTTCAC) was detected at 60°C in the fluorescein isothiocyanate channel. The amplicon was then submitted to agarose gel electrophoresis to evaluate its size.

SWALLOWING RECORDING

Experiments were performed on adult male Wistar rats weighting 350 g (Charles River, I'Arbresle, France), anesthetized with 0.6 ml of a mixture of ketamine (100 mg/ml; Merial, France) and xylazine (15 mg/ml; Bayer France), injected intraperitoneally in a proportion of 90% and 10%, respectively. The anesthesia was then continued by perfusion of the same mixture diluted at 10%, through a catheter inserted in the femoral vein, at a rate of 0.01–0.05 mL/h. After occipitoparietal craniotomy and removal of the posterior part of the cerebellum, the floor of the fourth ventricle appeared to lie in a horizontal plane. The surface of the medulla was exposed in order to allow the stereotaxic introduction of the microelectrode in the intermediate NTS containing the SwCPG for ODN injection, and of the stimulatory bipolar electrode into the ST. The medulla was covered with warm liquid paraffin. Swallowing was triggered by central stimulation of the ST corresponding to the entering of the sensitive fibers which convey through the superior laryngeal nerve. Stimulation with a long train of pulses produced several swallows [or rhythmic swallowing recorded by electromyography (EMG)], at a rhythm depending on stimulation frequency. In the present study, repetitive long trains of pulses (5 s duration at 15 Hz frequency every 30 s) were used. The pulse voltage, duration and frequency varied according to the animal (2.6–4.8 V; 0.02–0.6 ms) in such a way as to trigger around 4–6 rhythmic swallows. To monitor swallowing, the EMG activity of sublingual muscles (mainly the geniohyoid) was recorded by means of bipolar copper wire electrodes inserted into the muscles, using a hypodermic needle. The respiratory activity was recorded by means of a mechanotransducer placed around the thorax, and the electrocardiogram (ECG) by subcutaneous electrodes placed on each side of the thorax. Moreover, the electrocardiogram and swallowing EMG signals were fed to loud speakers for auditory monitoring. Rectal temperature was monitored and maintained around 37°C with a heating pad. The EMG, ECG and respiration signals were recorded on a computer using an analog-to-digital interface (PowerLab 8SP data acquisition software for Windows, AD Instruments, USA). A stable control sequence involving three 30-s trains of stimulations was performed before ODN injection. The mean values obtained during this sequence were used as control values. Afterwards, stimulations and recordings were maintained until recovery. ODN injection in the SwCPG is a brief application. ODN was delivered in the SwCPG by pressure ejections through glass pipettes (70 μm O.D. at the tip) using an injection device (PMI-200, Dagan Corp., Minneapoli, MN USA). The pressure ejection was adjusted between 150 and 200 kPa for pulses of 3 s in duration, and the injected volume was 100 nl.

STATISTICAL ANALYSIS

Comparisons between groups in **Figure 6** were performed with repeated ANOVA with Tukey's HSD (Honestly Significant Difference) test used for *post-hoc* analysis. *P*-values <0.05 were considered significant. Comparisons between groups in **Figure 7** were carried out with unpaired 2-tailed Student's *t*-test. *P*-values <0.05 were considered significant. S.E.M values were derived from at three independent experiments. For swallowing experiment (**Figure 8**), statistical analyses were performed using one way analysis of variance (ANOVA) followed by Fisher's protected least-significant difference *post-hoc* test. Differences

were considered significant when $P < 0.05$. Data were expressed as mean ± SEM. StatView for Windows 5.0.1; SAS Institute was used for statistical analysis.

RESULTS

ODN Immunoreactivity within the Caudal Brainstem is Associated with Glial Cells

ODN immunohistochemistry within the rat brainstem, visualized on horizontal sections, revealed a striking pattern of immunoreactivity within the DVC, which distinguished it from the surrounding brainstem nuclei (**Figure 1A**). Within the DVC, a distinct subregional distribution of ODN immunoreactivity was observed. Indeed, the AP appeared heavily labeled throughout its rostro-caudal extent while a moderate ODN labeling was observed in the NTS (**Figure 1A**). Noticeably, the border between the AP and NTS i.e., the *funiculus separens* appeared also strongly stained (**Figures 1A,B**). At this level, a bundle of thin ODN-positive processes which radiated rostro-caudally into the NTS parenchyma was observed. Preincubation of the ODN antiserum with synthetic ODN (10^{-6} M) resulted in a complete loss of the immunoreaction (**Figure 1C**). Hypothalamic sections were used as positive control of ODN immunohistochemistry. As previously described (Lanfray et al., 2013), ODN staining was mainly observable in tanycytes lining the 3th ventricles and tanycytes located within the median eminence (**Figure 1D**).

To investigate the possible glial identity of brainstem ODN positive cells, we next performed ODN and GFAP double labeling. ODN-positive processes extending horizontally at the NTS/AP interface were also GFAP-positive (**Figures 2A–D**). Within the AP, ODN staining did not co-localize with GFAP since GFAP labeling was virtually absent from this structure. The presence of a sub-population of ODN/GFAP-positive cells exhibiting atypical morphology prompted us to perform vimentin immunolabeling since this intermediary filament protein is known to be expressed by tanycytes-like cells present at the brainstem level (Pecchi et al., 2007; Langlet et al., 2013). In the DVC, vimentin immunoreactivity was mainly observed in the NTS/AP border and on the edge of the 4th ventricle (**Figures 2E–J**). As expected, immunohistochemistry revealed the presence of ODN/vimentin immunoreactivity at the border between the AP and the NTS (**Figures 2E–I**). XZ orthogonal projection of images acquired on horizontal sections confirmed the co-localization of ODN and vimentin in thin processes surrounding the AP (**Figure 2J**). Within the AP, a part of ODN expressing cells remained vimentin negative, suggesting the existence multiple ODN-positive

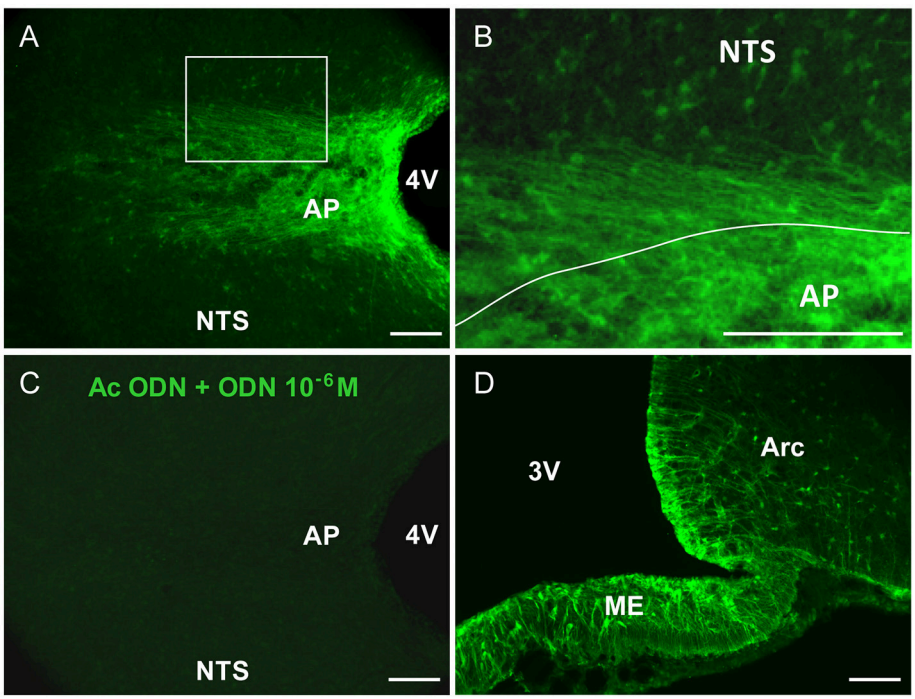


FIGURE 1 | ODN immunohistochemistry in the brainstem. **(A)** Confocal fluorescent micrographs illustrating ODN immunohistochemistry performed on horizontal brainstem sections. ODN immunostaining showed the presence of positive cells in the area postrema (AP), at the border between AP and nucleus tractus solitarius (NTS) and within NTS surrounding the AP. Lateral dorsal vagal complex was devoid of labeling. **(B)** High magnification microphotograph originating from image in A (rectangle in A) and illustrating the shape of ODN immunoreactivity within the AP and surrounding structures. **(C)** Pre-incubation of ODN antibody with an excess of ODN peptide resulted in the absence of staining. **(D)** Confocal fluorescent micrograph illustrating ODN immunohistochemistry performed on coronal hypothalamic sections. As expected ODN immunoreactivity was found in the median eminence (ME), tanycytes lining the 3th ventricle (3V) and arcuate nucleus (Arc). 4V, 4th ventricle. Scale bars: 100 μm.

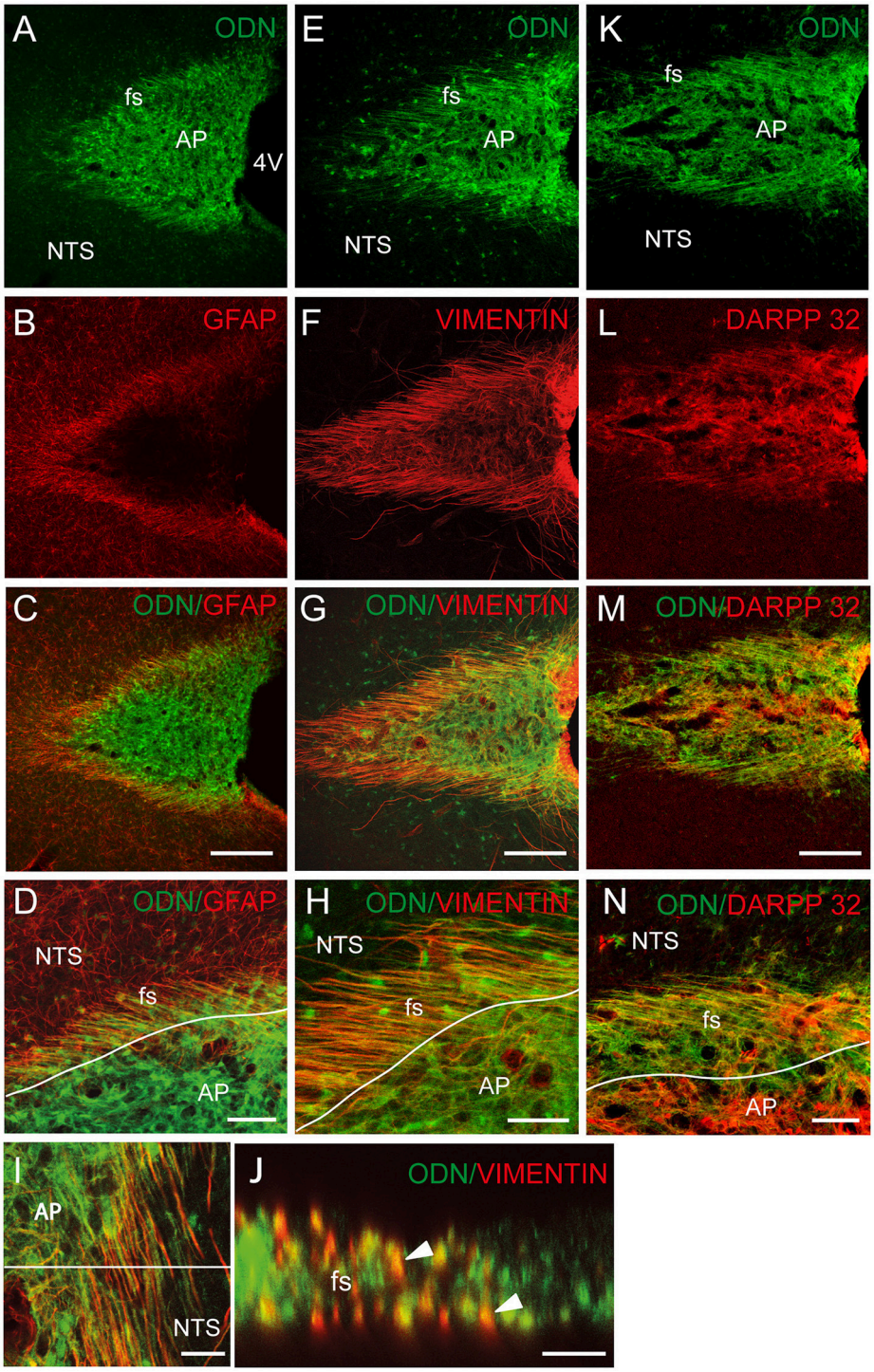


FIGURE 2 | Tanycytes-likes cells of the brainstem are ODN positive. **(A–C)** ODN partly co-localized with GFAP immunostaining within the dorsal vagal complex (DVC). The presence of ODN/GFAP-positive processes was visualized at the nucleus tractus solitarius/area postrema (NTS/AP) border **(A–C)**. High magnifications illustrated the presence of ODN/GFAP double labeled radial processes **(D)**. **(E–H)** ODN partly co-localized with vimentin within the AP, at the AP/NTS interface i.e., *funiculus separens* (fs) and on the wall of the 4th ventricle (4V). **(I)** ODN/vimentin-positive processes originating from cells lining the 4th ventricle and radiating rostro-caudally were observed on horizontal sections of the DVC. A partial co-localization was also observed within the AP. **(J)** XZ orthogonal projection of images acquired on horizontal sections confirmed the presence of ODN/vimentin-positive processes surrounding the AP (arrowheads). **(K–M)** ODN/DARPP 32 co-localization within the AP and NTS/AP border. **(N)** High magnification illustrating ODN/DARPP 32 co-localization in long, radiating processes and cells located within the AP. Lines in D, H and N symbolize the AP boundary. Scale bars: 100 μm in **(A–C)**, **(E–G)**, and **(K–M)**; 40 μm in (**D,H,N**; 5 μm in **I** and **J**.

subpopulations in this structure. The dopamine- and cyclic adenosine-3′:5′-monophosphate (cAMP)-regulated phosphoprotein (DARPP-32) was found to be present in hypothalamic ependymal tanycytes lining the walls and floor of the third ventricle or located within the median eminence (Meister et al., 1988). At the AP level, DARPP-32 partly co-localized with ODN both within the AP and in the thin processes located in the *funiculus separens* (**Figures 2K–N**). In addition to ODN positive fibers located in the *funiculus separens* border area, ODN staining was observed within the NTS. This labeling exhibited a rounded shape and was observed in the vicinity of the *f. separens*. This ODN staining progressively diminished as one get away from the NTS/AP border (**Figures 3A,C**). Similarly, the GFAP labeling was heterogeneously distributed within the NTS, with a lesser concentrated staining in the lateral parts of the nucleus (**Figures 3B,D**). High-magnification images revealed an ODN/GFAP co-localization in cells exhibited typical features of differentiated protoplasmic astrocytes were observed (**Figures 3E–G** and inset in G).

Electron Microscopy of the NTS/AP Border and ODN Immunoreactivity

We next performed electron microscopy at the NTS/AP border zone of coronal brainstem sections. Serial mapping with electron microscopy at low power magnification provided an overview of this area, and showed that it was organized with numerous glial processes (**Figure 4A**). Interestingly, these fibrous processes seemed to shape a continuous layer between the AP and the NTS. It seems likely that these processes observed in electronic microscopy match to the atypical and thin glial processes described above. This level of analysis revealed also that these glial processes originating from tanycyte-like cells located at the NTS/AP interface were rounded with very short ramifications (**Figures 4A**,**D–F**). These processes were clearly identified by numerous intracellular cytoskeleton filaments (**Figure 4B** and inset), exhibiting a compact organization with a similar orientation (**Figure 4C**). In some cases, short ramifications originating from fibrous processes were found in close apposition to neuronal elements such as somata, dendritic profiles, or axon terminals (**Figures 4D–F**). At the electron microscopic level, ODN immunoreactivity was also found exclusively in glial processes and cells (**Figure 5**). In addition to tanycyte-like processes located at the NTS/AP interface (**Figure 5A**), ODN staining was also found associated with protoplasmic astrocytes located in the subpostremal NTS (**Figure 5B**). In both cells types, ODN immunoreactivity was cytosolic. ODN-positive protoplasmic astrocytes were sometimes found in the close vicinity of synaptic profiles (**Figure 5C**).

Finally, the presence of DBI mRNA in rat DVC was investigated by RT-PCR analysis. A cDNA band of the expected size (95 bp) was detected in the reverse transcribed products from this structure. cDNA from hypothalamus, a structure known for its high ODN expression (Alho et al., 1985; Tonon et al., 1990; Malagon et al., 1993), were used here as a positive control (**Figure 5D**).

Fourth Ventricle Endozepine Administration Reduced Food Intake

To determine whether endozepines could modify food intake by acting at the brainstem level, we performed 4th ventricle injection. The DVC which is involved in the initiation of meal as well as in the satiety reflex lines the 4th ventricle in the caudal brainstem. A single administration of ODN or OP (2 μg/animal) resulted in a decrease of food intake consumed during refeeding [**Figure 6A**, $F_{(2,\ 26)} = 3.901$, $P = 0.0218$]. This effect presented a short latency, since it was significant in the first hour post-treatment and feeding behavior remained profoundly affected during the first 3 h post-treatment. Cumulative food intake measured over a period of 9 h, revealed that OP affected food intake more deeply and durably than ODN [**Figure 6B**, $F_{(2,\ 26)} = 18.006$, $P < 0.0001$].

Cellular Activation Induced by 4th Ventricle OP Administration

We next sought to confirm that 4th ventricle OP injection resulted in cellular activation within the DVC and determine whether this cellular activation spread out of the brainstem. Central structures activated in response to OP (2 μg/animal) administration were identified using the immune detection of the c-Fos protein. Animals were sacrificed 90 min after injection of either NaCl 0.9%. or OP 2 μg/animal. A very low basal level of c-Fos positive nuclei was observed in the brainstem of NaCl-treated rats (**Figure 7A**). OP-treated rats exhibited a strong rise in the number of c-Fos positive nuclei within the NTS whatever the rostro-caudal level analyzed (**Figure 7B**). At the brainstem level, other DVC regions such as the AP and the dorsal motor nucleus of the vagus nerve (DMNX) were devoid of labeling (**Figure 7A**). At the time point analyzed here i.e., 90 min post-treatment, pontine and hypothalamic structures did not exhibited significant c-Fos expression increase in OP-treated animals as compared to control rats (**Figures 7A,B**).

ODN Inhibited Swallowing Reflex in Anaesthetized Rats

Given the pattern of OP-induced c-Fos expression observed within the DVC, we next tested the impact of a brief central ODN injection on the swallowing reflex. The present results showed ODN microinjections induced a dose-dependent decrease in the number of swallows recorded during ST stimulation (**Figure 8A**), with a slight non-significant effect at 50 μM (5 trials, 3 rats; **Figure 8B**), and a significant effect at 100 μM (10 trials, 5 rats; **Figure 8B**). The inhibitory effect observed upon 100 μM ODN microinjections appeared with a latency of 4.4 ± 1.4 min and persisted during 41.20 ± 4.96 min. This inhibitory effect of ODN on rhythmic swallowing pattern after its central injection within the SwCPG was not associated with variation of either cardiac frequency or respiratory activity (data not shown).

DISCUSSION

In the central nervous system, endozepines exhibit a wide distribution as reported by several groups (Alho et al., 1989;

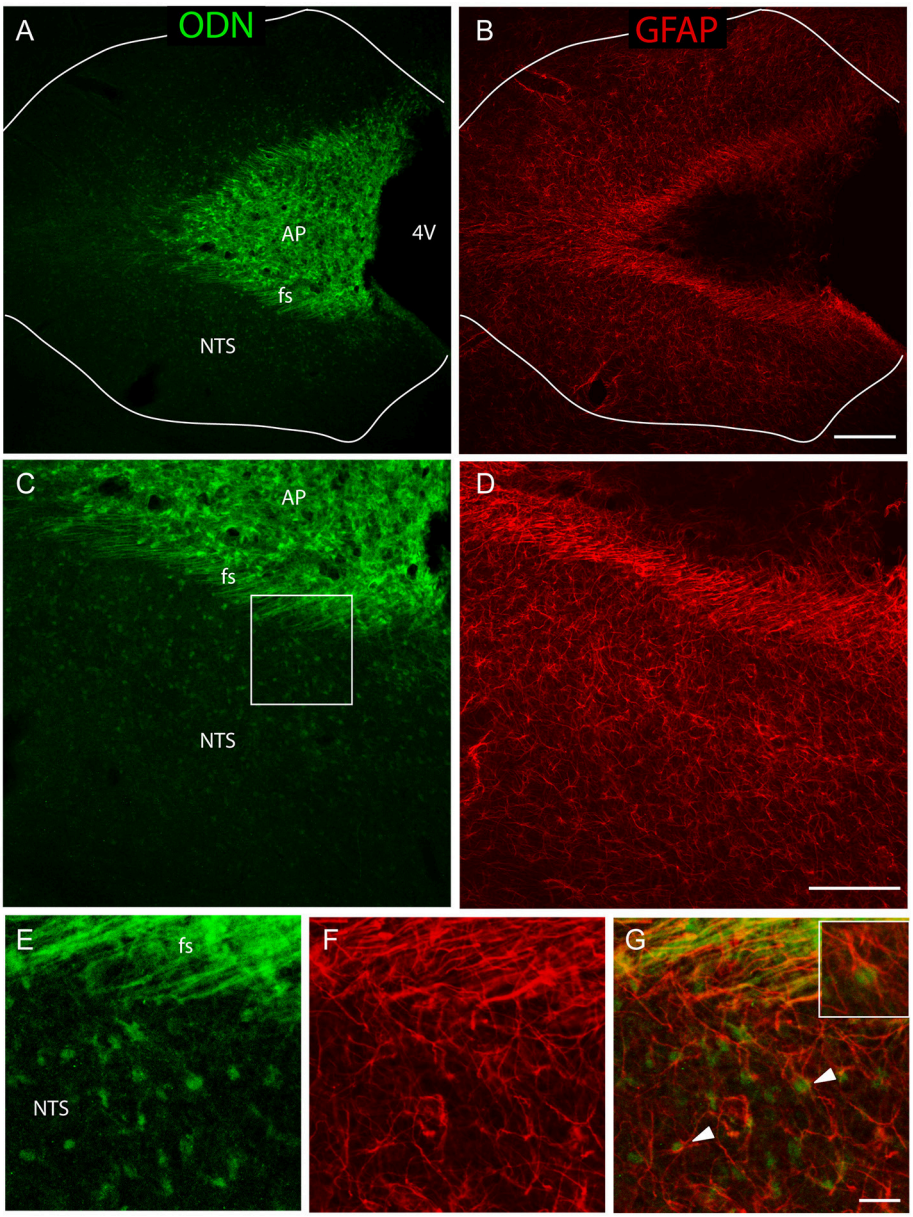


FIGURE 3 | A sub-population of NTS protoplasmic astrocytes expressed ODN. **(A–D)** Low-magnifications images illustrated the presence of a rounded ODN labeling within the NTS. This labeling was intense in the vicinity of the *funiculus separens* and progressively diminished in the lateral parts of the NTS **(A–C)**. Similarly, the distribution of GFAP labeling was heterogeneous within the NTS, with a lesser concentrated staining in the lateral part than in the areas surrounding the *funiculus separens* **(B–D)**. Lines in A and B symbolize the NTS boundaries. **(E–G)** High-magnification images illustrated the presence of ODN/GFAP double labeled protoplasmic astrocytes located within the subpostremal NTS bordering the AP (arrowheads in **G**). Inset in **G**: Detail of an ODN positive astrocytes**.** Squares in **(C,D)** indicate the area from where images **(E–G)** originate. Scale bars: 250 μm in **(A–B)**; 100 μm in **(C–D)**, 10 μm in **(E–G)**.

Tonon et al., 1990; Costa and Guidotti, 1991; Malagon et al., 1993). Indeed, using *in situ* hybridation or immunochemistry approaches, DBI or its major central processing product ODN was observed in many brain regions, such as olfactory bulb, cerebral, and cerebellar cortex, hippocampus, hypothalamus, inferior colliculus, and periaqueductal gray matter. Noticeably, very few data are available on brainstem DBI expression, with the exception of two *in situ* hybridization studies mentioning DBI expression within the AP (Alho et al., 1988; Tong et al., 1991). The present study was designed to perform a more comprehensive analysis of ODN expression at the brainstem level with a focus on the DVC. We observed a strong ODN expression in the AP, thus confirming previous observations (Alho et al., 1988; Tong et al., 1991). In addition, we reported a labeling in AP-surrounding regions i.e., the *funiculus separens* and the subpostremal/commissural NTS. From a functional

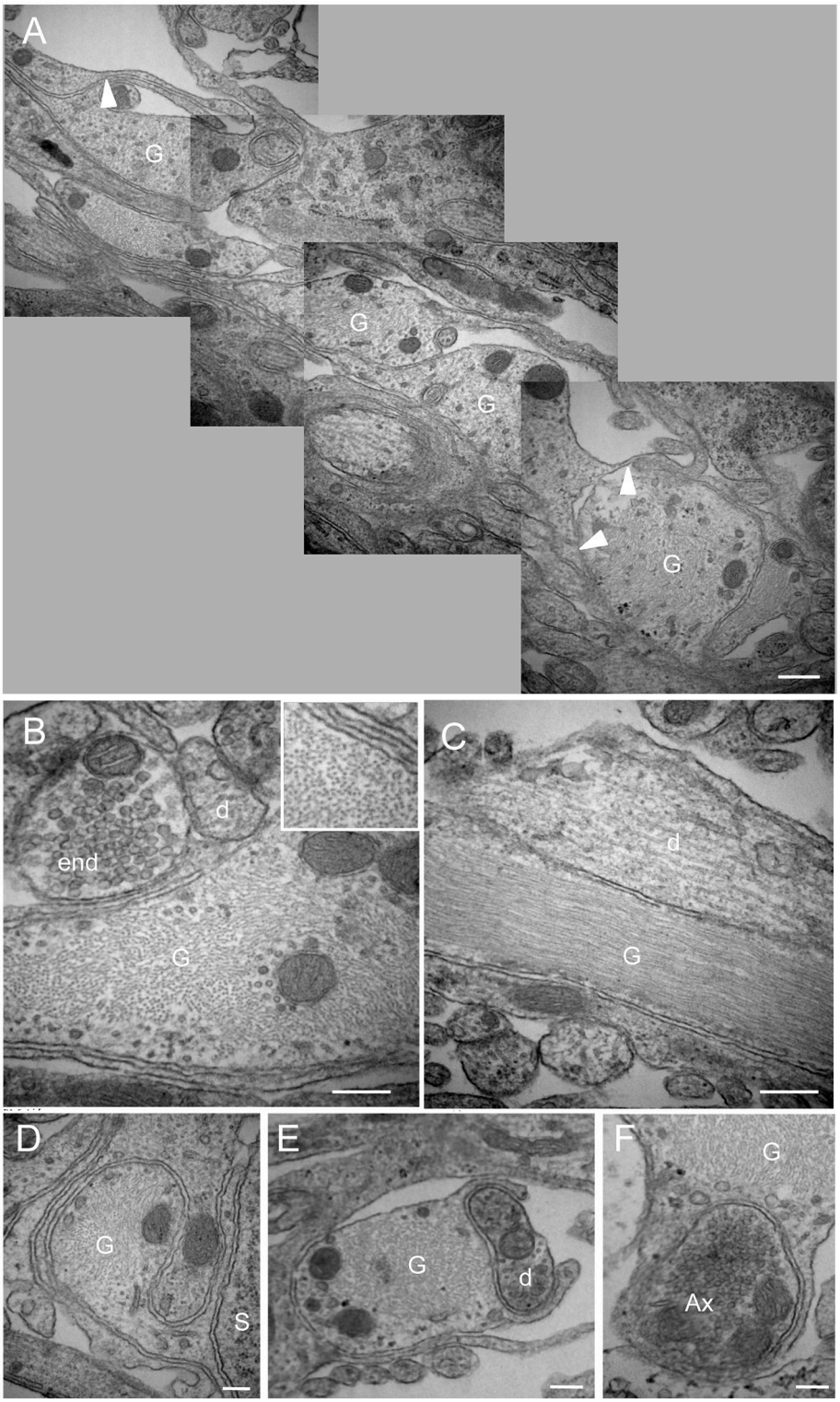


FIGURE 4 | Morphological organization of the NTS/AP border. **(A)** Electron micrographs performed on sagital dorsal vagal complex sections showing the presence of rounded, fibrous glial processes at the border between the area postrema (AP) and nucleus tractus solitarius (NTS). Note that these fibrous processes create a continuous layer between the AP and the NTS. Small ramifications (arrowheads) of the rounded processes could be observed. **(B,C)** These glial processes could be identified by their numerous cytoskeleton filaments visible on sagital **(B)** and horizontal **(C)** sections. Inset in **(B)** Illustration of the high cytoskeleton filaments density in rounded glial processes. **(D–F)** Close juxtapositions between fibrous glial processes and neuronal elements i.e., neuronal soma **(D)**, dendritic profile **(E)** and axon terminal **(F)** are noticeable. Scale bars: 500 μm in A; 200 μm in **(B–F)**. D, dendritic profiles; Ax, axon terminals; S, soma; G: glial process.

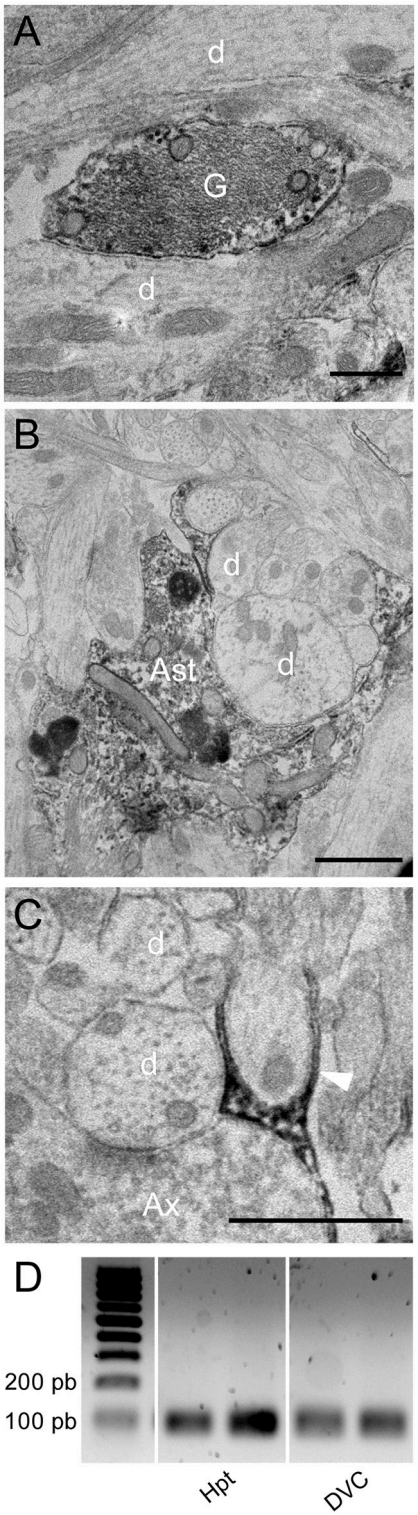


FIGURE 5 | Immunoelectron microscopy of ODN. **(A,B)** Electron micrographs taken from the subpostremal nucleus tractus solitarius (NTS) illustrating the ODN immunostaining in protoplasmic astrocytes. Note that ODN positive processes are sometimes strongly associated with neuronal elements **(B)**. **(C)** Electron micrograph originating from NTS/area postrema border showed a *(Continued)*

FIGURE 5 | Continued
fibrous glial process positive for ODN (arrowheads). **(D)** RT-PCR of DBI mRNA from hypothalamus (Hpt) and dorsal vagal complex (DVC) extracts. Scale bars: 500 nm in A; 1 μm in B and C. Ast, astrocytes; d, dendritic profiles; Ax, axon terminals; G, glial process.

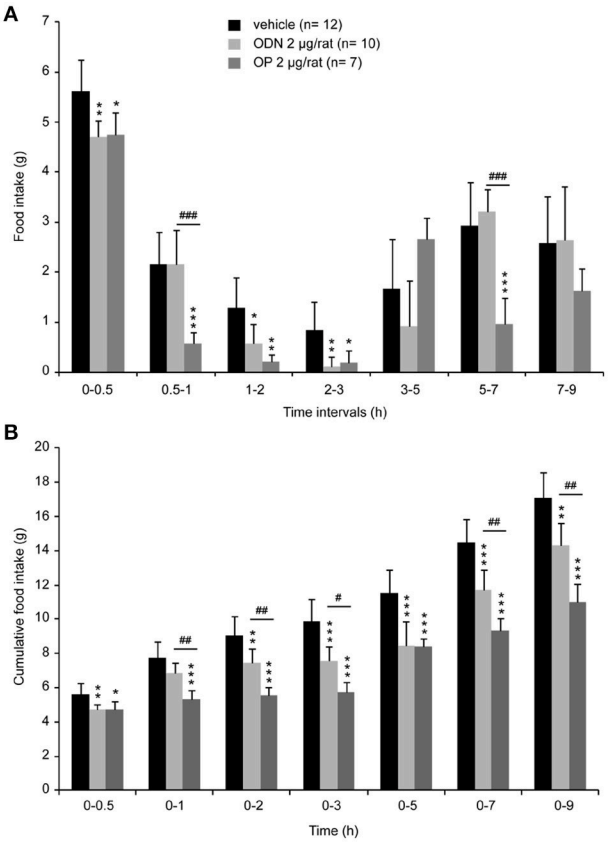


FIGURE 6 | Acute 4th ventricle endozepine administration modifies food intake. Food intake measured at different time intervals after treatment **(A)** and cumulative food intake **(B)** monitored over 9 h after central injection of either saline (vehicle), ODN or OP (2 μg/rat). Animals were fasted 18 h before the 4th ventricle injection, and food was presented 45 min after treatment. *, $\#P < 0.05$; **, $\#\#P < 0.01$ and ***, $\#\#\#P < 0.001$ significantly different from vehicle-treated animals, respectively for ODN and OP-treated rats.

point of view, the AP constitute a circumventricular organ of the brainstem necessary to relay the anorexic effects of circulating compounds such as amylin or leptin (Lutz et al., 2001; Liberini et al., 2016; Smith et al., 2016; Levin and Lutz, 2017). Furthermore, a subpopulation of AP neurons project heavily onto the immediately subjacent NTS i.e., subpostremal and commissural subnuclei (Shapiro and Miselis, 1985). The nervous peripheral input to the NTS through the vagus nerve exhibits a viscerotopographic organization (Loewy, 1990). The subpostremal and commissural NTS receive afferents from the gastrointestinal tract and integrate a wide variety of signals involved in the regulation of appetite and satiety (Shapiro and Miselis, 1985; Loewy, 1990). The brainstem ODN expression, we reported here, was thus associated with regions strongly involved

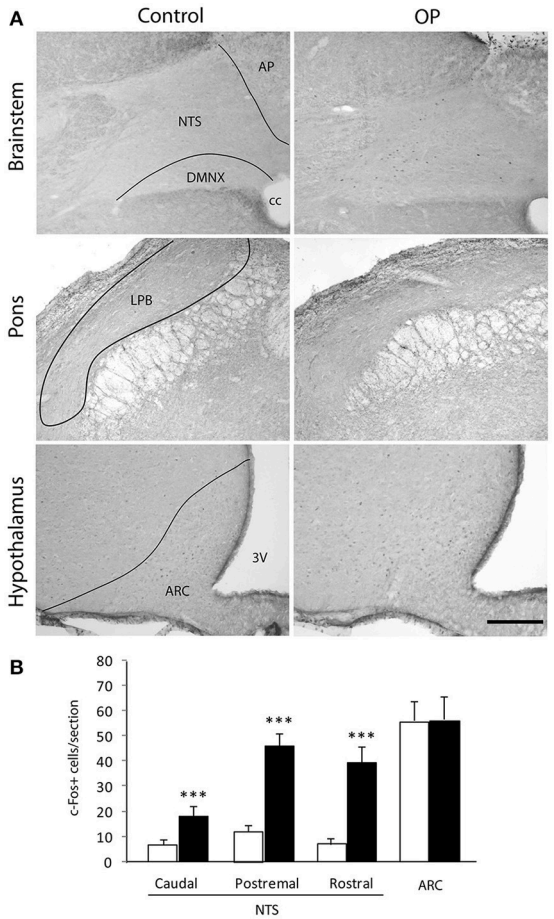


FIGURE 7 | Effects of 4th ventricle OP administration on c-Fos immunoreactivity. **(A)** Representative coronal sections illustrating the c-Fos labeling observed within brainstem, pons and forebrain regions of mice treated with NaCl 0.9% (left panel) or anorexigenic OP dose (right panel, 2 μg/rat) and sacrificed 90 min post-treatment. **(B)** Quantification of the number of c-Fos immunoreactive nuclei within brainstem, pons and forebrain nuclei observed in mice treated either with vehicle (NaCl 0.9%, white bars) or OP (2 μg/rat, black bars). ***$P < 0.001$ significantly different from vehicle-treated rats. Scale bar: 150 μm. 3V, third ventricle, AP, area postrema; ARC, arcuate nucleus; cc, central canal; LPB, lateral parabrachial nucleus; NTS, nucleus tractus solitarius; DMNX, dorsal motor nucleus of the vagus.

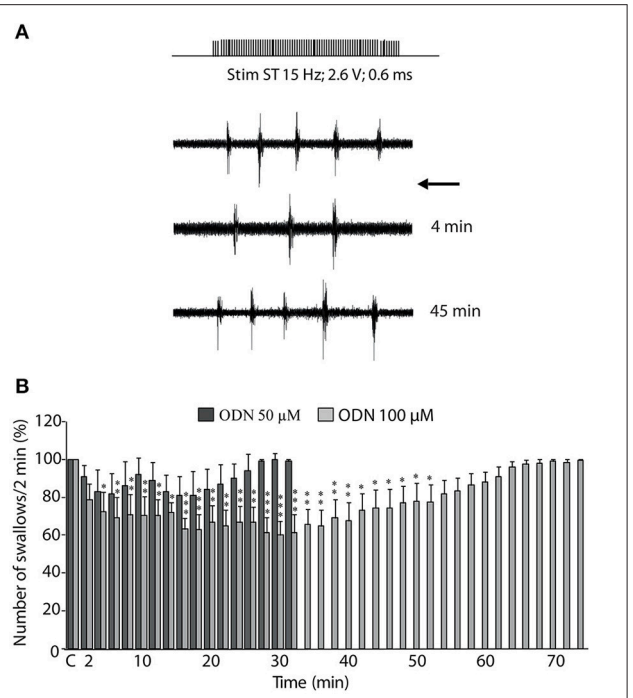


FIGURE 8 | ODN inhibition of swallowing reflex. **(A)** Effects of ODN on sublingual muscle electromyographical activity induced by solitary tract (ST) stimulation. ODN (100 μM, arrow) injected within the swallowing pattern generator (SwCPG) induced a powerful inhibition of the number of swallows. Note that this inhibitory effect intervened rapidly after ODN application ∼4 min. This effect was transient since the swallows recovered after 45 min. **(B)** Time course of the effects of ODN doses injected within the SwCPG at 50 μM (dark bars, 5 trials) and 100 μM (gray bars, 10 trials) on the number of swallows recorded over 2-min periods and until recovery. "C" represents the mean value of the control recorded before ODN microinjection (100%). Data are represented as means ± SEM normalized to the control value. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ significantly different from control.

in the integration of signals linked to the gastrointestinal tract and energy homeostasis.

We next sought to determine the cellular identity of ODN positive cells within the brainstem. Previous works using *in situ* hybridization have reported that in numerous brain areas, a specific labeling was associated with non-neuronal cells including ependymal and subependymal cells bordering the 3rd ventricle (Tong et al., 1991). By light microscope immunocytochemistry, ODN staining was also reported in glial and ependymal cells in the olfactory bulb, hypothalamus, hippocampus, periaqueductal gray, cerebral cortex, and the circumventricular organs (Alho et al., 1988; Tonon et al., 1990). These studies performed at the electron microscopic level confirmed the association of immunoreactive material with glial and ependymal cells (Alho et al., 1989). More recently, the identity of the endozepine-expressing cells within the hypothalamus was examined by immunohistochemistry. A strong ODN immunoreactivity was detected in DARPP 32/vimentin-positive thin processes, a labeling characteristic of tanycytes extending from the 3rd ventricle into the hypothalamic parenchyma (Lanfray et al., 2013). The results we obtained, at the brainstem level, showed that ODN and GFAP are co-expressed by protoplasmic astrocytes located in the subpostremal and commissural NTS subnuclei. Astrocytes from other parts of the NTS were devoid of labeling. In addition to ODN-positive astrocytes, ODN co-localized with GFAP in thin processes located within the *funiculus separens* known to originate from tanycytes-like cells i.e., vagliocytes previously described in this structure (Pecchi et al., 2007; Dallaporta et al., 2009). The cell bodies of these atypical glial cells are located at the border of the 4th ventricle or within the AP (Pecchi et al., 2007; Langlet et al., 2013). Co-staining of ODN with vimentin or DARPP-32 confirmed the ODN expression by DVC vagliocytes. The only partial overlapping between vimentin and DARPP-32 staining observed in the *funiculus separens* and the AP suggested

that different sub-populations of vagliocytes co-exist within the DVC. Interestingly, these cells were reported to express leptin receptor (Dallaporta et al., 2009). Electron microscopy showed a cluster of ovoid fibers forming a continuous layer at the AP and NTS border. These processes were obviously identified by their numerous intracellular cytofilaments showing a similar orientation and a high density. These glial processes could exhibit small and short lateral ramifications. Interestingly, fibrous processes or their ramifications were sometimes found in close apposition to neuronal elements such as dendritic profiles or axon terminals. The localization and the shape of these processes are evocative of the transection of vagliocytes processes and previously visualized by immunohistochemistry. ODN staining observed at the electron microscopic level confirms its expression by vagliocytes and protoplasmic astrocytes. ODN expressing astrocytes were also occasionally found in the immediate vicinity of synapses. In total, these data show that, at the brainstem level, ODN is expressed by multiple glial cell populations mainly located at the AP/NTS interface. The localization of these ODN expressing cells at a neurohemal interface, together with their juxtaposition with neuronal components strongly lead us to put forward the hypothesis that ODN expressing cells could act as sensors of circulating compounds and could in turn release ODN and modify excitability of NTS neurocircuitries.

Intracerebroventricular administration of ODN or its C-terminal octapeptide fragment OP in the 3rd ventricle has been shown to exert a potent anorexigenic effect in rodents (de Mateos-Verchere et al., 2001; do Rego et al., 2007). Moreover, Lanfray et al. (2013) reported the inhibition of food intake after a unilateral injection of the ODN agonist OP into the arcuate nucleus, supporting the view that endozepines may control arcuate neurons involved in feeding behavior. The NTS is known as a primary integration site for satiety signals involved in the termination of a meal (Grill and Kaplan, 2002). Direct information about meal size arising from the gastrointestinal tract conveys through the vagus nerve to reach the NTS. Vagal mechanosensors located in the gastrointestinal tract sense the volume of ingested food and locally released satiety hormones, such as cholecystokinin (Schwartz and Moran, 1994; Berthoud et al., 2001). Despite, this major role of NTS in the termination of food intake, functional evidence for a role of endozepines in the brainstem regulation of feeding is missing. The brainstem ODN expression we reported here, led us to evaluate the impact of 4th ventricle endozepine injections on refeeding-induced satiety, a condition known to strongly mobilize the NTS (Timofeeva et al., 2005). We demonstrate here that 4th ventricle ODN or its C-terminal iso-active fragment OP (Leprince et al., 1998) injections strongly reduced food intake. OP was significantly more effective to reduce food intake than ODN at the same 2 μg dose. However, it should be noticed that the OP molar dose was twice that of ODN and partially explains this difference in cumulative food intake. This effect was observable rapidly after ODN or OP injection into the 4th ventricle, suggesting a local endozepine action restricted to the DVC region. This hypothesis was confirmed by c-Fos expression mapping since at a time point where OP reduced food intake, cellular activation was confined to the NTS, whereas more rostral structures and particularly endozepine-sensitive hypothalamic nuclei did not exhibit significant cellular activation. Reductions in food intake caused by the administration of exogenous compound must be cautiously interpreted because this could be secondary to aversion and induced sickness behavior. The involvement of the NTS and AP in the development of conditioned aversions and gastrointestinal malaise was clearly established. The activation of NTS and AP neurons was reported in response to a variety of aversive inputs and stressful stimuli (Yamamoto et al., 1992; Swank, 1999; Spencer et al., 2012. Interestingly, we did not observe any significant difference between elicited c-Fos immunoreactivity in saline- and OP-treated mice in the AP. Moreover, Grill and Norgren (1978) reported that decerebrates rats, in contrast to controls, neither rejected nor decreased ingestive reactions to a novel taste after that taste had been repeatedly paired with lithium chloride-induced illness supporting the idea that the forebrain may be important for taste aversion learning. Here, we did not observed c-Fos expression in forebrain structures after OP administration. Altogether, these data lessened the possible presence of an OP-induced sickness behavior. Nonetheless, this point should be experimentally addressed in the future.

Cerebroventricular OP injection resulted in a significant increase in c-Fos expression within the interstitial solitary tract nucleus subnucleus. This subnucleus is known to be involved in the control of respiratory, cardiac and swallowing autonomic functions. Swallowing, the first motor component of ingestive behavior, allows the propulsion of the alimentary bolus from the mouth to the stomach. Swallowing is triggered by sensory afferent fibers conveyed by the superior laryngeal nerve that project through the solitary tract to the DVC and premotoneurons located within the interstitial and intermediate NTS constitute the SwCPG (Jean, 2001). Anorexigenic and orexigenic factors have been shown to modulate the swallowing reflex. For instance, we have previously shown that different anorexigenic factors, such as leptin (Félix et al., 2006), the growth factor BDNF (Bariohay et al., 2008), and the mycotoxin deoxynivalenol (Abysique et al., 2015) inhibit the swallowing reflex. Concurrently, the orexigenic cannabinoids are reported to induce a facilitation of the swallowing reflex (Mostafeezur et al., 2012). This led us to conceive a possible modulation of the swallowing reflex by endozepines. The present study constitutes the first demonstration that endozepines could inhibit the swallowing reflex. We studied the effect of ODN microinjections within the SwCPG and we reported a transient but significant inhibition of rhythmic swallowing. Extracellular ODN microinjection used here may concern both passing axons and NTS neurons surrounding the injection site. ODN could indeed reach multiple neural components but the observed inhibitory effect appeared to be specific since (i) swallowing inhibition was never observed with NaCl alone and (ii) in our experimental protocol, other autonomic functions regulated at the brainstem level such as cardiac and respiratory functions remained unaffected. Hence, in the light of the present data, endozepines join the group of

anorexigenic substances that both decrease food intake and inhibit swallowing.

In summary, the present work provides the first demonstration that endozepines are expressed by glial cells within the DVC. In this structure, ODN was found associated to protoplasmic astrocytes and tanycytes-like cells, located in regions known to integrate signals linked to the gastrointestinal tract and energy homeostasis. Moreover, the demonstration that endozepines affect food intake and swallowing reflex together with the close relationship of ODN expressing cells with neuronal elements strongly suggest that endogenous endozepines could act as local modulators of food intake behavior. The downstream ODN targets supporting the brainstem action of this peptide should be investigated in the future, with a particular attention to the melanocortin pathway (Lanfray et al., 2013).

AUTHOR CONTRIBUTIONS

FG, CG, AA, SG, RB, JV, and MD performed, analyzed and interpreted data for the work. JL provided ODN and OP peptide, MT provided ODN antibody. MT, JL, MD, AJ, JT, and BL designed the work. JL, MT, BL, and JT wrote the paper. All authors revised the final version and approved it to be published.

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MINI REVIEW
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Emerging Signaling Pathway in Arcuate Feeding-Related Neurons: Role of the Acbd7

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The understanding of the mechanisms whereby energy balance is regulated is essential to the unraveling of the pathophysiology of obesity. In the last three decades, focus was put on the metabolic role played by the hypothalamic neurons expressing proopiomelanocortin (POMC) and cocaine and amphetamine regulated transcript (CART) and the neurons co-localizing agouti-related peptide (AgRP), neuropeptide Y (NPY), and gamma-aminobutyric acid (GABA). These neurons are part of the leptin-melanocortin pathway, whose role is key in energy balance regulation. More recently, the metabolic involvement of further hypothalamic uncharacterized neuron populations has been suggested. In this review, we discuss the potential homeostatic implication of hypothalamic GABAergic neurons that produce Acyl-Coa-binding domain containing protein 7 (ACBD7), precursor of the nonadecaneuropeptide (NDN), which has recently been characterized as a potent anorexigenic neuropeptide capable of relaying the leptin anorectic/thermogenic effect via the melanocortin system.

Keywords: hypothalamus, leptin, POMC, ACBD7, food intake behavior

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INTRODUCTION

According to the World Health Organization (http://www.who.int/mediacentre/factsheets/fs311/en/) worldwide obesity prevalence has more than doubled since 1980. This situation is alarming given that obesity is often associated with costly diseases that include type-2 diabetes and cardiovascular diseases. In such a context, it appears urgent to improve the strategies to prevent or treat obesity, which cannot be elaborated without a deep understanding of the pathophysiology of excess fat deposition, hence the mechanisms whereby energy balance is regulated.

Obesity translates an imbalance between energy intake and energy expenditure leading to fat accumulation. In that respect, study of the causes of excess eating represents an inescapable step to understand obesity. Food intake as well as and energy expenditure are controlled by complex brain networks involving (i) cortical executive circuits, responsible for the self-control of eating and physical activity, (ii) corticolimbic reward pathways, which are involved in the integrations of hedonic and motivational signals, and (iii) autonomic hypothalamic and brainstem circuits that modulate the activity of the executive and reward structures while integrating peripheral homeostatic signals and controlling energy expenditure components (Gautron et al., 2015; Richard, 2015; Caron and Richard, 2017). Neurons of those networks produce numerous receptor types, neuropeptides and neurotransmitters that have been grouped into "pro-anabolic" (promoting obesity) or "pro-catabolic" (preventing obesity) chemical mediators.

AUTONOMIC REGULATION OF ENERGY HOMEOSTASIS

The autonomic circuits regulating energy balance mainly consist in two brain structures, namely the hypothalamus and brainstem, which coordinate their respective activity to control energy intake, by modulating the rostral forebrain appetite network (executive and reward systems), and to control energy expenditure, by for instance modulating brown adipose tissue (BAT) non-shivering thermogenesis. Due to their anatomic locations, hypothalamic and brainstem neurons of those two regions are able of sensing homeostatic hormones and nutrients translating energy balance fluctuations. Notably, the hypothalamus and brainstem are located near to circumventricular organs (CVOs), which are devoid of blood brain barrier allowing direct contact with nutrients and hormones (Schwartz et al., 2000).

The hypothalamus is constituted of nuclei comprising neurons involved in autonomic functions including the control of energy intake and energy expenditure (Gautron et al., 2015; Richard, 2015; Caron and Richard, 2017). Those neurons, which are still to be fully characterized are located in nuclei including the arcuate nucleus (ARC), ventromedial nucleus (VMH), dorsomedial hypothalamic nucleus (DMH), lateral hypothalamus (LH), preoptic area (POA), and paraventricular hypothalamic nucleus (PVH) (Schwartz et al., 2000). They for instance convey homeostatic signals between the hypothalamus and appetitive rostral forebrain systems (Gautron et al., 2015; Richard, 2015; Caron and Richard, 2017). The brainstem comprises the dorsal vagal complex (DVC), which includes interconnected neurons found in the nucleus of the solitary tract (NTS), area postrema (AP), and dorsal motor nucleus of the vagus nerve (Blevins and Baskin, 2010; Schwartz, 2010; Simpson and Bloom, 2010). Other brainstem structures including the pontine parabrachial nucleus (PBN), raphe pallidus (RPa), periaqueductal gray (PAG), and lateral paragigantocellular nucleus have been associated with SNS-mediated non-shivering thermogenesis, by mainly conveying information from the hypothalamus to the interscapular brown adipose tissue (iBAT) (Morrison and Nakamura, 2011).

The ARC in Energy Homeostasis

The hypothalamic nucleus that has been the most investigated in recent years with regard to energy homeostasis is undoubtedly the ARC (Gautron et al., 2015; Richard, 2015; Caron and Richard, 2017). The ARC is ventrally located on each side of the third ventricle just above the median eminence, a CVO allowing penetrance of peripheral hormones and nutrients (Schwartz et al., 2000; Richard, 2015). The ARC contains neurons producing proopiomelanocortin (POMC) and cocaine and amphetamine regulated transcript (CART) as well as neurons co-localizing agouti-related peptide (AgRP), neuropeptide Y (NPY), and gamma-aminobutyric acid (GABA), whose role in energy balance regulation have been acknowledged for years (Gropp et al., 2005; Luquet et al., 2005; Mayer and Belsham, 2009; Krashes et al., 2013). The NPY/AgRP/GABA- producing neurons exert anabolic effects while POMC/CART neurons are involved in catabolic processes. Those neurons have been referred to as "first order" neurons and project to "second order" neurons located in energy homeostasis-related nuclei, including the PVH and VMH, which individually form with the ARC prominent duets in the regulation of energy balance (Schwartz et al., 2000; Balthasar et al., 2005; Elmquist et al., 2005; Balthasar, 2006; Morton et al., 2006; Richard, 2015). Recently, single cell analysis performed in the hypothalamus revealed an important heterogeneity of ARC cells (Romanov et al., 2017), indicating that additional investigations will have to be make in order to fully characterized hypothalamic regulations of whole body homeostasis.

The Melanocortin System

ARC POMC/CART and NPY/AgRP/GABA neurons are major constituents of the melanocortin system, which is recognized as playing a genuine role in energy balance regulation (Adan et al., 2006; Butler, 2006; Cone, 2006; Ellacott and Cone, 2006; De Jonghe et al., 2011; Xu et al., 2011). POMC/CART neurons exert their hypophagic and thermogenic effects mainly by releasing the melanocortins α- and β-melanocyte-stimulating hormone (MSHs). α- and β-MSHs activate the melanocortin receptors 3 and 4 (MC3R, MC4R) to increase food intake and reduce energy expenditure (Cone, 2006). Interestingly, recent report indicate that POMC neurons are also able to released β-endorphin instead of MSHs indicating that those neurons should also act as orexigenic neurons (Koch et al., 2015). However, investigations performed on POMC knockout revealed morbid obesity resulting from hyperphagia as well as hypometabolism (Yaswen et al., 1999), indicating that POMC neurons mainly act as anorexigenic neurons. While the *Mc4r* knockout mice exhibit marked obesity, resulting from hyperphagia and hypometabolism (Huszar et al., 1997; Butler and Cone, 2003; Butler, 2006), genetic disruption of the *Mc3r* lead to a modest obesity phenotype (Chen et al., 2000; Butler and Cone, 2002, 2003), suggesting that MC4R constitutes the major MCR receptor involved in energy homeostasis. The prominent role of Mc4r in the hypothalamic regulation of energy balance has been confirmed in humans, in whom the *Mc4r* mutation leads to one of the most common forms of monogenic obesity (Coll et al., 2004).

NPY/AgRP/GABA neurons are also part of the melanocortin system. Together with inhibiting the activity of the POMC/CART neurons through a GABAergic effect (Pu et al., 1999), they release AgRP, a characterized anabolic peptide able to competitively inhibit α–MSH binding to the MC4R (Ollmann et al., 1997). AgRP has also recently been described as a biased agonist of MCR coupling to the inwardly rectifying potassium channel Kir7.1 (Ghamari-Langroudi et al., 2015). AgRP production is increased by fasting (Liu et al., 2012), supporting a physiological role for this peptide in the ARC control of energy homeostasis. Interestingly, non-conditional single KO $AgRP^{-/-}$ as well as double KO $AgRP^{-/-}$; $NPY^{-/-}$ display normal energy homeostasis (Qian et al., 2002), suggesting that NPY/AgRP neurons are dispensable in the hypothalamic control of energy homeostasis. However, the post-natal genetic disruption of AgRP induces hypermetabolism and hypophagia (Luquet et al., 2005), demonstrating some physiological relevance of the neuron

population. NPY has been considered as a robust orexigenic neuropeptide mainly by acting on Y1 and Y5 receptors (Richard, 2015). Interestingly, experiments performed in mice have indicated that the deletion of both Y1 and Y5 receptors induces anorexigenic effects (Nguyen et al., 2012), suggesting that Y1 and Y5 are receptors involved in energy balance. In contrast, the genetic disruption of *Npy* does not produce a lean phenotype or does not increase fasting-induced food intake, suggesting that *Npy* is not essential to the hypothalamic control of energy homeostasis. On the other hand, several investigations performed on *Npy* knockout mice revealed that those mice are less sensitive to high fat diet (Patel et al., 2006) (ref) and leptin (Erickson et al., 1996), which suggest that NPY could play a significant role in the hypothalamic integration of homeostatic signals. In that regard, future investigations appear require to further entirely decipher the role of NPY and its receptor in regulation of energy homeostasis.

The ARC as a Relay for Peripheral Homeostatic Signals

As alluded to above, ARC cells, including POMC/CART and NPY/AgRP/GABA neurons, are strategically located to act as relays between peripheral homeostatic signals and other hypothalamic circuits involved in energy balance. The homeostatic signals can be anabolic and catabolic circulating hormones and nutrients.

Among all characterized anabolic hormones, ghrelin has emerged as one of the most significant ones. Ghrelin, which is largely produced by stomach cells under fasting (Sanchez et al., 2004a,b; Vallejo-Cremades et al., 2004), promotes food intake and decreases energy expenditure (Ueno et al., 2005) by acting on the growth hormone secretagogue receptor type 1A (GHS-R) (Kim et al., 2003; Egecioglu et al., 2006; Bresciani et al., 2008). The physiological role of ghrelin in the energy homeostasis is supported by investigations performed in mice revealing that genetic disruption of *Ghrelin* or *Ghs-r* can prevent high-fat induced obesity (Lee et al., 2016). GHS-R is widely produced in the central nervous system, where ghrelin exerts its effects in several brain regions (Zigman et al., 2006). Furthermore, experiments performed in GHS-R-deficient mice have shown that specific re-expression of the GHS-R in ARC AgRP neurons partially restores the orexigenic effect of the ghrelin (Wang et al., 2014), which confirms the involvement of AgRP-producing neurons as relays in the hypothalamic ghrelin signaling pathway.

Among all key homeostatic hormones, leptin, which is produced mainly by white adipose tissue (MacDougald et al., 1995; Cinti et al., 1997; Niijima, 1998) (WAT), is considered as one of the most prominent catabolic circulating hormones (Elmquist et al., 1997; Friedman and Halaas, 1998; Elias et al., 1999; Friedman, 1999; Gautron et al., 2010; Gautron and Elmquist, 2011). Leptin levels increase with fat mass (Lonnqvist et al., 1997). Its access to the brain is insured by an active transport system apparently involving tanycytes (Balland and Prevot, 2014; Balland et al., 2014). Leptin acts by activating the LepRb receptor, which can be found in several populations of ARC cells including POMC/CART and NPY/AgRP/GABA neurons. Genetic disruption of the gene encoding leptin (*ob*) or its receptor (*Lepr*) leads to marked obesity, hyperphagia and reduced BAT thermogenesis in mice (Thenen and Mayer, 1976; Leiter et al., 1983; Garris, 1987, 1989; Malik and Young, 1996; Mizuno et al., 1998; Garris and Garris, 2004; Goncalves et al., 2009).

Notably, the obesity induced by the disruption of leptin signaling resembles that observed following *Pomc* or *Mc4r* nullification (Trevaskis and Butler, 2005). In such context one may argue the presence of a functional link between leptin and the melanocortin system, all the more so that POMC/CART and NPY/AgRP/GABA neurons express *LepR* mRNA (Baskin et al., 1999; Elias et al., 1999; Wilson et al., 1999; Williams et al., 2010) and that leptin increases and decreases the mRNA levels of *Agrp* and *Pomc* respectively (Elias et al., 1999; Cowley et al., 2001; van den Top et al., 2004; Takahashi and Cone, 2005). However, experiments performed in mice revealed that mice lacking *Lepr* on POMC neurons (e.g., *Pomc*-Cre, $Lepr^{lox/lox}$ mice) (Balthasar et al., 2004), on AgRP neurons (e.g., *Agrp*-Cre, $Lepr^{lox/lox}$ mice) (Tong et al., 2008) and on both POMC and AgRP neurons (e.g., *Pomc*-Cre, *Agrp*-Cre, $Lepr^{lox/lox}$ mice) (van de Wall et al., 2008) develop mild obesity, which suggests that the POMC/CART and NPY/AgRP/GABA could not be the only neurons interfacing the catabolic action of leptin. In that regard, the suggestion has been made that there could exist another population of neurons involved in the hypothalamic leptin signaling pathway. In that regard, mice lacking *Lepr* on GABA-producing neurons (Vong et al., 2011) (*Vgat*-Cre, $Lepr^{lox/lox}$ mice) develop strong obesity. Apparently, there are LepR-expressing GABAergic neurons, distinct from NPY/AgRP neurons that exert an inhibitory tone onto POMC neurons, which could be blunted by leptin (Vong et al., 2011). This presumption has however been challenged by experiments performed in obese model mice homozygous for the $Lepr^{S1138}$ allele, in which the ability to acutely decrease the GABA inhibitory tone is unaltered, despite the loss of the catabolic effects of leptin. In such a context, the characterization of the unidentified neurons capable of modulating POMC/CART neurons in response to leptin can be seen as major challenge of the current research in the neurobiology of obesity.

ARC ANORECTIC NEUROCHEMICAL CANDIDATES INTERFACING LEPTIN AND THE MELANOCORTIN SYSTEMS

The list of ARC neuromediators other than those released by POMC/CART and NPY/AgRP/GABA neurons, which could relay the catabolic message of leptin via the melanocrotin system, is rather short. It includes prolactin-releasing peptide (PrRP), neurotensin, diazepam-binding inhibitor/acylcoA binding protein (DBI/ACBP) and acyl-coA-binding domain containing protein 7 (ACBD 7).

PrRP is a potent anorexigenic neuropeptide acting via the Neuropeptide FF receptor 2 (NPFF2) receptor (Engstrom et al., 2003). It is expressed by ARC neurons harboring the LEPRs (Ellacott et al., 2002). Its expression is reduced in leptin-resistant Zucker rats, suggesting that leptin can directly activate PrRP

neurons (Ellacott et al., 2002). Moreover, a recent report revealed that PrRP was strongly enriched in LEPRs-positive neurons (Allison et al., 2015), indicating that PrRP positive neurons should play significant role in the leptin signaling pathway. However, mice lacking PrRP in LEPRs producing neurons (*PrRP*-Cre, and $Lepr^{flox/flox}$ mice) only develop mild obesity mainly due to lower energy expenditure (Dodd et al., 2014), which suggests that PrRP-producing neurons do not represent a major relay between leptin and the melanocortin signaling pathway.

Neurotensin is a 13-amino acid neuropeptide produced in the ARC, PVN, and DMH (Jennes et al., 1982; Beck et al., 1998). Its injection into the PVN decreases food intake (Stanley et al., 1983) and its production is increased by leptin injection (Beck et al., 1998). However, the evidence that leptin could mainly act through the ARC neurotensin-containing neurons is weak. Indeed, mice lacking the LEPR on NT neurons (*Nt*-Cre, $Lepr^{lox/lox}$ mice) develop only mild obesity (Leinninger et al., 2011).

The diazepam-binding inhibitor/AcylCoA binding Protein (DBI/ACBP) is a 87/88 amino acid polypeptide produced by astroglial cells in the rodent hypothalamus (Tonon et al., 1990). DBI/ACBP is processed into several gliopeptides, including the octadecaneuropeptide (ODN), a potent anorexigenic peptide (de Mateos-Verchere et al., 2001; do Rego et al., 2007; Lanfray et al., 2013, 2016). Although ODN is exclusively produced by astroglial cells in the hypothalamus (Tonon et al., 1990), it can be considered as a candidate in the leptin-melanocortin pathway since astrocytes have been shown to produce LEPRs (Hsuchou et al., 2009a,b). Interestingly experiments perform in laboratory rodents indicate that the anorexigenic effect of icv injection of ODN is relayed by central activation of the MC3/4R (Lanfray et al., 2013), suggesting that ODN could directly activate POMC/CART neurons. Moreover, it has been recently demonstrated that the pharmacological disruption of the endozepine metabotropic receptor blunts the anorexigenic effect of the leptin (Lanfray et al., 2016), indicating that ODN may be involved in the leptin-melanocortin pathway. However, it has been demonstrated that the *Dbi/Acbp* mRNA levels are not affected by leptin in mice (Compere et al., 2010), which suggests that ODN is probably not a major component in the action of leptin on the melanocortin system.

ACYL-COA-BINDING DOMAIN CONTAINING PROTEIN 7 (ACBD7) AS A NEUROMEDIATOR INVOLVED IN THE CENTRAL EFFECTS OF LEPTIN

ACDB7 Origin

ACBD7 is a member of the ACBD protein family, which includes proteins containing the acyl-coA-binding domain motif signature (Burton et al., 2005; Neess et al., 2015). This protein family contains the well-characterized ACBD1, also known as DBI/ACBP (see above), which is known to be involved in numerous intracellular processes including fatty acid, glycerolipid, and glycerophospholipid biosynthesis, cellular differentiation and proliferation, and β-oxydation. Several hypothetical related-proteins have been characterized *in silico,* including ACBD7, which represents the product of a well-conserved paralog gene of the DBI/ACBP. Interestingly, sequence analysis has revealed that ACBD7 contains all the residues relevant for DBI/ACBP stability and acyl-CoA binding efficiency (Burton et al., 2005). However, while the three-dimensional conformation of the ACBD7 has been characterized (Neess et al., 2015), its ability to bind acyl-CoA esters remains to be established.

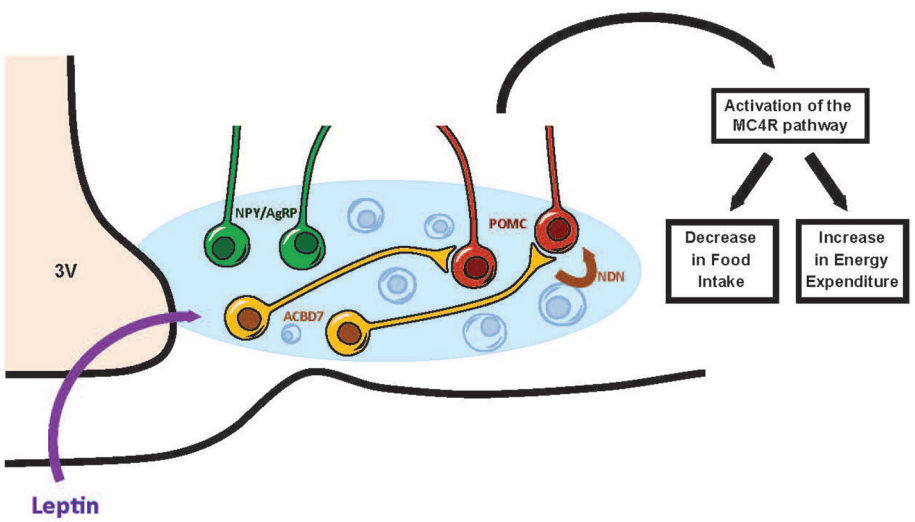


FIGURE 1 | Scheme tentatively summarizing the involvement of ACBD7 and its related product NDN in the effects of leptin in the ARC. According to this scheme, ACBD7-producing neurons would release NDN in response to an increase of leptin (Leptin) concentrations and stimulate the activity of POMC neurons. Activation of POMC neurons of the ARC would lead to the activation of the melanocortin signaling pathway and subsequently to a decrease in food intake and an increase in energy expenditure.

Considering the highly conserved exon/intron structure (Lanfray et al., 2016) of Acbd7, it has been postulated that the duplication of the ancestor gene occurs prior to the divergence of fish and higher vertebrates (450 Mya) (Burton et al., 2005). Interestingly, as for its paralog gene product (i.e., Dbi/Acbp), ACBD7 contains strongly conserved lysine allowing for the production of potential bioactive peptides, including a potential bioactive central fragment, released from a tryptic maturation process. By using a mass spectrometry-multiple reaction monitoring MS-MRM approach, we demonstrated that a 19-amino acid peptide-derived from ACBD7 (called nonadecaneuropeptide—NDN) was present in the mouse hypothalamus, demonstrating that Acbd7 was produced and processed *in vivo* (Lanfray et al., 2016).

The expression of Acbd7 in the hypothalamus suggests that ACBD7 may exert specific autonomic functions (Neess et al., 2015). We recently confirmed that Acbd7 was expressed by ARC and PVN neurons (Lanfray et al., 2016), two structures described above as key in the hypothalamic regulation of energy balance. Additionally, immunohistochemistry experiments have shown that ACBD7 is produced by ARC neuronal cells apparently differing from NPY/AgRP/GABA and POMC/CART (Lanfray et al., 2016). Additionally, our investigation indicates that ACBD7 immunoreactivity is co-localized with VGAT immuno-labeling (Lanfray et al., 2016), demonstrating that ACBD7 is produced by GABAergic neurons in the hypothalamus.

Effects of ACBD7 on Energy Homeostasis

The observation that ARC ACBD7 produced a fragment homologous to the anorexigenic DBI/ACBP-derived peptide ODN prompted us to hypothesize that ACBD7-containing neurons could be involved in energy balance, all the more so that there existed a *Acbd7* polymorphism that had been associated to obesity in humans (Comuzzie et al., 2012).

To determine the role of ACBD7 on energy homeostasis, we assessed the effects of NDN on both food intake and energy expenditure. Our investigations performed in mice revealed that intracerebroventricular (icv) administration of NDN induced an early and marked inhibition of food intake in fasted mice (Lanfray et al., 2016). Our investigations also demonstrated that the icv injection of NDN increased both O_2 consumption and UCP-1 expression in interscapular BAT (Lanfray et al., 2016), suggesting that NDN could also enhance energy expenditure. We also observed that the subchronic treatment with NDN (5 days) reduced food efficiency. Notably, the anorexigenic effect of NDN was blunted by the antagonism of the MC4R (Lanfray et al., 2016), the main effector of the melanocortin signaling pathway, suggesting that NDN acted upstream to the melanocortin signaling pathway.

We also demonstrated that Acbd7 mRNA levels, the ACBD7 protein levels and hypothalamic NDN levels varied with energy availability (Lanfray et al., 2016). This supports the notion that ACBD7-producing neurons are stimulated by one or several catabolic hormones/factors. In that regard, we demonstrated that the leptin treatment could increase the production of both ACBD7 and NDN, suggesting that leptin is able to stimulate ACBD7-producing neurons (Lanfray et al., 2016). Moreover, we demonstrated that the acute pharmacological disruption of the endozepines metabotropic receptor signaling blunted the anorexigenic effect of leptin (Lanfray et al., 2016).

Altogether the data accumulated so far suggest that (i) ACBD7 and NDN are produced by ARC GABAergic neurons different from POMC/CART and NPY/AgRP/GABA neurons, (ii) NDN is a anorexigenic peptide acting probably via the activation of ARC POMC/CART neurons, and (iii) NDN signaling contributes to the leptin-melanocortin pathway. While the identity of the uncharacterized endozepine metabotropic receptor remains to be fully established, we can postulate that the ARC ACBD7 producing neuron represents a significant relay between leptin and the melanocortin signaling pathway.

CONCLUSION

This review has focused on the recent discoveries regarding the hypothalamic leptin signaling pathway and on potential ARC anorectic neurochemical candidates interfacing leptin of the melanocortin system. Up until recently, it was though that leptin action on ARC POMC/CART and NPY/AgRP/GABA neurons was essentially mediated through the leptin receptors (LepR) found on those neurons (Elias et al., 1999; Balthasar et al., 2004; Zhang and Scarpace, 2006). However, recent data have indicated that the leptin effect on the melanocortin system can be relayed by an uncharacterized class of ARC neurons that are distinct from POMC/CART and NPY/AgRP/GABA neurons (Balthasar et al., 2004; van de Wall et al., 2008; Hill et al., 2010; Vong et al., 2011). In this context, the identification of ARC neuromediators other than those released by the POMC/CART and NPY/AgRP/GABA neurons that could relay the catabolic message of leptin via the melanocortin system, appeared justified in our understanding of the pathophysiology of obesity. We identified ARC ACBD7 and its anorexigenic maturation product NDN as playing a role in energy homeostasis. NDN acts mainly by stimulating the melanocortin signaling pathway while NDN signaling disruption blunts the anorexigenic effects of leptin (**Figure 1**). Future investigations to further examine the involvement of ACBD7 production by ARC Lepr-producing neurons in the hypothalamic leptin signaling pathway appear warranted.

AUTHOR CONTRIBUTIONS

DL contributed to manuscript preparation and manuscript definition of intellectual content. DR also contributed to manuscript preparation and followed by manuscript editing and revision.

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REVIEW
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Non-Neuronal Cells in the Hypothalamic Adaptation to Metabolic Signals

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Although the brain is composed of numerous cell types, neurons have received the vast majority of attention in the attempt to understand how this organ functions. Neurons are indeed fundamental but, in order for them to function correctly, they rely on the surrounding "non-neuronal" cells. These different cell types, which include glia, epithelial cells, pericytes, and endothelia, supply essential substances to neurons, in addition to protecting them from dangerous substances and situations. Moreover, it is now clear that non-neuronal cells can also actively participate in determining neuronal signaling outcomes. Due to the increasing problem of obesity in industrialized countries, investigation of the central control of energy balance has greatly increased in attempts to identify new therapeutic targets. This has led to interesting advances in our understanding of how appetite and systemic metabolism are modulated by non-neuronal cells. For example, not only are nutrients and hormones transported into the brain by non-neuronal cells, but these cells can also metabolize these metabolic factors, thus modifying the signals reaching the neurons. The hypothalamus is the main integrating center of incoming metabolic and hormonal signals and interprets this information in order to control appetite and systemic metabolism. Hence, the factors transported and released from surrounding non-neuronal cells will undoubtedly influence metabolic homeostasis. This review focuses on what is known to date regarding the involvement of different cell types in the transport and metabolism of nutrients and hormones in the hypothalamus. The possible involvement of non-neuronal cells, in particular glial cells, in physiopathological outcomes of poor dietary habits and excess weight gain are also discussed.

Keywords: hypothalamus, metabolism, energy, homeostasis, glia, inflammation

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INTRODUCTION

Our understanding of the neuronal circuits controlling metabolism has advanced in recent years and progress has been made in the development of potential treatments for obesity, particularly in specific monogenic forms of obesity (1). However, the brain is not composed of neurons alone; other cell

types actually outnumber these electrically excitable nerve cells and participate in and/or modulate all neuronal functions. In the hypothalamus, this includes the participation of non-neuronal cells in the modulation of neuronal circuits controlling appetite and metabolism.

Non-neuronal cells in the central nervous system (CNS), including glia, epithelial cells, pericytes, and endothelia, perform a wide spectrum of functions throughout the brain. Many of these functions are common in each brain area, although the specific outcomes are at least in part dependent on the neuronal circuits that are affected by their actions. Moreover, within each class of non-neuronal cell type there are generalized subclassifications that, although quite incomplete, indicate diverse functional states. There are also specialized cell types found only in specific areas of the brain. One important example that will be discussed in greater detail is tanycytes, specialized glial cells found lining the third ventricle and in close proximity to the neuroendocrine hypothalamus. The fact that there is wide heterogeneity within each non-neuronal cell type has become increasingly clear; however, we currently do not have the tools available to sufficiently distinguish between these subpopulations and this has clearly hindered advances in this field.

With the explosion in the prevalence of obesity that has occurred almost worldwide, investigation in the area of metabolic control has become a priority. This has led to an increase in our understanding of how non-neuronal cell types participate in the neuroendocrine control of appetite and energy expenditure, as well as in the response to increased weight gain and the development of secondary complications. Here, we have briefly outlined the different types of non-neuronal brain cells and some of their functions, both in general and those that are specific to the hypothalamus and metabolic circuits.

CLASSIFICATION OF NON-NEURONAL CELLS IN THE BRAIN

Astroglial Cells

Astrocytes were the first class of glial cells to be described (2) and they are also the most studied. One example of this is that a search of the word "astrocyte" in the PubMed Central database obtains approximately 48,000 results; typing "microglia" or "oligodendrocyte" receives less than 30,000 returns in either case. Astroglia are also the most abundant cell type in the CNS and were first thought to only constitute the physical and metabolic support for neuronal function (2). We now know that they are much more than just "neuron helpers" (3). Astrocytes do indeed transport nutrients and metabolic factors across the blood–brain barrier (BBB) and release them to the extracellular fluid where they can be used by neurons and other glial cells (4, 5) (**Figure 1**). However, it is now clear that this supply of energy substrates to other cell types is regulated with astrocytes responding to metabolic changes in order to maintain brain homeostasis (6–9). Astrocytes are also the only glial cells known to store energy through glycogenesis (10). In the synaptic cleft, they reuptake neurotransmitters and also can release gliotransmitters, forming part of what is called the "tripartite synapse" (11, 12). At the level of the BBB, astrocytes are involved in the formation and maintenance of some of the barrier properties (13) and can regulate vasodilatation, thus controlling the flow of blood-borne substances (14, 15).

Astrocytes are connected by gap junctions in their plasma membranes, which enable direct transport of small molecules between cells. Initially, it was thought that these channels allowed passive diffusion of substances; however, the transport through gap junctions is tightly regulated (16, 17). One important function of these gap junctions is the rapid transmission of calcium waves within the glial network, resulting in a form of non-neuronal signal transmission (18).

When employing classical labeling methods, astrocytes appear to have a star-shaped morphology, although two different forms, protoplasmic and fibrous, can be distinguished. The first are mainly found close to synapses and blood vessels, whereas the latter are frequently found within the white matter (19–21). The morphology of these glial cells also changes in respect to their functional or activational state. The fact that astrocytes differentially express certain proteins (e.g., receptors, enzymes, channels, etc.) depending on the brain area and the physiological or pathophysiological conditions to which they are subjected raises questions regarding the current definition and classification of astroglial cells (22). Growing evidence indicates that astrocytes are vastly heterogeneous (23–28). For example, Matthias and colleagues reported that within the hippocampus subsets of GFAP expressing cells expressed either glutamate transporters or glutamate receptors (23). Moreover, astrocytes throughout the brain differentially express connexins (24) and GABA and glutamate receptors (26) and different astrocyte populations are reported to differentially support developmental functions and synapse formation (28, 29). Thus, our understanding of the functions of astrocytes is advancing, but much is yet to be learned. Indeed, we are only now beginning to have the tools to understand the grand diversity of these glial cells.

Microglia

Microglial cells constitute the bulk of the immune system in the brain. There have been different systems suggested for the classification of microglia, with most engaging morphological features. The most general classification includes an amoeboid form, characteristic of early development, and a ramified form or "resting" microglia and reactive microglia (30–32). The phenotype of reactive microglia is defined by changes in morphology, to short and thick projections, and the release of factors like cytokines, nitric oxide, and reactive oxygen species (30, 31, 33, 34). This "activation" or change in phenotype can occur in response to brain damage, toxic substances, or injury due to harmful conditions like obesity or a high fat diet (HFD) (35, 36) and when this state is sustained, it can lead to a pathological chronic state of reactive microgliosis (37). However, the division that separates resting and reactive microglia has become more diffuse as we learn more about these cells (38).

One of the main functions of microglia is to "clean" the CNS by phagocytosis of cellular debris, foreign matter, and other wastes (39). In this manner, they participate in development and

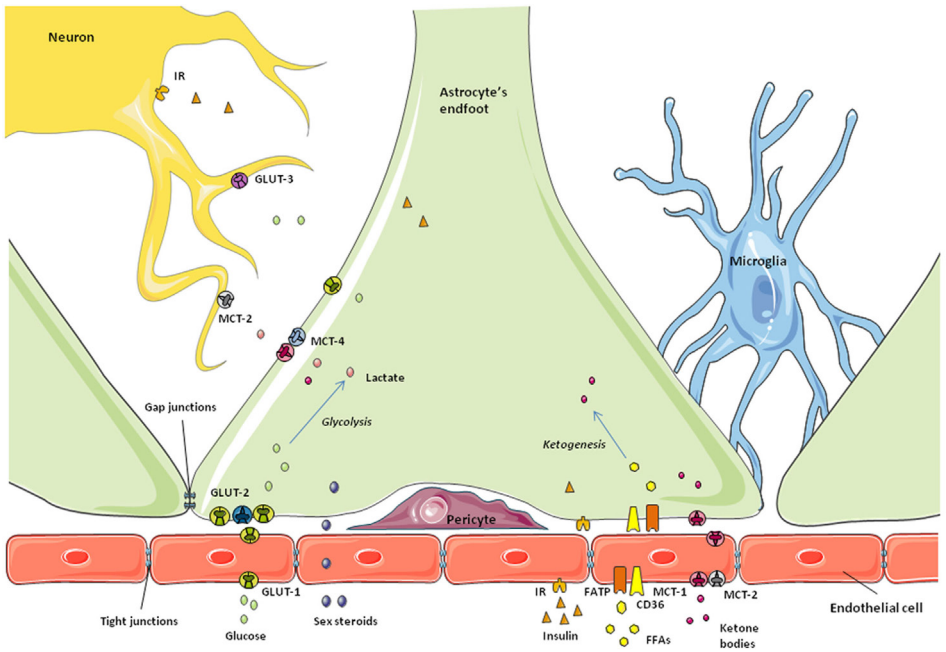


FIGURE 1 | Schematic representation of the blood–brain barrier. Astrocytic endfeet surround the microvessels and take up the nutrients and metabolic factors coming from the bloodstream. Endothelial cells at this level express specific receptors and transporters and restrict the passage of small molecules to the brain due to the tight junctions between them. Depending on the metabolic state, nutrients and factors are processed by astrocytes to control their access to neurons and maintain brain homeostasis.

synaptic plasticity (40–42). They can also release gliotransmitters and metabolic factors, contributing to maintain brain homeostasis (38, 43). Importantly, as part of the immune system, microglial cells respond to injury and harmful factors, including fatty acids, by releasing cytokines and to infection by presenting antigens to T-cells (35, 39, 43).

Oligodendrocytes

Oligodendrocyte projections wrap neuronal axons, forming the myelin sheaths in the CNS. To date, no direct link between these cells and systemic metabolic function has been verified, although some studies connecting metabolic signals with changes in myelination or oligodendrocyte survival suggest at least an indirect relationship with metabolism (44–48). However, it has been recently shown that oligodendrocyte precursors (NG2 glia) in the median eminence are important for the function of leptin receptor-expressing neurons, whose dendritic processes they contact (49).

Tanycytes

These specialized ependymal-like glial cells lining the ventral and ventrolateral part of the third ventricle (**Figure 2**) are proving to be very interesting as we know more about them. From dorsal to ventral, they are classified as subtypes α1, α2, β1, and β2. They are polarized cells: on the ventricle-side they express numerous receptors and transporters in their membrane and can be ciliated (not β2 tanycytes); and on the opposite side they present a long process that projects into the hypothalamic parenchyma or the median eminence (50). The β2 tanycytes can be found close to the median eminence, a subhypothalamic circumventricular organ. Capillaries on the median eminence are fenestrated, making the BBB permeable to many substances (50–52). The long processes of β2-tanycytes project into these fenestrated vessels, forming a blood–CSF barrier (BCSFB). The tight junctions between them, in addition to the specific transporters that they express, allow them to control the entry of many substances into the hypothalamus (53). They can also regulate the permeability of this barrier at this level of the brain, by the release of vascular endothelial growth factor-A in response to metabolic changes (54) and possibly by other mechanisms (55). Although astrocytes are the major cells expressing gap junctions, tanycytes also express these structures and can also produce calcium wave signaling (56). Tanycytes also possess stem cell properties (57) and participate in glutamate recycling (58), nutrient sensing (59, 60), and the conversion of thyroid hormones (TH) (61).

Pericytes

Pericytes are contractile cells surrounding the blood vessels (62, 63). In addition to their ability to modify blood flow due to their contractibility, brain pericytes have multiple roles in the development and maintenance of the BBB (64, 65), including macrophage-like functions and characteristics (66–68), angiogenic properties (69), and a role in neuroinflammation (70). Indeed, in response to brain injury, there is evidence that pericytes change to a microglia-like phenotype (68, 71), migrate

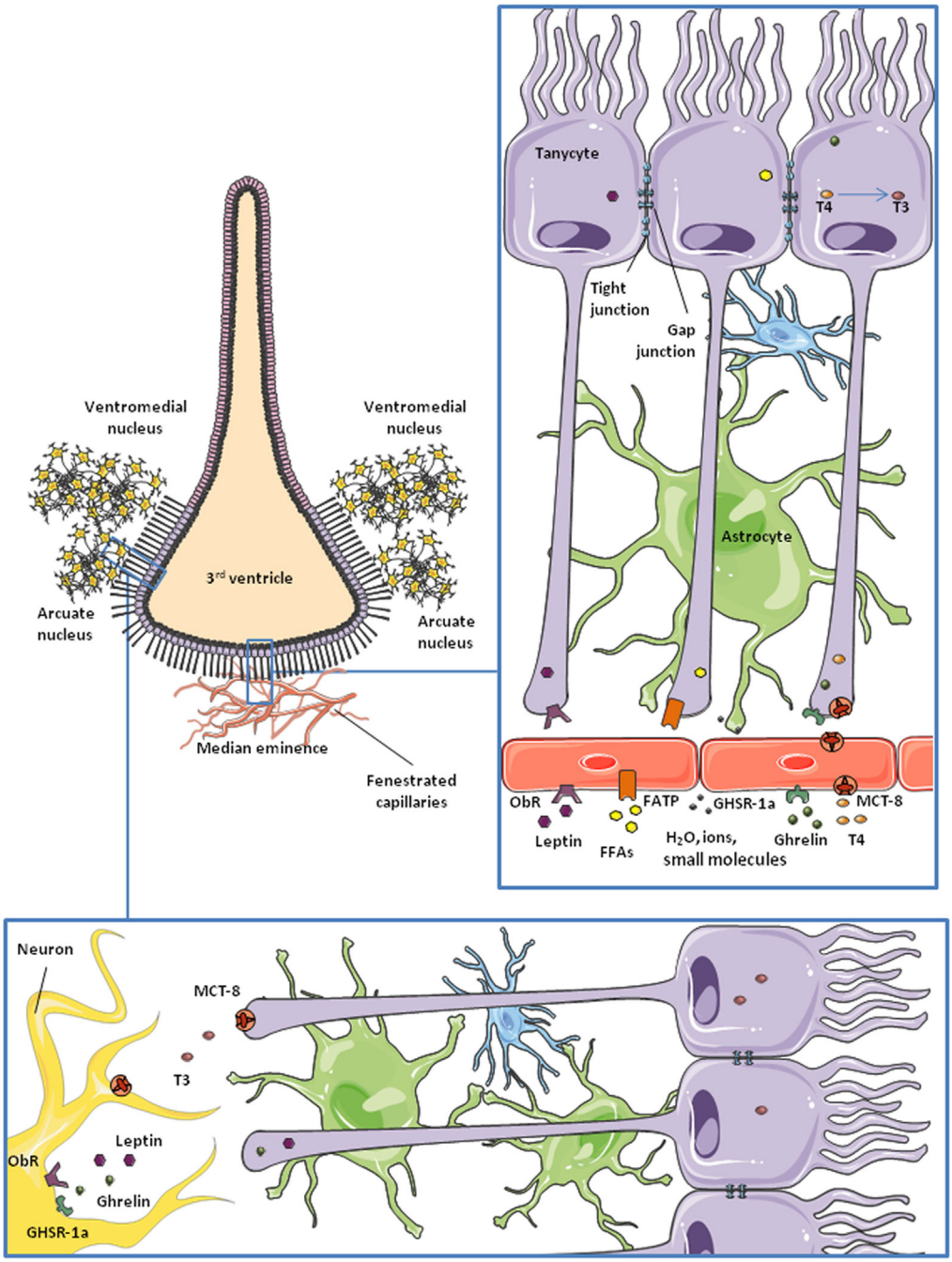


FIGURE 2 | Schematic representation of tanycytes lining the third ventricle. Microvessels of the median eminence are fenestrated, so they allow water, ions, and small molecules to freely enter the brain. β2-tanycytes present tight junctions between them, forming a blood–CSF barrier. They take up nutrients and factors from the microvessels and control their access to the cerebrospinal fluid (CSF) and the rest of the brain. This is probably the main route for some hormones and nutrients to the hypothalamus. Also, tanycytes are communicated through gap junctions, so some molecules can be transported to lateral β1 and α-tanycytes and gain access to the arcuate nucleus and the ventromedial nucleus of the hypothalamus.

to the brain parenchyma (72), and are involved in scar formation (73), antigen presentation (74), and the release of inflammatory factors (75, 76). Pericytes are also reported to be multipotential stem cells in the CNS (77). However, the identity of these stem cells is still a subject of controversy (78), due to the lack of reliable pericyte markers (79).

Endothelial Cells

Endothelial cells, along with pericytes, form the walls of the microvessels, taking part in the transport of metabolites through the BBB (80). The particularities of BBB endothelial cells, described below, allow for a strict control of the passage of substances from the blood into the CNS.

Epithelial Cells/Ependymocytes

In the CNS, epithelial cells can be found in the choroid plexus and lining the ventricles. They secrete the cerebrospinal fluid (CSF) that fills the ventricles and factors involved in neurogenesis and brain development (81–84). Epithelial cells of the CNS also express transporters for glucose, amino acids, and other molecules (85–87), as well as receptors for hormones such as sex steroids (88–90) and leptin (91). Moreover, they form a type of BCSFB due to the tight junctions between them (92). Ependymal cells are epithelial cells lining the ventricles. Their polarized organization and beating of numerous cilia are important for the movement of CSF (93, 94). They also possess precursor properties and, together with tanycytes, form the hypothalamic neurogenic niche (95).

FUNCTIONS OF NON-NEURONAL CELLS

Transport of Metabolic Signals into and within the Hypothalamus

The transport of nutrients and other metabolic signals is one of the best studied functions of non-neuronal cells in the nervous system. At the physiological level, nutrients from the diet, hormones, and other substances are delivered to all tissues through the bloodstream. However, due to its exceptional importance and vulnerability, the CNS protects its homeostasis by carefully controlling what can and cannot enter from the circulation. This function is carried out by the BBB, which is formed by specialized glia, pericytes, and endothelial cells expressing transporters, receptors, and sensors that allow them to select the information and nutrients accessing the nervous tissue (80) (**Figure 1**). As nutrients and metabolic signals are also found in the CSF, there is a BCSFB, formed by ependymal cells and tanycytes, in the third ventricle (50, 96, 97) (**Figure 2**). The distribution of tight junction proteins between tanycytes at this level is important in determining the permeability of the barrier, being lower at the median eminence, where there are fenestrated capillaries and higher next to the arcuate nucleus (98).

The first checkpoint for any substance to cross the BBB into the CNS is the endothelial cell, the bricks forming the capillary walls (**Figure 1**). Endothelial cells in the BBB are phenotypically different from those of peripheral vessels and restrict the access of blood-borne substances to the extracellular fluid of the CNS (80, 99). To achieve this, these endothelial cells have tight junctions between them, reduced endocytosis, no fenestrations, and specific transporters and receptors, in addition to a large number of mitochondria (65). Thus, brain capillary endothelial cells broadly determine the barrier permeability. Surrounding these capillaries are the astrocytic endfeet, along with pericytes and microglia (**Figure 1**). These other cells also participate in the regulation of nutrient and hormone entry, and thus metabolic signaling, from the periphery (80, 99, 100). Astrocytes and other non-neuronal cells can detect changes in the concentrations of specific nutrients and the presence of other signals and react consequently to maintain brain homeostasis, as described below.

Glucose

Glucose, the main energy source of the CNS, enters the brain from the bloodstream crossing the BBB through specific transporters. As normal brain function depends on its glucose supply, this step is highly regulated. That is, the transport of glucose across the BBB adapts in response to cerebral energy demand in order to maintain glucose homeostasis in the brain. The facilitative glucose transporter (GLUT)-1 is largely responsible for glucose transport across the BBB. This protein is expressed in non-neuronal cells throughout the CNS, especially in astrocytes and endothelial cells of the BBB (101), as well as in tanycytes along the BCSFB (50). However, GLUT-1 in endothelial cells is highly glycosylated, having a higher molecular weight than the isoform expressed in astrocytes and other glial cells (101, 102). As indicated in a recent review, some authors suggest different functional characteristics between the two forms of GLUT-1, although there is no consensus on this subject (103).

Changes in glucose concentration are rapidly detected in the hypothalamus, which adapts to such variations and emits a response to maintain glucose homeostasis not only in the brain, but also systemically as glucose-sensing neurons in the hypothalamus send signals to the autonomous nervous system, reaching peripheral organs such as the pancreas or the liver (104–107). There is more than one mechanism for central glucose sensing and different cell types are involved in this essential task (107–110). Two populations of glucose-sensing neurons have been identified: glucose-excited and glucose-inhibited neurons (GE and GI, respectively) (111) and glial cells also participate in these important glucose-sensing mechanisms. Astrocyte endfeet express GLUT-2 which, in addition to its transport functions, participates in glucose sensing (110, 112). This GLUT is highly expressed in tanycytes along the BCSFB (109), with these specialized glial cells also participating in glucose-sensing processes. In addition to expressing GLUT-2, astrocytes and tanycytes express sodium glucose transporter (SGLT)-1, glucokinase (GCK), and K_{ATP} channels (110), proteins that are all known to be involved in glucose-sensing mechanisms. Indeed, the classical mechanism for glucose sensing in pancreatic β-cells requires glucose uptake through GLUT-2 in rodents or GLUT-1 in humans, GCK, and activation of ATP-sensitive K^+ channels (112, 113). This system shares some similarities with glucose-sensing pathways in astrocytes and tanycytes.

One proposed model for glucose sensing in tanycytes involves glucose entering the cell through GLUT-2 and phosphorylation by GCK. Subsequently, glucose-6-phosphate undergoes glycolysis, producing pyruvate and, through the action of lactate dehydrogenase, lactate. Lactate is transported to the extracellular space by monocarboxylate transporter (MCT)-4 or MCT-1, and then taken up by neurons through MCT-2 (109). Depending on the kind of neuron, GE or GI, an excitatory or inhibitory signal

will be produced in the hypothalamus and sent to other brain areas and the autonomic nervous system (108). Tanycytes can also respond rapidly to glucose and other inputs by producing calcium waves, a process requiring ATP release and autocrine signaling through purinergic P2Y receptors (56, 59). The precise mechanisms involved in this tanycytic response are not yet fully elucidated, but it constitutes a possible model for tanycyte–neuron interaction.

Glucose sensing in astrocytes involves a similar process. Indeed, according to the "astrocyte-neuron lactate shuttle" hypothesis proposed by Pellerin and Magistretti over two decades ago (4), lactate from glucose or glycogen metabolism released by astrocytes is not only used by neurons as an energy source but can also signal energy availability to glucose-sensing neurons. Glucose transport into astrocytes is facilitated by GLUT-2 or occurs through gap junctions in a passive manner (112, 114–116). This glucose can be metabolized or stored as glycogen. However, it is still debated as to whether astrocytes secrete only lactate or also glucose to the extracellular fluid to act on glucose-sensing neurons and to be used as fuel (112). Moreover, astrocytes and tanycytes can respond to an increase in glucose or to other signals (i.e., some neurotransmitters) by secreting endozepines, anorexigenic peptides that act on hypothalamic neurons to maintain energy homeostasis (107, 117) and that also participate in unsaturated long-chain fatty acid metabolism in astrocytes (118).

The precise mechanisms of glucose transport and sensing in the hypothalamus are yet to be fully elucidated. For example, SGLT, an active sodium co-transporter, is reported to be involved in glucose sensing in the ventromedial nucleus of the rodent hypothalamus (119), although it is not clear whether this sensing occurs in glucose responsive neurons or in astrocytes. By using genetically engineered mouse models, García-Cáceres and collaborators recently demonstrated that insulin signaling in astrocytes plays a role in the regulation of systemic glucose homeostasis. Specific ablation of the insulin receptor (IR) in astrocytes was shown to impair their uptake of glucose and the ability to correctly respond to changes in glycemia (120). Other studies suggest a role of leptin in increasing (121) or ghrelin in reducing (122) glucose uptake by astrocytes, which might also affect glucose sensing. It thus appears that the transport of glucose by astrocytes is highly regulated by diverse nutrient and hormonal signals.

Ketone Bodies

Monocarboxylates are molecules with one carboxylate group; some examples with metabolic functions include not only lactate, but also pyruvate and ketones, all of which can be used by neurons as an alternative energy source in addition to acting as metabolic signals (123–126). The brain expresses MCTs-1, -2, and -4, with MCT-1 being found in endothelial and ependymal cells, as well as in astrocyte endfeet at the BBB (127, 128). MCT-2 is expressed in endothelial cells, but not in astrocytes, whereas MCT-4 appears to be specific for astrocytes (58, 129–131). Ketone bodies and other monocarboxylates from the bloodstream cross the BBB through specific MCTs present in both the luminal and abluminal sides of the endothelial cells (132, 133). Astrocyte endfeet not only takes up monocarboxylates through MCT-1 (132–134), but these glial cells are also able to synthesize ketone bodies from fatty acid β-oxidation and secrete them as an energy source for neurons and other glial cells (**Figure 1**). Tanycytes have also been suggested to transport lactate through MCT-2 in a photoperiodic model of Siberian hamster (58). These authors found that MCT-2 and the glutamate transporter GLAST were decreased during a short photoperiod, which could indicate a change in seasonal neurotransporter supply. In the rat brain, tanycytes were shown to express functional MCT-1 and MCT-4 in an anatomically specific manner (135), suggesting that these glial cells may also participate in lactate transport to neurons.

Regulation of the transport and production of ketone bodies in the brain is important in metabolic control as hypothalamic sensing of these monocarboxylates also participates in the regulation of food intake (126, 136). Indeed, after the initial HFD-induced hyperphagia, there is a reduction in food intake that is reported to be mediated, at least in part, by ketone body signaling to hypothalamic neurons. These ketone bodies are synthesized by hypothalamic astrocytes as products of fatty acid metabolism (136, 137).

Lipids

Lipid sensing in the hypothalamus is necessary for the correct regulation of energy balance (138). There are lipid sensing neurons that are excited or inhibited by fatty acids, depending on the type of neuron and also the metabolic state, i.e., fasting versus overfeeding (139). Although the role of glial cells in this process is not fully understood, astrocytes are the primary lipid metabolizers in the CNS. They also express proteins related to lipid sensing, such as transporter CD36 and peroxisome proliferator-activated receptor gamma, an important lipid-activated nuclear receptor that regulates transcription of numerous genes, including some involved in lipid metabolism (140, 141). In addition, astrocytic production of ketone bodies from fatty acids and their release to neurons could be one way by which an excess of fatty acids is signaled to metabolic neuronal circuits. Recent evidence suggests an increase in fatty acid β-oxidation in hypothalamic astrocytes from obese mice fed a HFD, as well as a role for tanycytes in restricting the passage of saturated fatty acids into the hypothalamus (142).

Although the brain produces lipids, it also has mechanisms to transport them from the bloodstream, but how they go through the BBB is not yet fully understood. Short and medium chain fatty acids appear to enter the CNS by simple diffusion through the plasma membrane (143). In contrast, long chain fatty acids (>12 carbons) need transporters to cross the BBB (144), with several fatty acid transport proteins (FATP) and fatty acid binding proteins (FABP) having been identified (145). *In vitro* studies indicate that FATP-1, FATP-4, and FABP-5 are the major isoforms expressed in microvessel endothelial cells and the gray matter of the human brain (145, 146). When the fatty acid translocase (FAT) CD36 is knocked-out in mice (CD36−/−), the uptake of monounsaturated fatty acids is significantly decreased, with no effect on polyunsaturated fatty acid uptake (147). In the CNS,

CD36 is expressed in endothelial cells, microglial cells, astrocytes, and in ventromedial hypothalamic neurons (148–151). Although it is not the most highly expressed FATP, studies indicate that CD36 is responsible for fatty acid sensing in the hypothalamus and is thus important for the control of energy homeostasis (136, 137, 152).

In addition to the passage of free fatty acids through the BBB, lipids can also enter or exit the CNS as lipoproteins. This process is mediated by apolipoprotein E (ApoE) interacting with lipoprotein receptors (153). In the CNS, ApoE is expressed in astrocytes and tanycytes and its levels are upregulated by both leptin and TH (154, 155), with this process being involved in the regulation of food intake and energy balance (156).

Hormone Transport and Signaling

Leptin

Leptin is an anorexigenic hormone that exerts part of its effects by inhibiting orexigenic neurons and activating anorexigenic neurons in the hypothalamus (157–159). It also has a role in the regulation of systemic lipid and glucose metabolism (160, 161). The leptin (or obesity) receptor (ObR), which has six isoforms, is highly expressed in brain endothelial cells, astrocytes and tanycytes (162–165), and endothelial and astroglial cells have been studied in attempt to unravel the mechanisms of leptin transport into the brain (163, 166). However, González-Carter and colleagues have recently reported that, in a human *in vitro* BBB model, leptin–ObR interaction is not necessary for the transport of this hormone across the BBB. They propose that lipoprotein receptor-related protein-2, expressed in endothelial cells at the BBB, is responsible for the passage of leptin across the BBB (167). Increasing evidence points to the BCSFB as the main pathway for entry of leptin into, at least, the hypothalamus (165, 168) (**Figure 2**).

The median eminence, a circumventricular organ close to the third ventricle, is the first site in the brain reached by blood-borne leptin (165). After an intraperitoneal leptin injection, there is a 1–2 h lag between the activation of leptin signaling pathways in the ventral and dorsal nuclei of the hypothalamus. This time-lag disappears when leptin is administered intracerebroventricularly, instead of intraperitoneally (169), suggesting that leptin transport from blood to the CSF is an important step in the action of this hormone in the brain and that it involves a delay in circulating changes reaching central target sites. Moreover, this process appears to be a finely regulated step in the control of energy balance as tanycytes act as "gatekeepers" for the passage of leptin into the mediobasal hypothalamus. Evidence suggests that leptin is first taken up by tanycyte processes in contact with the fenestrated capillaries at the median eminence (165) and that this uptake requires the activation of ObRb and the internalization of leptin by clathrin-coated vesicles (165). According to research carried out by Vincent Prevot and his team, this process involves signal transducer and activator of transcription (STAT)-3, protein kinase B (PKB)/Akt, and extracellular signal regulated kinase (ERK) phosphorylation, but is janus kinase-2 independent (165). Leptin is then transported toward the tanycyte cell body and, finally, released to the CSF and hypothalamic parenchyma (**Figure 2**) employing an ERK-dependent pathway (165). By using STAT-3 phosphorylation as an indicator of leptin signaling (170–172), Balland and collaborators reported that neutralization of leptin in the CSF impairs leptin signaling in mediobasal hypothalamic neurons (165), supporting the idea of the blood–CSF–hypothalamic gateway for leptin entry into the brain.

Taking into account the above mentioned studies, it appears that both endothelial cells and tanycytes contribute to the transport of leptin through the BBB and between different brain regions (163, 166, 167). In contrast, there is no clear evidence of the involvement of astrocytes in leptin transport, but a number of studies demonstrate that leptin signaling in astrocytes is important for energy homeostasis (173, 174).

Leptin transport into the brain is modulated by conditions including obesity and fasting, as well as metabolic factors. Obesity associated to HFD intake is reported to induce central leptin resistance. There are two main mechanisms or levels of leptin resistance suggested to occur: impairment of leptin transport into the brain (165) and reduction in the central response to leptin (175). Mice exposed long term to a HFD develop leptin resistance only when high levels of plasma leptin are reached (176). This suggests that hyperleptinemia is at least one of the causes of diet-induced leptin resistance. In addition, hypothalamic inflammation associated with diet-induced obesity could contribute to leptin resistance by altering the cellular networks and molecular pathways that control energy homeostasis (177). Nevertheless, recent studies suggest that leptin resistance does not imply a loss of responsiveness to endogenous leptin, but rather that there is a threshold above which exogenous leptin barely increases the response to leptin (178, 179). Glucose and insulin are reported to increase the transport of leptin across the BBB (180), while an increase in circulating triglycerides could impair leptin transport across the BBB (181). The latter suggests a possible mechanism for the reported reduction in leptin transport into the brain during fasting (182).

Ghrelin

Ghrelin is an orexigenic hormone produced and secreted in the stomach (183). It has similar targets as leptin in the CNS and also plays an important, but opposite, role in energy balance (184). There are two forms of ghrelin, acylated and unacylated, depending on the post-translational acylation with octanoic or decanoic acid (183, 185). This modification occurs mainly in the stomach, but there is evidence that it can also take place in target tissues (186). The acylated form of ghrelin exerts the majority of the metabolic effects of this hormone in the CNS and it binds more efficiently to the ghrelin receptor than the unacylated form (187). This receptor, also called the growth hormone secretagogue receptor 1a, is widely expressed in the hypothalamus (188). The mechanism underlying the passage of ghrelin across the BBB is not yet fully understood, but recent studies indicate that ghrelin possibly uses a similar route as leptin into the brain (189), i.e., through tanycytes in contact with the median eminence (**Figure 2**). Other studies indicate that this process is carried out by saturable transporters, at least for the acylated form, whereas

transport of des-acyl ghrelin is not saturable (190). Entry of acylated ghrelin into the CNS is increased by serum triglycerides and fasting and is decreased in obese mice (191), in contrast with leptin transport. Diet-induced obesity is reported to impair the hypothalamic response to peripherally or centrally administered ghrelin (192). The role of unacylated ghrelin on metabolism is largely unknown, but an increasing number of studies reveal that des-acyl ghrelin has similar and opposite functions as the acylated form (193–196).

Insulin

Insulin is a pancreatic hormone directly involved in glucose metabolism and homeostasis. Within the brain, it acts to increase energy expenditure and reduce food intake and energy storage (197). Insulin binds to its receptor in the plasma membranes of endothelial cells at the BBB and is internalized following a saturable pathway (198, 199). Recent studies have shown that IRs in astrocytes are involved in the entry of this hormone into the CNS (120). Also, as mentioned above, insulin signaling in astrocytes is necessary for the regulation of systemic glucose levels (120). Insulin transport into the brain is enhanced by satiation hormones like cholecystokinin (200). Although estradiol is known to impair insulin's actions in the brain, its effects appear to be unrelated to insulin transport (201). Some studies show that leptin increases insulin sensitivity in the hypothalamus at the molecular level (202), while others have found that leptin impairs insulin signaling in the brain (203). This discrepancy could be a matter of the experimental model employed, but further research is needed to understand the relationship between the effects of leptin and insulin at the level of the CNS. Leptin shares some signaling pathways with insulin, but the effects of these two hormones are not entirely parallel, as they exert opposite effects in some hypothalamic neurons (204). Saturated fatty acids induce insulin resistance in the hypothalamus (205), as has been previously described in peripheral tissues (206).

Sex Steroids

As hydrophobic molecules, estrogens, androgens, and progesterone can enter the brain by simple diffusion (207). Moreover, steroids are synthesized in the brain (208). These neurosteroids are produced in the CNS either from brain-borne cholesterol or from peripherally synthesized steroid precursors, like pregnenolone, deoxycorticosterone, and testosterone (209). The enzymes necessary for this synthesis are found in non-neuronal cells, including astrocytes, tanycytes, ependymal cells, and oligodendrocytes (210, 211), and in some neurons (212). As steroid hormones are known to regulate neurosteroid metabolism in glial cells (213–217) and also the expression levels of steroid receptors in the hypothalamus (218, 219), neurosteroids could have paracrine/autocrine functions within the brain.

Steroids and neurosteroids exert neuroprotective effects in the brain following brain injury, neurological disease, or inflammation (220–227). The expression of aromatase, the enzyme that catalyzes the conversion of testosterone into estradiol, is stimulated in reactive astrocytes after brain injury as a neuroprotective measure (228–230). Both microglial cells and astrocytes play an important role in the neuroprotective functions of steroids (231), as sex steroids diminish microglia reactivity (232–234) and astrocyte production of proinflammatory molecules (235–238).

Sex steroids, but specially estrogens, modulate energy homeostasis at the hypothalamic level decreasing food intake (239–241), increasing energy expenditure (242), and modulating the sensitivity to other metabolic hormones (243, 244). Their effect differs depending on the neuronal population (245, 246), but with an overall anorectic effect (247–249), although the underlying mechanisms are not yet fully understood. While nuclear estrogen receptors (ERs) are involved, especially ER α (247, 250–252), evidence indicates that estrogen responsive G-coupled membrane receptors can also regulate these effects (253, 254). The apparently contradictory results in the literature regarding the mechanism of action of estrogens on metabolism indicate a complex system for estrogens' function in energy homeostasis, where the different ERs could be acting in combination (255). Moreover, the mechanisms of action used by estrogens in metabolic control could be sexually dimorphic (256). The involvement of neurosteroids in energy homeostasis remains unknown.

Thyroid Hormones

The role of TH in increasing the metabolic rate has been known for more than a century (257). The involvement of these hormones in the control of energy homeostasis at the central level is a more recent discovery (258, 259). They promote lipogenesis at the level of the hypothalamus, which eventually leads to brown adipose tissue thermogenesis (259) and blockage of TH signaling in the hypothalamus reverts this process, leading to weight gain without an increase in feeding (259). Clinical studies and animal models with a pathological excess of TH synthesis and secretion (hyperthyroidism) have shed light on TH action in the hypothalamus and control of energy balance (260). Most hyperthyroid patients have an increased appetite and food intake and decreased body weight (261). Moreover, these same symptoms that are observed in animal models of hyperthyroidism are associated with the upregulation of orexigenic neuropeptides AgRP and NPY and downregulation of anorexigenic neuropeptides derived from POMC in the arcuate nucleus (259). There is evidence that TH are involved in brain inflammation, promoting survival, and processes growth in microglial cells and also in astrocytes (262–264). TH are also involved in systemic glucose homeostasis and insulin sensing (265, 266).

The thyroid gland produces and secretes mainly tetraiodo-L-thyronine or thyroxine (T4), which is generally transformed into triiodo-L-thyronine (T3) through deiodination at the level of peripheral tissues (267). Thus, deiodinase enzyme expression in peripheral tissues is important for the control of TH actions (268), as they catalyze the transformation of T4 into T3 and of both hormones into reverse T3 (rT3) and 3,5-diiodo-L-thyronine (T2), respectively (269). These two last forms are usually considered inactive, although in the last few years new roles have been proposed for them and other non-classical TH (270).

Thyroid hormones enter the hypothalamus mainly through MCT-8 (271) and organic anion transporting polypeptide-1C1 (272) in rodents (273). These transporters are expressed in endothelial cells of the BBB and epithelial cells of the choroid plexus (274), besides neurons and glial cells of the hypothalamus (275–277). Tanycytes act as gatekeepers for TH at the BBB (61) (**Figure 2**). These cells express the enzyme deiodinase II (DII) (278–280), catalyzing the formation of hormone T3 from the prohormone T4. Tanycytes uptake T4 from the capillaries and release T3 to the extracellular space in the hypothalamus, where this hormone can exert its central actions (258, 259, 281) (**Figure 2**). Modulation of deiodinase expression is a key point in TH homeostasis. For example, DII expression in tanycytes is promoted in fasting conditions (282). DII-expressing tanycytes are in direct contact with AgRP/NPY-expressing neurons of the arcuate nucleus. Upregulation of DII results in an increased production of T3, which activates AgRP/NPY neurons and, therefore, feeding behavior (283, 284). Tanycytes also express deiodinase III (DIII), which deiodinates T4 into reverse T3 which is biologically inactive, and T3 into T2. TH is important in the adaptation to different photoperiods in seasonal animals were, for example, there is a decrease in food intake and body weight during short photoperiods. The study of hypothalamic metabolism of TH during photoperiodic changes in seasonal mammals has shown that the there is an upregulation of DII during periods of long days, which would increase the levels of T3. In Siberian hamsters an upregulation of DIII in tanycytes has been shown to occur during short photoperiods, lowering active T3 levels (285). The retinoic acid pathway in tanycytes appears to be similarly regulated by photoperiodicity and also leads to modifications in energy expenditure (286, 287).

Thyroid hormone signaling usually occurs through nuclear thyroid receptors α and β (288) that function as transcription factors modulating gene expression (289). TH can also exert rapid non-genomic actions through membrane-associated receptors (290, 291). This signaling pathway could mediate TH effects on vasodilatation (292) and has been shown to be involved in neuronal excitability in the hippocampus (293, 294).

Centrally, THs control their own homeostasis in various ways, with non-neuronal cells having an important role, i.e., regulation of deiodinase expression (278) and inactivation of thyroid releasing hormone (295). Other hormones involved in metabolic control can enhance the secretion, synthesis, or sensing of TH, including leptin (296–298) and sex steroids (299–301).

Metabolism of Nutrients

Glucose

Perivascular astrocytes take-up blood-borne glucose that then undergoes glycolysis or glycogenesis (112). Lactate produced from glucose or glycogen metabolism in these cells is released to the extracellular space and enters neurons to be used as energy, constituting their primary energy source as suggested by some studies (302, 303). However, the question about the identity of the main energy source for neurons—lactate or glucose—is still debated. Tanycytes can metabolize and sense glucose in a similar manner (109).

Glucose storage as glycogen in astrocytes provides a way to guarantee energy release to neurons when it is needed, i.e., when faced with a raise in neuronal activity (304), by production of lactate from glycogenolysis. Several factors can regulate glycogen production and utilization in astrocytes, with insulin, insulin-like growth factor (IGF)-1 (305, 306), and leptin (203, 307) increasing their production of glycogen. More recently, ghrelin has been reported to possibly promote glycogenolysis in hypothalamic neurons (122).

Lipids and Ketone Bodies

It has been suggested that some fatty acids, like erucic acid (308, 309), suffer metabolic changes as they cross the BBB, whereas others do not (310, 311). Studies indicate that lipoproteins are hydrolyzed as they cross the BBB by the enzyme lipoprotein lipase associated to the membrane of endothelial cells (312–316).

In the absence of glucose and when glycogen stores are exhausted, such as in fasting conditions, astrocytes increase their uptake and utilization of fatty acids (136, 317, 318), which enter the mitochondria through carnitine palmitoyltransferase-1 to undergo β-oxidation (319). In the mitochondria, the enzymes 3-hydroxy-3-methylglutaryl-CoA synthase and lyase (320–322) transform fatty acids into β-hydroxybutyrate, a ketone body (323). Ketone bodies produced from this metabolic pathway are used by astrocytes themselves for fuel or secreted to be used by neurons and other glial cells (318).

Neurogenesis

Glial cells were first reported to participate in neurogenesis during development (324–326), but it later became apparent that they are also involved in this process in adulthood (327). In the developing hypothalamus of the rat, the birth of metabolically important neurons occurs between embryonic days 10.5 and 18.5 (328–330). Environmental changes during this period, including nutritional and hormonal disturbances, can modulate the normal process of hypothalamic neurogenesis and have an impact on later neuroendocrine function (330–332). For example, HFD intake by pregnant dams stimulates the proliferation, differentiation, and migration of orexigenic neuronal precursors and increases the density of orexigenic neurons at the level of the paraventricular nucleus in the offspring. This increase in the number and density of appetite-stimulating neurons and orexigenic neuropeptide expression leads to increased appetite, body weight, and propensity to develop obesity later in life (331).

The clear demonstration, as well as its acceptance by the scientific community, of neurogenesis in the adult hypothalamus is relatively recent and there is still much to be learned. Tanycytes form part of the pool of neuroprogenitor cells in the hypothalamus and these precursors are capable of differentiating into not only neurons, but also astrocytes both during development and in the adult brain (333, 334). These specialized glial cells form an important neurogenic niche in the vicinity of the median eminence (333) and can proliferate and differentiate under basal conditions and when stimulated by growth factors such as IGF-1 (57, 95), fibroblast growth factor (FGF)-2 (335), FGF-10 (336, 337), or even vitamins, as tanycytes have been shown to express

receptors, transporters, and other components of the vitamin A and C pathways (286, 287, 334, 338). FGF-10 positive tanycytes are reported to be important neural progenitors for NPY neurons in the arcuate nucleus, a function that may continue even during adulthood (337, 339). In addition, other isoforms of FGF are known to play a role in glucose homeostasis, inhibition of food intake, and body weight (340–343), with a possible involvement of glial cells (344–347). Although the generation of newborn neurons in the postnatal hypothalamus takes place at lower rates than during the embryonic period, it is physiologically relevant and has been shown to be regulated by diverse factors, including hormones and growth factors such as estradiol (332), FGF (335), and IGF-1 (348). Moreover, the nutritional status and dietary intake of an individual can modulate neurogenesis in hypothalamic metabolic circuits even in the adult animal (329, 333, 349–351).

The neurons composing the hypothalamic metabolic circuits experience a turnover rate such that approximately half of these cells are reported to be replaced between 4 and 12 weeks of age in mice (329). Diet-induced obesity suppresses this remodeling, at least in part, due to a decrease in actively proliferating cells in the hypothalamus with caloric restriction reversing this effect (329). Voluntary exercise is also reported to induce hypothalamic neurogenesis (352, 353). The effect of nutrient intake on the adult hypothalamus may be anatomically specific, as diets rich in fat are reported to inhibit neurogenesis in the mediobasal hypothalamic parenchyma (333), but to promote it in the median eminence in female mice (333). The enhanced neurogenesis that occurs at the level of the median eminence is suggested to be involved in the restoration of neurons that are damaged as a consequence of HFD intake (333); hence, impedance of this process could amplify the derogatory effects of poor dietary habits. There is tantalizing data indicating that hypothalamic neurogenesis in response to HFD differs between males and females (332, 333, 354), but whether this is involved in the sexually dimorphic metabolic response to HFD and weight gain requires further investigation.

Astrocytes are involved in the regulation of neuronal differentiation, proliferation, and synaptogenesis during development (3, 355). Microglia also actively participate in neurogenesis, both during development and adulthood, with most studies being performed in the hippocampus (356). Microglia not only phagocytize cells undergoing apoptosis in proliferative zones, but they also produce factors that can either inhibit or stimulate neuroprogenitor cells. The cross-talk between microglia and neuroprogenitor cells is an active area of investigation as this is a finely tuned process where these cells continuously interchange information (356). However, less is known regarding the role of astrocytes and microglia in neurogenesis in metabolic circuits of the adult animal.

Diet not only affects neurogenesis in the hypothalamus, but also in other brain areas such as the hippocampus, an area known to maintain active neurogenesis even in the adult (357). In the dentate gyrus of the hippocampus, HFD intake impairs neurogenesis (358) in addition to producing oxidative stress and lipid peroxidation (357). Palmitic acid (PA), a saturated fatty acid that is a major component of the majority of HFDs, was shown to reduce the proliferation of the neuroprogenitor cells (359) and the levels of brain-derived neurotrophic factor, indicating that PA-rich diets impair neurogenesis in the hippocampus. Caloric restriction and exercise increase neurogenesis in the hippocampus (350, 360, 361) and this has been associated with the anti-depressive effects of exercise (360).

Synaptogenesis, Synaptic Plasticity, and Synaptic Transmission

Astrocytes, in addition to participating in neuronal proliferation and differentiation, also regulate synaptogenesis during development (3, 355). In the hypothalamus, the neonatal and early prenatal hormonal and nutritional environments can affect the synaptic connectivity of metabolic circuits (189, 362). Astroglial coverage of neuronal cell surfaces has been shown to be inversely correlated with the number of synaptic inputs to their somas, with this astroglial ensheathment/synaptic input arrangement being physiologically relevant in the neuroendocrine hypothalamus (363–367). Thus, changes in astrocyte numbers or morphology in the hypothalamus might be expected to modify synaptic inputs both during development and in later life.

The generation and maturation of astrocytes is not fully complete until the third postnatal week in rodents (368, 369), so variations in the physiological levels of specific metabolic hormones or signals during early life could affect the development of these cells. For example, neonatal overnutrition and modifications in leptin levels or signaling affect the number and morphology of astrocytes in the arcuate nucleus in adulthood (121, 173, 370). The leptin peak that takes place between postnatal days 5 and 13 in rodents is essential not only for neuronal outgrowth and maturation, but also astrogenesis (368, 371, 372) and astrocyte development (373, 374). The timing and magnitude of this leptin surge can be modified by nutrition (371, 375, 376), as well as other conditions such as stress (377) and is one mechanism by which these early environmental influences can have long-term effects on metabolism.

Maternal dietary intake and body weight during gestation and lactation can also influence metabolic circuit formation in the offspring, including the astroglial ensheathment/synaptic input arrangement. For example, newborns from mothers fed a HFD during gestation and lactation have increased astroglial ensheathment of POMC neurons that is associated with a decrease in the resting mini inhibitory post synaptic currents of these neurons (121). The response of these POMC neurons to changes in glucose concentrations was also shown to be modified (121). Hence, alterations in the early nutritional environment could imply the modification of the appropriate development of neuron–glial interaction of metabolic circuits and therefore affect long-term metabolism.

Microglia are involved in synaptogenesis throughout the brain (378, 379); however, there is little information regarding the specific effects of microglia on the development of the synaptic interactions of metabolic circuits. These glial cells have been shown to have an active role in the sexual differentiation of behavior and masculinization of the brain (380), suggesting that

they may indeed be important for the development of endocrine circuits and possibly the sexual differentiation of some of these systems.

Modifications in the synaptic connectivity of metabolic circuits occur in postnatal life in response to metabolic and hormonal signals (241, 381–384) and are most likely involved in the adaptation to changes in energy inputs/conditions in attempt to maintain metabolic homeostasis, with astroglia participating in these synaptic rearrangements. HFD induced-obesity is associated with an increase in the glial coverage of both POMC and NPY cell bodies in the arcuate nucleus, which is coincident with a decrease in the number of synaptic inputs to the perikarya of these neurons (384). However, there is a decline in stimulatory inputs to NPY neurons and of inhibitory inputs to POMC neurons (384), which would result in an overall decline in orexigenic signaling. When first given a HFD, rodents experience a phase of hyperphagia that is normally followed by an attenuation of this rise in energy intake. The levels of polysialic acid (PSA) are rapidly increased in the arcuate nucleus in response to HFD (385). This cell-surface glycogen can attach to cell membrane proteins to weaken cell–cell interactions and facilitate synaptic reorganization (386). If PSA is enzymatically removed from neural-cell adhesion molecule (NCAM) in the hypothalamus, HFD induced modifications in metabolic circuits can be blocked and the adaptation to HFD-induced hyperphagia attenuated (385). In addition, studies in photoperiodic models have shown that PSA and NCAM levels in tanycytes are reduced during short photoperiods in conjunction with vimentin levels, modulating the plasticity for tanycyte connections with arcuate neurons (387).

Diverse hormonal/metabolic signals could be involved in these structural modifications, including leptin. This hormone rapidly induces synaptic changes in metabolic circuits (381), with some of these effects being mediated through astrocytes. These glial cells express different isoforms of ObR (163, 164), with the expression of this receptor being increased in astrocytes of obese rodents (163). Leptin can modify astrocyte morphology, inducing changes in the length and number of primary astrocytic projections and astroglial coverage of hypothalamic neurons (173, 388). The lack of leptin signaling due to the knock-out of this receptor in astrocytes changes synaptic inputs to POMC and NPY neurons, resulting in modifications in the function of these metabolic neurons and rendering the animals less susceptible to the effects of leptin (173). However, it remains unclear as to the mechanisms involved in the changes in neuronal/glial interactions, including identification of the initial step that triggers these morphological modifications.

Astrocytes modulate neuronal transmission by controlling glutamate concentrations in the synaptic cleft, which also plays an important role in preventing excitotoxicity (389). Leptin and ghrelin modulate glutamate uptake by these glial cells (121, 122) and could thus affect stimulatory signaling in metabolic circuits through this mechanism. Astrocytes also actively participate in synaptic transmission and plasticity by releasing gliotransmitters, including adenosine, ATP, D-serine, glutamate, and tumor necrosis factor α that directly activate postsynaptic receptors and by altering neurotransmitter release from presynaptic neuronal elements to induce short-term plasticity and to modulate synaptic efficacy (12, 390–393). Adenosine release by astrocytes inhibits the firing rate of AgRP neurons and food intake, modifying the response to metabolic hormones such as ghrelin (394).

Inflammatory Response

The inflammatory response to infection, foreign substances, mechanical damage, or any situation that could damage neurons is one of the best studied functions of glial cells (231, 395–398). However, the description of hypothalamic inflammation in obesity, as well as its association with the development of secondary complications, is more recent. In 2005, the group of Licio Velloso reported that inflammatory pathways were activated in the hypothalamus in HFD-induced obese rats (399). This same group went on to demonstrate that this hypothalamic inflammation was involved in the disruption of systemic glucose homeostasis (35). Numerous studies have since reported the link between hypothalamic inflammation and obesity-related comorbidities (36, 400–404). Hypothalamic inflammation is reported to be associated with the development of insulin resistance and type 2 diabetes (405) and increased cell death in the hypothalamus (329). Most studies analyzing hypothalamic inflammation have employed HFD-induced obesity models and suggest that dietary factors are involved in at least part of the inflammatory response. Indeed, hypothalamic inflammation is reported to occur even before an increase in adiposity or systemic inflammation are detected (36) and central administration of saturated fatty acids directly activates inflammatory signaling mechanisms in the hypothalamus (35, 406). However, increased weight gain can occur in response to genetic, epigenetic, and excess energy intake that is not due to increased fat consumption and the hypothalamic inflammatory/gliosis response differs depending on the underlying cause of weight gain (121, 195, 407, 408). These differential responses are most likely the result of dietary signals and the changes in metabolic signals associated to weight gain acting on both microglia and astrocytes. Sex may also be a factor, as the hypothalamic inflammatory response to chronic HFD-intake is reported to differ between males and females, with males being more susceptible (409). This could result from the greater rise in PA levels in the CNS of male mice compared to females, even though there is no sex difference in circulating fatty acid levels (410).

Inhibition of hypothalamic inflammation is reported to blunt or block the development of obesity-associated complications (400, 403) and dietary restriction can reverse central inflammatory processes (411–415). Exercise also protects against HFD-induced hypothalamic inflammation (416).

Microglia in Hypothalamic Inflammation

Microglia, the innate immune cells of the CNS, are the first line of defense in response to foreign substances (417, 418) and are activated in response to saturated fat consumption (36, 403, 408, 419). Indeed, these glial cells are suggested to dictate the inflammation that occurs in response to saturated fats (419). Microglia

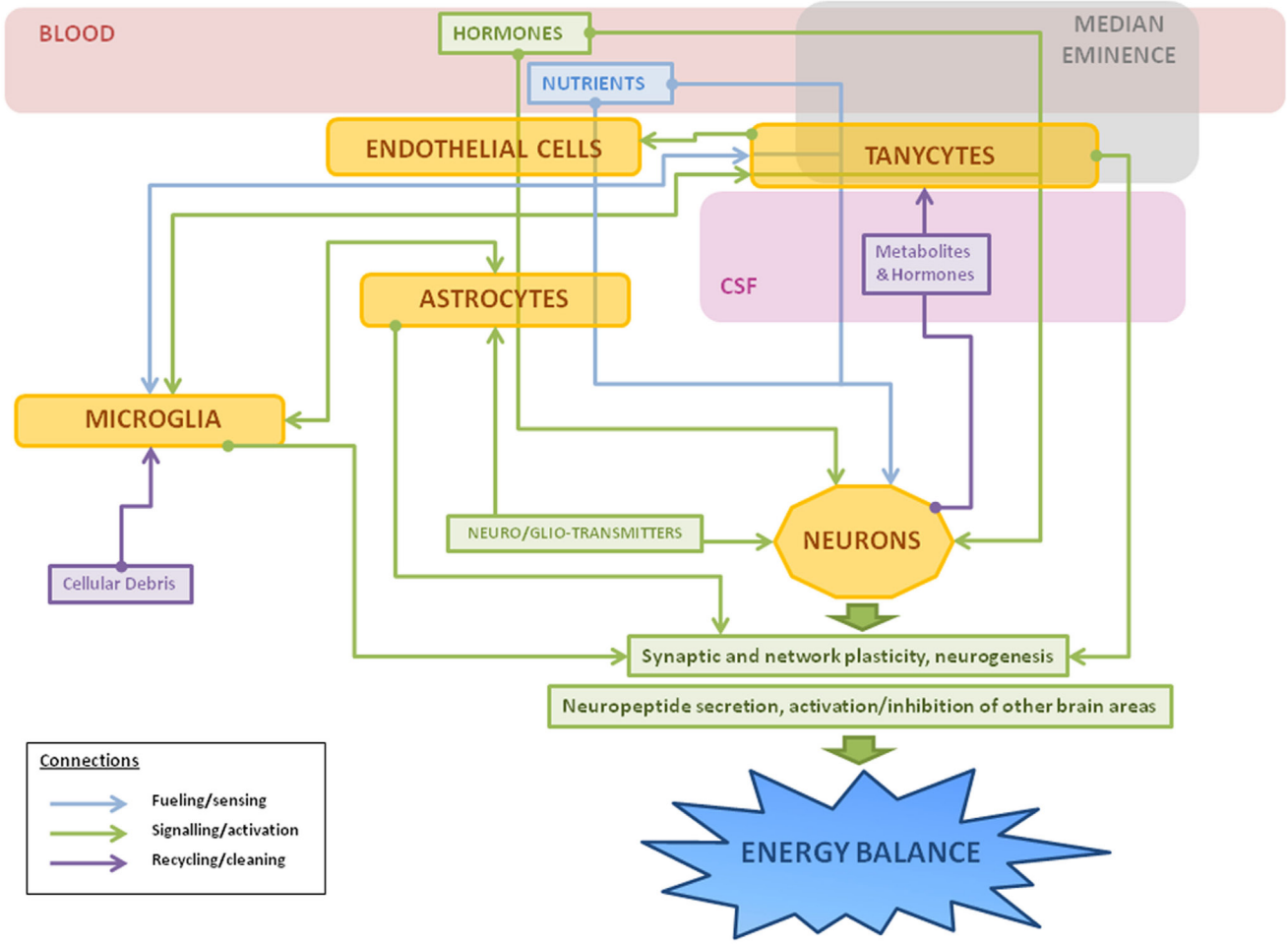


FIGURE 3 | Schematic representation of the main roles of hypothalamic non-neuronal cells in metabolism. Thin arrows represent the different connections between cells: fueling and sensing of nutrients (in blue); signaling through gliotransmitters, neurotransmitters, or other factors (in green); and recycling of molecules or cleaning of cellular debris (in purple). Hormones and nutrients from the bloodstream pass through one or more types of non-neuronal cells before reaching neurons, sometimes suffering metabolic changes during the process. In response to a metabolic imbalance (excess of saturated fatty acids, hyperleptinemia, etc), microglial cells change to an activated state, releasing inflammatory factors, such as cytokines, and activating astrocytes as a neuroprotective measure. If the insult continues, it can lead to gliosis, hypothalamic inflammation, and neuronal damage.

can also be activated when weight gain is due to excess intake of a normal diet and due to high fat intake, (402), indicating that not only dietary signals are involved. Leptin stimulates the release of inflammatory cytokines from microglia (420), suggesting that hyperleptinemia could be involved in microglial activation in obese subjects.

Astrocytes in Hypothalamic Inflammation

Astrocytes also respond to HFD intake (36, 384, 421) and can be directly activated *in vitro* by fatty acids (408, 409, 422). Hyperleptinemia associated with weight gain may also participate in the activation of glia in situation of obesity (121, 173, 388, 408, 423). Indeed, ob/ob mice, which are dramatically obese due to the genetic lack of leptin, do not exhibit astrogliosis and leptin-induced weight loss actually increases astrocytic profiles in the hypothalamus of these animals (408). However, we have found that in some situations of increased weight gain, such as increased carbohydrate intake in the form of sucrose, astrocytic markers may actually be decreased (407).

The astrogliosis response to HFD differs between males and females, as does the *in vitro* response to PA (409). The protective effects of estrogens are exerted through ERα in astrocytes (424), with estrogens protecting against PA activation of astrocytes *in vitro* (409). Morselli et al demonstrated that HFD-intake reduces hypothalamic ERα levels in males, but not in females, which may be involved in the decreased protection against diet-induced obesity in males.

Astrocytes have also been implicated in determining the preference for a HFD, with this mechanism involving cannabinoid receptor 1 (CB1) (425, 426). The intake of a HFD induces the preference for this type of diet and this appears to involve the production of endocannabinoids in the hypothalamus (426). Leptin signaling in astrocytes is regulated by CB1, with disruption of CB1 in these glial cells resulting in the inability of leptin to

regulate glycogen storage (307) and thus possibly affecting central energy storage and glucose sensing/signaling.

CONCLUSION

It is clear that non-neuronal cells are fundamental for the correct functioning of metabolic circuits, beginning with the essential process of regulating the nutrients and signals that reach these neurons. These cells are not only involved with the development, maintenance, and protection of their neuronal neighbors, but participate in all aspects of neuronal function (summarized in **Figure 3**). In the hypothalamus, numerous studies have shown how non-neuronal cells play an active role in the control of metabolism and in the pathological outcomes of poor metabolic control. Although the advances in laboratory techniques and genetically engineered animal models have increased our knowledge in this field, there is yet much to be learned regarding the mechanisms involved. Studies directed at developing markers to further identify different populations or subclasses of glial cells are of great importance in order to better understand the vast roles that these cells play in the different physiological functions controlled by the CNS. This increased knowledge will also hopefully add to our understanding of pathophysiological processes and future targets for treatments, including that of obesity and its secondary complications.

AUTHOR CONTRIBUTIONS

All authors have contributed to the writing and editing of this review. Figures were designed and made by AF-R.

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The Role of MicroRNA in the Modulation of the Melanocortinergic System

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The central control of energy balance involves a highly regulated neuronal network within the hypothalamus and the dorsal vagal complex. In these structures, pro-opiomelanocortin (POMC) neurons are known to reduce meal size and to increase energy expenditure. In addition, leptin, a peripheral signal that relays information regarding body fat content, modulates the activity of melanocortin pathway neurons including POMC-, Agouti-related peptide (AgRP)/Neuropeptide Y (NPY)-, melanocortin receptors (MC3R and MC4R)-expressing neurons. MicroRNAs (miRNAs) are short non-coding RNAs of 22–26 nucleotides that post-transcriptionally interfere with target gene expression by binding to their mRNAs. Evidence has demonstrated that miRNAs play important roles in the central regulation of energy balance. In this context, different studies identified miRNAs including miR-200 family, miR-103, or miR-488 that could target the genes of melanocortin pathway. More precisely, these different miRNAs can modulate energy homeostasis by affecting leptin transduction pathway in the POMC, or AgRP/NPY neurons. This article reviews the role of identified miRNAs in the modulation of melanocortin pathway in the context of energy homeostasis.

Keywords: microRNA, melanocortin, feeding behavior, hypothalamus, energy homeostasis

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INTRODUCTION

Overweight and obesity are significant risk factors for various chronic diseases, including cancer, heart diseases, and type 2 diabetes. In 2014, World Health Organization estimated that more than 1.9 billion adults were overweight. Of these over 600 million were obese. Dramatically, 41 million children under the age of 5 were overweight or obese in 2014. With such a high and expanding prevalence, and considering the associated diseases, obesity has an important economic impact on health care systems. For instance, the global medical costs related to obesity were estimated to reach up to 147 billion dollars per year in the USA (Bariohay et al., 2011). The direct health care costs linked to obesity in industrialized country can exceed 7% of the total health care costs (Bariohay et al., 2011). Environmental factors lead to an increase in the proportion of obese people. To date, the main treatment against obesity is to decrease caloric intake combined with an increase in the physical activity. The major limit of this treatment is the low achievement rate in the long haul, revealing the need for additional medical approaches. Then, given the expanding number of obese patients, obesity research is critical in the medication improvement field.

The control of energy homeostasis involves endocrine and neuronal mechanisms that modulate the balance between caloric absorption and energy expenditure. In this context,

the central nervous system (CNS) continuously follows modifications in metabolic parameters (i.e., glycemia or free fatty acids levels) or hormones (insulin, leptin, ghrelin, PYY3-36, GLP-1, and cholecystokinin) and elicits adaptive responses like food intake regulation or autonomic nervous system modulation of glucose homeostasis and energy expenditure (**Figure 1**). Among the brain regions involved in this regulation, the hypothalamus, and the dorsal vagal complex (DVC) in the brainstem play a pivotal role through specific neuronal networks (Berthoud, 2002, 2004; Morton et al., 2005, 2006; Schneeberger et al., 2014). More particularly, within the arcuate nucleus (ARC) of hypothalamus and the nucleus of the solitary tract (NTS) of DVC, pro-opiomelanocortin (POMC) neurons are important regulators of energy, and glucose homeostasis (Morton et al., 2006). In this context, leptin is an adipose-derived hormone that is crucial to maintain both normal body weight and insulin sensitivity by action in the hypothalamus (Balthasar et al., 2004; Coppari et al., 2005; Dhillon et al., 2006; Morton et al., 2006; van de Wall et al., 2008). This peripheral signal is detected by hypothalamic arcuate neurons expressing the anorexigenic peptide POMC or the orexigenic peptides Neuropeptide Y (NPY)/Agouti-related peptide (AgRP). These neurons project to melanocortin 3 and 4 receptor-expressing neurons located in hypothalamus and other brain structures (Morton et al., 2006). Together these neurons are called the melanocortin pathway and regulate feeding behavior, energy expenditure, and glucose homeostasis through activation of the autonomic nervous system and higher brain structures (Berthoud, 2002; Morton et al., 2006) (**Figure 1**).

One important goal of present research is to identify the molecular mechanism and the intracellular mediators allowing these POMC and NPY/AgRP neurons to respond to energy status variations. Then, it appears crucial to increase our knowledge of the mechanisms controlling the melanocortin system activity, particularly by the discovery of new signaling pathways involved in the control of POMC and AgRP genes expression by leptin. Such pathways should provide beneficial pharmacological targets, and lead to the development of new generation drugs that can safely and effectively treat overweight and obesity linked to leptin resistance. In this context, it has been recently discovered new mechanisms involved in the control of the expression of the melanocortin pathway's genes. In particular, epigenetic mechanisms, including DNA methylation, the modifications of histones, and specific microRNAs (miRNAs) expression, have been proposed to mediate the expression of the melanocortin system (Stevens et al., 2010, 2011; Funato et al., 2011; Cansell and Luquet, 2012; Schneeberger et al., 2015).

This review provides an insight into the new mechanisms of the regulation of the POMC, NPY, and AgRP genes and a focus on the function of the miRNAs in this process will be developed.

THE MELANOCORTIN SYSTEM AND REGULATION OF ENERGY HOMEOSTASIS

As mentioned above, POMC-expressing neurons moderate food intake, glucose homeostasis, and energy expenditure (Cowley et al., 2001; Parton et al., 2007; Mounien et al., 2009, 2010). The prohormone POMC is cleaved into α-melanocyte-stimulating hormone (α-MSH) that binds to the melanocortin 3 and 4 receptors (MC3R and MC4R) on neurons located in the nucleus of the hypothalamus as well as in the DVC (Cummings and Schwartz, 2000; Jégou et al., 2003; Coll et al., 2004; Rossi et al., 2011). The activation of MC4R induced a decrease of the food intake and an increase of the energy expenditure and this receptor is also involved in glucose homeostasis. MC4R agonists provide therefore a potential tool for the treatment of metabolic disorders as obesity (Rossi et al., 2011; Zechner et al., 2013). Conversely, mutations in the POMC, MC3R, or MC4R genes cause common or massive early-onset obesity in humans, further supporting a crucial role for the melanocortin pathway in energy homeostasis (Krude et al., 1998; Farooqi and O'Rahilly, 2000; Lee, 2009). It is important to notice that AgRP has been described as an endogenous antagonist or inverse agonist of the melanocortin receptors (Cone et al., 1996; Ollmann et al., 1997). Altogether, these neurons belong to the central melanocortin system, a family of diverse cells that comprise POMC-, AgRP-, MC3R-, and MC4R-expressing neurons. These neurons regulate peripheral metabolism through the activation of the autonomic nervous system and higher brain structures to control energy homeostasis but also the arousal and reward systems (Berthoud, 2002; Morton et al., 2006). Recently, by using optogenetic approach, Aponte et al. found that POMC and AgRP neurons have counter-regulatory roles on the regulation of food intake, confirming the pivotal role of these neurons in the control of feeding behavior (Aponte et al., 2011). Regarding the action of leptin on this melanocortin pathway, deletion of SOCS-3, a negative regulator of the action of this hormone, in POMC neurons, improved glucose homeostasis and insulin sensitivity as well as resistance to high fat diet (HFD) (Kievit et al., 2006). Lately, the simultaneous disruption of insulin and leptin receptors induced insulin resistance in mice (Hill et al., 2010). Altogether, these data showed the main role of POMC neurons in the integration of peripheral signals, as leptin, reflecting the energy status of organism. In addition, leptin is required for the accurate development of the POMC neurons (Bouret et al., 2004; MacKay and Abizaid, 2014).

In addition to the communication between brain and peripheral organs, intracellular metabolic-sensing mechanisms in CNS neurons are also crucial for the control of the energy balance. For instance, it has been established that AMP-activated protein kinase (AMPK), the mammalian target of rapamycin (mTOR), and SIRT1 deacetylase in the hypothalamus, are essential for leptin sensing and then energy homeostasis. More precisely, inactivation of AMPK in POMC neurons induced obesity while SIRT1 in POMC neurons is required for adaptations against diet-induced obesity (Claret et al., 2007; Ramadori et al., 2010). In addition, it has also been demonstrated that epigenetic mechanisms such as histone modifications or DNA methylation are acknowledged to modulate POMC gene activity under different nutritional status (Stevens et al., 2010; Funato et al., 2011). Then, these data established that POMC gene expression is highly and tightly controlled by different

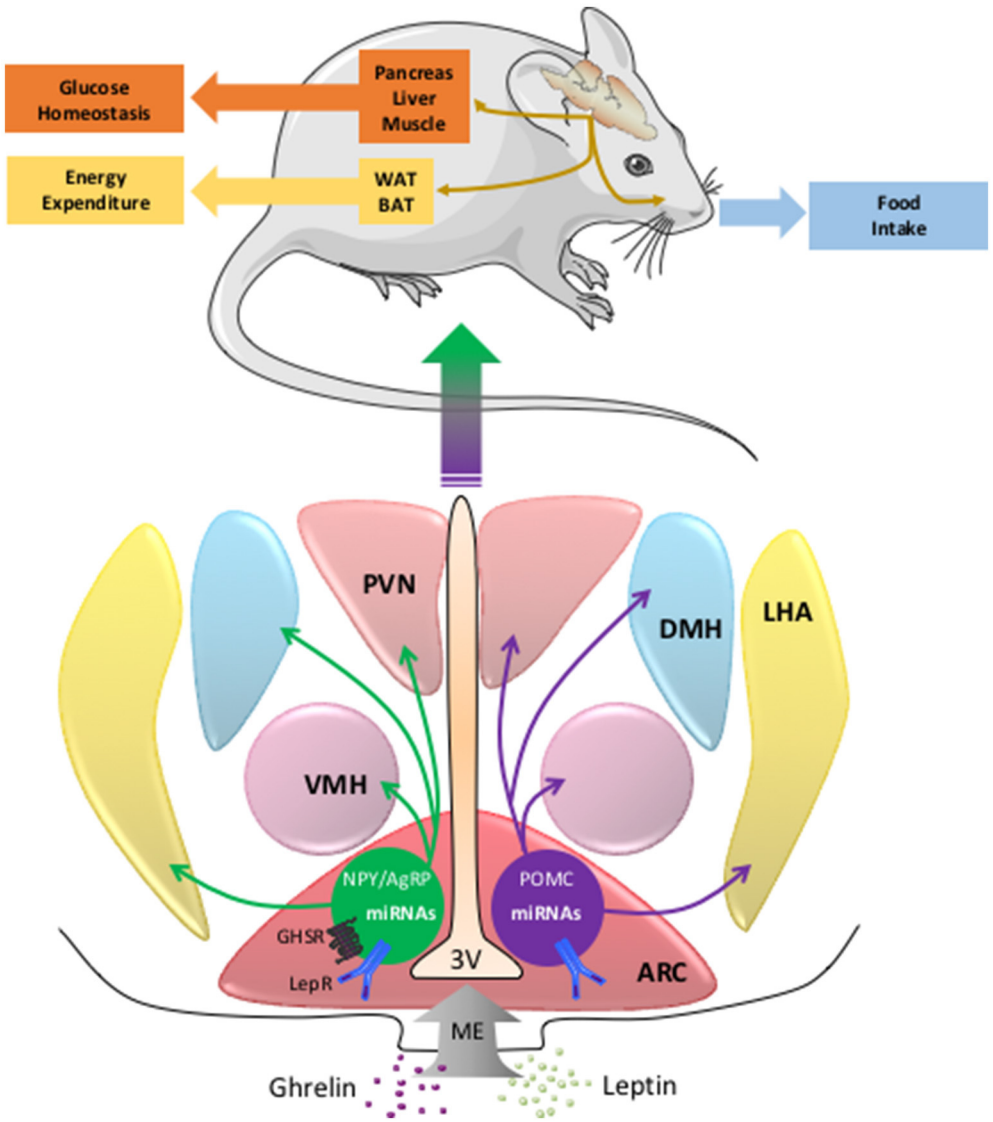


FIGURE 1 | The miRNAs in POMC and NPY/AgRP neurons and the central regulation of energy balance. The neurons of the melanocortin pathway integrate peripheral signals as ghrelin or leptin that modulate the expression of POMC, NPY, AgRP, and miRNAs genes. Then the melancortin pathway elicits adaptive responses like food intake, energy expenditure, or glucose homeostasis. AgRP, agouti related peptide; ARC, arcuate nucleus; BAT, brown adipose tissue; DMH, dorsomedial hypothalamus; LepRb, leptin receptor type b; LHA, lateral hypothalamus area; ME, median eminence; miRNA, microRNA; NPY, neuropeptide Y; GHSR, ghrelin receptor; POMC, pro-opiomelanocortin; PVN, paraventricular nucleus; VMH, ventromedial hypothalamus; WAT, white adipose tissue; 3V, third ventricle.

mechanisms in order to regulate energy homeostasis by the modulation of appetite and energy expenditure.

THE MICRORNA AND THE MELANOCORTIN SYSTEM

Gene expression can be controlled at the transcriptional or post-transcriptional levels as well as during and after the translation. In this context, it has recently been highlighted that small RNAs, miRNAs, play predominantly inhibitory regulatory roles by binding to the 3′ untranslated region (3'UTR) of message encoding RNAs. The miRNAs are small non-coding RNA molecules of 21 to 26 nucleotides that regulate gene expression (Bartel, 2004; Derghal et al., 2016). They were first discovered in *Caenorhabditis elegans* in 1993 and, later on, in vertebrates and plants (Lee et al., 1993; Wightman et al., 1993). These non-coding RNAs induced specific gene silencing by base pairing to 3'UTR of target messenger mRNAs. miRNAs exert their actions by inhibiting translation and by affecting mRNA stability and degradation (Bartel, 2004; Guo et al., 2010; Derghal et al., 2016). Based on computational algorithms, around 60% of human transcripts contain potential miRNA-binding sites within their 3′UTRs (Friedman et al.,

2009). A single miRNA can potentially bind to more than 100 target mRNAs, and multiple miRNAs can cooperate to finely tune the expression of the same transcript (Doench and Sharp, 2004; Grimson et al., 2007; Selbach et al., 2008). The miRNAs play key roles in numerous physiological processes including cell proliferation, apoptosis, neurodevelopment, and tissue differentiation but also in pathological processes as cancer (Bartel, 2004). Interestingly, defects in miRNA biogenesis and function have been shown to contribute to the development of metabolic disorders. For instance, mir-14, mir-278, and let-7 are involved in the metabolism of lipid and glucose respectively (Krützfeldt and Stoffel, 2006; Frost and Olson, 2011).

As indicated before, miRNAs are important for neurodevelopment but also neurotransmission or synaptic plasticity (Díaz et al., 2014). In the case of the hypothalamus, several studies demonstrated that the miRNA transcriptome is different at different stages of development. For instance, Zhang et al. showed that 30 miRNAs including miR-7 and miR-191 are differentially expressed in the hypothalamus of the pig between stages P60, P120, and/or P180 (Zhang et al., 2013). More recently, a nice work showed robust changes in the expression of numerous miRNAs during the period of functional organization of the ARC and median eminence between stages P8–P14 and stages P21–P28 (Doubi-Kadmiri et al., 2016).

As mentioned above, hypothalamus and DVC are important for the detection of circulating nutrients and hormones and in turn, these neuronal structures modulate the pancreas, liver, and adipose tissue physiology through efferent pathways. The function of miRNAs in the hypothalamus and DVC has not been clearly addressed. However, as in the other organs involved in energy homeostasis, miRNAs undoubtedly play a key role in hypothalamus and DVC neurons, and particularly in the function of melanocortin pathway. In accordance with this point, it has been shown that in the anorexia mouse model, *anx/anx*, there is an alteration of miRNA machinery expression. In particular, an up-regulation of RISC genes (Dgcr8, Ago2, Fmr1, Ddx6, and Pabpc1) has been observed in the hypothalamus of *anx/anx* mice (Mercader et al., 2012). However, the link between the phenotype of the *anx/anx* mice (anorexia, hyperactivity, and ataxia) and the differential regulation of RISC genes need to be clarified.

A large number of miRNAs are expressed in the brain, and deletion of Dicer, a specific enzyme involved in miRNA maturation, in specific brain structures or neuronal cell type can lead to behavioral defect and neurodegeneration (Schaefer et al., 2007; Cuellar et al., 2008; Olsen et al., 2009; Hébert et al., 2010; Tao et al., 2011). Recently, it has been shown that Dicer is essential for the central control of energy homeostasis. In fact, the neuron-specific deletion of Dicer induced obesity in mice (Mang et al., 2015). Interestingly, brain transcriptome analyses in this obese mice model identified several obesity-related pathways as leptin signaling (Mang et al., 2015). In the hypothalamus, deletion of Dicer in the ARC of adult mice induced hyperphagia and obesity (Vinnikov et al., 2014). The group of Dr Claret also showed that the hypothalamic expression of Dicer is modulated by fasting (Schneeberger et al., 2012). In contrast, the expression of Dicer is increased in diet-induced obesity model and *ob/ob* mice (Schneeberger et al., 2012). Altogether, these results suggest that the expression of Dicer is modulated by nutrient availability. Interestingly, Dicer is expressed in 94% of POMC and NPY/AgRP neurons suggesting an important function of Dicer and Dicer-derived miRNA in the modulation of the POMC, AgRP, and NPY genes expression (Schneeberger et al., 2012).

It has been established that each tissue exhibit a specific profile of miRNA expression (Babak et al., 2004; Lee et al., 2008). First studies revealed an enrichment of several miRNAs including let-7c, miR-7a, miR-7b, miR-124a, miR-125a, miR-136, miR-138, miR-212, miR-338, and miR-451 in the hypothalamus of rodents (Farh et al., 2005; Bak et al., 2008). These observations have been confirmed in ARC and paraventricular (PVN) nucleus of the hypothalamus by illumina sequencing technology (Amar et al., 2012). And in particular, expression was high or moderate for about 20 miRNAs as let-7, miR-7a and b that may be used to define a common ARC/PVN profile of male Wistar rats (Amar et al., 2012). In the line of this observation, it has been demonstrated that miR-7a is expressed preferentially in NPY/AgRP neurons (Herzer et al., 2012).

The functions of hypothalamic miRNAs are highly investigated. In particular, potential impact of leptin on hypothalamic miRNAs expression profile begins to be clarified. Recently, the group of Dr Taouis performed a large-scale expression analysis using Taqman Low Density Arrays methodology to analyse 524 rodent mature miRNAs on the hypothalamus of *ob/ob* mice (Crépin et al., 2014). They showed that the relative expression of only 11 out of 524 miRNAs were significantly modified in the hypothalamus of *ob/ob* mice compared to the control animals (Crépin et al., 2014). They confirmed the over-expression of miR-200a, miR-200b, and miR-429 in *ob*/*ob* mice as compared to control animals by real time PCR (Crépin et al., 2014). Interestingly, the expression of these miRNAs in *ob*/*ob* mice decreased after leptin treatment (Crépin et al., 2014). Importantly, the same group showed that overexpression of mir-200a in *ob/ob* mice can down-regulate Insulin receptor substrate-2 and leptin receptor hypothalamic expression that are involved in the insulin and leptin pathways (Crépin et al., 2014) (**Figure 2**). In other set of experiments, the group of Dr Taouis demonstrated that the defect in the leptin action in early life supports leptin resistance and disturbs the hypothalamic miRNA expression pattern in adulthood (Benoit et al., 2013). And in particular, daily injection of a pegylated rat leptin antagonist (pRLA) in newborn rats induced a modification of the hypothalamic miRNAs pattern expression at d28 (Benoit et al., 2013). Interestingly, after 1 month of HFD challenge, there is an up-regulation of miR-200a expression in the hypothalamus of pRLA (Benoit et al., 2013). These different observations suggest that miRNAs, and particularly miR-200a, are involved in the effect of leptin and insulin in the hypothalamus (**Figure 2**). In accordance with these studies, Sangiao-Alvarellos et al. demonstrated the alteration of the hypothalamic expression of a set of miRNAs, including let-7a, mir-9, mir-30e, mir-132, mir-145, mir-200a, and mir-218, after a chronic caloric restriction and a HFD in male rats (Sangiao-Alvarellos et al., 2014). The predicted targets of these miRNAs include different actors of key inflammatory and metabolic pathways, including such as nuclear

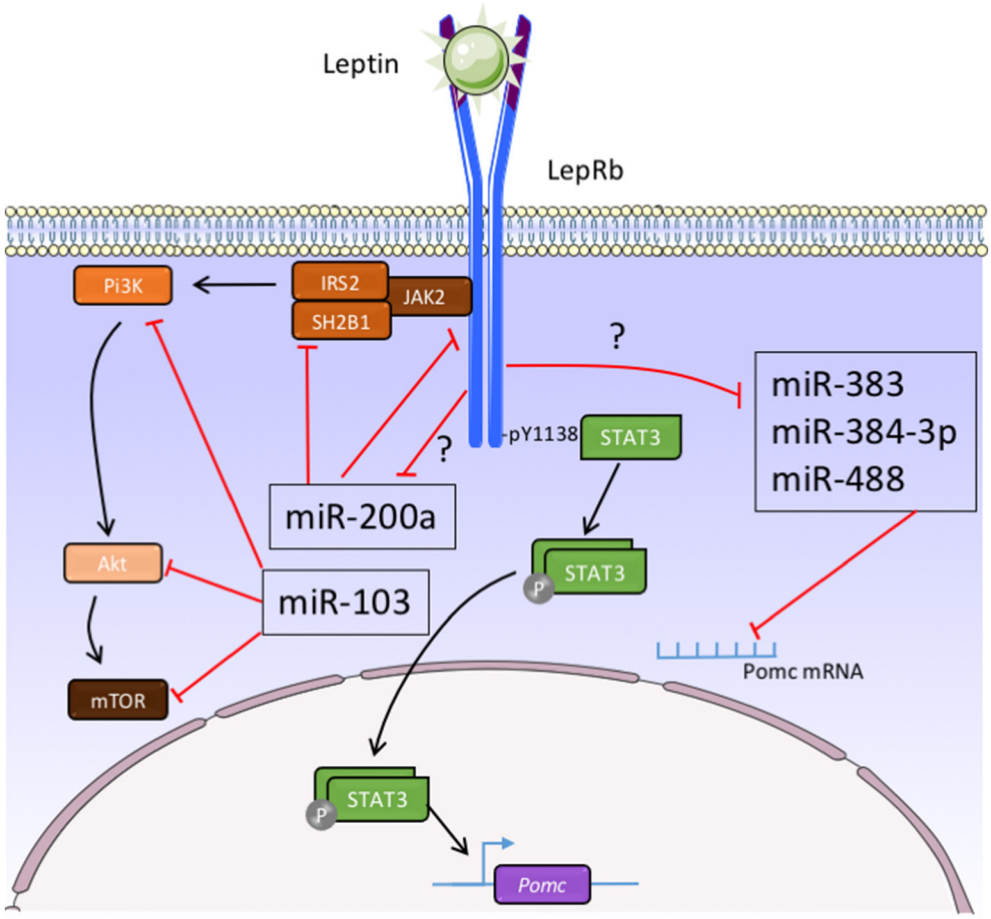


FIGURE 2 | Function of micro-RNAs in the control of melanocortin neurons activity by leptin. Akt, **s**erine-threonine protein kinase; IRS-2, insulin receptor substrate 2; JAK2, janus kinase 2; LepRb, leptin receptor type b; mTOR, mammalian target of rapamycin; Pi3K, phosphatidylinositol 3-kinase; POMC, pro-opiomelanocortin; SH2B1, Src homology 2 B adaptor protein 1; STAT3, signal transducer and activator of transcription 3; ?, unexplored pathways.

factor κβ, ILs, phosphatidylinositol 3-kinase (Pi3k)/serine-threonine protein kinase (Akt), insulin receptor, p70S6K, and Janus tyrosine kinase/signal transducer and activator of transcription (Sangiao-Alvarellos et al., 2014). Vinnikov et al. noticed that the injection of mir-103 mimic in the ARC reduced the obese phenotype of mice lacking Dicer in forebrain neurons (Vinnikov et al., 2014). The effect of miR-103 could be associated to Pi3K/Akt/mTOR signaling pathway (Vinnikov et al., 2014) (**Figure 2**).

Regarding the functions of miRNAs in the POMC neurons, it has been demonstrated that specific deletion of Dicer in POMC-expressing cells leads to obesity and diabetes which is associated with loss of POMC neurons in the ARC (Schneeberger et al., 2012; Greenman et al., 2013). In our group, we identified mir-383, mir-384-3p, and mir-488 that potentially bind the 3-UTR of POMC mRNA (Derghal et al., 2015) (**Figure 2**). Using *in situ* hybridization, we demonstrated that these three miRNAs are present in the POMC neurons of the ARC (Derghal et al., 2015). In addition, there is an increase of the expression of mir-383, mir-384-3p, and mir-488 in the hypothalamic structures of *ob/ob* and *db/db* mice models (Derghal et al., 2015) (**Figure 2**). The intraperitoneal and intracerebroventricular injection of leptin decreased the expression of these miRNAs in the hypothalamus of wild type and *ob/ob* mice suggesting a role of leptin in the expression of mir-383, mir-384-3p, and mir-488 (Derghal et al., 2015) (**Figure 2**). Altogether, these observations strongly suggest that miRNAs are important for the central regulation of energy homeostasis by melanocortin pathway.

CONCLUSIONS AND PERSPECTIVES

As indicated above, a large number of miRNAs are involved in the central regulation of energy homeostasis. We postulate that miRNAs are energy sensors involved in the hypothalamic control of systemic energy balance (**Figure 1**). Moreover, we cannot exclude a role of the miRNAs in the cortico-limbic system involved in the interaction of organism with the food-providing environment. The complete regulatory network

involving miRNAs is largely unknown. However, several studies have identified specific miRNAs that control the expression of the melanocortin pathway genes by leptin (**Figure 2**). Despite these promising observations, the specific roles of these different miRNAs in the melanocortin pathway neurons activity upon regulation of food intake, energy expenditure, and glucose homeostasis remain largely unknown. Great efforts should also be made to clarify this last point. To address this question, CRISPR/Cas9 as emerging genome editing tool in biology/medicine research could be used. Indeed, CRISPR/Cas9 shows a benefit in the specific control of crossing off-target impact on miRNAs in the same family or with highly similar sequences.

AUTHOR CONTRIBUTIONS

LM and AD wrote the manuscript. MD and JT helped with manuscript preparation.

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