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NUTRIENTS, NEUROTRANSMITTERS AND BRAIN ENERGETICS

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EDITORIAL
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Editorial: Nutrients, Neurotransmitters and Brain Energetics

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Editorial on the Research Topic

Nutrients, Neurotransmitters and Brain Energetics

Deficiency or overabundance of nutrients has a marked effect on brain functionality. Lasting effects are observed even when a nutritional insult occurs early in life, i.e., during brain development (Morgane et al., 1978; Guedes, 2011). Nutrient deficits, especially related to protein-energy undernutrition, have been extensively studied, but the specific effects of diet supplementation on brain functionality are growing in interest, particularly supplementation with lipids and their derivatives. Lifestyle and eating habits have changed worldwide over the last few decades. The consumption of high-palatable diets that are rich in carbohydrates and fats in association with a sedentary lifestyle has dramatically increased, and therefore, metabolic syndrome and neurodegenerative diseases are reaching epidemic proportions (Berthoud, 2012; O'Neil et al., 2014; Afshin et al., 2017).

The number of studies investigating the beneficial and deleterious effects of nutrients, especially fatty acids, on brain functions has increased, and dietary fatty acids are critical for brain excitability, metabolism, and behavior. From dietary manipulations of proteins, carbohydrates and lipids in *in vitro* studies of brain-cultured cells and clinical studies, the articles presented in this special issue investigate changes of brain excitability, metabolism, brain fatty acid membrane composition, and cognitive functions. The *in vivo* and *in vitro* studies that are presented below support the importance of lipids, or their derivatives, and proteins in normal brain excitability, development, cognition maturation and improvement and examine the role of novel food sources or nutraceutical compounds as emerging strategies to improve brain functionality at specific stages of the lifespan.

de Melo et al. studied diet manipulation with different fatty acid compositions in rats receiving diets supplemented with avocado (*Persea americana* Mill.) pulp and oil. Compared to the non-supplemented control group, maternal diet supplementation during gestation and lactation with avocado pulp and oil induced changes in the fatty acid composition of brain cell membranes, with higher total poly-unsaturated fatty acids (PUFA) in the brains of rat adult offspring. Avocado pulp supplementation resulted in a higher docosahexaenoic acid (DHA) proportion in the hippocampus. Oil- and pulp-supplemented diets anticipated in the offspring the appearance of reflex responses, and avocado pulp supplementation amplified six of seven tested reflexes. In another study, Queiroz et al. demonstrated memory improvement in rat offspring from pregnant and lactating dams that were treated with conjugated linoleic acid (CLA). Reflex maturation anticipation and reduced locomotor activity were also present in rats whose mothers received 3% CLA diet supplementation.

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Mendoza et al. and Lerner et al. studied the effects of fatty acids on brain excitability, metabolism and cell degeneration using a combination of cotinine plus krill oil administered via gavage or palmitoylethanolamide intraperitoneal injection, respectively. Krill oil is a source of PUFAs that is extracted from small marine crustaceans and exhibits higher DHA bioavailability than fish oil, and it has beneficial health effects on hyperlipidemia, inflammation, and cognitive function in rodents and humans (Xie et al., 2019). Cotinine is the main metabolite of nicotine, and it was used as a therapeutic agent to reduce neuroinflammation, enhance memory and reduce the effects of neurodegenerative diseases, such as Parkinson's disease (Barreto et al., 2015). C57BL/6J mice were subjected to restraint stress and treated for 21 consecutive days with a mixture of cotinine and krill oil. Treated animals exhibited a significant reduction in depressive-like behavior and cognitive impairment. An increased number of GFAP-positive cells was demonstrated in the hippocampal dentate gyrus of stressed mice treated with cotinine and krill oil. These results suggest that the association of cotinine and krill oil improves cognitive impairment and cell damage in the hippocampus of stressed animals and prevents depressive-like behavior and brain cells degeneration (Mendoza el al.).

Nutraceutical use of fatty acids and its derivatives in neuroprotection is increasing. Lerner et al. studied sub-chronic, prophylactic palmitoylethanolamide (PEA), which is a fatty acid amide present in a variety of food sources, such as egg yolks, soybeans, and peanuts, in C57BL/6J mice. Tissue lipidomic and transcriptomic analyses demonstrated that intraperitoneal administration of PEA differentially regulated neuroinflammatory pathways in the spleen and hippocampus. There was a downregulation of splenic pro-inflammatory signaling cytokines and a decrease in hippocampal neuroinflammatory pathways. A transcriptional downregulation of brain-derived neurotrophic factor (BDNF) and upregulation of Crh genes, which modulate hippocampal excitability, were also observed. These observations collectively represent new insights into the molecular changes of the prophylactic use of PEA to decrease the pro-inflammatory environment and modulate brain excitability, which suggests beneficial actions of this nutraceutical in neuroprotection, neuroinflammation, and convulsive disorders.

Behavioral and metabolic changes are also present during menopause due to ovarian hormone variability, which lead to a decreased metabolic rate and mood disorders (Gordon et al., 2015). Dornellas et al. studied the effects of high-fat diets enriched in lard or fish-oil on metabolism, behavior and the monoaminergic system were studied in ovariectomized rats. A lard (saturated fatty acid-rich) diet was more obesogenic than a fish-oil diet and had effects on the hippocampal monoaminergic system. A reduced expression of 5-HT_{1B} mRNA and increased expression of 5-HT_{2C} receptors was observed after treatment with a high-fat, fish oil-based diet. The serotonin-mediated hypothalamic hypophagia response was restored in ovariectomized rats receiving a high-fat fish-oil diet. Both diets reduced anxiety, but the lard diet induced a depressive-like behavior. Dietary long-chain omega-3 fatty acid consumption and behavioral changes in humans is an active research topic. Some evidence, as showed in this issue by Darcey et al., indicates a possible role of omega-3 fatty acids intake in modulating impulse control behavior in adolescents. Based on a Food Frequency Questionnaire and Go/No-Go functional magnetic resonance imaging (MRI) study, the authors evidenced an inverse relationship between the energy-adjusted omega-3 Index and activity in the dorsal cingulate cortex, which suggests that the intake of omega-3 fatty acids in adolescents is associated with a better ability to manage impulse control.

Two review papers in this issue examined diet-related nutritional imbalance and neuropsychiatric disorders: Meira et al. and Melo et al.. Melo et al. discussed the main role of dietary lipid-mediated tissue inflammation on cognitive deficits and behavioral changes. A cross-talk between peripheral proinflammatory cytokines, such as IL-6, IL-1β, and TNFα and activation of brain inflammation is suggested as part of the potential mechanisms involved in cognitive deficits due to an increased intake of diets rich in saturated fatty acids associated with a reduced intake of PUFAs. A very high omega-6/omega-3 ratio promotes brain inflammation, activation of microglia, and reduces tryptophan availability to produce serotonin, which may contribute to depressive-like symptoms. Further evidence demonstrated that omega-3 PUFAs prevented microglial activation and neuroinflammation and maintained normal synaptic function. Accordingly, Hu et al. demonstrated the neuroprotective effects of propionate, a short chain fatty acid precursor in lipid biosynthesis, in a study of neurite outgrowth in the SH-SY5Y neuroblastoma cell line treated with haloperidol. Neuropeptide Y mediated the neuroprotective effect of propionate on neurite outgrowth because treatment with NPY-siRNA (siNPY) suppressed the haloperidol-induced impaired neurite length, which indicates that propionate plays a role in neuroprotection.

Multiple pathways are involved in neuroinflammation/neuroprotection. For example, constant exposure to infrasound, which are inaudible to human ears (frequencies below 20 Hz), results in neuroinflammation and hearing damage. The role of astrocyte activation in mediating neuroinflammation was evaluated in rats and cultured astrocytes exposed to 16 Hz, 150 dB infrasound (Shi et al.). The results showed the participation of the fibroblast growth factor 2/fibroblast growth factor receptor 1 (FGF2/FGFR1) pathway, which inhibited astrocyte activation and reduced the levels of pro-inflammatory cytokines *in vitro* and *in vivo*. Neuroinflammation is also a characteristic of the epileptic brain. One of the most common treatments to manage refractory epilepsy is the use of ketogenic diets (KDs). A variety of long- and medium-chain fatty acids are used in KDs in the search for more appropriate combinations of anaplerotic fatty acids. Meira et al. present the classical mechanisms related to metabolic and neurotransmitter changes under KD, and new perspectives on the role of gut microbiota on the anti-seizure and anti-inflammatory effects of KD are mentioned. In their review article Poff et al. discuss new evidences of ketone bodies alone acting on anti-seizure properties of KD. Furthermore, Granado-Rojas et al. showed in neuronal cells of rat dentate gyrus an increased K+/Cl- cotransporter (KCC2) expression

which could be involved in the well-known GABA-mediated inhibition of brain excitability.

The consumption of protein-restricted diets during intrauterine and early postnatal life profoundly affects brain development and function. Current research presented in this issue in various papers shows the long-term effects of maternal protein-restricted diets in the first (F1) and second (F2) generations of rats. Mitochondrial function and neurotransmitter shifts were associated with anxiety-related behaviors and attentional deficits in adulthood. Mokler et al. showed that *in vivo* microdialysis of neurotransmitters to F1 restricted-protein rats revealed interhemispheric differences in the concentration of monoamines, with lower norepinephrine (NE) and dopamine (DA) levels in the right than left ventromedial prefrontal cortex (vmPFC) of malnourished rats. Increased levels of serotonin (5-HT) in the left vmPFC were also observed. In another investigation published in this issue, Newman et al. demonstrated attentional deficits in prenatal protein-malnourished rats, which were less distracted than well-nourished rats when confronted with predictable and unpredictable visual distractors. These results suggest more cognitive rigidity in malnourished rats. The noradrenergic system is associated with this cognitive rigidity because animals with selective noradrenergic deafferentation of the prelimbic cortex show the same behavioral change. Protein-restricted diets affect brain regions differently when malnutrition occurs during F1 or F2 rat generations, which was demonstrated in this issue by Santana et al.. A maternal low-protein diet affected brainstem mitochondrial bioenergetics in young male F1 rats, with the F1 progeny showing increased reactive species production and decreased antioxidant capacity. The antioxidant capacity was upregulated in adulthood in F2 generation rats. These data demonstrate an attempt of F2 generation rats to recover from the nutritional insult of the F1 generation. In this issue, Vega et al. using electroencephalographic data from children exposed to protein energy malnutrition (PEM) in the first year of life proposed a new classification procedure to evaluate the effects of early malnutrition on brain electrical activity.

Environmental factors tightly influence nutritional status and cognitive brain functions. Mendes et al. studied the association of soft (powder) diet, age, and environment impoverishment in young and aged mice. The reduction of masticatory activity due to intake of the powder diet changed mouse exploration patterns in the open field test, and environmental impoverishment contributed to the modification of exploratory patterns of locomotor activity, which declined with age.

The creation of new perspectives for improving nutritional status is represented in this issue by the search for safe food sources and supplements, as reviewed by Sinha et al.. These authors reviewed the nutritional properties of Spirulina and reinforced its use as a food source against protein malnutrition. Spirulina is a blue green alga that has been used as food source since the ancient times of the Aztecs and Mayans. Recent studies confirmed that Spirulina was an important source of protein (60 to 70%), essential fatty acids, vitamins, and minerals. Sinha et al. reviewed its effects and molecular mechanisms against oxidative stress, protein malnutrition, and neuroinflammation and showed beneficial effects of a Spirulina-supplemented diet in neuroprotection. Notably, the effects of plant derivatives, or synthetic anxiolytic/psychostimulant compounds, on behavioral disorders are also discussed in this issue. Stress, depression-like and anxiety-like behaviors are associated with imbalanced neurotransmitter systems and related cognitive deficits. The consumption of GABA-containing tea (2.01 mg/200 mL cup) had a positive effect in young people with self-reported stress and reduced heart rate variability (Hinton et al.). An experimental study demonstrated that matured hop bitter acids orally administered for 6 consecutive days improved depression-like behavior in mice and suppressed hippocampal neuroinflammation. Vagus nerve stimulation may mediate the effects of hop bitter acids and lead to increases in hippocampal norepinephrine levels via the *locus coeruleus* (Fukuda et al.). Zinc compounds also presented an antidepressant and anxiolytic effect in diabetic rats suggesting zinc supplementation as potential beneficial compound to improve neurobehavioral deficits in diabetic animals as demonstrated by Cavalcanti et al..

Francisco and Guedes demonstrated a cholinergic system action on behavioral parameters and brain electrical activity using chronic (21-day) pilocarpine administration in rats. The sub-convulsing dose (45 mg/kg) increased anxiety-like behavior and reduced the velocity of the propagation of cortical spreading depression (CSD), which is a pathophysiological phenomenon related to brain excitability disorders. Animals grown in unfavorable lactation conditions (suckling in a large litter size, 15 pups/dam) exhibited a more marked pilocarpine decelerating effect on CSD propagation compared to normal-sized litters (9 pups/dam), which suggests a differential change in brain excitability according to nutritional status. The same research group (Francisco et al.) demonstrated that these pilocarpine-effects also occurred at an older age. The authors demonstrated changes in blood glucose levels and the immunoreactivity for glial fibrillary acidic protein (GFAP)-containing astrocytes, and ionized calcium-binding adapter molecule 1 (Iba1)-containing microglia. Finally, these authors presented data suggesting that treatment with taurine attenuated the pilocarpine effects.

Changes in brain excitability were also reported in humans who were chronic alcohol consumers. This condition modified visual acuity in humans and led to deficits in spatial luminance contrast sensitivity and color vision. Visual impairment was not reversed after long-term alcohol abstinence, as demonstrated by Martins et al.. Furthermore, Lacerda et al. studying Amazonian population environmentally exposed to mercury contamination proved visual dysfunction mainly characterized by lower visual perimetric area in this population. Additional evidence suggests that ascorbate levels in retinal neuronal cells are fundamental to glutamatergic neurotransmission. The *in vitro* study in chick retinal cells (Portugal et al.) suggested a role of dopamine regulation of ascorbate release, which requires glutamate release and activation of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate receptors. Malnutrition and vitamin C deficiency are recurrent in chronic alcoholics, and mechanistic hypotheses, as presented by Portugal et al. are needed to better understand the role of

the bioavailability of ascorbate to neuronal cells in the retina and visual function.

Astrocytes play a central role in neuroprotection, brain metabolism, cellular communication, water transportation, regulation of neurotransmitters, ion concentrations, and brain diseases. Glucose destination in neural cells depends on cell metabolism, glucose supply and brain activity. Lactate produced and released by astrocytes during increased brain activity binds to monocarboxylate transporters (MCTs) or hydroxy-carboxylic acid receptor 1 (HCAR1) on post-synaptic neurons and astrocytic perivascular processes regulating synaptic communication and plasticity (Gonçalves et al.). Neuroplasticity mediated via brain-derived neurotrophic factor (BDNF) production during high-intensity interval training, which is characterized by exercise in relatively short bursts of vigorous activity, are also discussed in this issue by Jiménez-Maldonado et al..

Metabolic and vascular changes occur during the development of stroke and brain tumors, such as glioblastoma, which is the most aggressive CNS cancer. Regulation of the astrocytic expression of aquaporin 4 (AQP4) mediates the mechanisms of neuroprotection. Costa et al. used *in vivo* and *in vitro* approaches and demonstrated modulation of AQP4 expression via the thyroid hormone triiodothyronine (T3). This effect was observed during CNS development with T3 exerting a biphasic effect. AQP4 expression was reduced in postnatal early life, and its expression increased in the brains of young mice. T3 treatment to the GBM-95 glioblastoma cell line reduced AQP4 expression, which suggests that T3 reduction of AQP4 in astrocytes would prevent brain damage related to water flux changes in cerebral parenchyma. Data from rodent studies also showed improvement of brain functionality after stroke, which is a long-term disability related to brain hemodynamic and water transport changes, when animals were treated with *Panax ginseng* Meyer (Araliaceae). Neuroprotective effects were partially related to the action of ginseng on AQP4 reduced expression (Liu et al.). Therefore, astrocytic modulation of AQP4 expression seems to participate in the mechanisms implicated in the attenuation of brain damage related to stroke and glioblastoma cancer.

The articles presented in this special issue focus on dietary-related, metabolism-associated and neurotransmitter-dependent effects on brain excitability and behavioral changes, especially related to protein and fatty acid diet composition. The ingestion of a polyunsaturated omega-3-enriched diet showed beneficial effects, including improving metabolism, monoaminergic neurotransmitter system and depression-like and anxiety-like behaviors, and nutrient-deficient diets or high-fat saturated diets increased neuroinflammation and promoted attentional deficits, anxiety depressive-like behavior and cognitive rigidity. Regarding interventions based on the administration of nutritional compounds or drugs, additional studies demonstrating beneficial effects of the dose-response phenomenon known as hormesis, improving health and enhancing lifespan as a function of the adaptive plasticity of the organisms (Brunetti et al., 2020; Di Rosa et al., 2020), are surely wanted. We conclude that the interesting conjunction of the studies presented in this Research Topic provide a nice discussion about the underlying mechanisms of the neuroprotection/neuroinflammation relationship.

AUTHOR CONTRIBUTIONS

AX-d-S conceived of the presented idea. AX-d-S and RG wrote the manuscript. Both authors contributed to the article and approved the submitted version.

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Inhibitive Effects of FGF2/FGFR1 Pathway on Astrocyte-Mediated Inflammation *in vivo* and *in vitro* After Infrasound Exposure

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Infrasound, a kind of ambient noise, can cause severe disorders to various human organs, specially to central nervous system (CNS). Our previous studies have shown that infrasound-induced CNS injury was closely related with astrocytes activation and astrocytes-mediated neuroinflammation, but the underlying molecular mechanisms are still largely unclear. FGF2/FGFR1 (Fibroblast growth factor 2/Fibroblast growth factor receptor 1) pathway was reported to play an important role in anti-inflammation in CNS disorders. To further study the possible roles of FGF2/FGFR1 pathway in infrasound-induced CNS injury, here we exposed Sprague-Dawley rats or cultured astrocytes to 16 Hz, 150 dB infrasound, and explored the effects of FGF2 on infrasound-induced astrocytes activation and neuroinflammation. Western blotting, immunofluorescence and liquid chip method were used in this experiment. Our results showed that after 3- or 7-day exposure (2 h/day) of rats as well as 2 h exposure of cultured astrocytes to 16 Hz, 150 dB infrasound, astrocyte-expressed FGFR1 was downregulated *in vivo* and *in vitro*. FGF2 pretreatment not only inhibited infrasound-induced astrocyte activation in rat hippocampal CA1 region, but also reduced the levels of pro-inflammatory cytokines, such as TNF-α, IL-1β, IL-18, IL-6, and IFN-γ *in vitro* and *in vivo*. However, FGF2 significantly upregulated the expression of FGFR1. Furthermore, we showed that FGF2 could attenuate IκBα phosphorylation, NF-κB p65 translocation, pro-inflammatory cytokines levels, and neuronal loss in the CA1 region induced by infrasound. On the contrary, PD173074, a special antagonist of FGFR1, could reverse the effects above *in vitro* and *in vivo*. Taken together, our findings showed that FGF2/FGFR1 pathway may exert inhibitive effects on astrocyte-mediated neuroinflammation *in vitro* and *in vivo* after infrasound exposure.

Keywords: infrasound, hippocampus, astrocytes, FGF2, FGFR1, NF-κB, neuroinflammation

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INTRODUCTION

Infrasound is a common public health problem produced by all kinds of noise in the environment (Leventhall, 2007). Infrasound is also a sound of low frequency between 0.0001 and 20 Hz (Sand and Karlsen, 2000; Alves-Pereira and Castelo, 2007). Its essence is mechanical vibration. Infrasound comes from two aspects, natural world and artificial activities, including volcanic eruptions, tsunami, aircraft flight, and weapons (Angelis et al., 2016). Infrasound can cause a systemic disease, known as vibroacoustic disease (VAD) (Ferreira et al., 2006; Mendes et al., 2006; Alves-Pereira and Castelo, 2007), which may cause damages to many systems, such as the central nervous system (CNS) (Yuan et al., 2009; Cheng et al., 2012; Zhang et al., 2016), digestion system (Da et al., 2006), cardiovascular system (Pei et al., 2009), respiratory system (Castelo et al., 2003; Branco et al., 2007), and other systems.

Infrasound can cause VAD, though the specific mechanism is unknown. It has been suggested that infrasound can induce neuronal axon degeneration and neuronal damage by promoting Ca^{2+} influx (Cheng et al., 2012). Exposure to infrasound can remarkably impair the learning and memory ability of rats (Liu et al., 2010; Ma et al., 2015) and increase the neuronal apoptosis in rat hippocampus because of pro-inflammatory cytokines from neuroglial cells (Yuan et al., 2009; Shi et al., 2013; Cai et al., 2014). Under the infrasound with frequency 16 Hz and sound pressures ranging from 90 to 130 dB, the average escape latency of rats was increased, indicating that the more infrasound was intensified, the more learning and memory was damaged (Shi et al., 2013).

Our previous study revealed a relationship between infrasound-induced CNS injury and glia-triggered neuroinflammation (Shi et al., 2013). Glial cells were activated by infrasound exposure of 16 Hz, 130 dB and the levels of IL-1β and TNF-α in glial supernatants were increased, suggesting that anti-inflammation may be a new therapeutic approach to attenuate infrasound-mediated injury (Shi et al., 2013; Cai et al., 2014). However, we still have little knowledge about the mechanism underlying glial cell-mediated inflammation under infrasound exposure. Several lines of evidence revealed that FGF2/FGFR1 pathway played an important role in neuroinflammation as well as its effect on cell proliferation, differentiation, and neurogenesis (Yoshimura et al., 2001; Frinchi et al., 2010; Celik et al., 2016). For instance, exogenous FGF2 inhibited hippocampal inflammation induced by LPS and reversed the neuroinflammation-induced depressive-like behavior (Tang M.M. et al., 2017).

In this study, using infrasound-exposed rats and astrocyte culture, we respectively activated and blocked FGF2/FGFR1 pathway by FGF2 and FGFR1 antagonist PD173074 to study its effects on infrasound-induced inflammation. Our results revealed that infrasound exposure induced astrocyte activation and downregulated the expression of FGFR1. FGF2 administration could alleviate activated astrocyte and upregulate FGFR1 expression, and however, PD173074 reversed these effects.

In this study, to investigate whether FGF2/FGFR1 pathway attenuates astrocytes-mediated inflammation after infrasound exposure, we respectively activated and blocked FGF2/FGFR1 pathway by FGF2 and FGFR1 antagonist PD173074 to study its effects on infrasound-induced inflammation *in vivo* and *in vitro*. Given that astrocyte-mediated inflammation and anti-inflammation effects of FGF2, we proposed that infrasound may induced astrocyte-mediated inflammation by inhibiting FGF2/FGFR1 pathway and activating FGF2/FGFR1 pathway might be a promising way to treat infrasound-associated diseases.

MATERIALS AND METHODS

Infrasound Generation

High-intensity infrasound chamber and detection system, called as the infrasound system, were built by the Fourth Military Medical University and Aerospace Industry Corporation, described in our previous studies (Du et al., 2010; Shi et al., 2013). The infrasonic pressure chamber system used in this experiment is composed of an air compressor, an air flow modulator, an infrasonic cabin and a set of software analysis system. The detection system consists of three parts: a infrasound sensor, a set of data acquisition system for ultra-low frequency-noise signal and a set of computer simulation analysis system. The infrasound system uses the key techniques of high infrasonic excitation sources and realizes the continuous adjustable frequency of high-intensity infrasonic signal. The infrasound system can generate standard infrasonic waves with a frequency range from 4 to 20 Hz and a sound pressure level from 150 to 160 dB. In this experiment, a frequency of 16 Hz and a pressure level of 150 dB was performed.

Animals and Drug Administration

Male SD rats (clean grade) weighting 220–250 g were obtained from the Experimental Animal Center, Fourth Military Medical University. The animals were housed under controlled condition with 12-h light, 12 h dark. The rats were provided by normal administration of rodent diet and tap water. The protocol was approved by the Animal Ethics Committee of Fourth Military Medical University and Use Board. All invasive procedures were performed under anesthesia with pentobarbital sodium (40 mg/kg) and under physiological monitoring such as respiration and blood pressure, minimizing discomfort for rats.

In vivo, all rats were equally divided into the control group (the rats without infrasound exposure, $n = 6$), the infrasound (IS) group (those exposed to 16 Hz, 150 dB of infrasound for 1, 3, or 7 days, $n = 6$ each), the FGF2 groups (infrasound-exposed rats treated with FGF2, $n = 6$ each), the PD173074 groups (infrasound-exposed rats treated with PD173074, $n = 6$ each). For FGF2 administration, the rats were injected intraperitoneally (i.p.) with 0.1 mg/kg. For PD173074 administration, the rats were injected i.p. with 1.5 mg/kg. FGF2 was dissolved in saline (Graham and Richardson, 2009; Graham and Richardson, 2010). PD173074 was dissolved in saline containing 12.5% cremophor EL and 2.5% DMSO (Di Marco et al., 2014). The rats were received with four doses of either FGF2 or PD173074 before 24, 16, 8, and 2 h of infrasound exposure.

In vitro, cultured astrocytes were given with FGF2 (200 ng/ml) or PD173074 (2 μM) at 2 h before infrasound exposure, and then divided into the control group, the infrasound group, the FGF2

group, and PD group. The dosages of FGF2 and PD173074 used in the present study were based on the previous research and our pilot experiment (Williams et al., 2003; Weiss et al., 2010). The purified astrocytes were reseeded at a density of 3×10^4 cells/cm^2 onto glass coverslips in 96-well plates (for immunofluorescence) or 1×10^6 cells/cm^2 in 6-well plates (for western blot). For each group, at least three assays were repeated.

Reagents and Antibodies

Rabbit anti-FGFR1 was purchased from Abcam (Cambridge, United Kingdom), mouse anti-GFAP was from Novus (Colorado Springs, CO, United States). HRP goat anti-mouse IgG(H+L), HRP goat anti-rabbit IgG(H+L) were from Jackson ImmunoResearch (West Grove, PA, United States). Hoechst 33342 was from GeneCopoeia (Rockville, MD, United States). SDS-PAGE Gel Kit, SDS-PAGE Loading buffer were from Beijing ComWin Biotech, Co., Ltd. (Beijing, China). Recombinant rat fibroblast growth factor 2 was from Novoprotein (Shanghai, China). PD173074 dissolved in DMSO was from Selleckchem (Houston, TX, United States). NE-PER Nuclear and Cytoplasmic Extraction Reagents was from Thermo (Waltham, MA, United States). Milliplex MAP Kit Rat Cytokine Magnetic Bead Panel, Milliplex MAP Lysis buffer were from EMD Millipore (Millipore, Germany). DMSO was from Sigma (St. Louis, MO, United States). DMEM with glucose and L-glutamine was from Corning (New York, NY, United States). All the antibodies were placed at −20°C after dispensing, and stored at +4°C after used, except that the antibodies containing 50% glycerol were always placed at −20°C. All the antibodies were avoided freezing-thawing cycle.

Preparation of Brain Tissues

At the end of infrasound exposure, rats were anesthetized as described above. The protocol for obtaining brain slices was based on previously described (Melvin and Sutherland, 2010). Then rats were intracardially perfused with 150 ml normal saline at 30 r/min, 200 ml 4% paraformaldehyde (PFA) at 30 r/min and finally 200 ml 4% PFA at 4 r/min by a perfusion pump. After perfusion, brains were placed in 4% PFA, fixed for 6 h and replaced with 30% sucrose solution, until the brain was at the bottom of bottle. Subsequently brains were frozen and sectioned in 30 μm thick slices at −20°C by a Leica CM 1900 cryostat. The brains slices were placed in a 12-well plate filled with 1× PBS and transferred to a 24-well plate containing 70% glycerol and stored at −20°C. These brains slices were used for immunofluorescence.

Immunofluorescence

Double-labeling immunofluorescence was used to evaluate the co-expression of FGFR1/GFAP, cleaved caspase-3/NeuN and cleaved caspase-3/GFAP, as previously described (Kigerl et al., 2009). Cells and brain slices were washed with 1× PBS for 30 min, then blocked with 3% BSA for 30 min. The sections were incubated overnight at 4C in the following primary antibodies: rabbit polyclonal FGFR1 (1:200), mouse monoclonal GFAP (1:100), mouse monoclonal NeuN (1:800), Cleaved caspase-3 (1:200). After the sections were washed, primary antibodies were detected with dylight 488-conjugated goat anti-rabbit IgG or dylight 594-conjugated goat anti-mouse IgG secondary antibodies. Confocal images were obtained by using a Olympus FV1000 confocal microscope.

Primary Astrocytes Culture

Primary cultures of neonate rat hippocampus astrocytes were prepared from 1-day-old rats, as previously described (Li et al., 2006; Hansson et al., 2008). Briefly, the hippocampus tissue of rats was collected in small dishes containing D-hanks solution and blood vessels were carefully removed. The tissues were cut by ophthalmic scissors and then 2 ml 0.05% trypsin was added. After digestion for 3–5 min, 4 ml culture medium was added to stop digestion and the cell suspension was filtered. Then the filtered cell suspension was centrifuged at 1000 rpm for 5 min at 4°C, and the cell suspension was cultured in DMEM supplemented with 10% fetal bovine serum, 1% glutamine, 1% penicillin and streptomycin under a 37°C incubator. If the culture medium turned yellow, it should be replaced with new medium.

Western Blotting

Western blotting was done as earlier described (Block et al., 2013). Cytoplasmic protein and nucleoprotein was extracted from the primary culture cells in every group. According to the experimental procedure, equal amounts of proteins were loaded on 10% SDS-PAGE gels and transferred to membranes. Then the PVDF membranes were blocked with 5% skim milk for 2 h at room temperature and were incubated with rabbit polyclonal FGFR1 (1:400), rabbit polyclonal NF-κB p65 (1:500), rabbit monoclonal IκBα (1:1000), rabbit monoclonal p-IκBα (1:1000) overnight at 4°C. TBP (1:1000) and β-actin (1:2000) were used as an internal control. Corresponding second antibodies of HRP goat anti-rabbit IgG(H+L) and HRP goat anti-mouse IgG(H+L) were incubated for 1 h at room temperature. The enhanced chemiluminescence (ECL) system was used for detection to visualize proteins.

Proinflammatory Cytokine Detection

Milliplex MAP Rat Cytokine Kit was used to measure the following pro-inflammatory cytokines: TNF-α, IL-1β, IL-18, IL-6, and IFN-γ, according to manufacturer's instructions and as previously described (Arrode-Bruses and Bruses, 2012; Codices et al., 2012). All buffers and diluents were warmed to room temperature when used. The standard, quality controls, sample, buffer and beads were sequentially added in a 96-well filter plate and then were incubated for 2 h on the shaker at room temperature. For the detection of proinflammatory cytokine, the samples were respectively incubated with detection antibodies for 1 h and streptavidin–phycoerythrin for 30 min in the dark. After washing the beads twice with BioTek plate washer, the Median Fluorescence Intensity values were analyzed in the Luminex 200TM instrument by using the MILLIPLEX® Analyst 5.1 analysis software.

Data Analysis

All the data were expressed as means ± standard deviation (SD) and statistical analysis were performed using SPSS 16.0 software.

Differences between groups were analyzed with one-way ANOVA followed by Tukey test. Each of the analyses groups met the normal distribution before ANOVA was performed. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Infrasound Downregulated the Expression of FGFR1

To examine whether FGF2/FGFR1 pathway is involved in infrasound-induced inflammation in astrocytes, we first observed the changes in FGFR1 expression after infrasound exposure. We found that FGFR1 and GFAP showed a co-localized expression pattern, and FGFR1 immunoreactivity appeared granular and was distributed on the surface of $GFAP^+$ astrocyte membrane (**Supplementary Figure S1**).

Consistent with our previous study (Shi et al., 2013), astrocytic activation was evidence, as indicated by increased GFAP expression (**Figures 1A,C**) after infrasound exposure. By contrast, the expression of FGFR1 were significantly decreased after 3 days, 7 days post-exposure to infrasound, as compared with control group ($p < 0.01$) (**Figures 1A,B**). Compared with the control group (73.00 ± 7.00), the number of $FGFR1^+/GFAP^+$ cells was also decreased at 7 days post-exposure to infrasound (12.00 ± 4.00, $p < 0.01$) (**Figures 1A,D**). Furthermore, in primary cultured astrocytes, the expression of FGFR1 and the number of $FGFR1^+/GFAP^+$ cells were also reduced at 12 h (2.75 ± 0.48, $p < 0.05$), 24 h (1.81 ± 0.38, $p < 0.01$) post-exposure to infrasound, compared with the control group (4.61 ± 0.79) (**Figures 1E,F** and **Supplementary Figure S2**). Therefore, these results suggested that after infrasound exposure, astrocyte-expressed FGFR1 was decreased *in vivo* and *in vitro*.

FGF2 Attenuates Infrasound-Induced Astrocytic Activation

To investigate the effects of FGF2 on infrasound-induced astrocyte activation, the rats were treated with exogenous FGF2 intraperitoneally. At post-7d exposure, the morphology of astrocytes around hippocampal CA1 region revealed by GFAP immunofluorescence was observed. We found that the processes of astrocytes significantly were longer and their bodies became larger than those of the control group. After pretreatment with FGF2, the processes of astrocytes became shorter and bodies became smaller (**Figure 2A**). Consistently, GFAP immunoreactivity and $GFAP^+$ cells number were markedly increased after infrasound exposure ($p < 0.01$). FGF2 pretreatment attenuated infrasound-induced increased GFAP immunoreactivity ($p < 0.05$), and however, did not affect $GFAP^+$ cells number ($p > 0.05$) (**Figures 2B,C**). We also have preformed the double-staining of GFAP and cleaved caspase-3 (a marker for apoptotic cells), and found no overt colocalization after FGF2 pretreatment (**Supplementary Figure S3**). Thus, we proposed that the reduction of $GFAP^+$ area after FGF2 pretreatment did not result from the cells death but from the inhibition of astrocytes activation. Our results showed that infrasound exposure induced astrocyte activation and downregulated FGFR1 expression, and however, FGF2 administration could alleviate astrocyte activation, indicating that FGF2 may exert an inhibitory effect on infrasound-induced astrocyte activation.

FGF2 Inhibits Infrasound-Triggered Elevation of Pro-inflammatory Cytokines

Next, we examined whether FGF2 had an effect on glia-mediated inflammatory reaction, indicated by the levels of pro-inflammatory cytokines after infrasound exposure. Our results showed that the levels of TNF-α ($p < 0.01$), IL-1β ($p < 0.01$), IL-18 ($p < 0.01$), IL-6 ($p < 0.01$), and IFN-γ ($p < 0.01$) were significantly elevated in the hippocampus of rats after being exposed to infrasound at 7 days (**Figure 3**), compared with the control group. On the contrary, FGF2 administration inhibited the infrasound-mediated release of pro-inflammatory cytokines like TNF-α ($p < 0.01$), IL-1β ($p < 0.01$), IL-18 ($p < 0.05$), IL-6 ($p < 0.01$), and IFN-γ ($p < 0.05$), as compared with IS-7d group (**Figure 3**). To confirm these results, cytokines in the supernatant of infrasound-exposed astrocyte culture were examined. As expected, the levels of pro-inflammatory cytokines above were also remarkably increased after being exposed to infrasound at 12 h, compared with the control group (**Supplementary Figure S4**). However, cytokine levels were not statistically different between 12 and 24 h after infrasound exposure (data not shown). FGF2 pretreatment restrained the release of pro-inflammatory cytokines induced by infrasound (**Supplementary Figure S4**). Therefore, these results showed that FGF2 inhibited infrasound-triggered release of pro-inflammatory cytokines *in vivo* and *in vitro*.

FGF2 Upregulates FGFR1 Expression After Infrasound Exposure

Previous studies have showed that FGF2 could exert a positive feedback effect by upregulating FGFR1, its own receptor (Woodbury and Ikezu, 2014; Porta et al., 2017; Yuan et al., 2017). Therefore, we examined whether exogenous FGF2 also changed FGFR1 expression after infrasound exposure. As shown above, the expression of FGFR1 around the hippocampal CA1 region of rats was decreased to the lowest level at 7 days after infrasound exposure ($p < 0.05$), as compared with IS-3d group (**Figure 1A**). Therefore, we chose 7 days for the following study. FGF2 treatment increased FGFR1 immunoreactivity and $FGFR1^+/GFAP^+$ co-labeled cells around the CA1 region (**Figure 4A**). In addition, our *in vitro* results of Western blotting and immunofluorescence showed that after 12 h exposure to infrasound, FGFR1 expression in primary cultured astrocytes with FGF2 preconditioning was increased, as compared with that without FGF2 pretreatment (**Figure 4E** and **Supplementary Figure S5**). Taken together, our results showed that FGF2 remarkably upregulated FGFR1 expression both *in vivo* and *in vitro* after infrasound exposures.

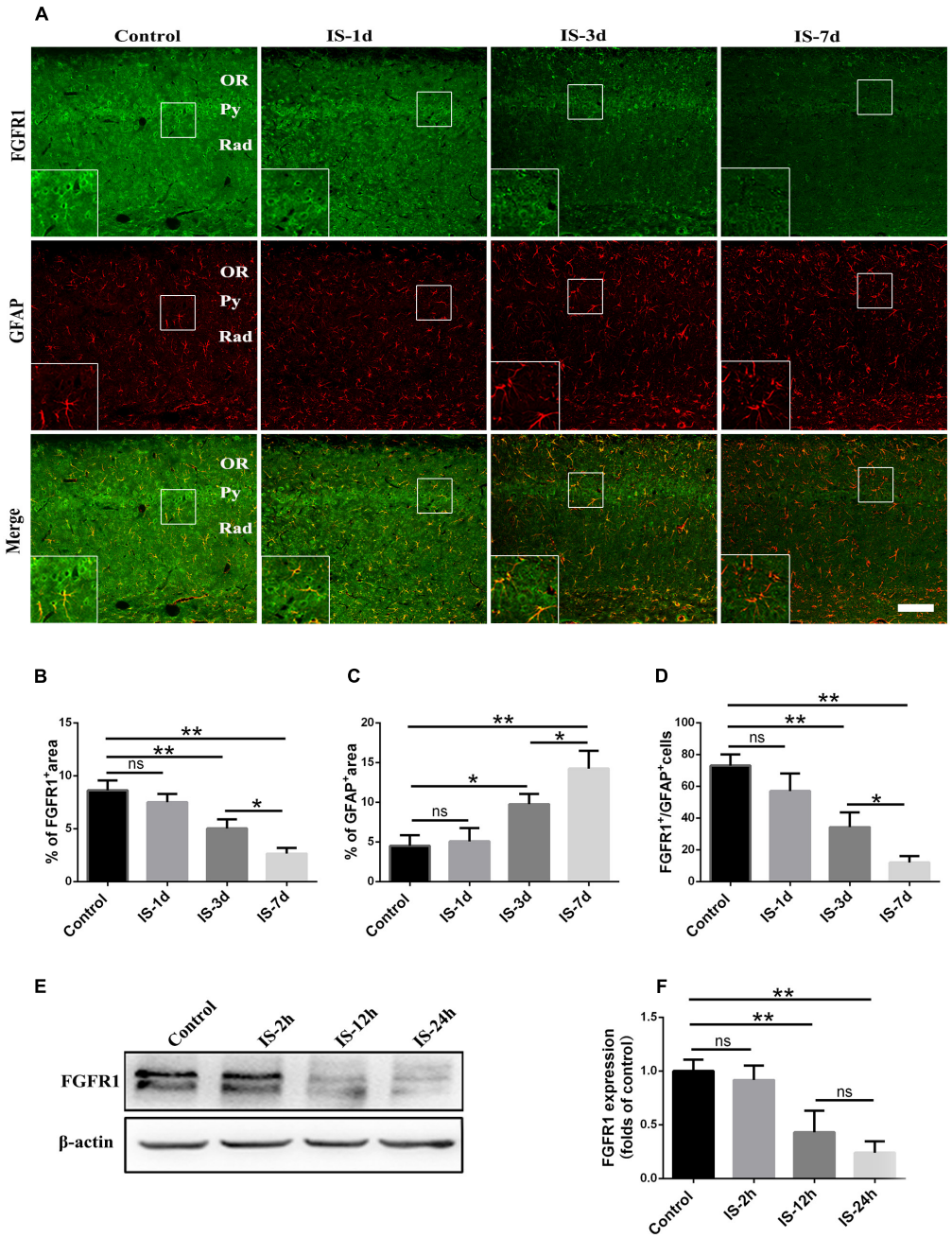

FIGURE 1 | Changes in the expression of FGFR1 after infrasound exposure. **(A)** The changes in the expression of FGFR1 (green) and GFAP (red) revealed by double immunostaining around the CA1 region of rats at 1d (IS-1d), 3d (IS-3d), or 7d (IS-7d) post-exposure to infrasound. OR, oriens layer of the hippocampus; Py, pyramidal cell layer of the hippocampus; Rad, stratum radiatum of the hippocampus. Scale bar: 100 μm. **(B,C)** Quantitation of the percentage of FGFR1$^+$ area and GFAP$^+$ area around the hippocampus CA1 region (620 μm × 620 μm). **(D)** Count of the number of FGFR1$^+$/GFAP$^+$ cells around the hippocampal CA1 region (620 μm × 620 μm). **(E,F)** Western blotting revealed the changes in FGFR1 expression in primary cultured astrocytes at 2 h (IS-2h), 12 h (IS-12h), or 24 h (IS-24h) post-exposure to infrasound. β-Actin is as an internal control. All the data are represented as means ± SD. *$p < 0.05$, **$p < 0.01$. ns, no significance.

FGF2 Inhibits IκBα Phosphorylation and NF-κB Nuclear Translocation After Infrasound Exposure

As aforementioned, exogenous FGF2 could increase FGFR1 expression, and attenuate astrocyte-mediated inflammation after infrasound exposure, but the specific mechanism was still not clear. It has been reported that FGF2 could accelerate rat skin wound repair and regulate inflammatory response by inhibiting NF-κB p65 expression (Xuan et al., 2016). Thus, next we investigated whether FGF2/FGFR1 pathway could

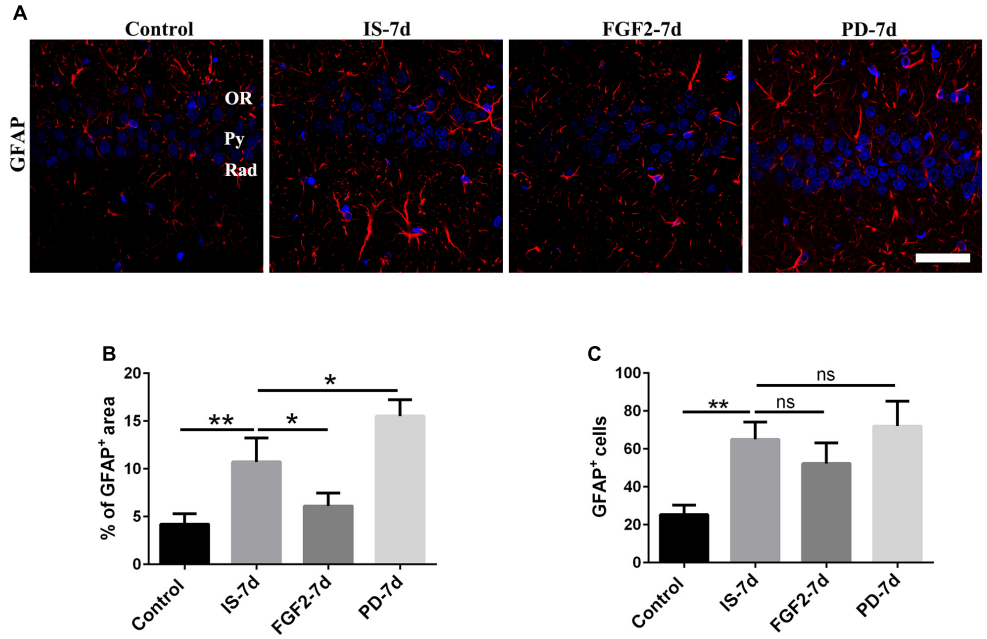

FIGURE 2 | FGF2 attenuates astrocyte activation around the CA1 region of rats after infrasound exposure. **(A)** The changes in cells morphology revealed by GFAP (red) immunostaining around rat CA1 region with FGF2 (FGF2-7d) or PD173074 (PD-7d) treatment after 7 days of infrasound exposure (IS-7d). OR, oriens layer of the hippocampus; Py, pyramidal cell layer of the hippocampus; Rad, stratum radiatum of the hippocampus. Scale bar: 50 μm. **(B)** Quantitation of the percentage of $GFAP^+$ area around the hippocampus CA1 region (620 μm × 620 μm). **(C)** Count of the number of $FGFR1^+/GFAP^+$ cells around the hippocampal CA1 region (620 μm × 620 μm). All the data are represented as means ± SD. $^*p < 0.05$, $^{**}p < 0.01$. ns, no significance.

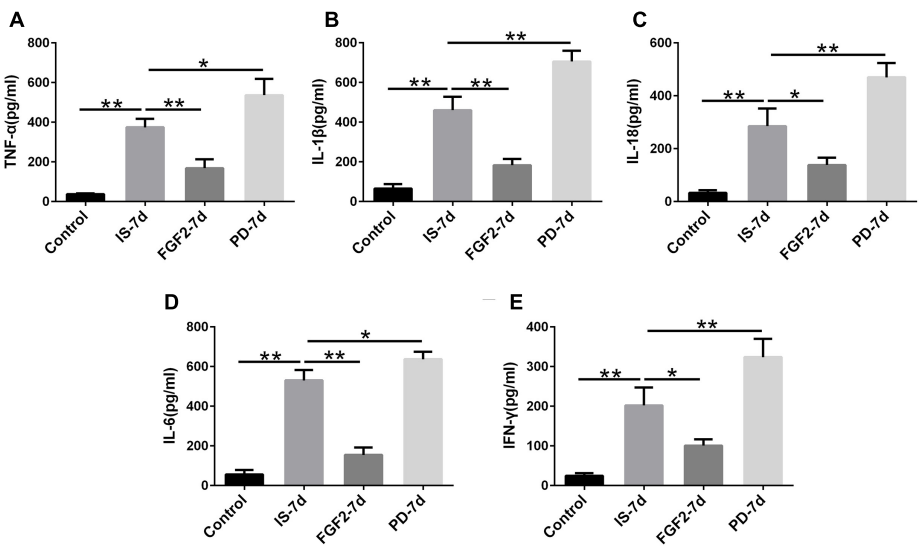

FIGURE 3 | The levels of TNF-α, IL-1β, IL-18, IL-6, IFN-γ in the hippocampus of rats. Effect of FGF2 on infrasound-triggered pro-inflammatory cytokines release, including TNF-α **(A)**, IL-1β **(B)**, IL-18 **(C)**, IL-6 **(D)**, IFN-γ **(E)**. IS-7d means 7d post-infrasound exposure, FGF2-7d means FGF2 treated for 7d post-infrasound exposure, PD-7d means PD173074 treated for 7d post-infrasound exposure. All the data are represented as means ± SD. $^*p < 0.05$, $^{**}p < 0.01$.

inhibit infrasound-triggered inflammation via NF-κB pathway as well.

As shown above, FGFR1 expression in cultured astrocytes decreased strikingly after 12 h post-exposure to infrasound, as compared with the control group ($p < 0.01$) (**Figure 1E** and **Supplementary Figure S2**), and then we chose 12 h as the time point for the following study. Immunoblotting results showed that the level of IκBα phosphorylation and the expression of NF-κB p65 in the nuclei were increased at 12 h post-exposure to infrasound ($p < 0.05$), compared with the control

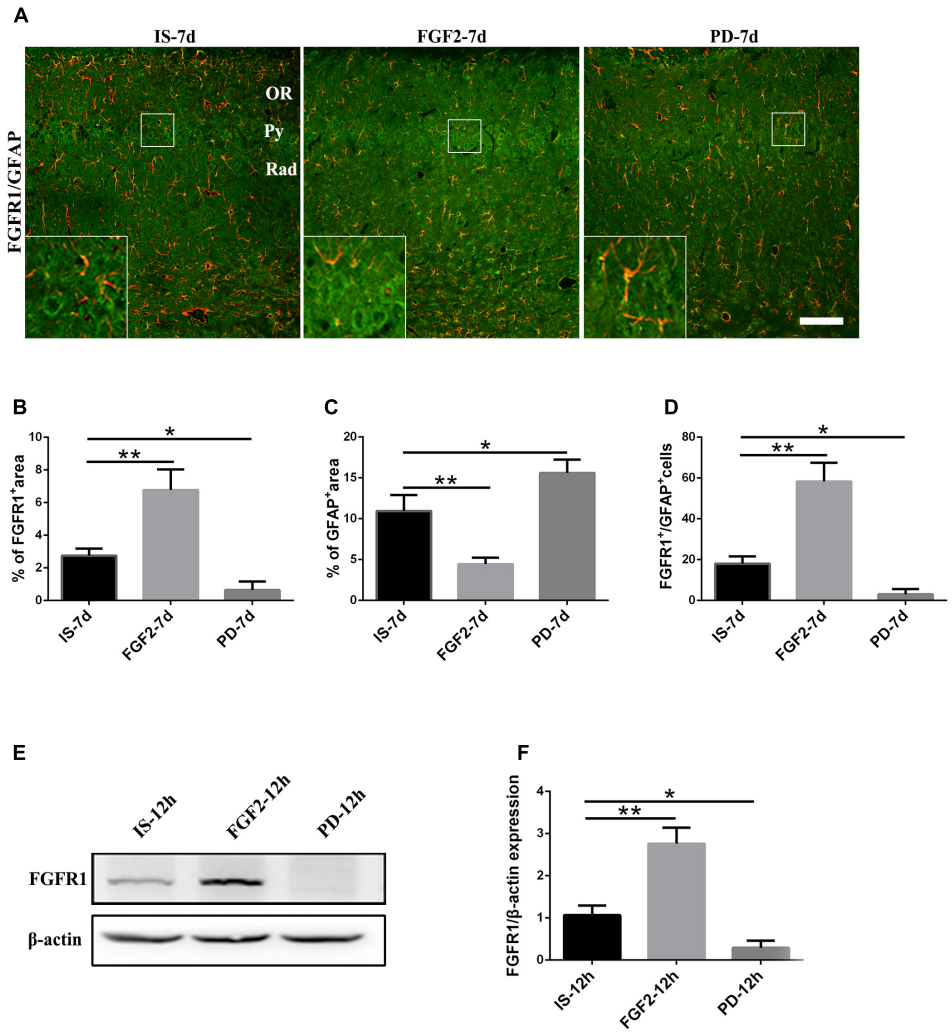

FIGURE 4 | Changes in the expression of FGFR1 after FGF2 administration. **(A)** The changes in the expression of FGFR1 (green) and GFAP (red) revealed by double immunostaining around the CA1 region in rats with FGF2 (FGF2-7d) or PD173074 (PD-7d) treatment after 7d of infrasound exposure (IS-7d). OR, oriens layer of the hippocampus; Py, pyramidal cell layer of the hippocampus; Rad, stratum radiatum of the hippocampus. Scale bar: 100 μm. **(B)** Quantitation of the percentage of FGFR1$^+$ area around the hippocampus CA1 region (620 μm × 620 μm). **(C)** Count of the number of FGFR1$^+$/GFAP$^+$ cells around the hippocampal CA1 region (620 μm × 620 μm). **(D,E)** Western blotting revealed the changes in FGFR1 expression in primary cultured astrocytes with FGF2 (FGF2-12h) or PD173074 (PD-12h) treatment after 12 h of infrasound exposure (IS-12h). β-Actin is as an internal control. All the data are represented as means ± SD. *$p < 0.05$, **$p < 0.01$.

group. On the contrary, after pretreatment of primary cultured astrocytes with FGF2, IκBα phosphorylation and nuclear p65 expression were obviously decreased ($p < 0.05$), compared with the infrasound group (**Figure 5**). Therefore, exogenous FGF2 may block infrasound-triggered NF-κB pathway.

FGF2 Alleviates Neuron Loss in Hippocampal CA1 Region After Infrasound Exposure

Our previous study showed that inflammatory cytokines from infrasound exposure can cause neuron loss (Shi et al., 2013). Consistently, our present data also showed that the number of neurons indicated by NeuN immunostaining was obviously decreased in rat hippocampal CA1 region at 7 days post-exposure to infrasound, compared with the control group. As expected, double-labeling immunofluorescence of cleaved caspase-3 and NeuN showed that infrasound exposure increased the neuronal apoptosis (**Supplementary Figure S6**). After pretreatment with FGF2, the number of neurons (64.33 ± 6.03) was more than that exposed to infrasound (40.00 ± 8.54, $p < 0.05$) (**Figure 6**). Taken together, FGF2 could alleviate neuron loss in hippocampal CA1 region after infrasound exposure.

FGF2/FGFR1 Pathway Inhibition Aggravates Infrasound-Induced Inflammation

In order to confirm the effects of FGF2/FGFR1 pathway after infrasound exposure, PD173074, a special antagonist of

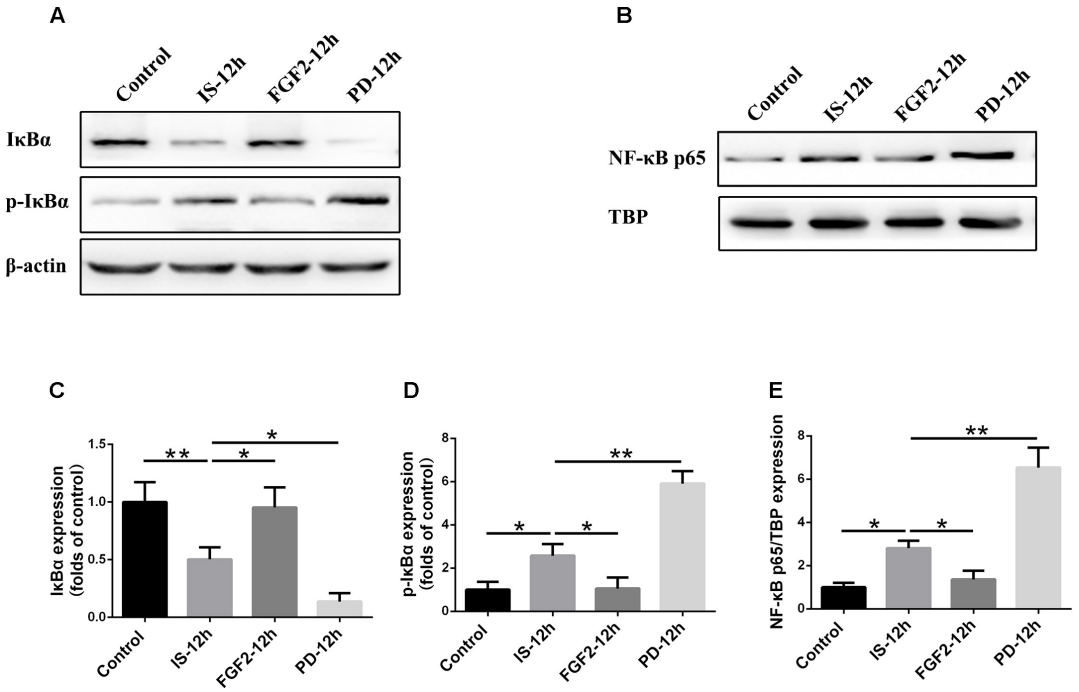

FIGURE 5 | Effect of FGF2 administration on the expression of IκBα and NF-κB in the cultured astrocytes after infrasound exposure. **(A)** Western blotting revealed the changes in IκBα and p-IκBα expression in the cytoplasm of cultured astrocytes with FGF2 (FGF2-12h) or PD173074 (PD-12h) treatment after 12 h of infrasound exposure (IS-12h). **(B)** Western blotting revealed the changes in NF-κB p65 expression in the nucleus of cultured astrocytes with FGF2 (FGF2-12h) or PD173074 (PD-12h) treatment after 12h of infrasound exposure (IS-12h). **(C–E)** The changes in IκBα, p-IκBα, and NF-κB p65 expression in primary cultured astrocytes revealed at IS-12h group, PD-12h group and FGF2-12h group. β-Actin is as an internal control of cytoplasmic protein **(A)**. TBP is as an internal control of nuclear protein **(B)**. All the data are represented as means ± SD. Data are all expressed as means ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$.

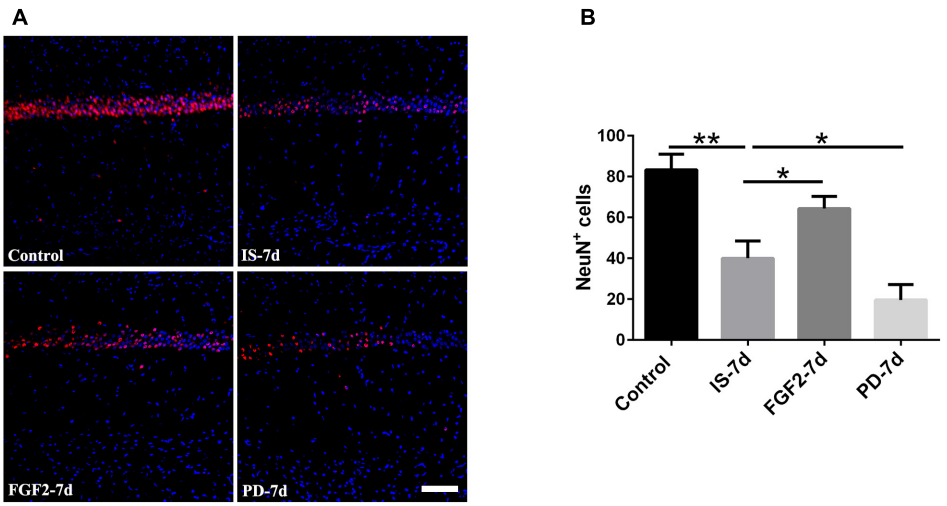

FIGURE 6 | Changes in the number of NeuN^{+} cells after FGF2 administration. **(A)** The changes in the number of neuron revealed by NeuN (red) immunostaining in rat CA1 region with FGF2 (FGF2-7d) or PD173074 (PD-7d) treatment after 7d of infrasound exposure (IS-7d). Scale bar: 100 μm. **(B)** Count of the number of FGFR1^{+}/GFAP^{+} cells in the hippocampal CA1 region (620 μm × 620 μm). All the data are represented as means ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$.

FGFR1, was administered. Our results showed that PD173074 aggravated infrasound-induced astrocyte activation and release of proinflammatory cytokines TNF-α, IL-1β, IL-18, IL-6, and IFN-γ *in vivo* and *in vitro* (**Figures 2**, **3** and **Supplementary Figure S4**). Moreover, PD173074 downregulated FGFR1 expression in astrocytes, compared with the infrasound group *in vivo* and *in vitro* (**Figure 4** and **Supplementary Figure S5**), and subsequently decreased IκBα phosphorylation ($p < 0.01$),

increased nuclear expression level of NF-κB p65 ($p < 0.01$) (**Figure 5**). Moreover, precondition with PD173074 aggravated neuron loss, as compared with that exposed to infrasound ($p < 0.05$) (**Figure 6**). Taken together, inhibition of FGF2/FGFR1 pathway could aggravate astrocyte-mediated inflammation after infrasound exposure.

DISCUSSION

In this study, we investigated possible effects of FGF2/FGFR1 pathway in infrasound-induced inflammatory injury. Our results revealed that the exposure to16 Hz, 150 dB infrasound *in vivo* and *in vitro* could result in astrocytic activation and a decrease of FGFR1 expression. Activating FGF2/FGFR1 pathway by FGF2 attenuated astrocytes-mediated inflammation while FGFR1 inhibition aggravated these effects after infrasound exposure.

Astrocytes are the most widely distributed cells in the brain of mammals and are the key components in the brain inflammatory response (Bellaver et al., 2015; Colombo and Farina, 2016; Gonzalez-Reyes et al., 2017). Astrocytes are known to be divided into two major types, protoplasmic astrocytes in gray matter and fibrous astrocytes in white matter which are associated with the blood–brain barrier (Chen and Swanson, 2003; Freeman, 2010). Protoplasmic astrocytes which play a crucial role, can differentiate into fibrous astrocytes during brain ischemia injury (Kajihara et al., 2001; Lukaszevicz et al., 2002; Bylicky et al., 2018). Activation of astrocytes can promote the production of pro-inflammatory cytokines in pathological conditions (Sofroniew, 2015; Jha et al., 2017; Bylicky et al., 2018). In the present study, we demonstrated that after infrasound exposure, astrocyte activation was significant and the levels of TNF-α, IL-1β, IL-18, IL-6, and IFN-γ were significantly increased *in vivo* and *in vitro*, compared to the control group. These pro-inflammatory cytokines had turned out to have a harmful effect on neurons, leading to neuronal apoptosis (Lu et al., 2005; Cai et al., 2014; Phuagkhaopong et al., 2017; Yin et al., 2017), which was confirmed by our present results.

For exploration of possible mechanism underlying astrocytes-mediated inflammation after infrasound exposure, we focused on FGF2/FGFR1 pathway due to its important role in neuroinflammation. FGFR1, a tumorigenic receptor tyrosine kinase, plays an important role in some physiological processes and progression of cancer (Woodbury and Ikezu, 2014; Haenzi and Moon, 2017; Tang C. et al., 2017). In some conditions, FGFR1 signaling can be stimulated by FGF2, which can lead to dimerization and dephosphorylation of FGFR1, exhibiting corresponding actions, not only including cell proliferation, cell growth, anti-apoptosis, but also anti-inflammation (Yoshimura et al., 2001; Zittermann and Issekutz, 2006; Sun et al., 2017; Tang M.M. et al., 2017). In addition, FGF2/FGFR1 signaling could regulate inflammation of hippocampus to exert a positive effect on some neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease, multiple sclerosis, and traumatic brain injury (Woodbury and Ikezu, 2014). In the present study, we showed that FGFR1 was expressed on astrocytes, and activated FGF2/FGFR1 signaling by FGF2 alleviated enhanced astrocytic activation and increased levels of TNF-α, IL-1β, IL-18, IL-6, IFN-γ induced by infrasound. Moreover, FGF2 could upregulate the expression of FGFR1 on astrocytes, implying that FGF2 maybe exert a positive feedback effect by upregulating its own receptor FGFR1 in infrasound-induced inflammation.

As for the mechanism underlying FGF2/FGFR1 signaling on anti-inflammation, NF-κB pathway may exert an important role (Xuan et al., 2016). NF-κB can regulate many processes involved in immune function and inflammation (Ho et al., 2012; Kim et al., 2017). Our results showed that after infrasound exposure, the expression of p-IκBα and nuclear NF-κB p65 was increased, suggesting that infrasound may activate NF-κB through degradation of IκBs and subsequent translocation of NF-κB p65 subunit into the nuclei, finally activating transcription of target genes, including various proinflammatory cytokines (such as TNF-α, IL-1β, IL-18, IL-6, and IFN-γ). After FGF2 administration, IκBα phosphorylation and p65 nuclear translocation were attenuated, the levels of pro-inflammatory cytokines were reduced, and the number of neuronal cells was increased, as compared with infrasound group. On the contrary, inhibition of FGF2/FGFR1 signaling by PD173074 reversed corresponding effects above. Thus, we proposed that FGF2 could attenuate the infrasound-induced inflammation by preventing the translocation of NF-κB into nucleus via the regulation of the IκBα.

Our results provided the first evidence that FGF2/FGFR1 pathway may exert an inhibitive effect on astrocyte-mediated inflammation *in vivo* and *in vitro* after infrasound exposure. Specifically, infrasound could downregulate FGFR1 expression and trigger astrocyte activation, NF-κB nuclear translocation, pro-inflammatory cytokine release and neuron loss. Activation of FGF2/FGFR1 pathway attenuated astrocytes-mediated inflammation while inhibition aggravated these effects induced by infrasound, suggesting that FGF2/FGFR1 pathway might be a promising target for treatment of infrasound-associated diseases.

CONCLUSION

Our results provided the first evidence that astrocytes-mediated inflammation was involved in infrasound-induced neuronal damage, and FGF2/FGFR1 pathway may exert an inhibitive effect on this process.

ETHICS STATEMENT

The experimental rules and regulations was approved by the Ethics Committee of the Affiliated Xijing Hospital of Fourth Military Medical University. The animal experiment for our study was approved by the Fourth Military Medical University Committee for Animal Research. All efforts were made to minimize animal suffering and to reduce the number of animals used.

AUTHOR CONTRIBUTIONS

LM and GZ conceived and designed the experiments. Y-JS performed the experiments. L-JX, LLi, and L-HZ analyzed the data. HH, C-YL, Q-JZ, and L-FZ contributed reagents, materials, and analysis tools. Y-JS and MS wrote the paper. All authors contributed to the final approval of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2018.00582/full#supplementary-material

FIGURE S1 | Co-localization of FGFR1 and GFAP around the CA1 region of hippocampus. FGFR1 (**A**, green) was expressed in astrocytes (**B**, red). Nucleus was represented by hoechst (**C**, blue). Co-localization of FGFR1$^+$/GFAP$^+$ **(D)** were shown as above. Scale bar: 10 μm.

FIGURE S2 | Changes in the expression of FGFR1 in the primary cultured astrocytes after infrasound exposure. **(A)** The changes in the expression of FGFR1 (green) and GFAP (red) revealed by double immunostaining of FGFR1 with GFAP at control group, 2 h (IS-2 h), 12 h (IS-12 h), 24 h (IS-24 h) post-exposure to infrasound. IS-2 h means 2 h post-infrasound exposure and so on. Scale bar: 100 μm. **(B–D)** Quantitation of the percentage of FGFR1$^+$ area, GFAP$^+$ area and FGFR1$^+$/GFAP$^+$ area (620 μm × 620 μm) in the primary cultured astrocytes at different time points after infrasound exposure. All the data are represented as means ± SD. $^*p < 0.05$, $^{**}p < 0.01$. ns: no significance.

FIGURE S3 | Double immunostaining of GFAP and cleaved caspase-3 around the CA1 region of hippocampus after FGF2 administration by infrasound exposure. GFAP (**A**, red) was not co-localizated with cleaved caspase-3 (**B**, green). Nucleus was represented by hoechst (**C**, blue). GFAP and cleaved caspase-3 (**D**) were not co-localizated. Scale bar: 10 μm.

FIGURE S4 | The levels of TNF-α, IL-1β, IL-18, IL-6, IFN-γ in the primary cultured astrocytes. **(A–E)** Effect of FGF2 on infrasound-triggered pro-inflammatory cytokines release, including TNF-α **(A)**, IL-1β **(B)**, IL-18 **(C)**, IL-6 **(D)**, IFN-γ **(E)**. IS-12 h means 12 h post-infrasound exposure, FGF2-12 h means FGF2 treated for 12 h post-infrasound exposure, PD-12 h means PD173074 treated for 12 h post-infrasound exposure. All the data are represented as means ± SD. $^*p < 0.05$, $^{**}p < 0.01$.

FIGURE S5 | Changes in the expression of FGFR1 after FGF2 administration by infrasound exposure. **(A)** The changes in the expression of FGFR1 (green) and GFAP (red) revealed by double immunostaining of FGFR1 with GFAP at 12 h post-exposure to infrasound (IS-12 h), FGF2 treated for 12 h post-infrasound exposure (FGF2-12 h), PD173074 treated for 12 h post-infrasound exposure (PD-12 h). Scale bar: 100 μm. **(B–D)** Quantitation of the percentage of FGFR1$^+$ area, GFAP$^+$ area and FGFR1$^+$/GFAP$^+$ area (620 μm × 620 μm) in the primary cultured astrocytes at different groups after infrasound exposure. All the data are represented as means ± SD. $^*p < 0.05$, $^{**}p < 0.01$.

FIGURE S6 | Co-localization of NeuN (**A**, red) and cleaved caspase-3 (**B**, green) around the CA1 region of hippocampus after infrasound exposure. Nucleus was represented by hoechst (**C**, blue). Co-localization of NeuN$^+$/cleaved caspase-3$^+$ **(D)** were marked by arrows. Scale bar: 10 μm.

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frontiers in Neuroscience

ORIGINAL RESEARCH
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Propionate Protects Haloperidol-Induced Neurite Lesions Mediated by Neuropeptide Y

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Haloperidol is a commonly used antipsychotic drug for treating schizophrenia. Clinical imaging studies have found that haloperidol can cause volume loss of human brain tissue, which is supported by animal studies showing that haloperidol reduces the number of synaptic spines. The mechanism remains unknown. Gut microbiota metabolites, short chain fatty acids including propionate, are reported to have neuroprotective effect and influence gene expression. This study aims to investigate the effect and mechanism of propionate in the protection of neurite lesion induced by haloperidol. This study showed that 10 μM haloperidol (clinical relevant dose) impaired neurite length in human blastoma SH-SY5Y cells, which were confirmed by using primary mouse striatal spiny neurons. We found that haloperidol impaired neurite length were accompanied by a decreased neuropeptide Y (NPY) expression, but no effect on GSK3β signaling. Importantly, this project research found that propionate was capable of protecting against haloperidol-induced neurite lesions and preventing NPY reduction. To confirm this finding, we used specific siRNAs targeting NPY which blocked the protective effect of propionate on haloperidol-induced neurite lesions. Furthermore, since NPY is regulated by the nuclear transcription factor CREB, we measured pCREB that was decreased by haloperidol and was normalized by propionate. Therefore, propionate has a protective effect against pCREB-NPY mediated haloperidol-induced neurite lesions.

Keywords: antipsychotic drug, haloperidol, neurite impairment, propionate, neuropeptide Y

INTRODUCTION

Antipsychotic drugs are the primary therapeutic agents used to treat schizophrenia and its allied mental disorders (Huang and Song, 2018). Among them, haloperidol is the first-generation antipsychotic drug and widely used to treat schizophrenia patients (Tardy et al., 2014). Haloperidol acts on the dopamine D2 receptors (D2R) and controls psychotic symptoms including

hallucinations, delusions, and aggressiveness (Jafari et al., 2012; Dold et al., 2015). However, it can also cause various side effects including extrapyramidal syndrome, tardive dyskinesia, and cerebrovascular events (Hufner et al., 2015). In brain morphology, chronic application of haloperidol has been reported to reduce brain volume (Ho et al., 2011). A number of studies have suggested that chronic or accumulative haloperidol administration can decrease synaptic spines and induce apoptosis (Frost et al., 2010; Nandra and Agius, 2012). A chronic application of haloperidol induces neurite lesion reported in human, animal, and cell-based studies (Kelley et al., 1997; Dorph-Petersen et al., 2005; Critchlow et al., 2006; Ho et al., 2011). A meta-analysis revealed that higher daily haloperidol intake in patients resulted in greater cortical gray matter reduction (Z, -2.31, $p = 0.02$) (Vita et al., 2015). Animal study shows that macaque monkeys treated with haloperidol for 17 to 27 months have a reduced brain weight by 8–11%, and these reductions were consistent across a number of brain areas (Dorph-Petersen et al., 2005). Another study shows that chronic administration of haloperidol at ∼0.35 mg/kg at 2-week intervals for 1 year significantly reduces neuronal cytoskeleton and spine-associated proteins in the cortices of rhesus monkey, where are rich in dopamine innervation and are implicated in the psychopathology of schizophrenia (Lidow et al., 2001). Therefore, there is an urgent need to search for a way to protect antipsychotic drug-induced neurite lesion.

Our previous study shows that haloperidol decreases neuropeptide Y (NPY) mRNA expression in the rat brain after haloperidol treatment (Huang et al., 2006). NPY is highly co-expressed in GABAergic neurons and is found to be a modulator of the neuroplasticity, neurotransmission, and memory (Gotzsche and Woldbye, 2016). Given these evidence, we have investigated whether or not NPY was involved in haloperidol-induced neurite lesion.

Short chain fatty acid (SCFA) including acetate, propionate, and butyrate are the metabolites produced by gut microbiome fermentation on dietary fiber. SCFA can enter the circulation via monocarboxylate transporters, cross the blood–brain barrier, and thereby enter the central nervous system (Pierre and Pellerin, 2005; Kekuda et al., 2013). More and more evidence show that SCFA regulate cell metabolism (Canfora et al., 2015), neurotransmitter synthesis and release (DeCastro et al., 2005; Shah et al., 2006), epigenetics (Yamawaki et al., 2012), and immune function (Correa-Oliveira et al., 2016). In particular, propionate and butyrate act as the histone deacetylases inhibitors (HDACi). HDACi regulates brain gene expression, improving the healthy state of patients suffering from Parkinson's disease, depression, and schizophrenia (Galland, 2014). However, the neurite protective, at high concentrations, propionate has also been reported to induce autism-like behavioral changes in rats (Macfabe, 2012). Collectively, these reports suggest that propionate may play an important role in neural function. Our study investigated whether or not propionate may be used to prevent haloperidol-induced neurite lesions. Furthermore, we have investigated the CREB-NPY signaling pathway in mediating the neurite protective effect of propionate in haloperidol-induced neurite lesion.

MATERIALS AND METHODS

Cell Culture and Treatments

The undifferentiated human SH-SY5Y neuroblastoma cell line were grown in Dulbecco's modified Eagle's medium (DMEM)-F12 supplemented with 1% penicillin–streptomycin and 10% heat-inactivated fetal bovine serum (FBS) from Bovogen Biologicals (Victoria, Australia). For differentiation, cells were seeded in culture plates coated with MaxGel™ ECM (E0282, Sigma Aldrich, Syndey). In the following day, media was removed and replaced with 10 μM retinoic acid (RA, R2625; Sigma-Aldrich) in DMEM-F12 with 1% FBS. Haloperidol (MP Biomedicals, Solon, OH) was dissolved in 100% dimethyl sulfoxide (Sigma-Aldrich). Sodium propionate was purchased from Sigma-Aldrich. Cells were treated with media containing either haloperidol or haloperidol with different concentrations of propionate. The neurite length was acquired in real time every 6 h for 24 h using IncucyteZoom Machine and analyzed with the Neuro Track software (Sartorius, Michigan).

Gene Transfection

The siNPYs (siNPY_001: 5′CAGACCTCTTGATGAGAGA3′; siNPY_002: 5′CGCTGCGACACTACATCAA3′; siNPY_003: 5′GAGGACATGGCCAGATACT3′) and respective negative control (NC) were synthesized (RiboBio, Guangzhou) and dissolved in the DEPC H_2O. Transfections of siRNAs were performed with the Lipofectamine 2000 (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. Medium was changed to the differentiation medium containing various treatments 6 h later.

Primary Striatal Neuronal Culture

Cultured striatal neurons were harvested from postnatal days 0 to 3 of C57Bl mice. Briefly, striatal neurons were gently dissociated with a plastic pipette after digestion with 0.5% trypsin (GIBCO, Los Angeles) at 37°C for 30 min. Neurons were cultured in neurobasal medium (GIBCO) containing B27 supplement (GIBCO) and 20 mM glutamine (Sigma Aldrich). After 24 h of culture, 5-fluoro-2′-deoxyuridine (Sigma Aldrich) was added at a final concentration of 10 μM to repress the growth of glial cells. Cultures were maintained at 37°C in a humidified 5% CO_2 incubator for 7 days (DIV7) prior to treatments. All experimental procedures for primary cell culture were approved by the Animal Ethics Committee, University of Wollongong, Australia, and complied with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

Western Blot

After 24 h treatments, cells were harvested with lysis buffer containing NP40 (Sigma-Aldrich), Protease Inhibitor Cocktail (Sigma-Aldrich), 1 mM PMSF (Sigma-Aldrich), and 0.5 mM β-glycerophosphate (Sigma-Aldrich). Total protein concentrations were determined by DC-Assay (Bio-Rad, Sydney) and detected with a SpectraMax Plus384 absorbance microplate reader (Molecular Devices, Sunnyvale, CA). Samples were heat-treated in Laemmli buffer at 95°C, loaded to

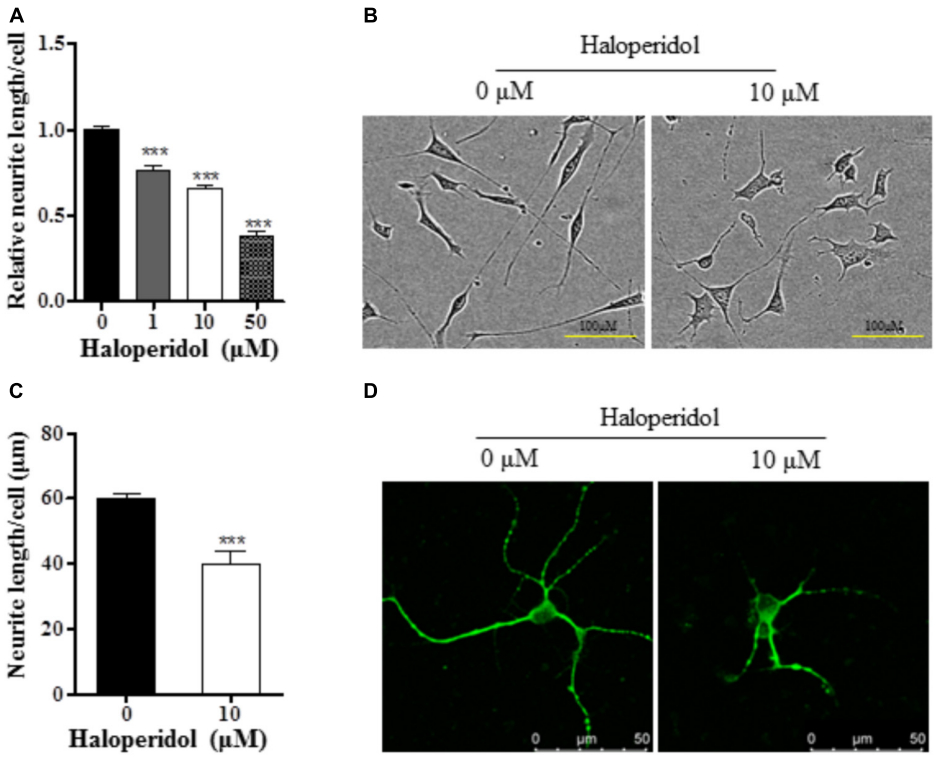

FIGURE 1 | Haloperidol inhibited neurite outgrowth. **(A)** Neurite length was reduced in a dose dependent manner by 1, 10, and 50 μM haloperidol treatments in SH-SY5Y cells for 24 h (n = 3–4); **(B)** representative images of cells with (10 μM) and without (0 μM) haloperidol treatments in SH-SY5Y cells; **(C)** haloperidol (10 μM) decreased neurite length/cell in primary striatal neurons (n = 6); **(D)** primary striatal neurons stained with MAP2 antibody and imaged with immunofluorescence confocal microscope. Mean ± SEM, ***$p < 0.001$.

10% SDS-PAGE gels (Bio-Rad) for fractionation, and then transferred into Immun-Blot TM PVDF membranes (Bio-Rad). The blocking buffer consisted of 5% slim milk in TBST. The membranes were incubated with NPY (sc-28943, Santa Cruz Biotechnology, Santa Cruz), phospho-GSK3β(Ser9) (#9323s, Cell Signaling Technology), and β-Catenin (#8480s, Cell Signaling Technology) antibodies in TBST containing 1% milk at 4°C overnight. Secondary antibodies were anti-rabbit IgG conjugated with horseradish peroxidase (Santa Cruz Biotechnology). For visualization, we used ECL detection reagents and obtained high resolution images with Amersham Gel Imager (GE Healthcare life Sciences).

Immunofluorescence Assay

Primary striatal neurons were grown to approximately 70% confluence on glass coverslips and treated with either negative control, haloperidol, haloperidol + propionate, or propionate for 24 h before being fixed in 4% formaldehyde for 15 min. Neurons were washed in PBS, and permeabilized with 0.3% Triton X-100 in PBS for 10 min. After blocking with 5% normal donkey serum for 1 h at room temperature, primary antibodies of MAP2 (M4403-2ML, Sigma-Aldrich), NPY, or GAD67 (MAB5406, Millipore, Bedford) were applied in 1% donkey serum in PBS at 4°C overnight. This was followed by incubation in a secondary antibody Alexa Fluor 488-conjugated donkey anti-mouse IgG (Invitrogen, Carlsbad, CA) or Alexa Fluor 488-conjugated donkey anti-rabbit IgG (Invitrogen, Sydney), Alexa Fluor™ 647-conjugated donkey anti-mouse IgG (Invitrogen), and Alexa Fluor 568 Phalloidin (A12380, Invitrogen) at room temperature for 2 h.

For SH-SY5Y cells, cells were seeded in the ibidi glass bottom dish (ibidi GmbH, Germany), treated with either haloperidol, haloperidol + propionate, propionate, or nil control in differentiated medium for 24 h, and then the above steps were followed by immunofluorescence assays. We applied the primary antibodies including pCREB (sc-101662, Santa Cruz Biotechnology) and MAP2 to cells at 4°C overnight, followed by incubation in a secondary antibody cocktail of Alexa Fluor 488-conjugated donkey anti-rabbit IgG (Invitrogen) and Alexa Fluor 647-conjugated donkey anti-mouse IgG (Invitrogen) at room temperature for 2 h. Cells were viewed using 40× or 63× oil immersion objective on a DMI6500B confocal microscope (Leica, Mannheim, Germany). The neurite length and protein expression were measured using the ImageJ Software.

Spine Morphology

Primary striatal neurons were cultured for 14 days (DIV14) and were used for spine morphology study. The procedure

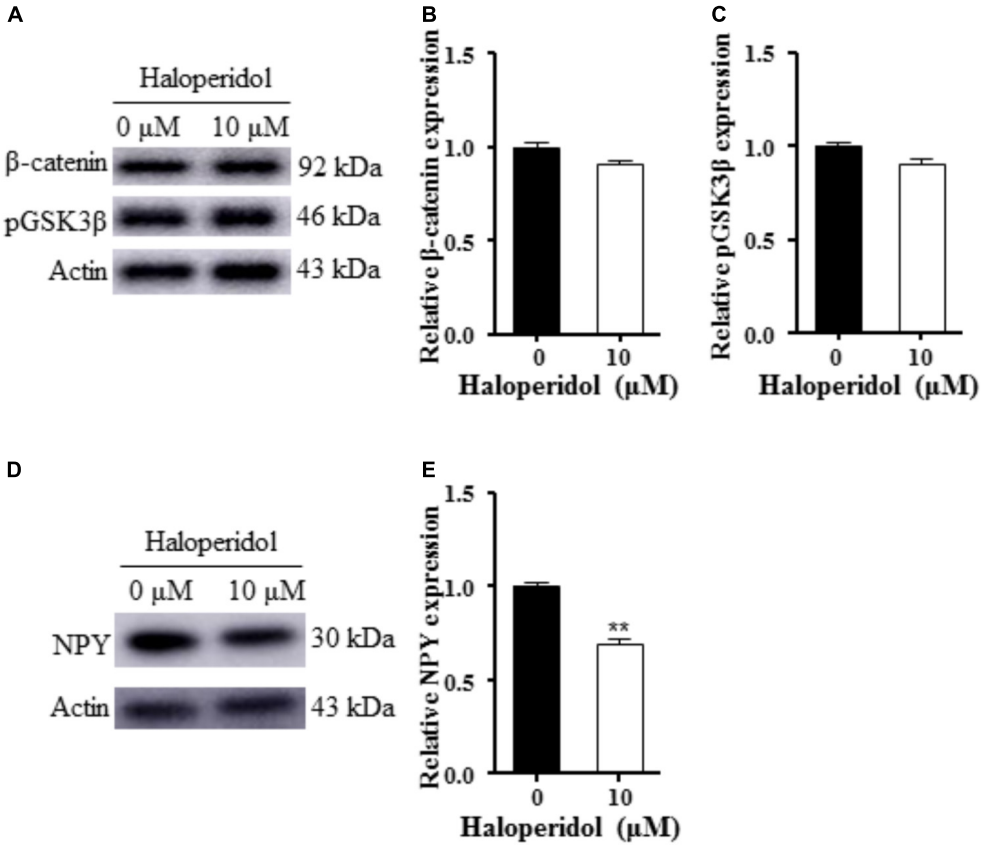

FIGURE 2 | Haloperidol reduced neuropeptide Y (NPY); however, haloperidol did not change GSK3β signaling. **(A–C)** Haloperidol (10 μM) reduced the neurite length, but no changes were found in pGSK3β_{Ser9} and β-catenin signaling in SH-SY5Y cells measured by Western blot (n = 3); **(D,E)** haloperidol (10 μM) reduced NPY expression in SH-SY5Y cells (n = 3). Mean ± SEM, $^{**}p < 0.01$.

was similar to the Immunofluorescence assay. After blocking, neurons were incubated with Alexa Fluor^{TM} 568 Phalloidin (A12380, Invitrogen) for 1 h and washed with PBS. Neurons were viewed using a 63× oil immersion objective on a DMI6500B confocal microscope (Leica, Mannheim, Germany). The number of synaptic spines was measured using ImageJ Software.

Statistics

SPSS program (version 21; Chicago, IL, United States) was used for statistical analysis. One-way analysis of variance (ANOVA) followed by *post hoc* Tukey's tests was performed for multiple comparisons. Data were expressed as mean ± SEM, and $p < 0.05$ value was considered statistically significant.

RESULTS

Haloperidol Inhibited Neurite Outgrowth

In order to investigate the effects of haloperidol on neurite morphology, RA-induced differentiated SH-SY5Y cells were incubated with various concentrations of haloperidol (0, 1, 10, 50 μM) for 24 h. The neurite length was measured in real-time. Haloperidol treatment significantly reduced the neurite outgrowth in a dose-response manner [$F(3,11) = 145.401$, $p < 0.001$, **Figure 1A**]. The neurite length was significantly shorter compared with control group after 1, 10, and 50 μM haloperidol treatments (all $p < 0.001$). As it is known that the plasma concentration of haloperidol-treated patients is between 2 and 10 ng/ml (Volavka et al., 1995), we used 3.8 ng/ml or 10 μM concentration of haloperidol. We repeated the results with 10 μM haloperidol and confirmed its effect on neurite inhibition (**Figure 1B**). To confirm these observations, we applied the same concentration of haloperidol in primary striatal neurons. We found that haloperidol treatment at 10 μM for 24 h significantly reduced neurite length visualized by MAP2 staining ($p < 0.001$, **Figures 1C,D**).

Haloperidol-Induced Neurite Lesions Did Not Alter GSK3β Signaling but Decreased NPY Expression

We examined GSK3β signaling since this signaling pathway is involved in neurogenesis and synaptic plasticity (Cole, 2013). No changes were detected in pGSK3β and β-catenin expression after 10 μM haloperidol treatment ($p > 0.05$, **Figures 2A–C**). However, we found a significant reduction of NPY expression

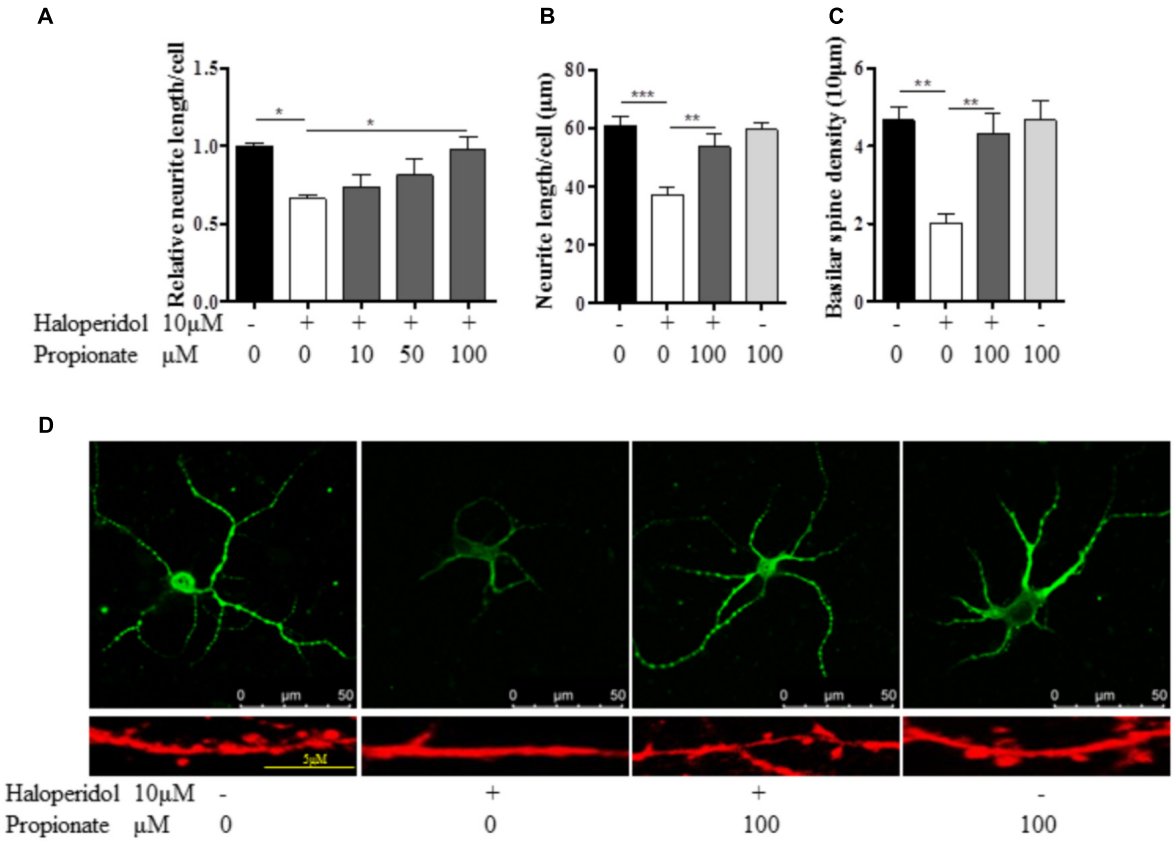

FIGURE 3 | Propionate protected neurite lesions. **(A)** Neurite lesions induced by 10 μM haloperidol were protected by 100 μM propionate in SH-SY5Y cells (n = 3–4); **(B)** Propionate prevented the haloperidol-induced neurite lesion in primary striatal neurons (n = 6); **(C)** Propionate prevented the haloperidol-induced synaptic spine reduction in primary striatal neurons (n = 6); **(D)** The striatal neurons were stained with MAP2 antibody (Top row). Synaptic spines were stained with Alexa Fluor™ 568 Phalloidin (Bottom row) and imaged with immunofluorescence confocal microscope. Cells were treated for 24 h. Mean ± SEM, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

after 10 μM haloperidol treatment in SH-SY5Y cells ($p < 0.01$, **Figures 2D,E**). The results suggested that NPY was associated with neurite reduction induced by haloperidol.

Propionate Prevented Neurite Lesion-Induced by Haloperidol

Previous studies suggest that propionate may play important roles in neurodevelopment. We tested whether or not propionate could prevent haloperidol-induced neurite deficit. We treated SH-SY5Y cells with sodium propionate at concentrations of 0, 10, 50, and 100 μM (Fasting plasma concentration in humans is about 24 μM.) for 30 min prior to 10 μM haloperidol treatment. We observed that propionate prevented neurite impairment induced by haloperidol [$F(4,11) = 5.357$, $p = 0.012$, **Figure 3A**]. Specifically, 100 μM propionate completely prevented neurite lesions induced by haloperidol ($p < 0.05$). Furthermore, we investigated if propionate could prevent neurite lesion in primary mouse striatal neurons. Our results showed that propionate prevented neurite lesion induced by haloperidol [$F(3,20) = 11.656$, $p < 0.001$, **Figures 3B,D**]. *Post hoc* analysis showed that the neurite lesions was prevented by 100 μM propionate (**Figure 3B**, $p < 0.01$). It has been reported that haloperidol can decrease the number of synaptic spines in the rat striatum (Kelley et al., 1997). We quantified the number of basilar synaptic spines. We found that propionate prevented the loss of synaptic spines-induced by haloperidol [$F(3,20) = 9.994$, $p < 0.001$, **Figures 3C,D**].

Propionate Prevented NPY Reduction Induced by Haloperidol

Since we observed that the neurons with neurite deficits induced by haloperidol have decreased NPY, we examined if propionate could prevent the reduced NPY. We found that propionate completely prevented NPY reduction induced by haloperidol in SH-SY5Y cells [$F(3,8) = 17.502$, $p = 0.001$, **Figure 4A**]. This result supported our above discovery that NPY is involved in haloperidol-induced neurite lesions and propionate prevented neurite lesions were involved in the regulation of NPY. Furthermore, we used primary mouse striatal neurons to validate our finding. Again, we found that haloperidol significantly reduced NPY, which could be prevented by propionate in

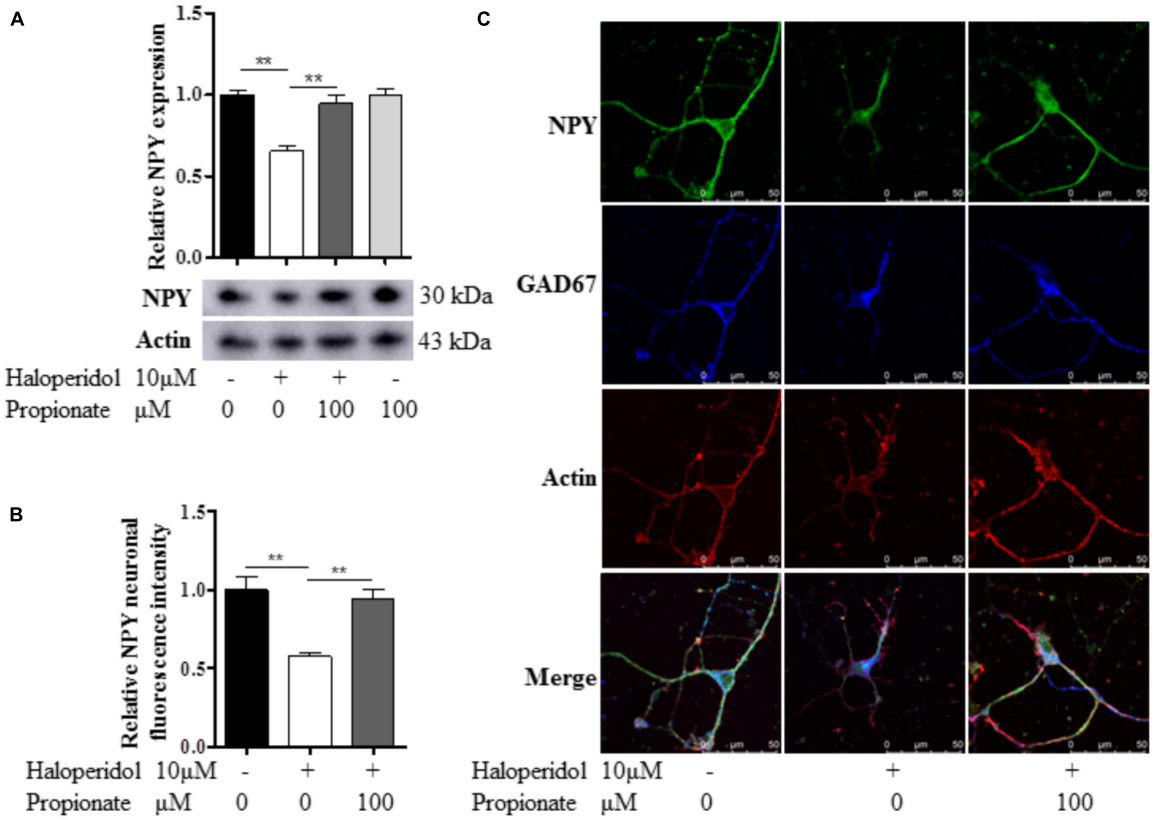

FIGURE 4 | Propionate prevented the NPY reduction induced by haloperidol. **(A)** Propionate (100 μM) protected haloperidol induced NPY reduction in SH-SY5Y cells (n = 3); **(B)** propionate prevented NPY reduction induced by haloperidol in primary striatal neurons (n = 6); **(C)** these striatal NPY neurons were GAD67 positive indicating that NPY and GABA are co-localized in these neurons. The morphological appearance showed these are medium sized spiny neurons with body size of ∼10 μM in diameter. Mean ± SEM, $^{**}p < 0.01$.

the striatal neurons [F(2,15) = 12.346, p = 0.001, **Figures 4B,C**]. Further characterization was performed using glutamic acid decarboxylase 67 (GAD67) antibody staining for GABA synthesis (Chattopadhyaya et al., 2007). We showed these haloperidol-responding striatal neurons contained both NPY and GABA (**Figure 4C**).

Furthermore, to confirm the role of NPY in propionate-induced neuroprotection against haloperidol-induced neurite lesions, we used specific siRNA to knock down NPY in SH-SY5Y cells (**Figures 5A–C**). In this case, propionate was no longer able to protect the neurite lesion-induced by haloperidol [F(3,8) = 11.788, p = 0.003, **Figure 5A**]. As shown that the neurite lesions induced by haloperidol ($p < 0.01$) was protected by 100 μM propionate ($p < 0.05$), while siNPY abolished the neural protective effects of propionate. Western blotting results showed that the NPY was reduced in NPY-siRNA treated cells compared with the cells without NPY-siRNA treatment ($p < 0.05$, **Figures 5B,C**). Similarly, propionate prevented neurite lesion in primary mouse striatal neurons but not after NPY-siRNA treatment [F(3,20) = 29.663, $p < 0.001$, **Figures 5D,E**]. These results indicated that propionate protective effect was mediated by NPY.

CREB Phosphorylation Was Involved in the Neuronal Protective Effect of Propionate

The cAMP responsive element binding protein (CREB) is a ubiquitous transcription factor located in CRE promoter regions, which modulates the transcription of genes with cAMP responsive elements (CRE). Since it is known that CREB gene transcription factor regulates NPY expression in neurons, we investigated possible correlations between phosphorylated CREB in our propionate treated cells. As expected, we observed that haloperidol decreased CREB phosphorylation, which was prevented by propionate; however, propionate alone did not alter CREB phosphorylation [F(3,20) = 14.741, $p < 0.001$, **Figures 6A,B**]. These data suggested that propionate could prevent the down-regulation of CREB phosphorylation induced by haloperidol, which in turn prevented down-regulation of NPY and neurite lesions (**Figure 7**).

DISCUSSION

Antipsychotic drugs are widely used in treating schizophrenia and allied disorders. However, antipsychotic drug treatment

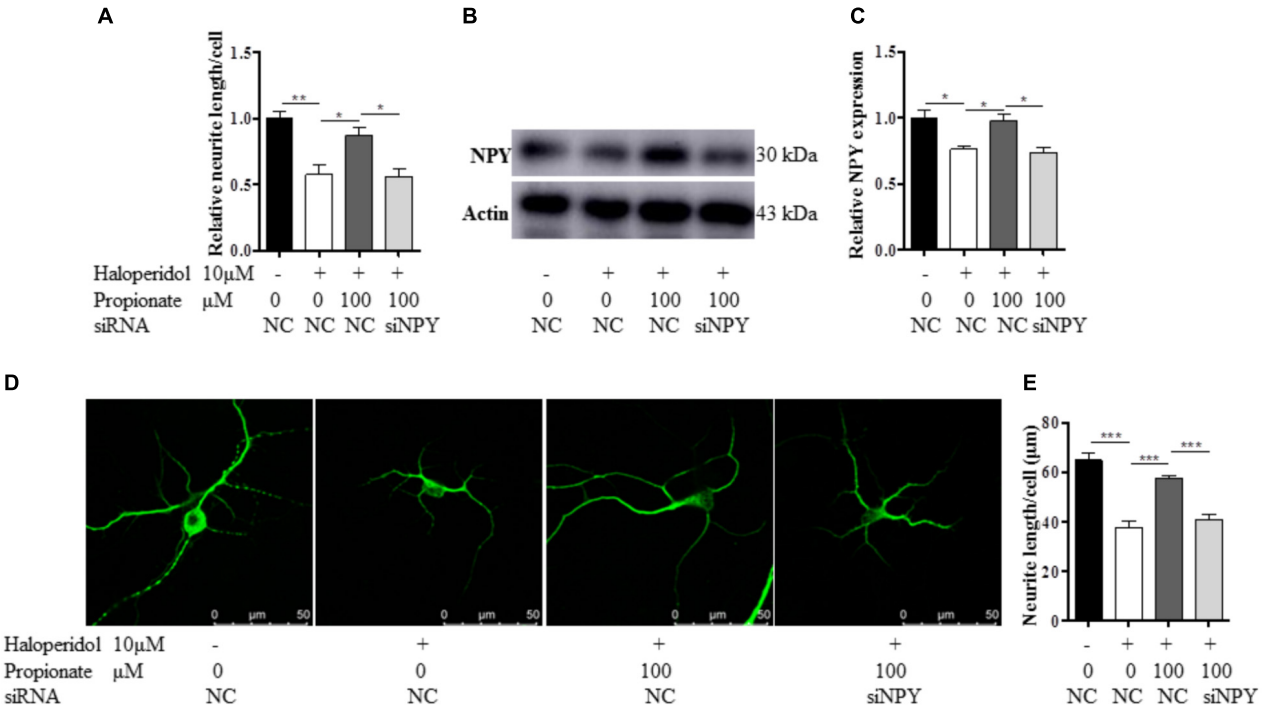

FIGURE 5 | The treatment of NPY-siRNA (siNPY) eliminated the protective effects of propionate on the neurite impairment-induced by haloperidol. **(A)** The quantification of neurite length of SH-SY5Y cells treated with/without siNPY ($n = 3$); **(B,C)** the treatment of siNPY eliminated the effect of propionate in preventing haloperidol induced NPY reduction in SH-SY5Y cells ($n = 3$); **(D)** fluorescence confocal images of striatal neurons stained for MAP2, and **(E)** quantification of neurite length showed that the treatment of siNPY eliminated the effect of propionate in preventing haloperidol-induced neurite lesions in primary striatal neurons ($n = 6$). Mean ± SEM, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

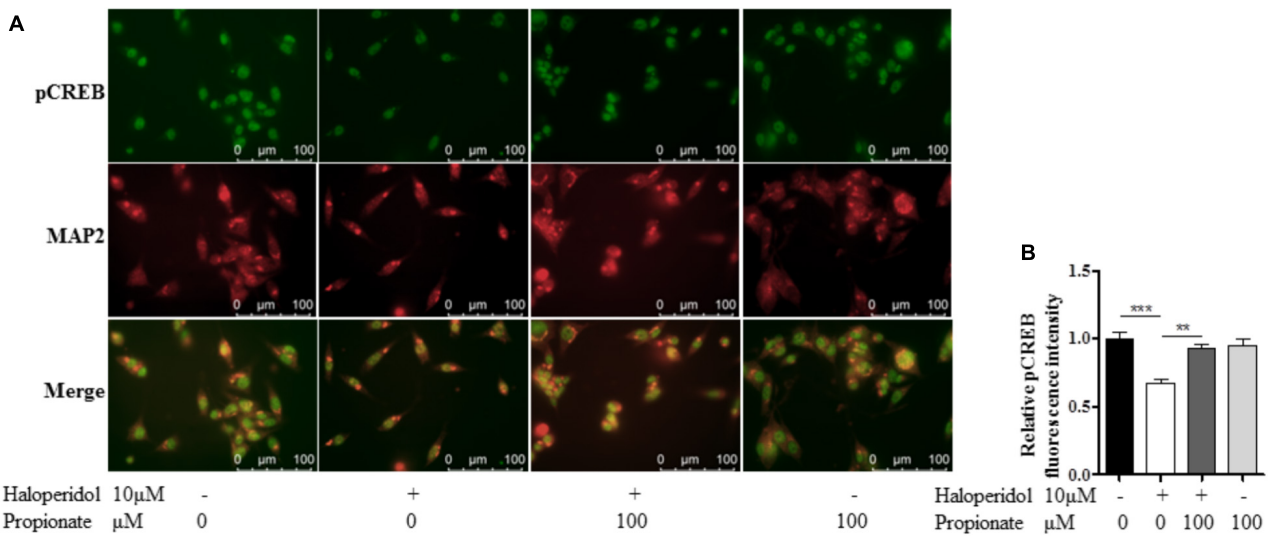

FIGURE 6 | Propionate protected the reduction of CREB phosphorylation (pCREB) induced by haloperidol in SH-SY5Y cells. **(A)** Fluorescence confocal images of SH-SY5Y cells showed that haloperidol reduced pCREB which was prevented by propionate (Top row), cell contour showed by MAP2 staining (middle row), and merge (bottom row). **(B)** The quantification of pCREB immunofluorescence intensity ($n = 6$). Mean ± SEM, $^{**}p < 0.01$, $^{***}p < 0.001$.

could result in neurite lesion in brain areas, rich in dopamine D2R (Huang and Song, 2018). This is because D2R plays an important role in neurite growth and synaptogenesis (Jia et al., 2013) and virtually all antipsychotic drugs have D2R antagonist properties (Jafari et al., 2012; Huang and Song, 2018). Haloperidol is a typical antipsychotic drug having strong D2R

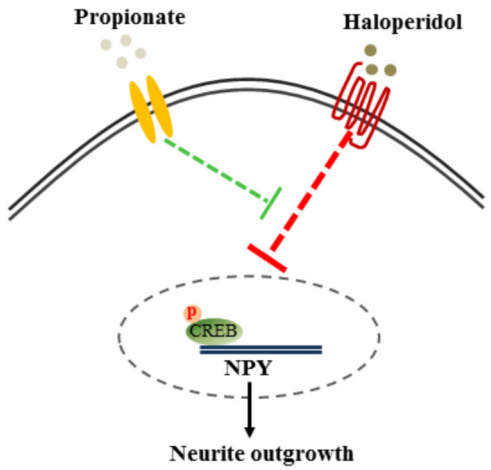

FIGURE 7 | Proposed mechanism of propionate preventing haloperidol-induced neurite lesion *in vitro*. Haloperidol inhibited pCREB-NPY signaling causing neurite deficit. Propionate prevented haloperidol-induced reduction of pCREB-NPY signaling, which reversed neurite deficits.

antagonist property. Plasma concentrations of patients treated with haloperidol are between 2 to 10 ng/ml (Volavka et al., 1995). This study used a clinical relevant dose 3.8 ng/ml (equivalent to 10 μM; molecular weight of haloperidol is 376 g/mol), which resulted in neurite lesions in both SH-SY5Y and primary striatal neurons. A previous study has shown that 0.1 μM haloperidol treatment decreases the dendritic spine density as well as spines-enriched proteins of rat hippocampal neurons (Critchlow et al., 2006). Similarly, chronic haloperidol treatment (1.5 mg/kg daily similar to human dose) results in 58% reduction of spine density in rat striatum (Kelley et al., 1997). These data provide strong evidence that clinically used doses of haloperidol may cause neurite lesion.

Propionate is a short chain fatty acid which is a product of gut microbiome fermentation of dietary fiber (Neis et al., 2018). Short chain fatty acids can affect brain function and behavior (Selkrig et al., 2014). Our study showed that propionate prevented neurite lesions-induced by haloperidol. Previous study has suggested that propionate can have significant effects on brain neurites. For example, children with autistic spectrum disorders are characterized by elevated concentrations of propionate, which may link to exaggerated neural synaptic spine formation (Penzes et al., 2011; Frye et al., 2015). Introducing propionate into brain ventricle could induce autism-like behavioral changes in rats (MacFabe et al., 2007, 2011). Our study showed that propionate protected the neurite and synaptic spine lesion induced by haloperidol supporting the effect of propionate on promoting neurite outgrowth.

NPY is highly expressed in striatal GABAergic neurons and regulates GABA and glutamate release potentially contributing to neuroprotection, learning, and memory (Gotzsche and Woldbye, 2016). The present study showed that haloperidol decreased NPY expression in both human blastoma SH-SY5Y and primary striatal neurons. This is in consistent with our early *in vivo* study, which we have demonstrated that haloperidol decreases NPY expression in the rat amygdala and hippocampus (Huang et al., 2006). Other studies have shown that haloperidol decreases NPY in the striatum, but increases in the hypothalamus (Gruber and Mathe, 2000). The present study showed that propionate prevented haloperidol-induced NPY reduction in SH-SY5Y and striatal neurons. When we used NPY-siRNA to decrease NPY activity, we found that the protective effect of propionate against haloperidol-induced lesion in neurites was eliminated. Collectively, these results support the idea that the NPY pathway was involved in the prevention of haloperidol-induced neurite lesion by propionate.

The mechanism underlying haloperidol decreasing NPY expression is not clear. One possibility could be via CREB signaling. CREB is a transcriptional coactivator involved in the regulation of synaptic plasticity and long-term memory through activation of gene transcription (Vecsey et al., 2007). It is known that the CREB pathway regulates NPY expression (Wand, 2005). It has also been demonstrated that D2R regulates CREB. For example, D2R agonist quinpirole stimulates CREB phosphorylation by activating protein kinase C and Ca^{2+}/calmodulin-dependent protein kinase (Yan et al., 1999).

In addition, GSK3β signaling pathway is involved in neurogenesis and synaptic plasticity (Cole, 2013). Previous studies have reported that haloperidol does not change the GSK3β pathway in SH-SY5Y cells and in primary hippocampal neurons (Park et al., 2009, 2011). In agreement with their studies, we did not find a change of GSK3β signaling after haloperidol treatment. Our study showed that haloperidol reduced CERB phosphorylation, which was prevented by propionate. Therefore, it is suggested that haloperidol may act on other D2R protein kinase dependent pathway to inhibit pCREB rather than the pGSK3β signaling pathway as per previous observations (Borroto-Escuela et al., 2013; Fuxe et al., 2014).

It is known that short chain fatty acids are ligands for G-protein coupled receptors GPR41 and GPR43 (Tan et al., 2014), which have no other known ligands (Tazoe et al., 2008). Until now, no GPR41 or GPR43 receptors have been reported in the brain. On the other hand, it is known that short chain fatty acids can directly enter brain and interact with neurons (Erny et al., 2015; Stilling et al., 2016). Our study showed that propionate prevented haloperidol-induced neurite lesions in a dose-dependent manner. The concentration of propionate is between 14 to 19 mM in human feces (Schwiertz et al., 2010) and 19.4 to 28.5 μM in human fasting plasma, which could vary depending on the detection method used or a person's dietary profile (De Filippo et al., 2010). It is possible that increasing propionate by either delivering highly concentrated propionate capsule or providing selected dietary fiber may provide a possible protective effect against neurite lesion.

CONCLUSION

Our study showed that haloperidol reduced neural pCREB-NPY signaling, which was involved in neurite and synaptic

spine lesions. Propionate prevented haloperidol-induced neurite lesions via increased pCREB-NPY signaling *in vitro*. Further study needs to be performed to examine if propionate could protect neurite lesion induced by haloperidol *in vivo*.

AUTHOR CONTRIBUTIONS

XH, MH, PZ, and YX provided substantial contributions to the conception or design of the work. MH performed the acquisition and analysis of data for the work. XH, MH, ZB, YY, RT, AJ, and KZ were involved in the interpretation of data and manuscript preparation for important intellectual content. All authors have read the paper and agreed to be authors on the paper.

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MINI REVIEW
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The Impact of High-Intensity Interval Training on Brain Derived Neurotrophic Factor in Brain: A Mini-Review

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The brain-derived neurotrophic factor (BDNF) is a protein mainly synthetized in the neurons. Early evidence showed that BDNF participates in cognitive processes as measured at the hippocampus. This neurotrophin is as a reliable marker of brain function; moreover, recent studies have demonstrated that BDNF participates in physiological processes such as glucose homeostasis and lipid metabolism. The BDNF has been also studied using the exercise paradigm to determine its response to different exercise modalities; therefore, BDNF is considered a new member of the exercise-related molecules. The high-intensity interval training (HIIT) is an exercise protocol characterized by low work volume performed at a high intensity [i.e., ≥80% of maximal heart rate (HRmax)]. Recent evidence supports the contention that HIIT elicits higher fat oxidation in skeletal muscle than other forms of exercise. Similarly, HIIT is a good stimulus to increase maximal oxygen uptake (VO_2max). Few studies have investigated the impact of HIIT on the BDNF response. The present work summarizes the effects of acute and long-term HIIT on BDNF.

Keywords: brain-derived neurotrophic factor, high-intensity interval training, brain, health, physical exercise

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INTRODUCTION

Physical exercise (PE) is considered a subcategory of the physical activity domain (Caspersen et al., 1985; Physical Activity Guidelines Advisory Committee , 2008). As opposed to physical activity, PE is characterized for being planned, structured, and repetitive, with the inherent goal of improving one or more components of the physical fitness, physical performance, or health (Caspersen et al., 1985;Physical Activity Guidelines Advisory Committee , 2008). The PE prescription is usually reported as exercise mode, intensity, frequency, and duration of the activity.

Several training modalities have been developed over the years with the aim of improving cardiorespiratory fitness, musculoskeletal function, and metabolic activity. Among these, aerobic, endurance, and/or resistance (i.e., strength exercise) are the most common exercise training modalities (Kang and Ratamess, 2014). The aerobic exercise (AE) is also known as moderate-intensity continuous training (MICT), and it is usually performed over long periods of time (e.g., ≥30-min to moderate intensity, performing exercises such as walking, cycling, jogging, and swimming) (Garber et al., 2011). Although the benefits of MICT on health related parameters in humans are well studied (Aldred et al., 1995; Poehlman et al., 2000; Mador et al., 2004;

Frøsig et al., 2007; Camargo et al., 2008; Bell et al., 2010; Fisher et al., 2015; Daabis et al., 2017); currently, the world population considers that lack of time as the main barrier to practice AE regularly (Weston et al., 2014; Fisher et al., 2015).

Scientists and exercise professionals have focused on studying the impact of short exercise bouts on human physiology with the aim of optimizing time use (Gibala et al., 2006); for instance, high-intensity interval training (HIIT). The HIIT refers to exercise characterized by relatively short bursts of vigorous activity, interspersed by rest or low-intensity recovery exercise. In general, HIIT is performed on a training session lasting ≤30-min, including warm-up and cool down stages (Gibala and Jones, 2013; Gillen and Gibala, 2013; Weston et al., 2014). The high-intensity bouts should be performed at near maximal effort, reaching intensities between 80 and 100% of the maximal heart rate (HRmax) (Gibala et al., 2014; Saanijoki et al., 2018). The exertion is performed no longer than 60-s (Gillen and Gibala, 2013), and the recovery periods (low-intensity exercise or rest) can be up to 4-min (Burgomaster et al., 2005, 2006; Gibala and McGee, 2008). Besides Gibala's protocols, others have reported different high- and low-exercise bout durations (Saucedo Marquez et al., 2015; Lira et al., 2017; Stöggl and Björklund, 2017); however, the training session has been kept within ≤30-min.

In addition, HIIT can be performed on cyclical exercises such as bicycling (Saucedo Marquez et al., 2015), running (Lira et al., 2017), swimming (Courtright et al., 2016), and whole-body exercise (Machado et al., 2017; Schleppenbach et al., 2017). Several physiological adaptations of HIIT have been reported to improve physical performance in humans (Burgomaster et al., 2005, 2006; Gibala et al., 2006; Talanian et al., 2007; Connolly et al., 2017). The effects of HIIT on brain function have been also reported; however, there available evidence is scarce (Afzalpour et al., 2015; Lucas et al., 2015; Coetsee and Terblanche, 2017; Santos-Concejero et al., 2017; So et al., 2017; Freitas et al., 2018; Robinson et al., 2018). Indeed, the current evidence showed a positive impact of HIIT in brain, specifically in neurotrophin expression and function. In this context, the aim of this work is to briefly describe the current knowledge regarding the acute and long-term effects of HIIT on brain-derived neurotrophic factor (BDNF) in brain. It is known that BDNF is a protein that plays a key role to maintain or improve several brain functions (Vaynman et al., 2003, 2004; Duman and Monteggia, 2006; Duman and Li, 2012; Fernandes et al., 2017).

HIGH-INTENSITY INTERVAL TRAINING (HIIT): AN EFFICIENT TOOL TO IMPROVE PHYSICAL PERFORMANCE, METABOLISM, AND BRAIN FUNCTION

As described, HIIT refers to exercise characterized by relatively short bursts of vigorous activity, interspersed by rest or low-intensity recovery exercise (Gibala and Jones, 2013; Gillen and Gibala, 2013; Weston et al., 2014). Previous scientific reports have indicated that HIIT is perceived as an exercise modality eliciting higher exhaustion compared to MICT (Saanijoki et al., 2015, 2018). However, HIIT is considered more enjoyable than MICT (Heisz et al., 2016); in agreement with this, HIIT has been proposed as an excellent strategy aimed to increase adherence to exercise programs in sedentary people (Heisz et al., 2016). In this section, the impact of HIIT on the human physiology (physical performance, metabolism, and brain function) will be briefly described. In sport, scientific reports indicate that HIIT was popularized by the runner Emil Zatopek around 1950 [see Billat's (2001) review on the historical analysis of HIIT]; in fact, several coaches think that HIIT played a key role on Zatopek's successful sport career. Similarly, recent evidence indicates that HIIT improves physical performance (e.g., speed, agility) in team sport athletes such as soccer and basketball (Iaia et al., 2015; Sanchez-Sanchez et al., 2018).

Regarding to metabolic dysfunctions in glucose and lipids induced by sedentary lifestyle and hypercaloric diets, several evidence showed that HIIT is an efficient stimulus to improve lipid and glucose metabolism. Concretely, Talanian et al. (2007), reported that seven sessions of HIIT increased fat oxidation in skeletal muscle in recreationally active women. Similarly, others demonstrated that HIIT interventions enhance insulin sensitivity, glucose control, and cardiorespiratory fitness in sedentary women (Connolly et al., 2016, 2017). In addition to research on women, others reported that HIIT increases muscle oxidative capacity in recreationally active men (Burgomaster et al., 2005, 2006; Gibala et al., 2006).

The central nervous system (CNS) response to HIIT has been reported in spinal cord (Astorino and Thum, 2018) and brain studies (Coetsee and Terblanche, 2017; Santos-Concejero et al., 2017; Robinson et al., 2018). For instance, a 16-week HIIT program elicited higher oxygen utilization and cerebral oxygenation than MICT in older people (Coetsee and Terblanche, 2017); similar results were found in younger adults (Robinson et al., 2018). In these studies, the BDNF's response was dependent of the exercise intensity. However, the molecular mechanisms explaining these brain adaptations to HIIT are yet to be elucidated.

BRAIN-DERIVED NEUROTROPHIC FACTOR (BDNF): A PROTEIN SENSITIVE TO EXERCISE IN BRAIN

BDNF Function in Brain and Periphery

The BDNF is a protein member of the neurotrophin family, and it is found in the nervous system and peripheral organs such as skeletal muscle (Funakoshi et al., 1993; Conner et al., 1997; Matthews et al., 2009). In the CNS, the neurons are the principal source of BDNF (Mowla et al., 2001), and evidence suggests that BDNF plays a key role in memory and learning processes (Erickson et al., 2011). Moreover, molecular evidence indicates that this neurotrophin through a tyrosine kinase b receptor (TrkB) increases long-term potentiation, neurogenesis, axonal growth, and synaptogenesis (Tyler and Pozzo-Miller, 2001; Vaynman et al., 2003, 2004; Fernandes et al., 2017). Besides

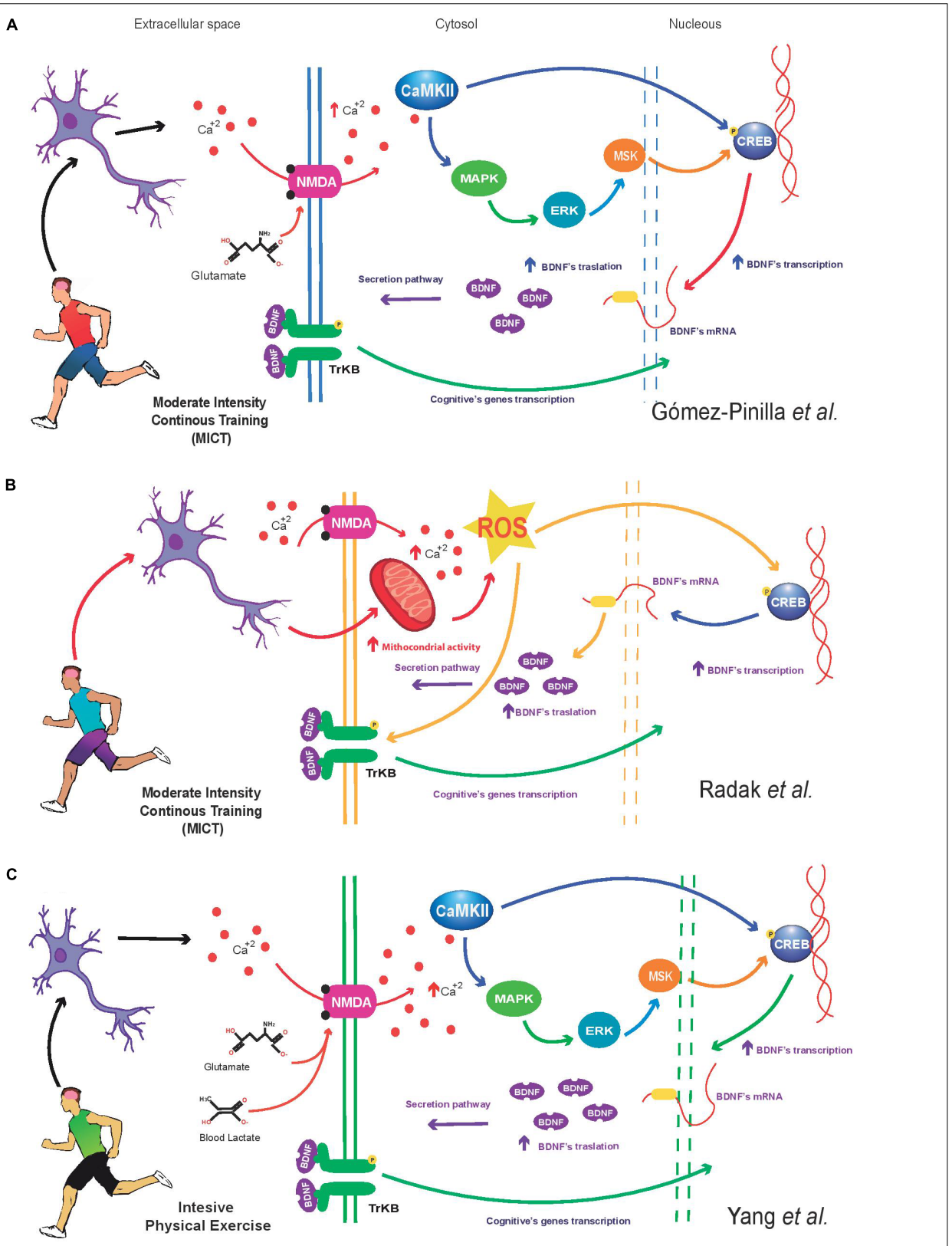

FIGURE 1 | (A) Moderate-intensity continuous training (MICT) increases intracellular calcium (Ca^{2+}) levels in neurons through the NMDA receptor. Intracellular Ca^{2+} increases the activity of calmodulin dependent kinase II (CaMKII), triggering the activation of the MAPK/ERK/MSK cascade signaling, resulting in the increase of the

(Continued)

FIGURE 1 | Continued
expression and phosphorylation of cAMP response element-binding protein (CREB). Finally, CREB enhance the *Bdnf* transcription. This molecular mechanism described above result in a higher BDNF protein, the neurotrophin is released by the neuron to induce transcription of cognitive genes. The present model is based on Gomez-Pinilla's studies (Fernandes et al., 2017). **(B)** MICT enhances the mitochondrial activity in neurons. Higher mitochondrial activity increases reactive oxygen species (ROS) from complexes I and III. The change in ROS levels modify and regulate a wide of signaling process including the CREB-BDNF signaling pathway. Once activated, BDNF regulates a positive feedback mechanism to induce the cognitive genes transcription. Additionally, the aerobic exercise increases the calcium concentration in neurons; this ion through the calpain and xanthine oxidase increases the ROS that consequently increase the CREB's activation and *Bdnf* expression. **(C)** Exercise performed at high intensity (≥80% HRmax) activates several metabolic pathways in muscle (including glycolysis), this condition generates a higher systemic blood lactate concentration reaching the brain, this metabolite can be oxidized by astrocytes or neurons to produce glucose (Dienel and Hertz, 2001). In addition, experimental evidence indicates that lactate increase NMDA activity and intracellular Ca^{2+} levels in neurons. Indeed, it is possible that the lactate in neurons enhance the CaMKII activity and the MAPK/ERK/MSK signaling to induce the CREB's activation and *Bdnf* expression. Finally, the BDNF activate a positive loop to induce the expression of cognitive genes (Yang J. et al., 2014).

the local effect of BDNF in the brain, some authors suggest that the brain is the major source of circulating BDNF at rest and during exercise (Rasmussen et al., 2009; Seifert et al., 2010). In the periphery, studies performed in rodent and human tissues have revealed that BDNF regulates other physiological pathways such as glucose metabolism (Hanyu et al., 2003; Jiménez-Maldonado et al., 2014), and fat oxidation (Matthews et al., 2009).

Molecular Mechanism Induced by Physical Exercise Increasing Brain BDNF

Several stimuli can increase BDNF's expression and function. In rodents, the kainic acid exposure increased hippocampal BDNF (protein) levels (Rudge et al., 1998), resulting from an enhancement in glutamatergic signaling. Other evidence suggests that intermittent hypoxia increases BDNF levels in neurons of the primary motor cortex (Satriotomo et al., 2016). In addition to these findings, it is widely known that PE is an effective stimulus to increase BDNF synthesis in the brain (Oliff et al., 1998; Vaynman et al., 2003, 2004; Erickson et al., 2011), and the periphery (Dinoff et al., 2016, 2017).

Regarding to the impact of the PE on increasing BDNF in brain, different molecular mechanisms have been proposed to explain how PE (mainly MICT) enhances BDNF synthesis in neurons. The Gomez-Pinilla's group suggests that PE increases the intracellular Ca^{2+} levels in neuronal cells (Fernandes et al., 2017). This ion activates CaMKII indirectly; and once active, this kinase increases the MAP-K pathway to phosphorylate CRE-binding protein and activate the CREB transcription, and consequently *Bdnf* transcription (Vaynman et al., 2004; Fernandes et al., 2017; **Figure 1A**). Another model suggests that PE induces BDNF synthesis in the brain by enhancing the activity of reactive oxygen species (ROS) (Radak et al., 2016). Based on Radak et al's. proposal, PE increases the mitochondrial activity in neurons; and it is known that higher mitochondrial activity produce excessive ROS. Thus, ROS enhance the activity of CRE-binding protein, to activate the CREB and *Bdnf* transcription (Radak et al., 2016; **Figure 1B**). Additionally, the Radak's model indicates that the exercise increases the Ca^{2+} in neurons; this ion through the calpain and xanthine oxidase induces higher ROS production in brain as well (Takuma et al., 1999; Kahlert et al., 2005). In addition to the previous mechanism, it has been suggested that systemic molecules such as the lactate synthesized during intensive PE (≥80% HRmax) can activate the BDNF production (Bergersen, 2015). However, this molecular mechanism of BDNF production is still poorly understood. Experimental evidence has shown higher NMDA receptor activity in the presence of lactate; furthermore, high lactate concentrations are associated to increased neuronal Ca^{2+} levels (Yang J. et al., 2014) and higher *Bdnf* transcription (Yang J.L. et al., 2014). It is likely that lactate synthesized during PE reach the neurons and increase the NMDA receptor activity to increase the Ca^{2+} concentration in neurons, and Ca^{2+} activates CaMKII, and consequently, the kinase phosphorylates activating the MAPK/ERK signaling pathway to enhance *Bdnf* transcription (**Figure 1C**).

THE EFFECTS OF HIIT ON BDNF

Animal Models

As previously described, HIIT is characterized by exercise bouts of high-intensity and low volume. Regarding exercise intensity, evidence in healthy rodents has shown that brain BDNF synthesis was higher in animals performing a high-intensity training compared to those animals performing a low-intensity training and sedentary rodents (de Almeida et al., 2013). However, the authors used a continuous training protocol; the training time was similar in both models (low- and high-intensity training, 30-min/session), which means that the HIIT characteristics were unattained.

There is evidence regarding the long-term effects of HIIT on BDNF synthesis in rodents (Afzalpour et al., 2015; So et al., 2017; Freitas et al., 2018). Thirty HIIT sessions significantly increased BDNF levels (protein) in the brain compared with continuous training protocol and a control group (Afzalpour et al., 2015). The authors discussed that HIIT increased hydrogen peroxide (H_2O_2) and Tumor Necrosis Factor Alpha (TNF-α) concentration in brain; and these molecules could activate the BDNF synthesis (Wang et al., 2006; Bałkowiec-Iskra et al., 2011) or CREB (Pugazhenthi et al., 2003), a transcription factor positively regulating BDNF synthesis. However, although the previous paper found a positive effect of HIIT on BDNF, the authors did not report a specific anatomical region sensitive to elevations on the neurotrophin following HIIT. Consequently, others evaluated with more detail the impact of HIIT on BDNF in the hippocampus (Freitas et al., 2018). In the study by Freitas et al. (2018), 36 sessions of HIIT elevated BDNF levels in the hippocampal region of healthy rats. However, the molecular

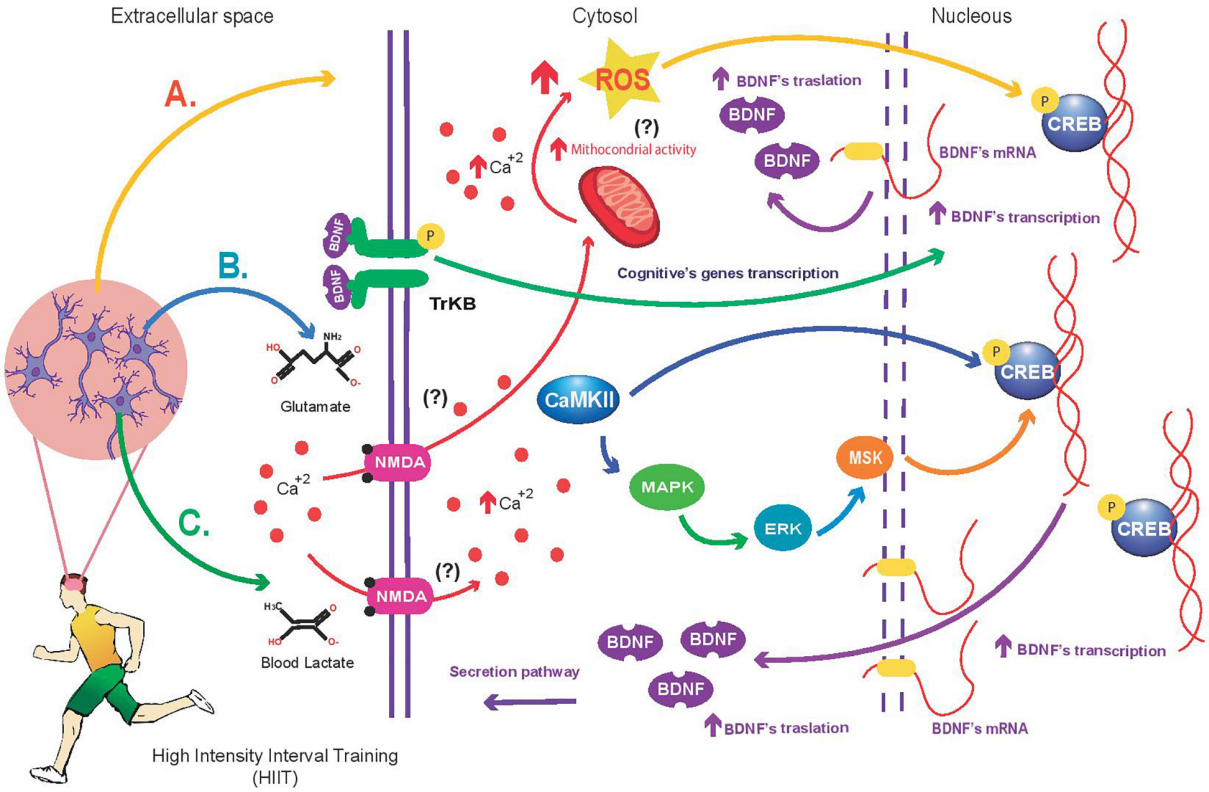

FIGURE 2 | Mechanism proposed about the High Intensity Interval Training (HIIT) impact on BDNF synthesis in brain. **(A)** HIIT increases mitochondrial activity (not reported) and ROS concentration in neurons (Afzalpour et al., 2015) compared with MICT. ROS induce higher *Creb-Bdnf* transcription and signaling than MICT (not reported). **(B)** HIIT causes greater Ca^{2+} concentration in neurons than MICT (not reported); this condition enhances the CaMKII activity and MAPK/ERK/MSK signaling to activate the *Creb-Bdnf* transcription and neuronal plasticity. Additionally, the intracellular calcium can increase the ROS generation in neurons. Once synthetized, ROS can activate *Creb-Bdnf* transcription. Currently, there is not experimental evidence to indicate that HIIT triggers more this mechanism than MICT. **(C)** HIIT elevate systemic blood lactate concentration, and consequently enhance the NMDA receptor activity to increase intracellular Ca^{2+} concentration in neurons (not reported). The ion activates the CaMKII activity and MAPK/ERK/MSK signaling to induce the *Creb-Bdnf* transcription and neuronal plasticity (not reported). (?): not reported.

mechanism responsible for increasing BDNF synthesis was not demonstrated in the study. In agreement with their results, the authors suggested that 36 HIIT sessions increased BDNF levels and attenuated hippocampal oxidative damage (Freitas et al., 2018).

Human Models

There are reports on the effect of a single HIIT session on BDNF (Saucedo Marquez et al., 2015; Cabral-Santos et al., 2016; Slusher et al., 2018). For instance, a single session of supramaximal HIIT elevated serum BDNF levels (Slusher et al., 2018), suggesting increases in BDNF secretion of the platelets unrelated to brain secretion (Slusher et al., 2018). Saucedo Marquez et al. (2015), found that HIIT was a more powerful stimulus to elevate systemic (serum) BDNF compared to MICT. The exercise modality employed in their study (cycle-ergometer) did not induce muscle damage (Saucedo Marquez et al., 2015). Therefore, the higher BDNF levels were not caused by platelet activation to increase the BDNF secretion (Saucedo Marquez et al., 2015), suggesting that PE itself is enough stimuli that lead to higher circulating BDNF levels. Thus, the higher serum BDNF levels following HIIT resulted from a greater synthesis of BDNF in the brain. The authors discussed that a single bout of HIIT induced higher brain H_2O_2 and TNF-α levels. These molecules activate the signaling of peroxisome proliferator-activated receptor-γ coactivator (PGC-1α) to enhance neuron BDNF synthesis (Saucedo Marquez et al., 2015). Similarly, a single session of HIIT significantly increased peripheral plasmatic BDNF levels immediately following the exercise (Cabral-Santos et al., 2016). However, after 60-min that the HIIIT session ended, BDNF concentrations returned to baseline levels (Cabral-Santos et al., 2016). Regarding that plasmatic BDNF levels reflect the BDNF secretion from the brain (Lommatzsch et al., 2005); the Cabral-Santos data reflect the HIIT impact on BDNF in brain. The authors suggested that brain hypoxia induced by HIIT was the main factor explaining their results (Cabral-Santos et al., 2016).

Finally, the long-term effects of HIIT on systemic (serum) BDNF levels have been also reported (Murawska-Cialowicz et al., 2015). In the study, participants performed whole-body exercises for 3 months, and the protocol was effective at increasing serum BDNF concentrations. However, the BDNF source was not elucidated.

PERSPECTIVES AND CONCLUDING REMARKS

Studies performed in rodents (Tsuchida et al., 2001; Hanyu et al., 2003; Yamanaka et al., 2006; Jiménez-Maldonado et al., 2014) and humans (Bulloì et al., 2007; Krabbe et al., 2007; Li et al., 2016) have demonstrated that BDNF participates in glucose and lipid metabolism (Matthews et al., 2009). Therefore, this molecule also is known as metabotrophin (Gomez-Pinilla et al., 2008). Several health conditions such as type 2 diabetes, obesity, metabolic syndrome, and cardiovascular diseases are mainly caused by dysfunctional metabolic mechanisms and sedentary lifestyle. Therefore, it is important to identify efficient stimuli to increase the BDNF function in population with high risk to suffer metabolic diseases or in people who are suffering metabolic diseases. Thus, HIIT seems be a good stimulus to enhance the BDNF action. However, the impact of HIIT on BDNF and its effect on glucose and lipid metabolism is poorly studied. Further experimental studies are necessary to elucidate the impact of HIIT on BDNF and its effect on glucose and lipid metabolism in humans with metabolic or cardiovascular diseases. In addition, during modern-life diseases (Type II diabetes, obesity, and metabolic syndrome); the brain function is also affected (Cisternas et al., 2015; Agrawal et al., 2016). Therefore, studies are needed to assess the impact of HIIT interventions on BDNF synthesis and signaling pathways in brain under morbid conditions. The current work proposes a model about the impact of HIIT on BDNF expression in the brain (**Figure 2**). It will be reasonable to use previous HIIT protocols that reported a positive impact in peripheral BDNF when thinking about the design of HIIT protocols aimed at increasing BDNF synthesis and brain signaling. For example, sprint interval training (60-s run at 100% VO_2max, interspersed with 60-s passive recovery) (Cabral-Santos et al., 2016). In addition, the peak power output (PPO-Watts-) can also be used to design a HIIT protocol (Saucedo Marquez et al., 2015); for instance, the protocol could consist in pedaling for 60-s at 90% of PPO, alternating with 60-s of active rest at 60 Watts (total duration of HIIT is 20-min) (Saucedo Marquez et al., 2015). Finally, a recent report performed in overweight subjects showed that a HIIT protocol designed using heart rate as the main variable to establish the workload intensities is not a good stimulus to increase the peripheral BDNF (Domínguez-Sanchéz et al., 2018). Further studies are needed to determine whether heart rate may be considered as a reliable physiological variable used to design a HIIT protocol aimed at increasing circulating BDNF in non-obese subjects.

AUTHOR CONTRIBUTIONS

AJ-M conceived the review focus, reviewed the literature, wrote the first draft, and finalized the manuscript. IR and PG-S reviewed the literature, wrote the first draft, and finalized the manuscript. JM-J and LF-R finalized the manuscript. All authors approved the final version of the manuscript.

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Sub-Convulsing Dose Administration of Pilocarpine Reduces Glycemia, Increases Anxiety-Like Behavior and Decelerates Cortical Spreading Depression in Rats Suckled on Various Litter Sizes

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Epilepsy and malnutrition constitute two worldwide health problems affecting behavior and brain function. The cholinergic agonist pilocarpine (300–380 mg/kg; single administration) reproduces the human type of temporal lobe epilepsy in rats. Pilocarpine-induced epilepsy in rodents has been associated with glycemia, learning and memory and anxiety disturbances. Cortical spreading depression (CSD) is a neural response that has been linked to brain excitability disorders and its diseases, and has been shown to be antagonized by acute pilocarpine. This study aimed to further investigate the effect of chronic pilocarpine at a sub-convulsing dose on weight gain, blood glucose levels, anxiety-like behavior and CSD. In addition, we tested whether unfavorable lactation-induced malnutrition could modulate the pilocarpine effects. Wistar rats were suckled under normal size and large size litters (litters with 9 and 15 pups; groups L_9 and L_{15}, respectively). From postnatal days (PND) 35–55, these young animals received a daily intraperitoneal injection of pilocarpine (45 mg/kg/day), or vehicle (saline), or no treatment (naïve). On PND58, the animals were behaviorally tested in an open field apparatus. This was immediately followed by 6 h fasting and blood glucose measurement. At PND60–65, CSD was recorded, and its parameters (velocity of propagation, amplitude, and duration) were calculated. Compared to the control groups, pilocarpine-treated animals presented with reduced weight gain and lower glycemia, increased anxiety-like behavior and decelerated CSD propagation. CSD velocity was higher ($p < 0.001$) in the L_{15} groups in comparison to the corresponding groups in the L_9 condition. The results demonstrate an influence of chronic (21-day) administration of a sub-convulsing, very low dose (45 mg/kg) of pilocarpine on CSD propagation, anxiety-like behavior, glycemia and body weight. Furthermore, data reinforce the hypothesis of a relationship between CSD and brain excitability. The lactation condition seems to differentially modulate these effects.

Keywords: anxiety-like behavior, blood glucose, brain excitability, litter size, nutritional deficiency, pilocarpine, spreading depression, unfavorable lactation

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INTRODUCTION

Epilepsy is the third most common chronic brain disorder (Landgrave-Gómez et al., 2016), being more prevalent in developing countries (Hackett and Iype, 2001). The rodent model of epilepsy that is based on pilocarpine treatment has been widely used to reproduce the histological, biochemical, behavioral, and electrophysiological manifestations found in humans with temporal lobe epilepsy (Duarte et al., 2013). Pilocarpine is an alkaloid extracted from the leaves of the jaborandi plant (*Pilocarpus jaborandi*). It is a muscarinic non-selective cholinergic agonist capable of inducing status epilepticus in rodents with only a single intraperitoneal (i.p.,) dose (300–380 mg/kg) (Turski et al., 1989; dos Santos et al., 2000). Evidence suggests that epilepsy influences emotional responses in human beings (Devinsky, 2004; Jackson and Turkington, 2005), and anxiety has been the most common psychological disorder among people with epilepsy (Beyenburg et al., 2005). Pilocarpine-induced experimental epilepsy in rodents has been associated with glycemia, learning and memory disturbances and behavioral disorders such as depression and anxiety (Cardoso et al., 2009; Duarte et al., 2014; Castelhano et al., 2015; Cossa et al., 2016). Furthermore, although sub-convulsing doses of pilocarpine do not cause behavioral or electrocorticographic changes indicative of seizure, they are able to antagonize the propagation of the excitability-related phenomenon known as cortical spreading depression (CSD) along the cortical rodent tissue (Guedes and Vasconcelos, 2008).

First described by Aristides Leão, CSD represents a reduction in the spontaneous and evoked electrical activity of the cerebral cortex in response to an electrical, chemical, or mechanical stimulation of a point on the brain (Leão, 1944). This fully reversible neural response propagates very slowly (propagation velocity in the order of few mm/min) from the initially stimulated point to more and more remote parts of the tissue (Lima et al., 2017). CSD has been linked to brain excitability disorders and its diseases such as migraine with aura (Peeters et al., 2007; Vinogradova, 2018), multiple sclerosis (Pusic et al., 2015), epilepsy (Tamaki et al., 2017), traumatic brain injury (Mayevsky et al., 1998; Hartings et al., 2009), subarachnoid hemorrhage (Dreier et al., 2006; Sugimoto et al., 2016), and malignant ischemic stroke (Woitzik et al., 2013; Pinczolits et al., 2017). CSD has been demonstrated not only in experimental animals (Guedes et al., 2017; Accioly and Guedes, 2017) but also in humans (Carlson et al., 2016; Lauritzen and Strong, 2016). CSD propagation can be facilitated under unfavorable suckling conditions (pups being suckled in large litters; Lima et al., 2017), and this can modulate the effect of other treatments (Francisco and Guedes, 2015). Measuring CSD velocity of propagation along the cortical tissue has been largely used in our laboratory as a useful physiological index that helps to understand the electrophysiological aspects of brain functioning in health and disease (Guedes, 2011; Guedes et al., 2012). Increased or reduced CSD propagation velocity indicates a greater or lesser respective ability of the cortical tissue to propagate CSD, which can be associated with anxiety-like behavior (Francisco and Guedes, 2015; Lima et al., 2017) and glycemic changes (Francisco and Guedes, 2015; Souza et al., 2015).

In addition to epilepsy, malnutrition is an important public health problem in a number of developing countries, with economic and sociocultural implications. It is believed that the changes caused by both epilepsy and malnutrition, when in association, can potentiate their deleterious neural effects (Porto et al., 2010). However, the hypothesis that malnourished humans would present a higher incidence of epilepsy compared to well-nourished humans needs much investigation. Previous studies on well-nourished rats described significant effects of a convulsing dose of pilocarpine on body weight and glycemia (Cossa et al., 2016), as well as on the propagation of CSD (Guedes and Cavalheiro, 1997). However, it is not known whether this convulsing compound would act relevantly on behavioral and electrophysiological parameters, when chronically applied in very low sub-convulsing doses, i.e., when producing a situation of chronic muscarinic cholinergic activation. In this study, we investigated in the rat the repercussion of chronic administration of a sub-convulsing dose of pilocarpine on the functioning of neural tissue in order to answer the two following questions: (1) Does the chronic treatment with a sub-convulsing dose of pilocarpine induce glycemia and anxiety-like and electrophysiological (CSD) alterations? (2) Are such effects of pilocarpine influenced by unfavorable lactation conditions?

MATERIALS AND METHODS

Animals

All experimental procedures were previously approved by the Institutional Ethics Committee for Animal Research of our University (Approval protocol no. 23076.015655/2015-99), whose norms comply with those norms established by the National Institutes of Health Guide for Care and Use of Laboratory Animals (Bethesda, MD, United States). Newborn Wistar rats of both genders, born from different dams, were assigned to be suckled under normal or unfavorable conditions, represented, respectively, by litters with nine pups (L_9 groups) and litters with 15 pups (L_{15} groups). Weaning occurred on postnatal day (PND) 21, when pups were separated by sex and housed in polypropylene cages (51 cm × 35.5 cm × 18.5 cm; three rats per cage) under a 12-h light:12-h dark cycle (lights on at 6:00 a.m.), controlled temperature (23 ± 1°C), and with free access to water and the same commercial lab chow, with 23% protein, that was offered to their dams (Purina Ltd). In this study, we analyzed data from male pups only: 27 L_9 and 27 L_{15} rats. The animals were weighed on PND7, PND21, PND35, PND49, and PND60.

Administration of Pilocarpine

Pilocarpine hydrochloride and scopolamine methyl nitrate were purchased from Sigma-Aldrich (St Louis, MO, United States). From PND35 to PND55, L_9 and L_{15} rats received a single daily intraperitoneal injection of pilocarpine (45 mg/kg/day dissolved in saline; n = 9 L_9 and 9 L_{15} rats), as previously

described (Guedes and Vasconcelos, 2008), or vehicle (saline; n = 9 L_9 and 9 L_{15} rats). One additional L_9 and one L_{15} group received no treatment (naïve groups; n = 9 L_9 and 9 L_{15} rats). Scopolamine methyl nitrate, a muscarinic receptor antagonist, was administered i.p., (1 mg/kg/day dissolved in 0.9% saline) in both groups 30 min before pilocarpine or saline administration to prevent the peripheral cholinergic effects elicited by pilocarpine (Peixinho-Pena et al., 2012). Immediately following pilocarpine administration, the animals were observed over 1 h for detection of spontaneous seizures as measured by the Racine (1972) scale with the following stages: (0) No abnormality; (1) Mouth and facial movements; (2) Head nodding; (3) Forelimb clonus; (4) Rearing with forelimb clonus; (5) Rearing and falling with forelimb clonus. At this low dose of pilocarpine, no behavioral signs of epilepsy were detected in our animals.

Open Field Test

On PND58, the rats were individually placed in the center of a circular arena (diameter 89 cm and height 52 cm). The apparatus was located in a room with dim light and sound attenuation. Rats were positioned in the center of the arena, and their movements were recorded using a digital camera for 5 min. Between each test, the open field apparatus was wiped with a paper cloth soaked with 70:30 ethanol:water solution. The video-recorded activity was stored in a computer and subsequently analyzed with the software ANY maze^{TM} (version 4.99 m), as previously described (Lima et al., 2017). The following parameters were considered: number of expelled fecal boluses, total distance traveled, total immobility time, number of entries in the central zone and the time spent in the central zone.

Analysis of Blood Glucose

As previously reported (Francisco and Guedes, 2015), after the open field behavioral test, the animals were fasted for 6 h, after which a drop of blood was collected from the animal's tail and used for measuring the blood glucose level using a portable glucose meter (G-TECH free).

CSD Recording

On the day of the electrophysiological recording (PND60–PND65), each animal was anesthetized with a mixture of 1 g/kg urethane plus 40 mg/kg chloralose injected intraperitoneally. Three trephine holes were drilled on the right side of the skull, aligned in the frontal-to-occipital direction and parallel to the midline. One hole was positioned on the frontal bone (2 mm in diameter) and used to apply the stimulus (KCl) to elicit CSD. The other two holes were positioned on the parietal bone (3–4 mm in diameter) and used to record the propagating CSD wave. CSD was elicited at 30-min intervals by a 1-min application of a cotton ball (1–2 mm in diameter) soaked with 2% KCl solution (approximately 270 mM) to the anterior hole drilled at the frontal region. Rectal temperature was continuously monitored and maintained at 37 ± 1°C by means of a heating blanket. The DC slow potential change accompanying CSD was recorded for 4 h using two Ag–AgCl agar–Ringer electrodes (one in each hole) against a common reference electrode of the same type, placed on the nasal bones. We calculated the CSD velocity of propagation from the time required for a CSD wave to pass the distance between the two cortical electrodes. In the two recording locations, we used the initial point of each DC-negative rising phase as the reference point to calculate the CSD velocities. In addition, we calculated amplitude and duration of the CSD waves, as previously reported (Lima et al., 2017).

Statistics

Results in all groups are expressed as the means ± standard deviations (SD). Body weight, anxiogenic-like behavioral activity, blood glucose level and CSD propagation rate were compared between groups using ANOVA, including the following as factors: lactation conditions (L_9 and L_{15}) and treatment (naïve, vehicle, pilocarpine), followed by a *post hoc* test (Holm–Sidak) where indicated. A $p < 0.05$ was considered significant.

RESULTS

Body Weight

As shown in **Figure 1**, in all treatment groups ANOVA showed a main effect of the lactation condition on body weight ($p < 0.001$). The L_{15} animals presented with lower body weights compared with the L_9 groups. The weight reduction ranged from 20.1 to 36.5% and was independent of the treatment. In the normal (L_9) lactation condition, intergroup difference was observed in PND49 only [$F(2,41) = 22.502$; $p < 0.001$]. The treatment with pilocarpine was associated with weight reduction, compared to the respective L_9 control groups. In the unfavorable (L_{15}) lactation condition, pilocarpine reduced body weight at PND49 and PND60 [$F(2,39) = 14.785$; $p < 0.001$].

Blood Glucose Level

In the L_{15} control groups, glycemia was significantly lower than the corresponding L_9 groups [$F(1,35) = 22.990$; $p < 0.001$].

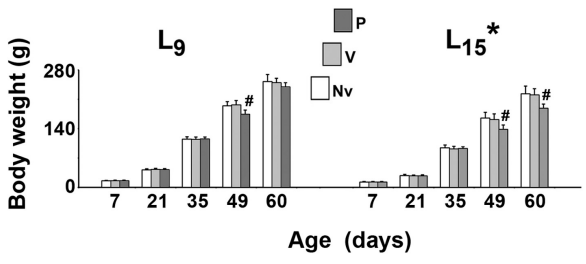

FIGURE 1 | Suckling under large litter size and pilocarpine administration reduced body weight of male rats. Animals were previously suckled in litters with 9 and 15 pups (respectively, L_9 and L_{15} condition). Naïve (Nv), no treatment; Vehicle (V), scopolamine methyl nitrate 1 mg/kg/day dissolved in 0.9% saline + 0.9% saline; Pilocarpine (P), scopolamine methyl nitrate 1 mg/kg/day + 45 mg/kg/day of pilocarpine; both dissolved in 0.9% saline. Note that since pilocarpine or vehicle intraperitoneal administration occurred from postnatal day 35–55, a differentiation between the three groups at day 7 and 21 can be done only by the assignment to the groups, but not by treatment. Data are mean ± standard deviation. *$p < 0.001$ compared with the corresponding L_9 condition. #$p < 0.001$ compared with control groups in the same lactation condition (ANOVA plus Holm-Sidak test).

Pilocarpine treatment reduced blood glucose levels in the L_9, but not in the L_{15} groups [$F(2,35) = 9.709$; $p < 0.001$] compared with the corresponding control groups (naïve and vehicle). Data on glycemia are illustrated in **Figure 2**.

Behavioral Activity in the Open Field

The effect of administration of pilocarpine on the behavioral activity in the open field test is shown in **Figure 3**. Compared with the naïve (Nv) and vehicle-treated (V) controls, the L_9 group treated with pilocarpine (P) entered in the center area a lower number of times (P, 4.3 ± 1.9 vs. Nv, 9.6 ± 2.4 and V, 9.6 ± 5.0; $p < 0.001$), expelled a higher number of fecal boluses (P, 5.9 ± 1.1 vs. Nv, 2.9 ± 1.5 and V, 2.8 ± 1.0; $p < 0.001$), traveled a shorter distance in the circular arena (P, 19.5 ± 6.2 m vs. Nv, 28.1 ± 9.7 m and V, 29.1 ± 5.8 m; $p < 0.001$) and remained in immobility for a longer time (P, 49.1 ± 23.9 s vs. Nv, 27.2 ± 11.8 s and V, 26.4 ± 11.7 s; $p < 0.001$). No difference was observed for the time in the center area (P, 12.7 ± 4.9 s vs. Nv, 18.9 ± 5.0 s and V, 17.7 ± 11.8 s).

In the L_{15} condition, the pilocarpine-treated group entered a lower number of times in the center area (P, 3.1 ± 1.3 vs. Nv, 6.7 ± 1.8 and V, 5.0 ± 2.7), remained a shorter time in the center area (P, 11.4 ± 4.1 s vs. Nv, 19.7 ± 4.9 s and V, 19.7 ± 6.4 s; $p = 0.007$), expelled a higher number of fecal boluses (P, 5.4 ± 1.4 vs. Nv, 2.6 ± 1.8 and V, 2.9 ± 2.2), traveled a shorter distance in circular arena (20.81 ± 4.70 m vs. 29.09 ± 3.31 m and 28.55 ± 6.04 m) and remained longer in immobility (P, 52.3 ± 13.9 s vs. Nv, 25.4 ± 8.2 s and V, 24.3 ± 8.2 s).

CSD Parameters

In all groups, topical application of 2% KCl at one point of the frontal cortical surface for 1 min elicited, as a rule, a single CSD wave that propagated without interruption and was recorded at the two parietal recording points (**Figure 4A**; see the recording points in the skull diagram). During the 4-h recording session, the slow DC potential change, which consistently appeared after KCl stimulation, confirmed the presence of CSD. In the ECoG recordings, no signs of hyperexcitability (i.e., spikes, sharp waves, paroxysmal depolarization shifts etc.) were observed.

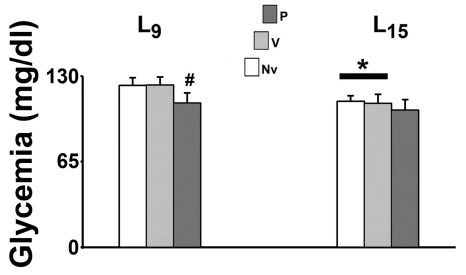

FIGURE 2 | Suckling under large litter size and pilocarpine administration decreased blood glucose levels of 58 day-old male rats. Animals were previously suckled in litters with 9 and 15 pups (respectively, L_9 and L_{15} condition). Naïve (Nv), no treatment; Vehicle (V), scopolamine methyl nitrate 1 mg/kg/day dissolved in 0.9% saline + 0.9% saline; Pilocarpine (P), scopolamine methyl nitrate 1 mg/kg/day + pilocarpine 45 mg/kg/day; both dissolved in 0.9% saline. Pilocarpine or vehicle intraperitoneal administration occurred from postnatal day 35–55. Data are mean ± standard deviation. *$p < 0.001$ compared with the corresponding L_9 condition. #$p < 0.001$ compared with control groups in the same lactation condition (ANOVA plus Holm-Sidak test).

In the L_9 animals, CSD velocities (mean ± SD in mm/min) in the naïve, vehicle and pilocarpine groups were, respectively, 3.69 ± 0.13, 3.70 ± 0.10, and 3.11 ± 0.15. In L_{15} animals, the CSD velocities for the naïve, vehicle and pilocarpine groups were, respectively, 4.20 ± 0.17, 4.18 ± 0.18, and 2.93 ± 0.23. ANOVA indicated a main effect of the lactation condition [$F(1,46) = 33.575$; $p < 0.001$], and *post hoc* (Holm–Sidak) test comparisons showed that the velocities were higher in the L_{15} groups compared to the L_9 groups. ANOVA also detected a main effect of treatment [$F(2,46) = 173.067$; $p < 0.001$], and *post hoc* testing revealed that pilocarpine treatment significantly lowered the CSD propagation velocity compared with the corresponding naïve and vehicle controls. The pilocarpine decelerating effect on CSD propagation was more intense in L_{15} than L_9 rats [$F(2,46) = 22.658$; $p < 0.001$], indicating an interaction between the treatment with pilocarpine and the lactation condition. Data on CSD velocity are shown in **Figure 4B**.

Table 1 shows data on amplitude and duration of the negative slow potential change, which is the hallmark of CSD. ANOVA indicated a main effect of the lactation condition on the CSD amplitude [$F(1,42) = 8.165$; $p = 0.007$], and a *post hoc* (Holm–Sidak) test comparison showed that the amplitudes were higher in the naïve and vehicle L_{15} groups compared to the corresponding L_9 groups. The factor treatment also affected the amplitude [$F(2,42) = 9.490$; $p < 0.001$], and a *post hoc* test showed that the amplitude was lower in the L_{15}, but not in the L_9 pilocarpine-treated group, compared with the corresponding naïve and vehicle controls. ANOVA also confirmed an interaction between both factors [$F(2,42) = 8.999$; $p < 0.001$].

Analysis of CSD duration indicated a main effect of the lactation condition [$F(1,47) = 84.197$; $p < 0.001$] and treatment [$F(2,47) = 3.262$; $p = 0.047$] as well as an interaction between these two factors [$F(2,47) = 3.674$; $p = 0.033$]. The Holm-Sidak test indicated a shorter duration in the L_{15} groups compared with the corresponding L_9 groups and a longer duration in the L_{15} pilocarpine-treated animals compared with the corresponding naïve and vehicle controls.

DISCUSSION

The present data demonstrate that treatment for 21 days with a sub-convulsing dose of pilocarpine reduces glycemia, promotes anxiety-like behavior and decelerates CSD in rats suckled on two litter sizes. In some measurements (see "Results"), the data also suggest an interaction between lactation condition and pilocarpine treatment, indicating that the pilocarpine effect is more evident in the nutritional deficiency condition (L_{15}). Data on weight gain and open field behavior using a relatively low, sub-convulsing dose of pilocarpine constitute novel evidence of the pilocarpine action in rats, whereas data on CSD confirm a previous study

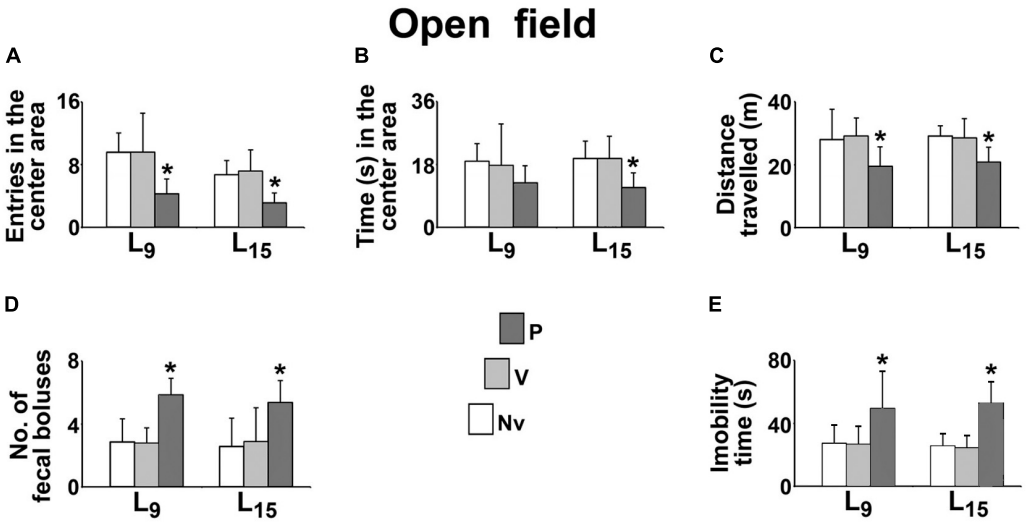

FIGURE 3 | Sub-convulsing dose administration of pilocarpine results in anxiogenic-like behavioral activity in the open field test of 58 day-old male rats previously suckled in litters with 9 and 15 pups (respectively, L_9 and L_{15} condition). Anxiogenic-like activity was characterized as a decrease of the following parameters: number of entries into the central area **(A)**, time spent in the central area **(B)** and distance traveled **(C)**, as well as an increase of the number of fecal boluses expelled **(D)**, and immobility time **(E)**. Naïve (Nv), no treatment; Vehicle (V), scopolamine methyl nitrate 1 mg/kg/day dissolved in 0.9% saline + 0.9% saline; Pilocarpine (P), scopolamine methyl nitrate 1 mg/kg/day + 45 mg/kg/day of pilocarpine; both dissolved in 0.9% saline. Pilocarpine or vehicle intraperitoneal administration occurred from postnatal day 35–55. Data are mean ± standard deviation. *$p = 0.007$ for time in the center area compared with control groups in the same lactation condition and $p < 0.001$ for other parameters (ANOVA plus Holm-Sidak test).

(Guedes and Vasconcelos, 2008). The stress of the treatment procedure cannot be the cause of the reported alteration because the groups that received intraperitoneal injection of vehicle (saline and scopolamine) presented values similar to the naïve control. The results emphasize the effectiveness of pilocarpine in modifying behavioral and electrophysiological functioning of the brain.

Our findings on body weight difference between the L_9 and L_{15} lactation condition confirm previous evidence on the efficacy of increasing litter size in producing nutritional deficiency (Francisco and Guedes, 2015; Lima et al., 2017). Increasing the litter size during the suckling period (L_{15} condition) effectively impairs the pups' nutritional status, as judged by their reduced body weights (Rocha-de-Melo et al., 2006). This is in line with evidence from others indicating that body weight diminution reflects weight reduction in the brain and other important organs, which is usually accompanied by alterations in the organs' function (Morgane et al., 1978, 1993). Furthermore, the reduction in blood glucose levels in the L_{15} groups, compared with the corresponding L_9 groups, supports this conclusion.

Regarding the action of pilocarpine, previous work reported impairment in the growth and development as well as high blood glucose levels in pups of pilocarpine-treated epileptic dams (Cossa et al., 2016). In the present work, pilocarpine treatment of the pups did reduce the animals' body weight (**Figure 1**) and their blood glucose levels (**Figure 2**). While the data on body weight are coherent, differences in the blood glucose levels could be attributed to distinct pilocarpine treatment paradigms: administration of convulsing doses to the mothers (Cossa et al., 2016) vs. treatment of the pups with sub-convulsing doses (present work).

The present open field findings (**Figure 3**) are coherent with our previously published data (Accioly and Guedes, 2017; Lima et al., 2017). In contrast, some studies reported lower anxiety-like behavior in animals suckled in large litters in comparison with the control group (Bulfin et al., 2011; Clarke et al., 2013). This discrepancy could be due to methodological differences such as the number of pups per litter (12 and 20 pups vs. 9 and 15 pups in our work), type of behavioral test (elevated plus maze vs. open field in our work) and age of testing (PND25 or PND77, vs. PND58 in our work). In addition, those authors subjected the animals to other experimental procedures such as anesthesia with isoflurane and imaging (dual energy X-ray absorptiometry system) for whole body composition; this could likely contribute to the differences discussed here.

In the open field test, the treatment with a sub-convulsing dose of pilocarpine was associated with a more anxious behavior than that observed in the control animals (**Figure 3**). Of relevancy, the cholinergic system is implicated in emotional regulation (Hoeller et al., 2016). Both sub-convulsing (Duarte et al., 2010, 2013; Hoeller et al., 2016) and convulsing doses of pilocarpine (Leite et al., 2016) can induce short-lasting and long-lasting anxiogenic responses in rats. Taken together, these data allow us to suggest an important behavioral role for the cholinergic system and cholinergic drugs, which would lead to low preference for social novelty, as indicated by longer immobility after pilocarpine administration (Castelhano et al., 2015).

Over the last three decades, our group has characterized the accelerating effect of nutritional deficiency on CSD quite well

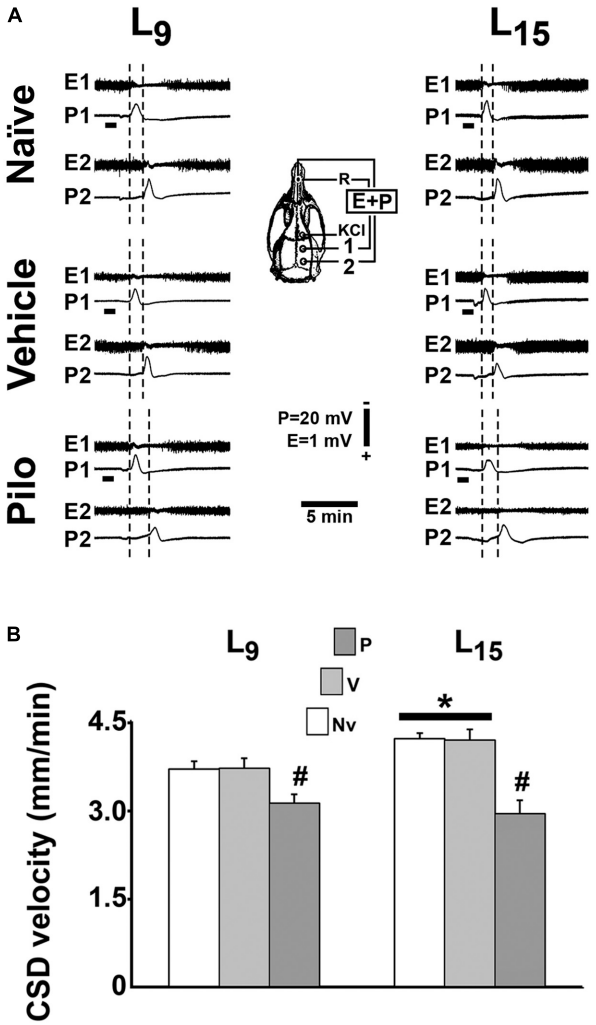

FIGURE 4 | Repeated daily administration (21 days) of a sub-convulsing dose of pilocarpine reduced, whereas unfavorable lactation increased the propagation velocity of CSD in 60–65 day-old male rats previously suckled in litters with 9 and 15 pups (respectively, L_9 and L_{15} condition). Pilocarpine or vehicle intraperitoneal administration occurred from postnatal day 35–55. **(A)** Electrocorticogram (E) and slow potential changes (P) during the passage of CSD, recorded at two cortical points (1 and 2) in three L_9 and three L_{15} rats. The diagram of the skull shows the recording positions 1 and 2, from which the traces marked at the left with the same numbers were obtained. The position of the common reference electrode (R) on the nasal bones and the application point of the CSD-eliciting stimulus (KCl) are also shown. CSD was elicited in the frontal cortex by chemical stimulation (a 1- to 2-mm diameter cotton ball soaked with 2% KCl) applied for 1 min on the intact dura mater, as indicated by the horizontal bars. The latencies are shorter in the L_{15} groups compared with the corresponding L_9 groups. In the groups treated with pilocarpine, the latencies are increased when compared with the respective naïve and vehicle controls. **(B)** CSD propagation velocity. Data are mean ± standard deviation for 8–9 rats per group. $^*p < 0.001$ compared with the corresponding L_9 condition. $\#p < 0.001$ compared with controls groups in the same lactation condition (ANOVA plus Holm-Sidak test).

TABLE 1 | The unfavorable lactation condition increases amplitude and diminishes duration of the negative slow potential change of CSD, whereas chronic application of a sub-convulsing dose of pilocarpine exerts the opposite effect.

Group	Amplitude (mV)	Duration (s)
L_9		
Naïve	8.4 ± 1.8(9)	69.6 ± 2.3(9)
Vehicle	8.5 ± 1.1(9)	70.1 ± 2.6(9)
Pilocarpine	8.4 ± 1.1(9)	69.7 ± 1.3(9)
L_{15}		
Naïve	11.1 ± 2.5(6)#	64.4 ± 0.9(8)#
Vehicle	11.0 ± 1.5(7)#	64.8 ± 1.1(9)#
Pilocarpine	7.0 ± 1.2(8)*	67.2 ± 1.2(9)*#

Male rats were previously suckled in litters with 9 and 15 pups (respectively, L_9 and L_{15} condition). Naïve, no treatment; Vehicle, scopolamine methyl nitrate 1 mg/kg/day dissolved in 0.9% saline + 0.9% saline; Pilocarpine, scopolamine methyl nitrate 1 mg/kg/day + 45 mg/kg/day of pilocarpine; both dissolved in 0.9% saline. Data are expressed as the mean ± standard deviation, with the number of animals in parentheses. $^*p < 0.001$ compared with control groups in the same suckling condition. $\#p < 0.001$ compared with the corresponding group in the L_9 condition (ANOVA plus Holm-Sidak test).

(Guedes et al., 1987; Rocha-de-Melo et al., 2006; Guedes, 2011; Francisco and Guedes, 2015; Accioly and Guedes, 2017; Lima et al., 2017). The present data (**Figure 3**) confirm this effect, whose underlying mechanisms are still a subject of investigation. When acting during the critical period of brain development, malnutrition can reduce the number and/or size of brain cells, the amount of myelin, the number of synapses and can alter the functioning of neurotransmitter systems (Morgane et al., 2002; Guedes, 2011). Of note, the reduced brain uptake of glutamate (Feoli et al., 2006) and the increased activity of key enzymes such as glutamic acid decarboxylase (Díaz-Cintra et al., 2007) has also been reported in malnourished animals, and both processes facilitate the propagation of CSD (Peeters et al., 2007; Tottene et al., 2009).

The possible relationship between CSD and brain excitability has been investigated in our laboratory using the acute pilocarpine administration paradigm; both convulsing (Guedes and Cavalheiro, 1997) and sub-convulsing dosing with a single injection of pilocarpine (Vasconcelos et al., 2004; Guedes and Vasconcelos, 2008) decelerated CSD. In the present study, we treated young animals (PND35) over 21 days with a sub-convulsing dose of pilocarpine and confirmed its CSD decelerating action. Recently, Mendes-da-Silva and co-workers treated well-nourished and malnourished younger rats (PND7-28) with the same dose of pilocarpine and found similar CSD outcome (Mendes-da-Silva et al., 2018), suggesting that the time-window for the pilocarpine action on CSD is not narrow. These reports are pioneering in demonstrating the action of chronic pilocarpine on CSD both under favorable and unfavorable conditions of lactation. In comparison to the pilocarpine protocol usually described in the literature to provoke seizure in rodents (300–380 mg/kg), the sub-convulsing dose of pilocarpine used in the present work (45 mg/kg) represents, on average, 12–15% of the convulsing dose. At this low dose, no behavioral signs of epilepsy were detected in our animals. Although sub-convulsing, our pilocarpine dose was effective in counteracting CSD propagation, as evaluated by the alteration in CSD parameters (lower propagation velocity, negative DC amplitude, and longer duration) in the

pilocarpine-treated group in comparison with the controls. Pilocarpine displaces the balance between neural excitatory and inhibitory mechanisms toward a hyperexcitability state (Morimoto et al., 2004; L'amoreaux et al., 2010), which makes elicitation and propagation of CSD more difficult (Guedes and Cavalheiro, 1997). The relationship between changes in brain excitability and CSD is still a matter of controversy. On one hand, in humans hyperexcitability has been associated with CSD appearance in migraine (Pietrobon and Moskowitz, 2014; Vinogradova, 2018); on the other hand, animal data demonstrate that CSD does not invade a cortical region in which hyperexcitability has been produced by repetitive electrical stimulation (Koroleva and Bureš, 1979). Regarding the probable biochemical mechanisms underlying the CSD impairment that is associated with pilocarpine treatment, one possibility would be based on the metabolic adaptation that increases brain efficiency to remove extracellular potassium under conditions of hyperexcitability (Heinemann and Lux, 1975; Koroleva and Bureš, 1980). Interestingly, hyperexcitability reduction by benzodiazepines restores the CSD proneness of the pilocarpine-induced resistant brain (Guedes and Cavalheiro, 1997). Nevertheless, additional factors, such as the action of excitatory amino acids and the participation of disinhibition mechanisms (Silva-Gondim et al., 2017), that also modulate brain excitability could contribute for the observed CSD effects of pilocarpine. The glutamatergic system, via *N*-methyl-D-aspartate receptors (NMDARs), importantly participates in developmental and excitability processes in the young brain (Szczurowska and Mareš, 2013) and influences CSD (Marrannes et al., 1988). On the other hand, disinhibition mechanisms, via depressing GABAergic interneurons, could also participate in hyperexcitability generation (McMahon and Kauer, 1997) and in CSD, as recently suggested (Silva-Gondim et al., 2017). Furthermore, GABA release has been found to reduce CSD amplitude (Richter et al., 2014). Further investigation shall confirm whether these two mechanisms are involved in the pilocarpine effects and are mutually exclusive or might act together.

Regarding the pilocarpine/malnutrition interaction, Cabral et al. (2011) reported that malnourished rats require a lower pilocarpine dose to become epileptic in comparison with well-nourished animals, suggesting that malnutrition early in life reduces the threshold for pilocarpine-induced epilepsy. Our CSD findings using a sub-convulsing dose of pilocarpine are consistent with these findings (see **Figure 4**). Taken together, the data confirm the hypothesis of an interaction between pilocarpine and malnutrition in the rat brain.

CONCLUSION

In conclusion, this study documents the metabolic, behavioral and electrophysiological effects of a low dose of pilocarpine and suggests that early malnutrition modulates the pilocarpine effects. The findings support the following three conclusions. First, chronic treatment with sub-convulsing doses of pilocarpine produced anxiety-like behavior and reduced the propagation velocity of CSD. Second, increasing litter size caused nutritional deficiency, reduced fasting blood glucose and accelerated CSD, confirming previous studies. Third, unfavorable lactation conditions (L_{15} condition) differentially modulated the pilocarpine effects on blood glucose levels and CSD. The present data might advance understanding the relationship between pilocarpine action on neuronal excitability, anxiety-like behavior and nutritional deficiency.

AUTHOR CONTRIBUTIONS

EF performed the experiments, analyzed the data and participated in conceiving the study, and writing and reviewing the manuscript. RG conceived the study, provided the funds, analyzed the data, and wrote the manuscript.

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Cotinine Plus Krill Oil Decreased Depressive Behavior, and Increased Astrocytes Survival in the Hippocampus of Mice Subjected to Restraint Stress

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Restraint stress (RS) is a condition affecting millions of people worldwide. The investigation of new therapies to alleviate the consequences of prolonged RS is much needed. Cotinine, a nicotine-derivative, has shown to prevent the decrease in cerebral synaptic density, working memory deficits, anxiety, and depressive-like behavior after prolonged restraint stress (RS) in mice. Furthermore, post-treatment with cotinine reduced the adverse effects of chronic RS on astrocyte survival and architecture. On the other hand, the nutritional supplement krill oil (KO), has shown to be beneficial in decreasing depressive-like behavior and oxidative stress. In this study, in the search for effective preventative treatments to be used in people subjected to reduced mobility, the effect of co-treatment with cotinine plus KO in mice subjected to prolonged RS was investigated. The results show that cotinine plus KO prevented the loss of astrocytes, the appearance of depressive-like behavior and cognitive impairment induced by RS. The use of the combination of cotinine plus KO was more effective than cotinine alone in preventing the depressive-like behavior in the restrained mice. The potential use of this combination to alleviate the psychological effects of reduced mobility is discussed.

Keywords: depression, cotinine, anxiety, restraint stress, krill oil, astrocytes

INTRODUCTION

Stress is generated when an individual is unable to cope with overwhelming physical or psychological demands. Although, the hormonal and behavioral changes that occur in response to threatening stimuli is crucial for survival and can be beneficial in recruiting adaptive responses to cope with a stressful situation, however, prolonged stress can result in maladaptive changes that may lead to mental illness, cognitive and motor deficits (Yehuda et al., 1998; Hammen, 2003; Luine et al., 2007).

In the clinical realm, the emotional and physical alterations induced by RS further diminish the quality of life of people with restricted mobility as stress/induced Neuroinflammation induce

depression (Iwata et al., 2013; Muscatelli et al., 2017). A meta-analysis including 354 studies, 18,374 individuals revealed that more than 70% of depressed individuals showed signs of stress such as elevated cortisol levels in plasma(Stetler and Miller, 2011).

Stress response when engaged for extended durations or activated by a traumatic event; it is linked to the dysregulation of the Hypothalamus-pituitary-adrenal (HPA) axis (Bauer et al., 2001). This dysregulation results in altered levels of glucocorticoid hormones and neurotransmitters in the brain and may lead to psychological depression (Hayase, 2011). For example, since the HPA axis has bidirectional relationships with the serotoninergic system its dysregulation can result in decreased levels of serotonin leading to depression and irritability. Also, neurons from the amygdala (AMY), which are responsive to the corticotropin-releasing hormone (CRH), project to the raphe nuclei, the main serotonin source to the forebrain. In addition, dysregulation of the HPA axis correlates with morphological alterations of the brain such as the reduction of hippocampal volume observed in individuals with major depressive disorder (MDD) and posttraumatic stress disorder (PTSD) (Sheline, 2000; Bonne et al., 2001; Czeh et al., 2001; Schmitz et al., 2002; Villarreal et al., 2002; Drevets et al., 2008; Felmingham et al., 2009; Apfel et al., 2011; Filipovic et al., 2011; Gonul et al., 2011; Teicher et al., 2012; Admon et al., 2013; Ahmed-Leitao et al., 2016).

Chronic stress has many other harmful effects on the brain including neuroinflammation, oxidative stress, microgliosis, reduced neurogenesis, and diminished numbers and architectural complexity of neurons. Chronic stress also evokes synaptic alterations including spine number and shape (Kassem et al., 2013; Bennett and Lagopoulos, 2014; Scharfman and MacLusky, 2014). In rodents, restraint stress (RS) affects spatial memory (Bowman et al., 2002; Kleen et al., 2006) and long-term potentiation, a cellular model of learning and memory processes, in the hippocampus (Pavlides et al., 2002). Such effects have been associated with the retraction of apical dendrites, as well as the loss of synapses in the CA1, CA3, and dentate gyrus (DG) sub-regions of the hippocampus (McEwen et al., 1997; Magarinos et al., 2011). These cellular changes and brain connectivity, contribute to the development of PTSD in people subjected to trauma and suffering with reduced mobility. As a result, several neurological symptoms appear such as working memory loss, impulsivity, aggressive behavior, and depression in traumatized individuals who developed PTSD (Tafet and Bernardini, 2003; Reagan et al., 2008; Conrad and Bimonte-Nelson, 2010; Luine, 2016; Moreira et al., 2016).

Numerous studies have emphasized the role of astrocytes in mediating brain homeostasis by supporting the blood-brain barrier function, sustaining neuronal energy metabolism (Stobart and Anderson, 2013), and neurotransmission (Schousboe et al., 1992). Furthermore, astrocytes modulate synaptic plasticity processes including synaptogenesis, neurogenesis, and learning and memory (Honsek et al., 2012; Bernardinelli et al., 2014; Haydon and Nedergaard, 2014). For these reasons, astrocytes are considered useful therapeutic targets for several neurological disorders (Garzon et al., 2016; Gonzalez-Giraldo et al., 2017).

Astrocytes have been classified according to their cellular morphologies (cell body size, and number, length, thickness, direction, and length of processes) and location, in two main subtypes, protoplasmic or fibrous (Sofroniew and Vinters, 2010). Protoplasmic astrocytes are found in the gray matter and present numerous stem branches that originate from several branching processes in a regular sphere-like distribution. Fibrous astrocytes have elongated processes and are present in the white matter (Sofroniew and Vinters, 2010). It has been proposed that the morphology of astrocytes can be a good indicator of their functions (de Filippis, 2011). Recently, Choi et al. studied the molecular and morphological changes of astrocytes induced by fear conditioning; the results showed a significant decrease of the immunoreactivity (IR) for the glial fibrillary acidic protein (GFAP) in the hippocampus of fear conditioned rodents (Saur et al., 2016). Our team found a similar reduction in the number of GFAP+ astrocytes in the CA1, CA3, and DG of the hippocampus after prolonged restraint stress (RS) in mice (Perez-Urrutia et al., 2017). Previous studies indicated that in the hippocampal formation posttreatment with cotinine normalized the number of GFAP+ astrocytes astrocyte and GFAP IR, in all hippocampal regions being the effect especially significant in the DG.

In this study, our goal was to assess the effect of co-treatment with KO plus cotinine on GFAP+ IR in the DG, and depressive-like behavior and working memory in a mouse model of prolonged RS. The results highlight the advantage of using cotinine plus a natural antioxidant nutrient to diminish stress-derived depressive behavior, cognitive impairment, and astrocytes abnormalities in the hippocampus after prolonged RS.

MATERIALS AND METHODS

Animals

Two-month-old male C57BL/6J mice (obtained from the University of Chile), weighing 25-30 g, were maintained on a 12-h (h) light/dark cycle (light on at 07:00 h) with *ad libitum* access to food and water and maintained at a controlled temperature (25 ± 1°C). Upon arrival, mice were group housed and acclimated for 7 days before behavioral testing. Experiments were completed during the light period of the circadian cycle. Animal handling and care were performed in compliance with the Guide for the care and use of laboratory animals adopted by the National Institute of Health (USA), according to a protocol approved

Abbreviations: AMPA, α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; ANOVA, Analysis of variance; CRH, corticotrophin releasing hormone; FS, Forced swimming; GFAP, Glial fibrillary acidic protein; HPA, Hypothalamus-pituitary-adrenal; IR, Immunoreactivity; KO, Krill oil; MD, Major depression; MAOIs, Monoamine oxidase inhibitors; MGV, Mean gray values; NOR, Novel object recognition; OF, Open field; PBS, Phosphate buffered saline; PFC, Prefrontal cortex; PTSD, Posttraumatic stress disorder; ROI, Region of interest; SSRIs, Region serotonin-reuptake inhibitors; SNRIs, Serotonin-norepinephrine reuptake inhibitors; TBS, Tris-buffered saline; TBST, TBS with 0.1% Tween 20.

by the ethical committee of the Universidad San Sebastian, Chile.

Drug Preparation

Cotinine ((5*S*)-1-methyl-5-(3-pyridyl) pyrrolidine-2-one) (Sigma-Aldrich Corporation, St. Louis, MO, USA) was prepared by dissolving the powdered compound in sterile phosphate buffered saline (PBS). KO capsules (300 mg) were purchased from Walgreens (Superba, USA). Soft gels contain 300 mg KO (90 mg omega-3 fatty acids, 50 mg eicosapentaenoic acid, 24 mg docosahexaenoic acid, 130 mg phospholipids). Manufacturers provided no information about the Astaxanthin content in the soft gels.

Experimental Groups and Drug Treatments

Initially, the mice were acclimated for 1 week and subjected to handling to reduce stress. Then, mice were randomly divided into five groups ($n = 8$ mice/condition) and treated via gavage with 0.05 ml of vehicle (PBS, pH 7.4), Cotinine (Cot), KO or Cot plus KO dissolved in 0.05 ml of vehicle as detailed: (1) control non-restrained mice treated with vehicle; (2) restrained mice treated with vehicle; (3) restrained cotinine-treated mice (5 mg/kg in vehicle); (4) restrained KO-treated mice (143 mg/kg in vehicle); (5) restrained mice treated with cotinine (5 mg/kg) plus KO (143 mg/kg, in vehicle). Mice were treated via at the same time of the day, 30 min (min) before restraint and continuously until euthanasia. After 21 days (d) into treatments, mice were tested for behavior in the order from less stressful test to more stressful as indicated in **Figure 1**, with 24 of resting between tests to permit adequate resting of mice and to prevent interference between test. After the end of experiments, mice were euthanized using cervical dislocation by well-trained personnel.

Behavioral Procedures

Restraint Stress

RS is a reliable method to model chronic stress that mimics the effects of restraint stress on brain functions and behavior in rodents (Pare and Glavin, 1986; Jaggi et al., 2011). Mice were gently introduced into 50-ml conical transparent plastic tubes (Corning Inc.) containing non-protruding perforations in both ends and in the walls to permit ventilation. Mice were kept inside these tubes, allowing slight movements, for 6 h a day for 21 d at room temperature (RT). After the daily restraint time, mice were returned to their home cages and permitted to move freely for the rest of the day. Following the 3 weeks of RS, mice were tested for locomotor activity and depressive-like behavior.

Open Field Test (OF)

The open field (OF) test (Belzung and Griebel, 2001) was conducted as previously described with minor modifications (Norcross et al., 2008) to identify changes in locomotor activity in response to stress and drug treatments. Mice were individually placed in a corner and permitted to freely explore an open square arena (40 × 40 × 35 cm) for 10 min (**Figure 2A**). Total distance traveled, speed and time spent in the center zone were measured under moderate lighting using the video tracking software (ANY-Maze, Stoelting Co.).

Forced Swim Test

The forced swim (FS) is a broadly used task to assess depressive-like behavior in rodents (Dalla et al., 2010). The FS is performed introducing each mouse in the surface of a transparent and inescapable cylinder two-thirds filled with water at 26 ± 1°C (**Figure 3A**). Mice engage in periods of intense movement followed for increasing periods of immobility. The immobility time is considered an expression of depressive-like behavior. Immobility time is defined as no longer exhibiting any escape behavior, motionless or moving only to keep floating. Immobility time during a 5-min trial was recorded and quantified by two investigators blind to the treatment groups.

Novel Object Recognition (NOR)

This task evaluates recognition memory, and it is based on the natural preference of rodents for novel objects when exposed to new and previously encountered objects (de Bruin and Pouzet, 2006). During the task, favored exploration of the novel object provides a measure of recognition memory. After a habituation step in a square arena (40 × 40 × 35 cm), each mouse was placed in the same arena but containing two identical transparent objects located equidistant to each other (familiarization phase) and led to explore the objects for 5 min (**Figure 4A**). Then, mice were returned to their cages and permitted to rest for 30 min. After resting, mice were placed back in the arena containing one of the familiar objects and a new object (**Figure 4B**). The time exploring the two objects was recorded for 5 min. Exploratory behavior was recorded and the time of exploration of each object was normalized for animal activity by calculating the exploration index (EI) that corresponds to the time spent by the mouse exploring one of the equal objects or the new object/ total time spent exploring both objects × 100%. The behavioral recording and analysis were performed using the (ANY-Maze, Stoelting Co.).

Sucrose Preference Test

The sucrose preference test was used to assess anhedonia, an indicator of depressive-like behavior. The sucrose preference test was performed as previously described (Serchov et al., 2016) with some modifications. The mice were habituated given two identical bottles with tap water in their cage. Then, mice were given free access to both, one with 2% sucrose solution and another with tap water for 3 days. The position of bottles was switched daily to avoid place preference in drinking behavior. Bottle weights were taken every day to determine solution intake. The preference for sucrose was calculated as a percentage of the volume of sucrose intake over the total volume of fluid intake and averaged over the 3 days of the test.

Morphological Analyses of Astrocytes in the Dentate Gyrus

Brain Tissue Preparation

For the protein analyses, mice were euthanized, and brains removed. The left hemisphere of brains was dissected out to collect the regions of interest and quickly frozen for later biochemical analyses. For the immunohistochemistry (IHC) and fluorescent IHC (F-IHC) analysis, the right hemisphere of each

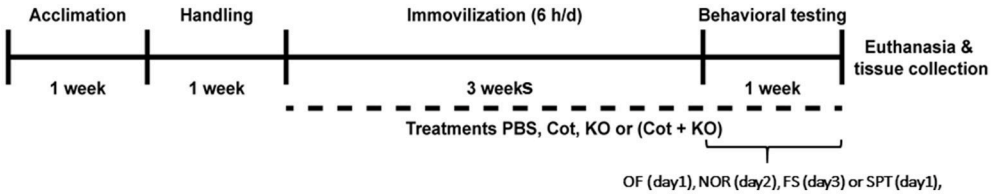

FIGURE 1 | Experimental design. Mice were subjected to restraint stress 6 h/day for 21 days and co-treated with PBS, krill oil (KO), Cotinine (Cot) or Cot plus KO. After restraint and under continuing treatments, mice were tested for locomotor function, recognition memory using the novel object recognition test (NOR) and depressive-like behavior using the forced swim (FS) test and the sugar preference test (SPT).

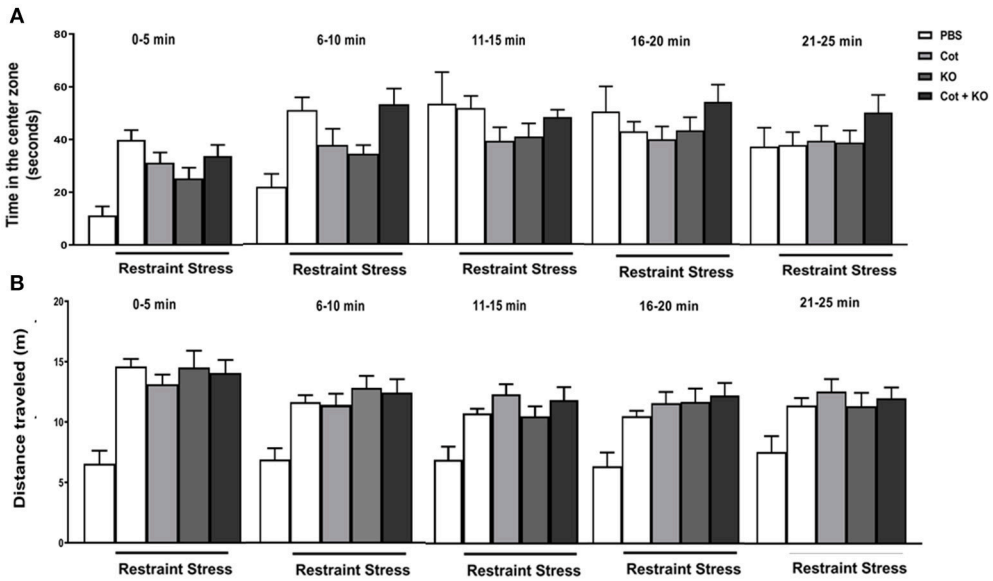

FIGURE 2 | Co-treatment with cotinine and krill oil does not affect locomotor activity in mice. After prolonged restraint stress (RS) and co-treatment with vehicle (PBS), cotinine (Cot, 5 mg/kg), krill oil (KO, 143 mg/kg) or (Cot plus KO), mice were tested for locomotor activity in the open field test for 25 min. The results show the effect of RS and treatments on. Time in the center zone **(A)** and total distance travelled **(B)**.

mouse brain was placed in 4% paraformaldehyde in PBS pH 7.4 at 4°C for 24 h. The tissues were embedded in 2% agarose molds for vibratome sectioning. The region of interest was located using the Paxinos Atlas as a reference (Franklin and Paxinos, 1997), and serial sections of 20 μm ($n \geq 2$/mouse) were collected using the Vibratome Leica VT1000S and placed on positively charged slides (Biocare Medical, Concord, CA).

Immunofluorescence and Confocal Microscopy

For the F-IHC, samples were washed three times for 7 min with Tris-buffered saline (TBS), pH 7.8. The primary antibody anti-GFAP (1:50, BioSB) was diluted in diluent buffer, containing TBS supplemented with 1% bovine serum albumin (BSA) and 0.2% Triton X-100 and incubated with the tissue sections overnight (ON) at 4°C. After three washes with TBS for 10 min, sections were incubated with the secondary antibody, Cy2-conjugated rabbit anti-mouse IgG (1:200, Jackson Immuno Research, Pennsylvania, USA) diluted in TBS containing 1% BSA for 2 h at RT. The samples were counterstained with Hoechst (1:1,000) and mounted with fluorescence mounting medium (Prolong, Invitrogen). Confocal z-stacks were acquired using an LSM 780 confocal microscope (Zeiss, Oberkochen, Germany), z-stacks were normalized to maintain a consistent signal intensity through the depth of the sample, confocal z-stack image series were superposed in maximum intensity projections by ImageJ (National Institute of Health, Bethesda, MA, USA) for the measurements.

Morphometric Analysis and Cell Counting

In each image, regions of interest (ROI) were defined on the dentate gyrus using free-hand drawing. For each ROI, the mean gray values (MGV), representing the area immunoreactive for GFAP, were measured. To measure GFAP immunostaining in the DG, maximum fluorescence intensity projections of confocal z-stacks acquired from sagittal brain sections were converted into 8-bit greyscale images with 256 scales (pixel intensity 0 corresponding to no signal and 255 to maximal signal) by Image J software. To calculate the area fraction of GFAP+ IR, binary images the immunoreactive area of the thresholded images was divided by the total of the ROI. For the GFAP+ cell counting, cell

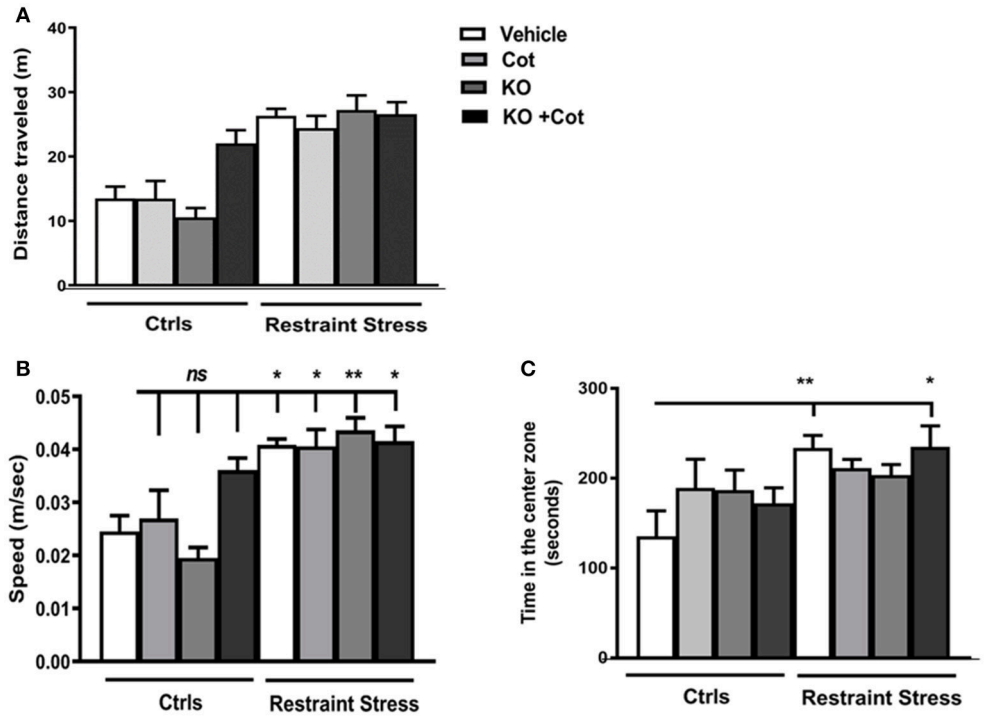

FIGURE 3 | Co-treatment with cotinine and krill oil does not affect locomotor activity in mice. After prolonged restraint stress (RS) for 10 min and co-treatment with vehicle (PBS), cotinine (Cot, 5 mg/kg), krill oil (KO, 143 mg/kg) or (Cot plus KO). The results show that RS did not significantly affect locomotor activity in the stressed mice. **(A)** Total distance traveled. **(B)** Mean speed (meters/seconds) **(C)** Time in the center zone. $^{*}P < 0.05$, $^{**}P < 0.01$.

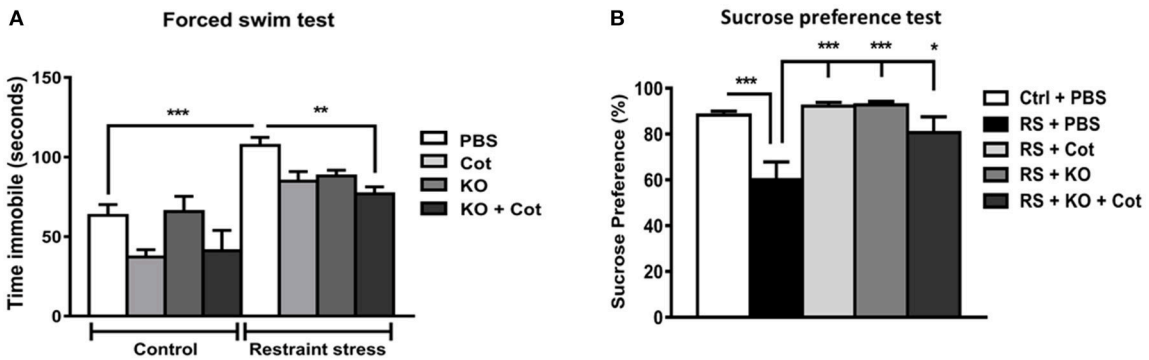

FIGURE 4 | Co-treatment with cotinine plus krill oil prevented the restraint stress-induced depressive-like behavior in mice. After three-week restraint and co-treatment with vehicle (PBS), cotinine (Cot, 5 mg/kg) or krill oil (KO, 143 mg/kg), mice were tested for depressive-like behavior in the forced swim tests (5 min). **(A)** or the sucrose preference tests **(B)**. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

to be counted must have at least half of the cell nucleus visible on the edge of the ROI and cells to be included in the analysis must be not adherent to blood vessels.

Statistical Analysis

To analyze group and treatment effects differences in the means between groups one-way analysis of variance (ANOVA), and *post hoc* Dunnet's test was performed using the GraphPad Prism software to assess difference significance between groups. Differences were considered significant with $P < 0.05$.

RESULTS

Effect of Krill oil and Cotinine on Locomotor Activity

To determine the changes in locomotor activity induced by co-treatments during RS, mice were tested using the OF test for 25 min. The results show that stressed mice showed higher time spent in the center zone than non-stressed controls in the first 10 min of the tests, but the differences diminished after this time between groups. The time spent in the center zone by the different groups was significantly different in the first 5 min of

the OF test [$F_{(4,63)} = 6.42$, $P < 0.0003$; **Figure 2A**]. At the contrary, the distance traveled show marked differences between non-stressed mice and all groups of stressed mice, independent of treatments (**Figure 2B**). Considering these results we proceed to analyze the changes in locomotor activity in the OF during the first 10 min of testing. One-way ANOVA analysis revealed significant differences in the distance traveled in the first 10 min [$F_{(7,67)} = 16$, $P < 0.0001$; **Figure 3A**] and speed [$F_{(7,76)} = 8.91$, $P < 0.0001$; **Figure 3B**]. There were no statistically significant differences in distance traveled or speed between non-stressed mice treated with vehicle, cotinine or KO. However, they showed significantly lower distances than non-stressed mice treated with KO plus cot ($P < 0.05$), and restrained mice treated with vehicle, cotinine, KO or KO plus Cotinine ($P < 0.0001$).

The increase in distance traveled was accompanied by a significant increase in speed in the mice subjected to RS when compared to non-stressed vehicle-treated mice (& RS, $P < 0.05$; & RS plus Cot $P < 0.05$; & RS, KO, $P < 0.01$; & RS, KO plus Cot, $P < 0.05$; **Figure 3B**). Also, the time spent in the center zone during the first 10 min of testing was analyzed, when anxiety behavior was more evident. The results showed that in the first 10 min there was a non-significant increase in time spent in the center zone between control non-stressed mice and the mice subjected to RS [$F_{(7,67)} = 2.11$, $P < 0.053$] (**Figure 3C**). However, these differences were significant between treatment groups after 5 min of testing [$F_{(4,67)} = 9.17$, $P < 0.0001$; (**Figure 2A**) with significant differences between non-stressed mice and stressed-mice independent of treatments (& RS, $P < 0.0001$; & RS plus Cot $P < 0.001$; & RS, KO, $P < 0.001$; & RS, KO plus Cot, $P < 0.0001$).

Effect of Krill Oil and Cotinine on Depressive-like Behavior

To investigate the potential anti-depressant effects of cotinine, KO and KO plus cotinine when administered during stress, at the last day of RS mice were tested for depressive-like behavior using the forced swim test and sucrose preference test (**Figure 4**). A two-way ANOVA showed a significant impact of prolonged RS on the level of depressive-like behavior, expressed as a general increase in the immobility time during the FS test by the restrained mice when compared to control mice [$F_{(1,38)} = 15.35$, $P = 0.0004$]. Also, this analysis revealed a significant effect of treatments on depressive-like behavior [$F_{(3,38)} = 5.23$, $P = 0.004$]. Multiple comparison tests showed that mice subjected to RS and co-treated with Cot ($P < 0.05$), KO ($P < 0.05$) or KO plus cotinine ($P < 0.01$) showed lower levels of immobility than vehicle-treated restrained mice (**Figure 4A**).

To corroborate the antidepressant effect of treatments, a sucrose preference tests were performed. Also, the sucrose preference test revealed significant differences between treatments. One-way ANOVA revealed a significant effect of chronic stress in sucrose preference [$F_{(4,28)} = 8.65$, $P = 0.0001$]. Multiple comparison tests showed significant decrease in sucrose preference in the vehicle-treated restrained mice when compared to vehicle-treated non-stressed mice. However, treatments showed antidepressant effects and the stressed mice treated with cotinine ($P < 0.001$), KO ($P < 0.001$) or cotinine plus KO ($P < 0.05$) showed significantly lower levels of anhedonia and higher sucrose consumption than the restrained mice treated with vehicle (**Figure 4B**).

Effect of Krill Oil and Cotinine on Recognition Memory

To assess whether co-treatment with cotinine during RS influenced recognition memory, mice were tested in the novel object recognition NOR test. Non-significant differences were found between non-stressed and restrained mice in the familiarization step of the task, and all mice explored each of the two objects almost 50% of the time, no showing a preference for any of them (**Figure 5A**). However, when mice were exposed to one old object and a new object in the arena, one-way ANOVA analysis revealed significant differences between groups on recognition memory [$F_{(4,48)} = 4.29$, $P = 0.0049$]. Multiple comparison tests showed a significant difference in the time spent with the new object between the vehicle-treated non-restrained mice and the vehicle-treated restrained mice ($P < 0.05$). However, restrained mice treated with cotinine plus KO showed no differences in recognition working memory reflected as a similar level of preference for the new object than vehicle-treated non-restrained mice ($P > 0.05$) (**Figure 5B**).

Analysis of Changes in Morphology and Cell Viability of GFAP+ Astrocytes

Cell Counting

To assess changes induced by RS and treatments on astrocyte architecture and numbers. The number of GFAP+ cells, GFAP IR and area fraction were investigated in the DG, one of the region more affected in those parameters by RS (Perez-Urrutia et al., 2017).

Multiple comparison tests revealed no significant effects of treatments in GFAP IR in the non-stressed mice, but significant differences in the restrained mice. One-way ANOVA analysis of GFAP+ cells in the DG showed a significant effect of treatments on the number of GFAP+ cells in the DG [$F_{(7,46)} = 4.88$, $P = 0.0004$]. GFAP+ cell densities in the DG were significantly reduced in the vehicle-treated restrained mice when compared to vehicle-treated non-stressed mice ($P < 0.001$). Treatment with KO alone did not induced changes in the number of GFAP+ cell in the KO-treated restrained mice, when compared to vehicle-treated restrained mice. However, a substantial increase of GFAP+ IR cells was observed in the cotinine-treated restrained mice or KO plus cotinine, when compared to vehicle-treated restrained mice ($P < 0.05$) (**Figures 6A,B**).

Mean Gray Value

One-way ANOVA analysis of mean GFAP IR in the GFAP+ cells in the DG showed a significant effect of treatments on GFAP IR intensity in the DG [$F_{(7,33)} = 5.10$, $P = 0.0005$]. Multiple comparison tests revealed no significant effects of treatments on the mean GFAP IR in the non-stressed mice. However, a significant decrease in the mean IR intensity was found in the vehicle-treated restrained mice group when compared to vehicle-treated non-restrained mice ($P < 0,05$). Nonsignificant changes in GFAP IR were found in the KO-treated restrained mice when compared to the vehicle-treated restrained mice ($P > 0.05$). On the other hand, a significant increase of IR

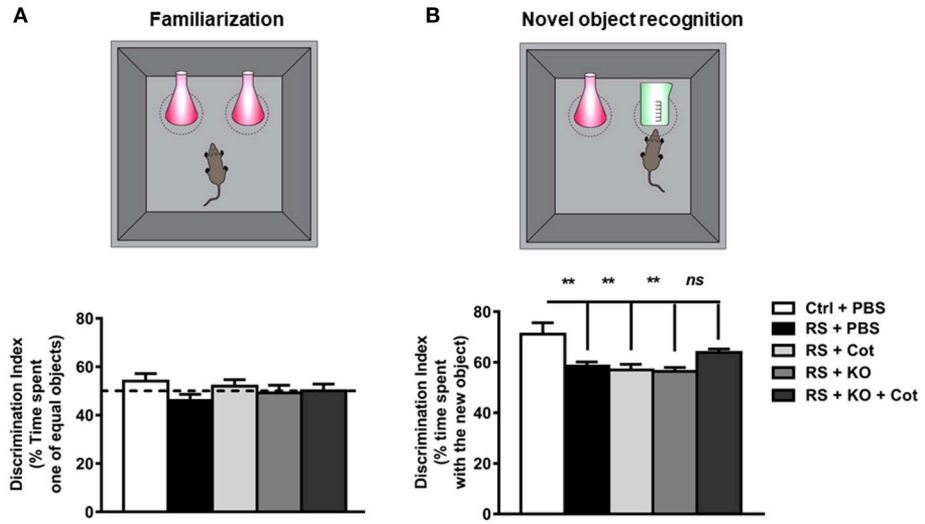

FIGURE 5 | Co-treatment with cotinine decreased the restraint stress-induced deficit in recognition memory. After restraint and co-treatment with vehicle (PBS), cotinine (Cot, 5 mg/kg) krill oil (KO, 143 mg/kg) or Cot plus KO, mice were tested for locomotor activity in the open field test and next day mice were tested for recognition memory in the novel object recognition test (NOR). **(A)** Familiarization: mice were individually exposed to two identical objects. **(B)**, Novel object recognition step: after 30 min of rest, mice were exposed to one of the old objects and a new object. Chronic restraint stress impaired novel object recognition. Co-treatment with KO plus Cot preserved recognition memory abilities in the stressed mice to levels non-significantly different from control non-stressed mice. $^{**}P < 0.01$.

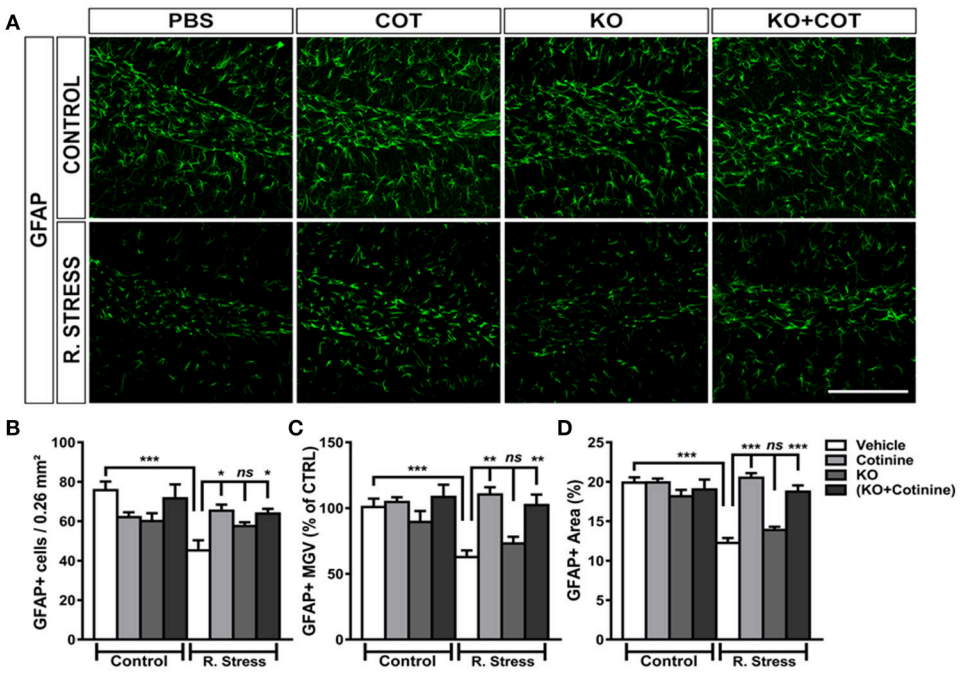

FIGURE 6 | Analysis of the effect of cotinine plus krill oil on astrocytes in the dentate gyrus of the hippocampus. A figure representing the changes in cell GFAP+ cells numbers and morphology in the dentate gyrus region of the hippocampus in male mice subjected or not to restraint stress (R. Stress) **(A)**. Graph depicting the changes in the number of GFAP+ IR cells **(B)**; main gray values (MGV) **(C)**; area of GFAP IR in the DG of vehicle-treated non-retrained mice or restrained (RS) mice, cotinine (Cot, 5 mg/kg) or Cot plus KO (143 mg/kg) (Cot + KO) **(D)**. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

intensity was found in the cotinine-treated restrained mice when were compared to vehicle-treated restrained mice ($P < 0.01$). Also, there was a significant increase in GFAP IR in the KO plus cotinine-treated restrained mice when compared to vehicle-treated restrained mice ($P < 0.01$; **Figure 6C**).

Area Fraction

The analysis of the percent area fraction occupied by GFAP+ cells revealed significant effects of treatments in the DG [$F_{(7,34)} = 17.28$, $P < 0.0001$]. Multiple comparison analysis showed that vehicle-treated restrained mice show a significant decrease of the GFAP+ IR area in comparison to non-stressed vehicle-treated mice ($P <$ 0,001). Nevertheless, a significant increase in the GFAP+ IR fraction area was found in the cotinine and KO plus cotinine-treated restrained mice when compared to vehicle-treated restrained mice ($P < 0.001$) in the DG (**Figure 6D**).

DISCUSSION

Chronic immobilization or reduced mobility stress can result from obesity, paralysis induced by vascular events such as stroke, spinal cord injury, advanced age, and many neurodegenerative conditions such as arthrosis, and ataxia. These events result in depression and cognitive impairment in the affected individuals. RS is a broadly used model of stress-induced depressive-like behavior (Buynitsky and Mostofsky, 2009). Prolonged RS results in morphological changes in the brain such as retraction of processes in hippocampal neurons and astrocytes (Magarinos et al., 1997; McEwen et al., 1997), neuroinflammation (Bauer et al., 2001; de Andrade et al., 2012; Tymen et al., 2013), and cognitive deficits (Thorsell et al., 2000; Abidin et al., 2004; Conrad et al., 2004; Cherian et al., 2009; Mika et al., 2012) and depressive-like behavior in rodents (Buynitsky and Mostofsky, 2009; Chiba et al., 2012). It has been shown that cotinine administered before and after RS, reduces the depressive-like behavior, synaptic deficits, astrocyte alterations, and cognitive impairment in mice (Grizzell et al., 2014; Grizzell and Echeverria, 2015; Perez-Urrutia et al., 2017). In this study, it was investigated the effect of co-treatment with cotinine combined with KO, during chronic RS, on the development of depressive-like behavior and cognitive impairment induced by chronic stress in mice. RS provoked a decrease in recognition memory abilities and depressive-like behavior in the mice, however, cotinine plus KO prevented these behavioral changes. These results showed a synergistic beneficial effect of both cotinine and KO in preserving mood stability and cognitive abilities under conditions of chronic RS.

At the neurochemical level, chronic stress induces a deficit in glutamatergic neurotransmission by mechanisms involving a decrease of NMDA (N-Methyl-D-aspartate) and AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptors in the postsynaptic site in the prefrontal cortex and the hippocampus, two brain regions that are fundamental for mediating declarative and working memory abilities. This reduction in the number of synaptic glutamate receptors induces a decrease in the activity of brain networks controlling stress behavior including the prefrontal cortex-amygdala-hippocampus pathways. Some evidence suggests that loss of glutamate receptors in neurons of the prefrontal cortex after repeated stress in rats, it is due to increased ubiquitin–proteasome-dependent degradation of these receptors (Joels et al., 2004; Yuen et al., 2012). Furthermore, previous studies using rodent models of chronic stress found a reduced proliferation of glial progenitor cells, and a decrease of GFAP+ cells in several brain regions, including the hippocampus and prefrontal cortex in rats. In rats, glucocorticoids can diminish the expression of GFAP in the PFC, resulting in >20% reduction in GFAP expression that was accompanied by a decrease of the GFAP mRNA levels (Zschocke et al., 2005). Also, chronic RS inhibits the glutamate uptake by astrocytes enhancing excitotoxicity and long-term depression (Yang et al., 2005). Furthermore, some evidence shows that rats exposed to early-life stress have a decrease in astrocytes levels in the frontal cortex in adulthood, indicating a long-term effect of stress on glial cells development (Leventopoulos et al., 2007). It is reasonable to propose that a deficit in astrocyte's function plays a crucial role in the higher susceptibility to PTSD observed in persons with a previous history of child abuse.

We have previously found a protective effect of intranasal cotinine administered against RS-induced astrocytes decrease. In this study, we found that co-treatment of mice with oral cotinine plus KO prevented the decrease in the number and complexity

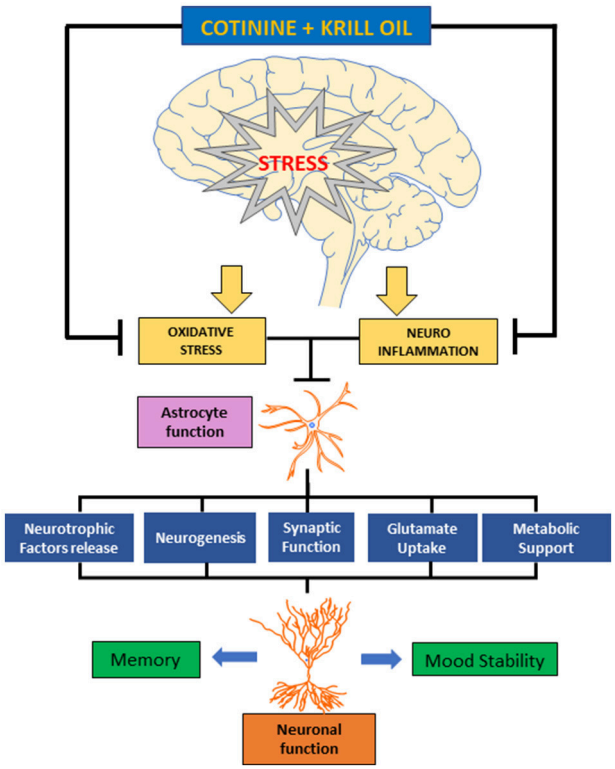

FIGURE 7 | Diagram representing the effect of cotinine and krill oil preventing the effects of chronic stress on astrocyte and neuronal function and behavior. Cotinine plus KO may counteract the neuroinflammatory and oxidative processes triggered by chronic stress in the brain. This effect may prevent the reduction in astrocyte affecting their role supporting the neuronal plasticity processes that are required for working memory and mood stability affected by chronic stress.

of astrocytes in the DG of mice subjected to RS. In this study, we observed a beneficial effect of cotinine and cotinine plus KO but not KO alone in preserving the number and arbor complexity of astrocytes under conditions of RS.

We have previously shown that, in the absence of stress, long-term cotinine treatment for up to 8 months did not induce significant differences in sensory-motor abilities or anxiety in mice (Zeitlin et al., 2012). Alike these results, no significant changes in locomotor activity in the mice treated with cotinine, KO or cotinine plus KO and subjected to RS were found. Thus, the superior effect of the combination of cotinine plus KO increasing the escape-oriented behavior in the FS test, cannot be explained by an increase in locomotor activity induced by the mix. The open field test is a good test to assess locomotor activity. However, the interpretation of the time spent in the center zone as a measure of anxiety has proven to be misleading and sometimes contradictory (Belzung, 2001a,b; Prut and Belzung, 2003). For example, it has been shown that many clinically effective non-benzodiazepine anxiolytics, except 5-HT1A agonists, these anxiolytic drugs exhibit extremely variable effects in the open field tests. Alike other anxiolytic drugs cotinine, KO or cotinine plus KO did not diminish the increased locomotor activity or time spent in the center zone during the first 10 min of the test, that is considered a measure of anxiety behavior after prolonged restraint stress.

It is appealing that comparable results were obtained in the behavioral parameters tested, with a more significant effect of the mix cotinine plus KO than the individual components in the mix. The connection between changes in astrocytes and depressive-like behavior has been reported before. For example, a previous study reported that the decrease in astrocytes numbers in the frontal cortex induced by L-alpha-aminoadipic acid provoked depressive-like behavior in rodents (Lee et al., 2013). This evidence demonstrated that astroglia ablation in the PFC is sufficient to prompt depressive-like behaviors comparable to the one induced by chronic RS. This data strongly suggests that the loss of astroglia may be a key factor contributing to the development of long-lasting depression (Lee et al., 2013).

The effect of cotinine in the mix preventing the effect of stress on mood can be the result of the action of cotinine as an anti-inflammatory compound inhibiting microgliosis and neuroinflammation as well as promoting neuronal and astrocyte survival throughout the activation of pro-survival cell signaling pathways.

Increased levels of astrocytes may provide neurons with more energy substrates, glutamate precursors, and neurotrophic factors. Also, astrocytes can decrease the toxic effect of the abnormal increase in glutamate release induced by corticosteroids at the presynaptic level, by uptaking the glutamate from the synaptic space. On the other hand, KO components such as omega-3 and astaxanthin can prevent oxidative stress and diminish the deleterious effects of stress and Neuroinflammation on brain function (Barros et al., 2014; Polotow et al., 2015; **Figure 7**).

A more detailed study of the effect on cotinine and KO on astrocyte function is guaranteed in the light of the present results.

CONCLUSIONS

In this work, the effect of an oral formulation of cotinine plus KO to prevent the cognitive and depressive-like behavior induced by chronic restraint stress. The results show that at the dose tested, the cotinine plus KO prevented depressive-like behavior, memory impairment and the astrocytes decrease induced by RS, and suggests that this formulation may be useful in humans and non-primates mammals suffering from restraint stress due to aging or other pathological and traumatic conditions. Clinical studies are needed to confirm this hypothesis.

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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Maternal Protein Malnutrition: Current and Future Perspectives of Spirulina Supplementation in Neuroprotection

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Malnutrition has been widely recognized as a grave burden restricting the progress of underdeveloped and developing countries. Maternal, neonatal and postnatal nutritional immunity provides an effective approach to decrease the risk of malnutrition associated stress in adulthood. Particularly, maternal nutritional status is a critical contributor for determining the long-term health aspects of an offspring. Maternal malnutrition leads to increased risk of life, poor immune system, delayed motor development and cognitive dysfunction in the children. An effective immunomodulatory intervention using nutraceutical could be used to enhance immunity against infections. The immune system in early life possesses enormous dynamic capacity to manage both genetic and environment driven processes and can adapt to rapidly changing environmental exposures. These immunomodulatory stimuli or potent nutraceutical strategy can make use of early life plasticity to target pathways of immune ontogeny, which in turn could increase the immunity against infectious diseases arising from malnutrition. This review provides appreciable human and animal data showing enduring effects of protein deprivation on CNS development, oxidative stress and inflammation and associated behavioral and cognitive impairments. Relevant studies on nutritional supplementation and rehabilitation using Spirulina as a potent protein source and neuroprotectant against protein malnutrition (PMN) induced deleterious changes have also been discussed. However, there are many futuristic issues that need to be resolved for proper modulation of these therapeutic interventions to prevent malnutrition.

Keywords: protein malnutrition, fetal programming, oxidative stress, inflammation, nutritional supplementation, Spirulina, neuroprotectant

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INTRODUCTION

Rapid industrialization and commercialization have changed our living standard and dietary habits. A proper nutrition provides a flexible infrastructure for the brain development which forms the basis of human growth and economic progress. The hunger level is measured globally as Global Hunger Index (GHI) by Welthungerhilfe and concern worldwide to evaluate the progression in hunger (Von Grebmer et al., 2018). 12.3% of world's population is undernourished with 27.9% stunted and 9.3% wasted children as reported by GHI, 2018. Every country in the world is facing

a grave burden of malnutrition where South Asia and Africa is striving hard with under serious hunger category with GHI of 30.5 and 29.4, respectively. The mortality rate of children aged under five, category is 4.2%. However, GHI score of almost all countries from 2000 to 2018 decreased substantially except Central African Republic which shows no advancement. India ranks 103 out of 119 countries assessed with a GHI score of 31.1 (serious category). Still today, 20–50% of the patients admitted in hospital are found to be malnourished (Kirkland et al., 2013) with poor recovery rate from illness (Marshall et al., 2014). In simple words, malnutrition is a condition of imbalanced or inadequate nutrient intake by a person. It includes undernutrition (wasting, stunting, and underweight), over-nutrition (overweight and obesity) and micronutrient related malnutrition (micronutrient deficiency or excess). Malnutrition is a serious health problem associated with the increased susceptibility to mortality and morbidity. It is now widely accepted as silent executioner. In this review, we use the term 'malnutrition' to refer simply to a deficiency of nutrition particularly protein. Developmental Origins of Health and Disease (DOHAD) hypothesis suggest the link between early life developmental patterns and late onset of diseases (Barker et al., 2002; Shih et al., 2004). Many epidemiological and experimental reports suggest that nutritional status during fetal development plays an important role in maintaining energy metabolism at later life (Silveira et al., 2007; McMillen et al., 2008). The nutritional status of the mother strongly affects the brain development and cognitive abilities in the offspring. All the members in a community are affected by maternal nutritional patterns, but children and infant are at the highest risk as they need more nutrition for growth and development. Similarly, pregnant and lactating women are at risk and the babies born will be more prone to any disease at later life. Maternal and infant malnutrition is found to be associated with behavioral and cognitive impairment throughout the childhood and adulthood that makes them more vulnerable to neuropsychiatric disorders (Naik et al., 2015). Several studies demonstrated that maternal malnutrition alters the fetal genome and increases the risk of neuropsychiatric disorders including depression, schizophrenia, aggression, hyperactivity and anti-social behavior (Duran et al., 2005; Galler et al., 2005). In addition, it also reduces the thickness of the visual cortex, parietal neocortex, dentate gyrus, CA3 and cerebellum (Noback and Eisenman, 1981; Díaz-Cintra et al., 1990; Ranade et al., 2012). The compromised physical development, impaired spatial learning and memory, compromised astrogenesis and oligodendrogenesis leading to hypo-myelination have all been reported in an intra-generational protein restriction rat model (Naik et al., 2015, 2017; Patro et al., 2018). Protein malnutrition (PMN) is also found to be associated with free radical overproduction, decreased antioxidant defense system (Feoli et al., 2006; Khare et al., 2014) and immune impairments that initiate a cascade of inflammatory reaction (Welsh et al., 1998; Yamada et al., 2016).

Despite the high prevalence of the serious consequences of maternal PMN, a very little information dealing with potent nutritional rehabilitation strategies against malnutrition is available. There is an urgent need of food or a food supplement which imparts both nutritional and medicinal benefits to the society. Such types of dietary supplements are called as nutraceuticals or functional foods. Nowadays, microalgae are gaining popularity as a dietary supplement because of low cost, high nutritional value and enormous health benefits. Spirulina contains significant amount of proteins, vitamins, beta-carotene, minerals, polysaccharides, glycolipids and sulfolipids (Campanella et al., 1999; Blinkova et al., 2001; Watanabe et al., 2002; Colla et al., 2004). Various bioactive peptides are now derived from Spirulina which can be used as efficient nutraceutical ingredients in novel food designing (Ovando et al., 2018). A large number of studies evidence the neuroprotective role of Spirulina in variety of diseases like ischemic brain damage, Parkinson's disease, LPS induced inflammation and rheumatoid arthritis (Bhat and Madyastha, 2001; Stromberg et al., 2005; Patro et al., 2011). Thus, dietary complementation with Spirulina could be a cost-effective way to enhance the balanced food security in a natural way. This review is focused on the effects of malnutrition on fetal programming, CNS development, oxidative stress, inflammation and various nutritional supplementation and rehabilitation strategies with special attention on neuroprotective role of Spirulina against PMN induced changes.

MATERNAL NUTRITION AND FETAL PROGRAMMING

The development of mammalian central nervous system is a complex process that begins *in utero* and continues throughout the adolescence and adulthood. It is tightly regulated by both genetic and environmental factors. The prenatal and postnatal environment affects the brain development, maturation and function both positively and negatively depending on the type of environmental stimuli (Kolb and Whishaw, 1998; Lenroot and Giedd, 2008). Several studies were conducted using different animal models, protocols, sources and types of enrichment to analyze how prenatal environmental enrichment (EE) affects the development of fetus (Kiyono et al., 1985; Mychasiuk et al., 2012; Rosenfeld and Weller, 2012). In most of the studies, EE is reported to exhibit beneficial effects on neuronal growth and maturation, neurocognitive abilities, cortical thickness, dendritic arborization, synaptic integrity, vascular inflammation, and neurogenesis (Nilsson et al., 1999; Olson et al., 2006; Reynolds et al., 2010; Simpson and Kelly, 2011; Mesa-Gresa et al., 2013; Caporali et al., 2014). Such favorable effects appears to be a common denominator for the remedial efficacy of an EE against experimental pathologies including trauma, cerebral ischemia, astroglial degeneration, and glioma growth (Passineau et al., 2001; Dahlqvist et al., 2004; Anastasía et al., 2009; Yuan et al., 2009; Cao et al., 2010; Jain et al., 2013; Rodriguez et al., 2013; Garofalo et al., 2015). Both the embryological and the postnatal development of the offsprings are governed by *in utero* environment (Barbazanges et al., 1996; O'donnell et al., 2009). An enriched *in utero* environment probably helps the fetus to survive in a specific environment as newborns are more prone to a variety of infections. Neonatal infection is a predominant cause of childhood mortality and morbidity causing 40% of mortality

in under five age group (Liu et al., 2012; Bhutta and Black, 2013; Blencowe et al., 2013; Lawn et al., 2014).

Any adverse environmental condition imposed during the most vulnerable period of development (gestational and lactational period) affects the epigenetic status and also alter the covalent modifications of the DNA and histones of embryos, fetuses, and neonates (Reynolds and Caton, 2012; Wu et al., 2012). These changes may influence the complete life cycle of an offspring and may be transmitted from one generation to another and thus could result in the origin of '*fetal programming*' or '*neonatal programming*' concept (Barker and Clark, 1997; Ji et al., 2016). Imbalanced maternal nutrition often leads to the pathophysiological condition termed as intrauterine growth restriction (IUGR). It exerts detrimental effects on preweaning survival and developmental rate, growth rate, food intake, organ structure and function (Wu et al., 2006; Rekiel et al., 2014) and onset of diseases at adulthood and senility, including type-2 diabetes, hypertension, hyperglycemia, and cardiovascular disease (Alberti et al., 2005; Longo et al., 2013; Langley-Evans, 2015). It also affects the transfer of nutrients and oxygen from pregnant mother to fetus, vascular growth and functional capacity of placenta (Wu et al., 2004; Leddy et al., 2008). Maternal stress or infection during the prenatal or perinatal period also alters the physiological and behavioral profile of the stress response progression in offspring. One important pathway for the transmission of prenatal stress is through the secretion of corticosterone from mother to fetus via placenta, a proximal symbol of the outer environment. Prenatal inhibition of maternal corticosterone secretion exerts no difference between progeny of stressed and non-stressed mothers, thus signifying a possible mechanism of maternal stress transmission via corticosterone (Barbazanges et al., 1996; Cicchetti, 2016). The placenta plays a key role in maternal nutrient transport to the fetus, thus placental abnormalities may inhibit the nutrient support to fetus. The nutrient supply depends on the size of placenta, morphology, blood flow and nutrient transporters (Fowden et al., 2006; Higgins et al., 2011). Thus, proper modulation of placental function either through gene expression or supplementation (nutrients or hormones) is necessary to fulfill the energy demands of growing fetus (Belkacemi et al., 2010). Both undernutrition and overnutrition contributes to the metabolic syndrome and chronic life-threatening diseases in adults (Boney et al., 2005; Leddy et al., 2008; Rkhzay-Jaf et al., 2012). To elucidate this, a number of studies have been conducted across the world using rat as a model organism to mimic the conditions of protein calorie malnutrition, total calorie undernutrition, maternal protein restriction and anemia (Ozanne and Hales, 2002; Armitage et al., 2004). Out of all these models, maternally protein deprived rat model is the most extensively studied model which involves the feeding of a low protein diet (5–8% protein) to pregnant dams in comparison to dams fed with a control diet (20% protein). The F1 progeny born from protein restricted mothers presented low birth weight and were more prone to cardiovascular diseases and psychiatric disorders (Fernandez-Twinn et al., 2003). Moreover, maternal over-nutrition before and during gestation also resulted in abnormal weight gain during pregnancy as compared to the normally nourished mothers which further increases with each successive pregnancy (Castro and Avina, 2002). Such anomalies further contribute to the progression of gestational diabetes along with long lasting negative health consequences for the infant (Ostlund et al., 2004). The excess protein supplementations to pregnant mothers also result in preterm delivery and enhanced perinatal mortality (Say et al., 2003). It is also observed that high fat diet during pregnancy decreases the mitochondrial copy number in kidney, thus changing the glucose homeostasis leading to a condition in parallel with mammalian metabolic syndrome counting endothelial dysfunction, hypertension, altered serum lipid status and adiposity (Armitage et al., 2005). Similarly, maternal glycemia also result in increased body weight and chances of diabetes in offspring (Franks et al., 2006).

PROTEIN MALNUTRITION AND CNS DEVELOPMENT

The proper placental development, fetus growth and all other changes occurring in mother's body during pregnancy necessitate amino acids for protein formation. Both mother and fetus are actually competing for this 'scarce resource.' Major proportions of the amino acids in the fetus are transported from the maternal circulation through active transport (Regnault et al., 2005a,b). Both placenta and fetal tissues are the disposal sites of amino acids present in the fetus, which is further involved in fetal amino acid metabolism (Brown et al., 2011). Thus, because of great involvement of amino acid in CNS functioning (amino acid as precursors of neurotransmitter, structural proteins, enzymes, peptide hormones), studies on the effect of PMN in developing CNS is gaining acclamation. Generally, casein content is modified in the diet to design protein restricted animal model. The protein deprived diet consists of 5–9% protein, while 16–25% protein is present in the normal diet.

Brain development is tightly regulated by both environmental and genetic factors. The period of rapid brain growth is considered as most critical period of development as brain is more vulnerable to any insult during this period. Any insult incurred during this period of brain development may result in long lasting negative effects on brain function, behavior and cognition. Nutrition is one of the most important epigenetic regulators that can affect brain function and behavior. Nutritional insults during pregnancy changes the epigenetic of the fetal genome and may leave a permanent devastating effect. The severity of the effects depends on the developmental timeline and magnitude of insult, i.e., earlier the insult, the more permanent the effects are. Vast literature indicate that malnutrition influences the cell count, synapse formation, dendritic arborization, cellular differentiation and proliferation, cell migration, growth factor synthesis and myelination and all these changes in turn result in impaired motor and cognitive functions (Georgieff, 2007; Prado and Dewey, 2014). Other studies also reported a reduction in dendritic basal number and processes, spine density, thickness of dendrites, somal size and neuronal loss following malnutrition (Salas and Nieto, 1974; Garcia-Ruiz et al., 1993; Lister et al., 2005). Such changes in malnourished animals may lead to permanent disturbances

in dendritic arborization, architecture and synaptic efficiency (West and Kemper, 1976; Desmond and Levy, 1988). *In utero* protein deprivation also leads to reduced body and brain weight (Dickerson et al., 1967; Smart et al., 1973; Peeling and Smart, 1994). In nutshell, the nutritional inadequacies affect the neuroanatomy, neurochemistry, neurophysiology and neuropathology of the nervous system. A balanced nutrient supply which includes protein, iron, zinc, folate, choline, vitamin A and polyunsaturated fatty acids, governs brain function, behavior and cognitive development (Rao and Georgieff, 2001). Rats born to protein deprived mothers have shown increased arterial blood pressure and high cardiovascular sympathetic tone (Ozaki et al., 2001; Barros et al., 2015). It is observed that periconceptional and prenatal nutritional insult affects the postnatal brain maturational events (Morgane et al., 2002). Early life nutritional insult is also reported to be associated with enhanced risk for schizophrenia development (Brown and Susser, 2008; Bale et al., 2010). Hippocampus is adversely affected by early malnutrition. A significant reduction in the size of cells of the dentate gyrus, reduction in the degree of dendritic branching, and reduction in the number of granule cells is evident in hippocampus (Levitsky and Strupp, 1995).

Salas and Nieto (1974) reported that PMN also affects the size of cerebellum and dendrites of cortical pyramidal cells. Cell generation time is also increased in undernourished conditions (Deo et al., 1978). In cerebellar cortex, granule cells and basket cells per Purkinje neuron were reduced in number along with hypoplasia of glial cell following PMN (Clos et al., 1977). Severe lactational undernutrition is also reported to decrease the number of cells both in external germinal layer and internal granular layer (Barnes and Altman, 1973).

More recently, Gould et al. (2018) have hypothesized that maternal PMN before implantation can cause the adverse developmental programming leading to behavioral deficits and short-term memory loss. This was correlated with reduced neural stem cell and progenitor cell numbers through suppressed proliferation, defective neurosphere formation and increased apoptosis. Relevant to this, one more study has shown impaired acquisition and memory following maternal protein restriction during pregnancy and/or lactation. Such memory impairment has been closely associated with altered glucocorticoid production, reduced hippocampal mossy fiber area and decreased basal dendritic length (Reyes-Castro et al., 2018). Additionally, Gianatiempo et al. (2018) have shown that perinatal PMN alters the mother-offspring interaction which further disrupt the maternal behavior and delay the acquisition of developmental landmarks and neurological reflex development. These behavioral alterations were not restricted to F1 generation only but also transmitted to following generation. These recent reports have further strengthened the idea that PMN results in neurobehavioral and epigenetic alteration leading to growth restriction and hypertension (de Brito Alves and Costa−Silva, 2018).

Our group is also engaged in working on the intragenerational PMN model of rats, i.e., on pregestational, gestational, and lactational PMN model (8% protein) which corresponds well with IUGR clinical conditions of poor socioeconomic group of human females. We have reported a compromised physical development (decreased body weight and brain weight), delayed neurological reflex development (cliff avoidance and negative geotaxis reflex), hyperactivity, poor neuromuscular strength, impaired spatial learning and memory and low anxiety in F1 progeny of low protein fed rats (Naik et al., 2015). All these observed behavioral and cognitive impairments are signature mark of neurological disorders including autism and schizophrenia. We have also assessed the astrocytic density and turnover number in LP-F1 progeny using standard immunohistochemical procedures and qRT-PCR assay. Expression of GFAP protein (astrocytic marker) was not evident until E18 in LP rats, whereas numerous stars shaped GFAP+ cells were reported in E18 HP brain which suggested delayed astrogenesis following PMN. The same trend was also recorded in A2B5 (glial restricted precursor) and BLBP (secondary radial glia) immunolabeling where LP brains showed reduced labeling which further indicated low progenitor pooling in the LP-F1 brain (Naik et al., 2017). We further investigated the oligodendrocyte genesis, differentiation, maturation and myelination through immunohistochemistry and quantitative PCR using the expression of myelin associated glycoproteins (MAG), proteolipid protein (PLP), myelin oligo glycoprotein (MOG) and platelet derived growth factor receptor α (PDGFRα) and found reduced expression of myelin proteins depicting impaired myelination and linked behavioral dysfunction following intragenerational protein restricted model (Patro et al., 2018). Our findings clearly demonstrated that the detrimental changes in astrogenesis and oligodendrogenesis were reflected in the neurobehavioral and cognitive outcome in the LP-F1 rats. These results thus support that the early life adversities are the main cause of later life impairments and neurodevelopmental dysfunctions (Anderson et al., 2003; Patro et al., 2013).

OXIDATIVE STRESS IN PROTEIN MALNUTRITION

Oxidative stress generally occurs due to an imbalance between free radical, i.e., pro-oxidant content (hydrogen peroxide, superoxide, hydroxyl radical, alkoxyl and peroxyl radicals) and anti-oxidant (both enzymatic and non-enzymatic) response system of body. This pro-oxidant/anti-oxidant balance is necessary for proper body functioning. Both free radical and mitochondrial theories of aging are the most widely accepted theories of aging which speculate that reactive oxygen species (ROS) alter mitochondrial function by interfering with the replication and transcription machinery of mitochondrial DNA (mtDNA) resulting in more ROS generation, which in turn damage mtDNA. In accordance with the above theories, an aged tissue presents more ROS production suggesting ROS as a critical contributor in aging (Sawada and Carlson, 1987; Sohal and Dubey, 1994; Hamilton et al., 2001; Capel et al., 2005). ROS is responsible for generating DNA lesions which causes genetic instability. The most dominant DNA lesion formed by ROS is 7,8-dihydro-8-oxo-deoxyguanosine (8-oxo-dG) which causes G:C to T:A transversions (Grollman and Moriya, 1993; Dizdaroglu et al., 2002). These devastating outcomes of ROS production

can be neutralized by enhancing antioxidant defense system. Numerous studies have shown the inverse relationship between oxidative stress and life span. The enhanced expression of catalase (CAT) and superoxide dismutase (SOD) exhibits extended life expectancy in Drosophila (Orr and Sohal, 1994). Consistently, decreased life span is also observed in those *C. elegans* which are more susceptible to oxidative stress (Larsen, 1993; Ishii et al., 2004) whereas, antioxidant mimetics (SOD/CAT) reverse these changes and enhances *C. elegans* life span (Melov et al., 2000). The increased oxidative stress is also known to promote autophagy in Alzheimer disease (AD), Parkinson disease (PD), amyotrophic lateral sclerosis (ALS), Huntington disease (HD) brain samples, suggesting the possible role of autophagy in the pathophysiology of these diseases (Hirai et al., 2001; Nixon et al., 2005; Rubinsztein et al., 2005; Cherra and Chu, 2008).

Golden and Ramdath (1987), proposed that free radical overproduction is involved in pathogenesis of kwashiorkor leading to haemolytic anemia. Increased oxidative and nitrosative stress is reported to be involved in neurological disorders like AD, PD, HD, and aging (Sies, 1985; Calabrese et al., 2003; Mariani et al., 2005; Valko et al., 2007). de Brito Alves et al. (2016) examined the association between maternal PMN induced hypertension and oxidative stress. They noticed that oxidative dysfunction and impaired antioxidant defense system in ventral medulla might contribute to progression of hypertension following maternal protein restriction. Another study by Verma et al. (2017) also reported increased serum malondialdehyde levels leading to oxidative stress in severe acute malnourished children as compared to control group. Prenatal and lactational PMN in rats is found to be associated with increased levels of thiobarbituric acid reactive substance (lipid peroxidation product) in the cerebellum and cerebral cortex and decreased CAT activity in the cerebellum (Feoli et al., 2006). Similarly, increased plasma malondialdehyde (by-product of lipid peroxidation), protein carbonyl (by-product of protein oxidation) and decreased anti-oxidants (ascorbic acid, glutathione, SOD, ceruloplasmin) were reported in a study conducted over 193 malnourished children of age group 6 months to 5 years in Eastern Uttar Pradesh, India (Khare et al., 2014). Increased red cells SOD was also observed in malnourished children with kwashiorkor and marasmus (Rana et al., 1996; Ashour et al., 1999). However, majority of the studies showed reduced antioxidant activity following PMN (Sive et al., 1993; Houssaini et al., 1997).

In response to any oxidative stress, antioxidant level either decreases due to their depletion during scavenging of free radicals or increases to overcome the oxidative stress development and this shift further depends on multiple factors including type and source of oxidative stress, duration of exposure, toxicant concentration, intensity and model organism. These findings indicate that increased oxidative stress and compromised anti-oxidant defense system following PMN may be a risk factor for developing serious neurological and neurodegenerative disorder at later life.

MALNUTRITION AND INFLAMMATION

Insufficient dietary intake of nutrients is the leading cause of immunodeficiency worldwide. A synergistic relation exists between malnutrition and infection where nutritional inadequacy increases the risk to infection by impairing both innate and adaptive arms of immune response (Neumann et al., 2004). Infection also affects the nutritional status of an individual by reducing food intake and impairing nutrient absorption (Woodward, 1998; Solomons, 2007). The severity of infection depends on health status, type of infection and dietary intake. PMN is associated with immune impairments that initiate a cascade of inflammatory reaction (Welsh et al., 1998). Undernutrition is also considered as a pro-inflammatory state with increased expression of IL-6 (Dulger et al., 2002). However, PMN induced inflammatory response is still a controversial subject.

Some studies in malnourished children demonstrate a decreased expression of inflammatory markers while others report inflammatory response similar to the healthy children (Bondestam et al., 1988; Malave et al., 1998). Leptin (adipocyte derived cytokine) is considered as a central mediator between nutrition, neuroendocrine system and immunity (Fernandez-Riejos et al., 2010) and PMN has been reported to result in a decreased concentration of leptin, thus increasing the susceptibility to infection (Matarese, 2000). Compromised cellular components of immune response (IFN-Y, TNF-α, nitric oxide) have also been reported following PMN. These components are crucial for protection against *Mycobacterium tuberculosis* (Chan et al., 1996). Breast feeding enhances the infant immunity and provides protection against gastrointestinal and respiratory infections (Chien and Howie, 2001). Epidemiological data favors the association between breast milk feeding and reduced risk of type 1 diabetes, asthma, eczema, rheumatoid arthritis, multiple sclerosis and bowel disease (Brandtzaeg, 2003; Hanson et al., 2003). It contains a variety of lymphocytes, macrophages, neutrophils, cytokines, chemokines, growth factors and long chain polyunsaturated fatty acids (PUFAs) which aid in promoting the neonatal immune system development. Primarily, inflammation is a defensive response against any infection or stress but its exaggerated response may exert negative consequences to the infants. Breast milk is rich in both proinflammatory (IL-1β, IL-6, IL-8, and TNF-α) and anti-inflammatory (IL-10) cytokines. The only limitation in understanding the complete association between breast milk components and infant immunity is the variation that exists amongst women and period of active lactation. Higher fat content is observed in the milk of malnourished women as compared to the well nourished women, although difference is not statistically significant between the two groups (Spring et al., 1985). Alternatively, decreased protein, lactalbumin and lactoferrin concentration is reported in severely malnourished mothers (Lonnerdal et al., 1976; Forsum and Lonnerdal, 1980), whereas high protein diet results in enhanced nitrogen contents of milk (Emery, 1978). The rats fed with a very low protein diet (VLPD) develop systemic inflammation and vascular calcification as revealed in a recent publication (Yamada et al., 2016).

TABLE 1 | Protective effects of Spirulina against oxidative stress and neuroinflammation.

Parameter	Test species	Main findings	Reference
Protein malnutrition induced oxidative stress and neuroinflammation			
Oxidative stress	Rats	Increased CAT and decreased SOD activity in marasmic-kwashiorkor rats	Akinola et al., 2010
	Human	Increased lipid peroxidation product (MDA) and decreased anti-oxidant level (GSH, Zn-SOD)	Khare et al., 2014
	Human	Reduced blood levels of CAT, SOD, GSH, vitamin C and increased MDA concentration	Aly et al., 2014
	Rats	Increased MDA level, reduced SOD enzyme activity and metabolic dysfunction	Vega et al., 2016
	Human	Increased serum MDA levels	Verma et al., 2017
Neuroinflammation	Mice	Decreased serum protein levels and reduced superoxide anion production	Redmond et al., 1991
	Human	Reduced anti-oxidant level (glutathione and vitamin E) in kwashiorkor patients and increased concentration of IL-6 and soluble receptor of TNF-α	Sauerwein et al., 1997
	Rats	High circulating concentration of TNF-α, increased expression of TNF-α mRNA in liver, reduced phagocytic activity of neutrophils and increased superoxide anion production	Silva et al., 2010
	Rats	Increased serum TNF-α and urinary 8-hydroxydeoxyguanosine level	Yamada et al., 2016
	Human	Increased serum levels of pro-inflammatory cytokines and reduced serum levels of Zn, Ca, and Mg	El-Maksoud et al., 2017
Anti-oxidant and anti-inflammatory activities of Spirulina			
Anti-oxidant activity	Human	Reduced plasma level of MDA and increased SOD activity	Lu et al., 2006
	Rat	Increased level of antioxidants (GSH, SOD and CAT)	Jeyaprakash and Chinnaswamy, 2007
	Mice	Decreased lipid peroxidation (LPO) level and antioxidants concentration (SOD and CAT) were restored to near normal level	Sharma et al., 2007
	Mice	Reduced LPO level in hippocampus, striatum and cortex, increased CAT and glutathione peroxidase activity	Hwang et al., 2011
	Rat	Reduced lipid peroxidation and decreased percentage of DNA fragmentation	Hassan et al., 2012
Anti-inflammatory activity	Human	Reduced plasma MDA level, decreased LDL-cholesterol and IL-6 expression	Lee et al., 2008
	Mice	Inhibited humoral and cell mediated immune response and decreased TNF-α production	Rasool and Sabina, 2009
	Human	Increased indoleamine 2,3-dioxygenase (IDO) level and ameliorated senescence	Selmi et al., 2011
	Rat	Reduced expression of TNF-α, IL-1β, and IL-6	Abdel-Daim et al., 2015
	Human	Increased CD4 cell count and significant reduction in viral load	Ngo-Matip et al., 2015

Proinflammatory cytokines in particular TNF-α and IL-6 are reported to affect child growth in chronic inflammatory disorders, which could be rescued when these cytokines are blocked, stressing the correlation of abnormal expression of these cytokines with stunted growth (Sederquist et al., 2014). This was evident from a recently published report by El-Maksoud et al. (2017) demonstrating the increased serum levels of proinflammatory cytokines and C-reactive protein in nutritionally stunted Egyptian children. Thus, there is a possible link between the malnutrition and inflammation, although the

exact mechanism by which early life protein deprivation governs neuroimmunity and also how it integrates with neuroendocrine system needs further attention.

NUTRITIONAL SUPPLEMENTATION FOR PROTEIN MALNUTRITION

Dietary supplements generally exert protective effects against diet related diseases (obesity, diabetes, cardiovascular disease, and osteoporosis). The successful implementation of nutritional therapies demands a suitable target (pregnant women and/or children), which needs to be properly monitored and supplemented at critical window of development. Diverse studies across the world have proven the positive health benefits of oral nutritional supplementation (ONS) in the subjects suffering from adult malnutrition (Baldwin and Weekes, 2012; Collins et al., 2012). These ONS are enriched in high quality nutrition. Recently it has been reported that ONS improves the muscular strength of leg among malnourished and sarcopenic older patients (Cramer et al., 2016). A number of nutritional interventions during pregnancy were designed and studied to prevent IUGR. Maternal folic acid supplementation either alone or in combination with other vitamins decreases the incidences of neural tube defects in the offspring (Rieder, 1994; Pitkin, 2007; Bhutta et al., 2013). Similarly, maternal intake of cod liver oil during gestational and lactational period is associated with higher intelligence as measured by higher mental processing composite source (Helland et al., 2003). Both oral and intravenous arginine administration in IUGR pregnancies lead to body weight gain in fetus (Sieroszerski et al., 2004; Xiao and Li, 2005). Intra-amniotic or maternal protein supplementation could be a possible way to direct fetal growth. However, only few studies have focused on how maternal intake of individual amino acid or protein affects the embryo-fetal development especially in terms of brain structure, function and behavior.

SPIRULINA: A WONDER NUTRACEUTICAL

Nowadays, commercial food sector is interested in food or food products with nutritional and medicinal benefits. These types of food products come under an umbrella term 'nutraceutical.' They are also called as functional foods, medical foods, nutritional supplements or designer foods. Out of a huge range of available nutrients, microalgae (particularly Chlorella and Spirulina) are gaining special attention as a food supplement due to its easy availability, rapid growth, low cost and high nutritive value. Spirulina is extensively studied for its anti-oxidant, anti-inflammatory, anti-bacterial, anti-viral and immunomodulatory properties (Mallikarjun Gouda et al., 2015; Wu Q. et al., 2016; Finamore et al., 2017) along with its potent role in preventing IUGR related abnormalities as summarized in **Table 1**.

Spirulina is a progeny of first photosynthetic life form that was created by nature 3.6 billion years ago and belongs to the phylum Cyanobacteria. The name Spirulina is derived from a Latin word meaning tiny spiral. It is microscopic, photosynthetic, filamentous, spiral shaped and dark-blue in color due to the presence of pigment called phycocyanin. Surprisingly, it doubles its biomass in every 2–5 days and grows naturally in ponds of brackish or alkaline water. Very few microorganisms are capable of surviving in such extreme conditions in which Spirulina develops which in turn ensures crop hygiene. W.H.O designated it as 'Food of the Future' because of its high protein content and rapid growth. Moreover, it is also called as a 'Super food' or 'Wonder food' and various published scientific studies reveal how it boosts the immune system and improves health. It is approved in Russia as '*Medicine food*' for treating radiation induced effects whereas, NASA considered it as a '*Best food*' for space travel, as its small quantity contains a range of nutrients.

The most commonly used species of Spirulina are *Spirulina platensis* and *Spirulina maxima*. It has been used as a food for human consumption for centuries and consumed in many different countries such as Germany, Brazil, Spain, France, Canada, United States, Ireland, Philippines, Argentina, India, and Africa. The cell wall of Spirulina is devoid of cellulose and mainly composed of mucopolysaccharides, which makes it easily digested, assimilated and effective for the people suffering from intestinal malabsorption (older people and victims of kwashiorkor). It exhibits various positive biological activities including anti-viral, antibacterial, anti-fungal, anti-parasitic, free radical scavenging (anti-oxidant) and anti-arthritic effect. Our lab demonstrated the protective efficacy of Spirulina against collagen-induced arthritis in (CIA) rats. Various antioxidant constituents (phycocyanin, carotenoids, vitamins) present in Spirulina suppresses the physiological, histological and biochemical changes produced during CIA in rats (Kumar et al., 2009). Use of Spirulina three times a day in fish feed (*Maylandia lombardoi*) is reported to increase growth rate and seed production as compared to Spirulina intake once a day (Karadal et al., 2017). It is widely used in treating nutritional deficiencies, recovering from malnutrition, immune enhancement and in correcting iron anemia. It stimulates hematopoiesis especially erythropoiesis. It meets all international food quality and has applications in health foods and therapeutics. Its impressive protein content and rapid growth have attracted the attention of both researchers and industrialist. Thus, Spirulina supplementation during pregnancy and lactation may be of great potential value as it contains all hematopoietic nutrients that will ultimately benefit both mother and fetus.

NUTRITIONAL COMPOSITION OF SPIRULINA

Spirulina is one of the most potent sources of nutrition. The protein content of Spirulina varies between 60 and 70% of its dry weight. It also contains vitamins (vitamin B-12, beta carotene, vitamin E), various mineral substances (iron, calcium, phosphorus, magnesium, and trace minerals), essential

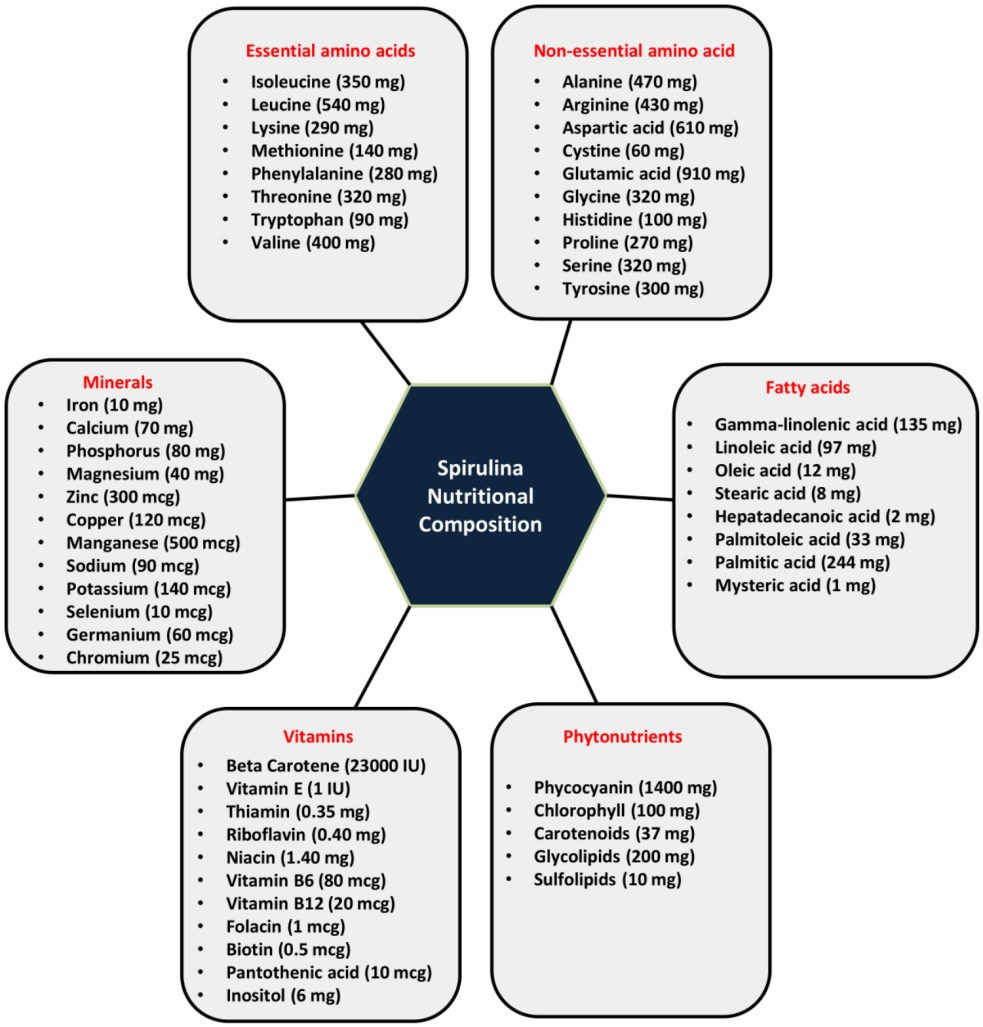

FIGURE 1 | Nutritional composition of Spirulina (serving size-10 gm of Spirulina): Spirulina is incredibly rich in proteins (60–70%) and contains a wide range of essential amino acids, non-essential amino acids, vitamins, minerals, phytonutrients and fatty acids.

fatty acids (gamma-linoleic acid, palmitic acid, linoleic acid, oleic acid, etc.), polysaccharides (rhamnose and glycogen), glycolipids and sulfolipids, enzymes (SOD responsible for quenching free radicals) and various pigments like phycocyanin, chlorophyll, carotenoids (Campanella et al., 1999; Blinkova et al., 2001; Watanabe et al., 2002; Colla et al., 2004; Khan et al., 2005; Earthrise, 2006) as represented in **Figure 1**. Phycocyanobilin (phycobilin-protein complex) is an inhibitor of NADPH oxidase. This enzyme is involved in oxidative stress in various neurological disorders. Thus, Spirulina intake decreases the activity of NADPH oxidase and has therapeutic interventions in many vascular diseases, cancers, diabetes, neurodegenerative and inflammatory disorders (McCarty, 2007). It has been shown that carbohydrates present in Spirulina increases cell nucleus enzyme activity (particularly endonucleases) and DNA repair synthesis (Baojiang, 1994). It also positively influences both the humoral (antibodies and cytokines) and cell-mediated immunity (T cell and macrophages). Downregulation of inflammatory and oxidative stress markers is observed in rats with Spirulina rich diets both in aging and neurodegenerative disorders making it more suitable as a natural drug for the treatment of neurological disorders.

SPIRULINA AS THERAPEUTIC INTERVENTION AGAINST INFLAMMATION AND OXIDATIVE STRESS IN AGING AND NEURODEGENERATIVE CONDITIONS

The predominant factors responsible for aging and neurodegeneration are inflammation and oxidative stress. There is a decline in the normal antioxidant and anti-inflammatory defense mechanisms in both aging and neurodegeneration that

makes the brain more susceptible to the deleterious effects of oxidative stress (Finkel and Holbrook, 2000). There are considerably strong evidences elucidating that most neurological disorders (AD, PD, HD, ALS, inflammatory injuries, and senility) are the result of oxidation and/or inflammation. Various nutraceuticals as well as pharmaceuticals have been extensively investigated for their anti-inflammatory and anti-oxidant potential. With respect to nutraceutical, several dietary supplementations (blueberries, spinach and Spirulina) have been reported to protect CNS by downregulating the markers of inflammation and oxidative stress and thus reducing neurological deficits. Spirulina consumption also improves life span with numerous health benefits, increases locomotor activity and reduces HSP70 (indicator of cellular stress) and Jun-N-terminal kinase signaling (JNK signaling involved in modulating the life span) in DJ-1$\beta^{\Delta 93}$ flies (a Parkinson's disease model) in *Drosophila melanogaster* (Kumar et al., 2017). A myriad of studies demonstrate that this dietary supplementation increases cerebellar glutathione levels, reduces malondialdehyde levels, decreases pro-inflammatory cytokines and improves spatial and motor learning in senile rats (Bickford et al., 2000; Gemma et al., 2002).

Thus, although an immense literature is available specifying the widely documented use of Spirulina as a functional food, yielding health promoting properties and/or reducing the risk of disease (Wu Z. et al., 2016; Furmaniak et al., 2017) only a very few and discrete studies have focused to report the adverse effects of Spirulina. More recently, the genome and proteome analysis of *Arthrospira platensis* has clearly mentioned the absence of genes responsible for the synthesis of various toxins (Furmaniak et al., 2017), supporting the statement that Spirulina shows no toxicity, i.e., neither acute nor chronic, making it safe for the human use (Gutierrez-Salmean et al., 2015). However, the minor adverse effects of Spirulina, reported include headache, gastrointestinal discomforts, muscle pain and cramps, skin rashes, etc. (Iwasa et al., 2002; Mazokopakis et al., 2008; Marles et al., 2011). In one study, the Spirulina was reported to be a causative factor for acute rhabdomyolysis in a young human patient (Mazokopakis et al., 2008). Although we have not come across any more studies specifying the adverse effects of Spirulina, but keeping in view the increasing use of Spirulina as dietary supplement, more studies are warranted to focus this issue.

SPIRULINA AS A NEUROPROTECTANT

Phenotypic outcomes are generally governed by epigenetic processes suggesting a possible connection between food quality and neurological disorders. Neuroprotective effects of Spirulina are well evidenced in ischemic brain damage with progressive decline in TUNEL positive cells and caspase-3 activity in the ischemic hemisphere (Wang et al., 2005). Brain ischemia or cerebral ischemia is a condition marked by the cerebral hypoxia that leads to the generation of free radicals, reactive oxygen or nitrogen species and energy crisis. Phycocyanin and phycocyanobilin present in the Spirulina have strong anti-cyclooxygenase-2 and anti-oxidant activities that reduce peroxynitrite induced oxidative damage to DNA (Bhat and Madyastha, 2001). Further advances and intervention studies in omics technology may provide useful information in understanding the mechanism of microglia mediated neuroinflammation (Patro et al., 2016) and the possible role of nutritional approaches in regulating microglia aging (Wu Z. et al., 2016).

Dietary supplementation with Spirulina in rat model of Parkinson's disease results in significant reduction in lesion volume and decreased microglial activation (Stromberg et al., 2005). Anti-inflammatory effects of Spirulina have also been investigated against LPS-induced inflammation in rodent model. LPS insult causes increased astrogliosis with prominent activation of GFAP in existing cells and decreased proliferation of neural progenitor cells (NPCs). However, diet supplemented with 0.1% Spirulina for 28 days before LPS administration prevents the LPS induced decrease in NPC proliferation and astrogliosis (Bachstetter et al., 2010). Researchers at University of Yaounda (Azabji-Kenfack et al., 2011) found that food supplementation with Spirulina for 12 weeks in malnourished adults infected with HIV stimulates weight gain and increase fat free mass as compared to soya beans. In addition, anti-retroviral treatment (ART) along with Spirulina showed more beneficial effects than ART coupled to soyabeans (increased CD4 cell counts and decreased viral load in Spirulina group). Spirulina platensis was also found to suppress the peripheral sensitization, improve motor coordination and restore motor activity in collagen-induced arthritic rats by reducing NF-200 accumulation in spinal cord neurons suggesting a possible neuroprotective role of Spirulina for the treatment of rheumatoid arthritis (Patro et al., 2011). Spirulina also supports the viability of astrocytes (Kim et al., 2012). Interestingly, polycaprolactone Spirulina nanofiber mat (composite nanomedicine) was proved to be effective against CNS injury as it reduces the astrocyte activation which in turn, could reduce inflammation induced by astrogliosis. Neuroprotective role of Spirulina is also marked in alpha-synuclein model of Parkinson's disease, where increased expression of tyrosine hydroxylase (TH) positive and NeuN positive cells was observed. Accordingly, reduced number of activated microglia was also reported as determined by the reduced OX6 (MHC-II) immunostaining (Pabon et al., 2012).

It is thus apparent that dietary complementation with Spirulina could be beneficial to the patients suffering from neurodegenerative disorders. It maintains proliferation, differentiation and migration of NPCs which may lead to improved brain functioning and body health. The elevated response of human stem cells in terms of proliferation potential was also reported in Spirulina fed group. In normal conditions, CX3CL1 and CX3CR1 are expressed at high levels in brain, but as age advances their expression decreases. Spirulina intake shows the increased expression of CX3CR1, suggesting a possible mechanism of action in neuroprotection (Pabon, 2011). Generally, non-steroidal anti-inflammatory drugs act by suppressing the immune activation but Arthrospira enhances both the innate and adaptive immunity thereby

increasing cellular and humoral adaptive immunity. There are now accumulating evidence that constituents of Spirulina have both anti-oxidant and anti-inflammatory activities that inhibits ROS formation and decreases the cytokine mediated neuroinflammation, thereby making it a suitable and effective therapeutic target for combating neurodegenerative disorders (**Figure 2**).

NUTRITIONAL REHABILITATION USING SPIRULINA – SUPER FOOD TO FIGHT AGAINST MALNUTRITION

Hunger can be either due to the non-availability of food or due to the lack of micronutrients. Inadequate food sources and improper nutritional awareness program in developing countries are the major causes of malnutrition. High protein content of Spirulina makes it a suitable adjunct to combat malnutrition. Both *Spirulina platensis* and *Spirulina maxima* have been extensively studied due to its high protein content, micronutrient composition, vitamins and minerals, easy and rapid reproducibility, inexpensive and non-toxic nature. Mass culture and utilization of Spirulina as a potent protein source against hunger, malnutrition and starvation was first explored and published by Rodulfo (1990). Many studies have shown a strong association between malnutrition and anemia thus suggesting malnutrition as a common cause of anemia in both children and elderly (Mitrache et al., 2001; Anticona and San Sebastian, 2014; Sahin et al., 2016).

Numerous studies have been conducted to test the efficacy of Spirulina supplementation in rodent model covering a range of variables including body growth, protein efficiency ratio (PER), hematological status and toxicity (Maranesi et al., 1984; Tranquille et al., 1994; Salazar et al., 1996; Narayan et al., 2005). Enhanced skeletal muscle proteins were also reported in Spirulina supplemented young rats (Voltarelli and de Mello, 2008). These animal based studies were further extended to clinical studies in human subject. In this context, various non-government organizations (NGO) and international organizations have chosen to work for reducing public health problems and prevention of malnutrition in developing countries. Azabji Kenfack and associates in 2011 examined the effect of daily Spirulina intake for 12 weeks on 56 malnourished HIV infected adult patients and concluded that Spirulina consumption effectively improved weight and body mass index (BMI) among undernourished HIV sufferers. Spirulina supplementation at a dose of 10 gm per day was found to improve the nutritional status as well as to increase the corpuscular hemoglobin and hematocrit levels in malnourished children in Democratic Republic of the Congo (Matondo et al., 2016). Another research on 550 malnourished children showed the beneficial effects of Spirulina in combination with Misola (millet, coja, and peanut). This combination was proved to be more effective than Spirulina or Misola alone (Simpore et al., 2006). In addition to these, Spirulina platensis also exerts a positive impact against immunosenescence and anemia because of the presence of active components such as folic acids, vitamin B12, phycocyanin, essential amino acids and iron content, which in turn play central role in Erythropoiesis (Kapoor and Mehta, 1998; Mani et al., 2000; Khan et al., 2005; Selmi et al., 2011). Subsequently, one more study was conducted in Burkina Faso evaluating the effects of Spirulina consumption on 84 HIV positive and 86 HIV negative anemic children, and the results indicated that 81.8% of HIV negative and 63.6% HIV positive children recovered from anemia, thus speculating potent role of Spirulina even in patients with compromised immune system (Simpore et al., 2005). Effectiveness of Spirulina against child malnutrition has also been reported in a study in Zambia, where 10 gm daily Spirulina consumption significantly improved HAZ (height-for-age-z) score in malnourished children (Masuda et al., 2014). From the above reports, it can be safely inferred that Spirulina supplementation can effectively combat the malnutrition.

NEUROPROTECTIVE EFFECTS OF SPIRULINA CONSUMPTION DURING PREGNANCY AND/OR LACTATION

Majority of the available studies have demonstrated the neuroprotective effects of Spirulina in adult animals. However, only few studies have been designed to investigate how maternal Spirulina supplementation would influence the developmental profile of an offspring. Spirulina enriched diet (0.1% Spirulina) to lactating mothers, 1 day prior to LPS treatment in offspring maintains the p38 and IL-1β levels thereby regulating neuroinflammation and antioxidant defense system in the offspring (Patil et al., 2018). Furthermore, Spirulina consumption by pregnant women in Dakar region is proved to be more effective than iron and folic acid (IFAC) supplementation in terms of weight gain and improved hemoglobinemia in the newborn (Niang et al., 2016). It has also been reported that supplementing pregnant hyperglycemic albino mice with Spirulina improves fertility rate, reproductive performance and reduces teratogenicity associated with diabetes (Pankaj, 2015). Furthermore, Banji et al. (2013) have reported that consumption of this edible alga from embryonic day (ED) 6 to postnatal day (PND) 15 reduces fluoride toxicity in developing brain, promotes antioxidant formation and minimizes the risk of neurodevelopmental disorders. These evidences mark the beneficial health effects of Spirulina in offsprings following Spirulina supplementation to pregnant mothers. However, the exact mechanism by which Spirulina supplementation during pregnancy imparts health benefits to the offsprings remains to be elucidated.

To the best of our knowledge, there is no complete study evaluating how Spirulina supplementation to malnourished pregnant and lactating mother would affect the oxidative functioning, inflammatory response and mental skills among children. Thus, enriching maternal environment with potent protein source (Spirulina) can be an effective way to reduce oxidative stress, neuroinflammation, behavioral and cognitive deficits induced by maternal PMN. This strategy of using

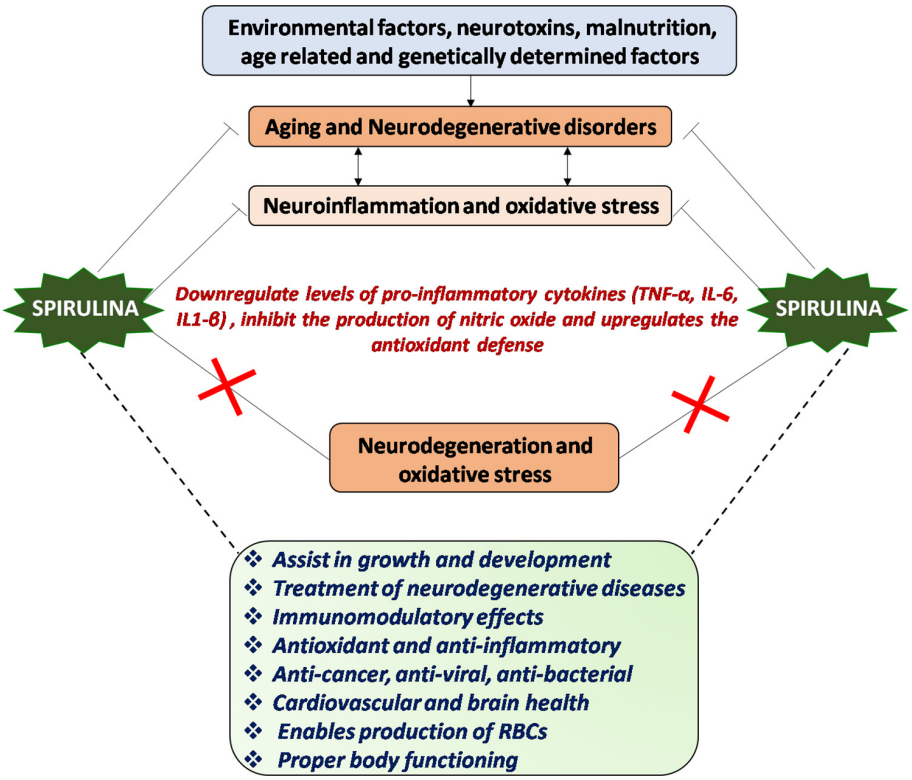

FIGURE 2 | Mechanism of action of Spirulina: Environmental and genetic factors regulate aging process and progression of neurological disorders, chiefly characterized by neuroinflammation and oxidative stress or vice-versa. Spirulina has both antioxidant and anti-inflammatory activities and downregulates the proinflammatory cytokines, which in turn might inhibit the neurodegeneration and oxidative stress thereby aids in maintaining proper brain and body health.

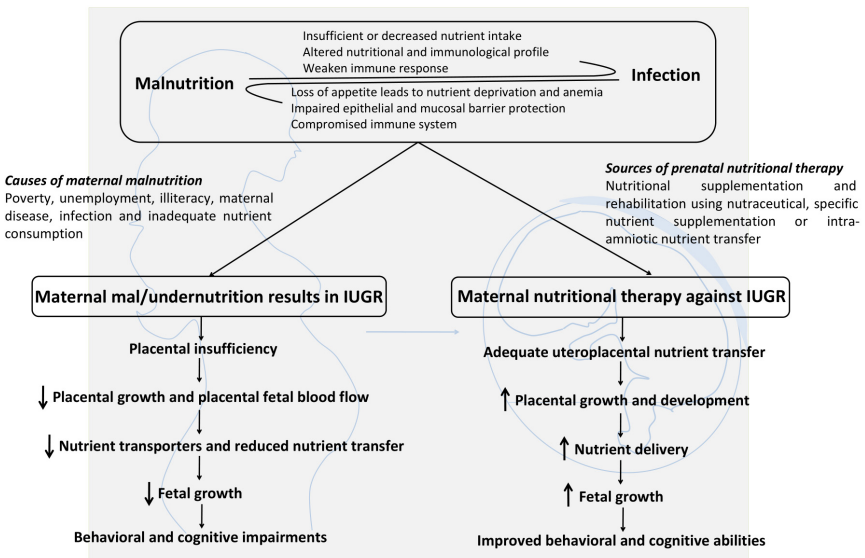

FIGURE 3 | Maternal malnutrition causes, consequences and possible nutritional supplementation strategies to treat IUGR: A synergistic relationship exists between malnutrition and infection. Maternal malnutrition results in decreased body growth and impaired behavioral and cognitive abilities. Prenatal nutritional therapy represents a promising approach to treat IUGR by enhancing uteroplacental nutrient transfer, placental growth and nutrient transport, fetal growth which ultimately results in improved behavioral and cognitive abilities.

Spirulina during pregnancy and lactation period would be advantageous for treating abnormalities during IUGR pregnancy.

CONCLUSION AND RECOMMENDATION

Any environmental stress during critical periods of CNS development affects the developmental profile of an individual. Brain homeostasis and neuronal communication may be disturbed specifically in conditions of protein deprivation. Increased reactive oxygen and nitrogen species, impaired antioxidant defense system, altered glial cell physiology and inflammatory response are postulated to be the possible drivers in PMN induced neurocognitive decline. Diets enriched in foods with high ORAC could be used in reverting age and poor diet associated behavioral, cognitive and neurochemical impairments and maintaining cellular homeostasis. Spirulina contains a combination of nutrients (β carotene, vitamin B12, tocopherols, essential fatty acids, polysaccharides, glycolipids, sulfolipids and phycobiliprotein) which exerts more neuroprotective effects than single nutrient source. The present review has compiled the numerous studies conducted on Spirulina to establish its implications as a potent source of nutrition to combat against micronutrient deficiency, PMN and neurological disorders. The data discussed in this review suggests that exposure to malnutrition during critical developmental timeline where developmental plasticity is at peak, results in long lasting irreversible behavioral and cognitive abnormalities which further increases the risk of neurological disorders.

However, this review has certain limitations. More research is needed in understanding how Spirulina consumption affects neuron-glia communication? What is the exact molecular mechanism of Spirulina action? Is Spirulina supplementation enough for completely combating the detrimental effects of malnutrition? What are the important subcomponents of Spirulina necessary for making it as a neuroprotective agent and its dose response? What are the possible molecular targets while considering pharmaco-therapeutic applications of Spirulina? Does it exert any effect on human epigenome? How Spirulina supplementation regulates placental function? All these questions need to be resolved in order to make Spirulina as an ideal natural drug with neuroprotective properties. Various nutritional inputs including nutritional supplementation, rehabilitation and therapy, nutritional education and specific nutrient supplementation (vitamins, mineral, and micronutrient) are necessary to overcome the detrimental effects of IUGR for better fetal growth and development (**Figure 3**). A combination of nutrition education and nutritional supplementation could exert more beneficial effects than any of the mentioned nutritional inputs alone. Inconsistency in research studies focusing on prenatal therapies and long term nutritional intervention programs are the major barriers to design effective strategies to fight malnutrition. Thus, a special attention should be given to animal studies involving both pre- and early postnatal nutritional supplementation along with intra-amniotic nutrient transfer strategies to ameliorate fetal growth and metabolic functioning during IUGR pregnancy. Further advances and elucidation of the mechanism of action of dietary supplements and their effects on microbiota-gut brain axis will therefore open up new windows for therapeutic intervention against neurological disorders arising from malnutrition.

AUTHOR CONTRIBUTIONS

SS wrote this review with input from NP and IP.

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Dietary Long-Chain Omega-3 Fatty Acids Are Related to Impulse Control and Anterior Cingulate Function in Adolescents

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Impulse control, an emergent function modulated by the prefrontal cortex (PFC), helps to dampen risky behaviors during adolescence. Influences on PFC maturation during this period may contribute to variations in impulse control. Availability of omega-3 fatty acids, an essential dietary nutrient integral to neuronal structure and function, may be one such influence. This study examined whether intake of energy-adjusted long-chain omega-3 fatty acids [eicosapentaenoic acid (EPA) + docosahexaenoic acid (DHA)] was related to variation in impulse control and PFC activity during performance of an inhibitory task in adolescents ($n = 87$; 51.7% female, mean age 13.3 ± 1.1 years) enrolled in a longitudinal neuroimaging study. Intake of DHA + EPA was assessed using a food frequency questionnaire and adjusted for total energy intake. Inhibitory control was assessed using caregiver rating scale (BRIEF Inhibit subscale) and task performance (false alarm rate) on a Go/No-Go task performed during functional MRI. Reported intake of long-chain omega-3 was positively associated with caregiver ratings of adolescent ability to control impulses ($p = 0.017$) and there was a trend for an association between intake and task-based impulse control ($p = 0.072$). Furthermore, a regression of BOLD response within PFC during successful impulse control (Correct No-Go versus Incorrect No-Go) with energy-adjusted DHA + EPA intake revealed that adolescents reporting lower intakes display greater activation in the dorsal anterior cingulate, potentially suggestive of a possible lag in cortical development. The present results suggest that dietary omega-3 fatty acids are related to development of both impulse control and function of the dorsal anterior cingulate gyrus in normative adolescent development. Insufficiency of dietary omega-3 fatty acids during this developmental period may be a factor which hinders development of behavioral control.

Keywords: impulse control, response inhibition, dorsal anterior cingulate gyrus, omega-3 fatty acids, DHA, EPA, adolescents, fMRI

INTRODUCTION

Development of cortical gray matter follows a regionally-specific, non-linear maturation pattern, whereby gray matter volume generally increases in childhood, peaks in late childhood/early adolescence and declines into young adulthood (Giedd et al., 1999; Gogtay et al., 2004; Lenroot and Giedd, 2006). Gray matter thinning during adolescence is thought to reflect, at least in part, synaptic pruning and refinement (Huttenlocher and Dabholkar, 1997), and is associated with improvements in cognitive function and behavior (Casey et al., 2000). Within the PFC, these dynamic developmental processes occur rapidly during the adolescent years and are thought to underlie improvements in executive function, including impulse control (Rubia et al., 2000; Tamm et al., 2002). Response inhibition, an individual's ability to inhibit his/her actions, is one executive function that is integral to developing the ability to delay gratification (Steinbeis et al., 2014) – a cornerstone of long-term achievements (Mischel et al., 1989). Response inhibition improves from childhood, through adolescence, and into young adulthood (Luna et al., 2010). This maturational process is supported by regionally specific changes in activation within the PFC (Rubia et al., 2006; Ordaz et al., 2013) and the protracted development of the PFC may reflect a period of vulnerability to various environmental and biological factors.

Omega-3 fatty acids are a class of long-chain polyunsaturated fats that can only be obtained via diet. Docosahexaenoic acid (DHA), an omega-3 fatty acid found in marine sources, is the only fatty acid of its class relevant to the central nervous system (Stillwell and Wassall, 2003). Variation in dietary DHA is reflected in variation of DHA content of phospholipids measured both in the peripheral tissues and in the central nervous system (Connor et al., 1990; Moriguchi and Salem, 2003; Hulbert et al., 2005). Within the central nervous system, the distribution of DHA is particularly concentrated in the neuronal membranes of the PFC (Bradbury, 2011), highlighting its importance in a region that is critical for executive function. DHA accrues rapidly in the PFC from the perinatal period though the first 18 years of life, with little increase in PFC DHA content after the second decade of life (Carver et al., 2001) suggesting that the adolescent years are part of a crucial period from infancy through young adulthood to ensure adequate accrual of DHA in the PFC.

Docosahexaenoic acid plays a key role in neuronal functions critical to development. DHA promotes membrane fluidity and the interaction of embedded proteins (Stillwell and Wassall, 2003), neuronal signaling and the resolution of inflammation (Mitchell et al., 1998; McNamara and Carlson, 2006), is associated with greater neuronal size (Ahmad et al., 2002), enhances dendritic spine density thereby promoting synaptic formation (Wurtman et al., 2009), and facilitates cortical pruning (de Velasco et al., 2012). Low levels of omega-3 fatty acids in the diet reduce DHA incorporation in synaptic membranes (Hulbert et al., 2005), which may be detrimental to function of the PFC and inhibitory control over the lifespan. In adults, omega-3 fatty acid status is associated with greater anterior cingulate cortex (ACC) thickness (Zamroziewicz et al., 2015) and volume (Conklin et al., 2007a). In children, blood DHA levels were related to PFC activity during sustained attention, activity which is enhanced with supplementation, including within the ACC (McNamara et al., 2010). Compared to boys with high blood DHA, boys with low DHA displayed reduced functional connectivity in cortical attention networks, including the right ACC, during sustained attention (Almeida et al., 2017). Moreover, the neural effects of omega-3 fatty acid status during development may be long-lived. Compared to children who did not receive supplementation as infants, children who received formula supplemented with essential fatty acids (including EPA and DHA) during infancy displayed differential patterns of brain activity via EEG during a Go/No-Go task (Liao et al., 2017). Behavioral outcomes have also been associated with omega-3 fatty acid status. Low blood levels of DHA are associated with Attention Deficit Hyperactivity Disorder in children (Stevens et al., 1995; Burgess et al., 2000; Chen et al., 2004) and self-reported impulsivity in adults (Conklin et al., 2007b). Moreover, low intake of dietary sources of omega-3 fatty acids produces impulsive behaviors in animal models (Levant et al., 2010) and is associated with externalizing behaviors in children (Gispert-llaurado et al., 2016). Supplementation has been shown to improve impulse control task performance in adults (Fontani et al., 2005). Furthermore, while evidence suggests that DHA is the long chain omega-3 fatty acid of greatest relevance to the central nervous system, reports from ours and other groups also implicate its long chain precursor, eicosapentaenoic acid (EPA) in neural and cognitive outcomes (Bauer et al., 2013, 2014; Pottala et al., 2014; Darcey et al., 2018). Taken together, these studies suggest that omega-3 fatty acids may be important for prefrontal structure and function, particularly in the anterior cingulate cortex.

Dietary profile of polyunsaturated fat intake by Americans has changed dramatically over the last century, resulting in a net decrease in effective dietary omega-3 fatty acids (Blasbalg et al., 2011) and adolescent diets have been found to be poor in sources of omega-3 fatty acids (Cutler et al., 2009). Decreased intake of omega-3 fatty acids among adolescents may be of particular concern given that DHA rapidly accumulates in membranes of PFC gray matter primarily during the first two decades of life (Carver et al., 2001). Low intake during a critical window of DHA accrual in a brain region undergoing major dynamic development has the potential to negatively impact cortical function and related developing behaviors such as impulse control.

In the present study, we investigated the relationship between intake of long chain omega-3 fatty acids and prefrontal function during impulse control in a cross-sectional sample of typically developing adolescents. Adolescent participants completed a food frequency questionnaire from which an energy-adjusted Omega-3 Index was computed. This Index was then related to prefrontal activity and task performance during a Go/No-Go task while undergoing fMRI, as well as caregiver-rated ability of the adolescent to inhibit impulses. Given the evidence reviewed above, we expected that greater intake of energy-adjusted Omega-3 Index would facilitate task performance. Specifically, we hypothesized that higher levels of Omega-3 Index would be associated with lower PFC activity (i.e., greater efficiency) and

increased ability to inhibit prepotent responses (i.e., correct No-Go's). We also predicted higher levels of Omega-3 Index would be associated with better inhibitory behavior as rated by caregivers.

MATERIALS AND METHODS

Participants were recruited as a part of a longitudinal neuroimaging study, the Adolescent Development Study (ADS), aimed at identifying neurobiological precursors and consequences of early drug and alcohol initiation and escalation. Full details of the methods are described elsewhere (Fishbein et al., 2016). In brief, adolescents in a narrow age range (11–13 years old) were recruited and the main exclusionary criteria included prior substance use, left-handedness, conditions rendering MRI unsafe, history of head trauma, and neurodevelopmental disorders. Participants taking psychostimulant (centrally acting) medications were permitted to enroll in the study if study visits could be scheduled during normally occurring medication "holidays." This study was carried out in accordance with the recommendations of the Georgetown Institutional Review Board with written informed assent and consent obtained from all adolescent and adult participants, respectively, in accordance with the Declaration of Helsinki. The protocol was approved by the Georgetown Institutional Review Board.

Food Frequency Questionnaire

Adolescents completed a paper-based food frequency questionnaire called the Harvard Youth/Adolescent Food Frequency Questionnaire (YAQ) to assess usual diet over the past year. The YAQ is a widely-used, scantron questionnaire validated for ages 9–18, which provides a dietary analysis based on a retrospective assessment of usual frequency and portions of 152 food items consumed over the past 12 months (Rockett et al., 1997). Questionnaires were completed at the end of study visits. Participants were paid $15 via Amazon gift card for completing the 30-min survey. Nutrient output was compiled using 2011 nutrient tables (Rockett et al., 1997).

We calculated dietary Omega-3 Index via summation of reported EPA and DHA intakes (Harris and Von Schacky, 2004), then adjusted for total energy consumed [(EPA grams + DHA grams)/total calories] (Subar et al., 2001) and then scaled by 1000 calories to represent long chain omega-3 fatty acid consumption per 1000 calories of intake. Reported intake of Omega-3 Index is adjusted for total energy intake to reduce extraneous variation (diets higher in total calories may also be higher in fats consumed) (Willett et al., 1997). A square root transformation was applied to the energy-adjusted Omega-3 Index in order to minimize influence of a few participants reporting highest omega-3 intakes. A subsample of adolescents participated in a validation sample, providing both YAQ responses and blood samples for whole blood essential fatty acid analysis (n = 19). Energy-adjusted dietary Omega-3 Index and Omega-3 Index observed in blood (EPA + DHA) were highly correlated (r_s = 0.660, p = 0.002), similar to other studies (e.g., Marangoni et al., 2007; Dahl et al., 2011; Almeida et al., 2017).

Additional Adolescent Measures

Participants' intelligence was assessed using a developmentally appropriate battery (Kauffman Brief Intelligence Questionnaire; K-BIT) (Kaufman and Kaufman, 1990). To account for potential differences in physical maturation, adolescents completed the Pubertal Development Scale (Petersen et al., 1988; Carskadon and Acebo, 1993) consisting of a series of questions about progress of physical development, asking respondents to evaluate the degree to which a specific physical change (such as skin/voice changes, growth spurt, breast development, and facial hair) has occurred. During study visits, body mass index (BMI) (kg/m^2) sex and age-specific z-scores and percentiles were calculated using weight measured with a digital scale (Health-O-Meter Professional 394KLX) and height measured via stadiometer (SECA 216 Wall-mount Mechanical measuring rod; triplicate measures within 0.5 cm, averaged) applied to 2000 CDC Growth Charts (Kuczmarski et al., 2000).

Family Socioeconomic Status

Caregivers of participants were interviewed to collect information on parental education and income to calculate an index of household socioeconomic status (SES index) using a method adapted from Manuck et al. (2010). Maternal and paternal cumulative years of education were averaged and a standardized education z-score was calculated for each participant. Reported level of total annual household income prior to taxes (ranging from less than $5,000 to greater than or equal to $200,000) was converted to standardized income z-score for each participant. SES index was computed by averaging standardized values for the income and education variables for each participant, then subsequently re-standardizing to achieve a distribution with a 0-centered mean and standard deviation of 1 for the full sample (N = 135).

ASSESSMENTS OF RESPONSE INHIBITION

Behavior Rating Inventory of Executive Function (BRIEF)

An 86-item psychometrically validated questionnaire (Gioia et al., 2000) to assess facets of executive abilities was administered to primary caregivers of participants. Caregivers rated the frequency that their child's behaviors were problematic as "never," "sometimes," or "often." The questionnaire yields 8 non-overlapping scales, of which the Inhibit subscale, reflecting the ability to control impulses or stop behavior, was of interest to the current study. Higher scores suggest higher level of dysfunctional behavior. Normative values for age and sex (t-scores) are reported here. No responses were classified as "inconsistent" and all were included in the analysis.

Go/No-Go Task

Adolescents completed a simple Go/No-Go functional MRI task, which elicits neuronal activity related to response inhibition (Menon et al., 2001; Simmonds et al., 2008). This task uses a

hybrid design with alternating blocks of event-related Go/No-Go (45 s) and Fixation (12–16 s) each repeated 5 times (total time: 5:02 min). During the Go/No-Go blocks, a series of 30 letters is presented for 200 ms each, followed by a 1300 ms fixation. Subjects are instructed to press the button held by their right hand as quickly as possible for every letter ("Go" trials) except the letter 'Q' ("No-Go" trials). A total of 150 trials are presented in this design of which 27 events (18%) are No-Go trials. The task was implemented in E-prime and completed during MR imaging.

While relative accuracy of performance on the Go/No-Go is an indication of attention, errors of commission (response to the target when the correct action is a withheld response) are an indication of poor response inhibition (Riccio et al., 2002). Thus, we analyzed the percentage of incorrect No-Go's (also known as false alarms), and reaction time to Go trials (reflecting processing speed). Responses faster than 150 ms were excluded from behavioral analysis and not modeled in the fMRI analysis (below) to minimize analysis of anticipatory responses (i.e., unlikely to reflect true stimulus processing). Hit rate, or response rate to correct Go (limited to responses longer than 150 ms), was used to determine whether participants were adequately engaged with the task and a hit rate of at least 70% of Go trials was required for inclusion in the analyses.

MRI PROTOCOL

Data Acquisition

The MRI scans were performed on a 3 T scanner (Siemens Tim Trio) at the Center for Functional and Molecular Imaging, Georgetown University (Washington, DC, United States) using a 12-channel radio frequency head coil. For anatomical localization and spatial normalization a structural MRI acquisition was collected using a 3D T1-weighted MPRAGE image with the following parameters: TR/TE = 1900/2.52 ms, TI = 900 ms, 176 slices and slice resolution = 1.0 mm^3. FMRI acquisition used T2*-weighted gradient-echo planar imaging (EPI). The blood oxygenation level dependent (BOLD) functional MRI acquisition parameters were: TR/TE 2500/30 ms, 90° flip angle, in-plane resolution 3.0 mm^2, 47 slices and slice thickness = 3.0 mm. Data for the Go/No-Go task were collected in one run.

fMRI Data Preprocessing

Image processing and statistical analysis was carried out using SPM8[1] including correction for sequential slice timing and realignment of all the images to the mean fMRI image to correct for head motion artifacts between images. Realigned images were then co-registered with the anatomical MPRAGE. The MPRAGE was segmented and transformed into the Montreal Neurological Institute (MNI) standard stereotactic space using affine regularization and non-linear registration with tissue probability maps. Lastly, these transformation parameters were applied to normalize the fMRI images into MNI space, after which the data were spatially smoothed using a Gaussian kernel of 6 mm^3 full-width half maximum (FWHM). A scrubbing algorithm utilizing framewise displacement (FD) was used to assess participant movement during the fMRI scans (Power et al., 2012). Participants were excluded from analyses if they had more than 1 mm frame-wise displacement in over 20% of their volumes during the fMRI scans.

[1] http://www.fil.ion.ucl.ac.uk/spm

First-Level Analysis

Regressors for the first-level analysis included vectors coding for correct Go and No-Go and incorrect Go and No-Go trials. Six regressors of no interest from the motion correction parameters were included to minimize signal changes related to head movement. The fMRI responses were convolved with the canonical hemodynamic response function and a 128-s temporal high-pass filter was applied to the data to exclude low-frequency artifacts such as MRI signal drift. The contrast of interest was successful inhibitions to examine activation associated with cognitive control of behavior (i.e., Correct No-Go > Incorrect No-Go).

fMRI Statistical Analysis

First level contrasts were entered into a linear regression with the square root transformed energy-adjusted Omega-3 Index. Given the relative importance of DHA to PFC function, second-level group analyses were constrained to a specific search region with an explicit mask of bilateral frontal lobe gray matter, created using AAL, Wake Forest Pick Atlas (Maldjian et al., 2003). Clusters were defined in SPM8 using an uncorrected threshold of $p = 0.001$ with a cluster extent of 10 voxels and were determined to survive correction for multiple comparison at the cluster level using FWE of $p < 0.05$. Peak MNI coordinates were extracted from each surviving cluster and anatomical descriptions were identified using the AAL atlas.

Data Reduction/Exclusions

Of 135 participants enrolled in the parent study, 126 completed the food frequency questionnaire. Seven of these responses were excluded from analysis for the following reasons: one participant for implausibly high caloric intake [>13,000 kcal per day (Cutler et al., 2009)]; three for leaving excessive numbers of questions blank; three due to factors revealed post-enrollment that may affect neurodevelopment ($n = 2$ reporting drug/alcohol prior to study enrollment, $n = 1$ Tourette's syndrome diagnosis).

Of 119 participants with eligible food frequency questionnaire data, thirteen were excluded from the behavioral analysis (three due to technical data collection errors; ten for hit rates < 70%). Of 106 participants remaining with eligible diet surveys and Go/No-Go behavior, 19 were excluded on the basis of imaging data (4 due to braces, 3 were missing full imaging data, 2 were missing part of the brain image, and 10 for excessive motion). Final analyses were restricted to participants with eligible imaging, behavioral and dietary data ($n = 87$).

Statistical Analyses

Data analysis was conducted in IBM SPSS Statistics 24 (IBM Corp. Released 2016. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY, United States: IBM Corp.). The

distributions of dependent variables were confirmed via Shapiro–Wilk test. The BRIEF Inhibit *t*-score, false alarms, and processing speed possessed skewed/kurtotic distributions, as indicated by *Spearman's Rho* (r_s). Non-parametric analyses were therefore used for these three DVs. The distribution of the independent variable, energy-adjusted Omega-3 Index, was transformed by taking the square root in order to minimize influence of a few participants reporting high omega-3 intakes. Multiple linear regressions were calculated using rank-transformed data to examine the relative contribution of energy-adjusted Omega-3 Index and demographic predictors (i.e., age, socioeconomic status index) on behavioral outcomes for significant correlations.

RESULTS

Participant characteristics are presented in **Table 1**. Energy-adjusted Omega-3 Index was unrelated to age (r = 0.182; p = 0.091; n = 87), SES index z-score (r_s = 0.089, p = 0.420, n = 84), pubertal development score (r = 0.044, p = 0.686, n = 87), BMI z-score (r = −0.072, p = 0.505, n = 87) or KBIT IQ score (r_s = 0.150, p = 0.174, n = 84). Males and females reported similar intakes of Omega-3 Index (Mann–Whitney-U test p = 0.363).

Age was unrelated to any behavioral variable (BRIEF Inhibit t-score [r_s < −0.001, p = 0.998, n = 86], false alarms [r_s = −0.142, p = 0.190, n = 87], and processing speed [r_s = −0.130, p = 0.231, n = 87]). Though SES was unrelated to task performance (false alarms [r_s = −0.136, p = 0.218, n = 84] and processing speed [r_s = 0.109, p = 0.323, n = 84]), greater family SES was associated with better caregiver-rated inhibitory control (BRIEF Inhibit t-score [r_s = −0.311, p = 0.004, n = 83]).

BRIEF Inhibit Subscale (Parental Report)

Mean and median percentile scores for this sample were 59 and 58%, respectively, indicating that the current sample is marginally more impulsive than peers of the same gender and age. Greater energy-adjusted intake of Omega-3 Index was associated with better ability to inhibit behavior as rated by caregivers (BRIEF Inhibit subscale t-score) (r_s = −0.257, p = 0.017; n = 86) (**Figure 1**).

A multiple linear regression was calculated using rank-transformed data to predict the Inhibit subscale ratings based on Omega-3 Index and SES Index. Age was not a significant correlate of the Inhibit subscale score and thus was not included in the model. A significant regression equation was found [$F(2,80)$ = 8.272, p = 0.001], with an R^2 of 0.171 (adjusted R Square 0.151). Standardized coefficients (β) were −0.274 (t = −2.678, p = 0.009) for Omega-3 Index and −0.281 (t = −2.743, p = 0.008) for SES Index, indicating both SES index and energy adjusted Omega-3 Index were significant predictors of BRIEF Inhibit subscale scores.

Go/No-Go Performance

Median false alarm rate (Incorrect No-Go) was 37%. The relationship between false alarm rate and energy-adjusted Omega-3 Index did not reach statistical significance (r_s = −0.194, p = 0.072, n = 87). Median reaction time (Correct Go's) was 317 ms. Reaction time was not significantly related to energy-adjusted Omega-3 Index (r_s = 0.157, p = 0.145, n = 87).

TABLE 1 | Participant characteristics.

	Central tendency		
	Mean (*SD*)	**Median**	**Range**
N	87		
Sex (% females)	45 females (51.7%)		
Age	13.3(1.1)	13.3	11.1–16.1
Intelligence [n = 84]	110.9(14.7)	111	71–138
Race and ethnicity			
Caucasian	52.9%		
African American	28.7%		
Hispanic or Latino	9.2%		
Other	9.2%		
Socioeconomic Status index (z-score) [n = 84]	0.115(0.967)	0.371	−2.589–1.511
Parental education (years, mean)	16.5(2.7)	17	7–22
Household income (median)	$50,000–$74,999	$100,00–$149,999	<$5,000–>$200,000
Pubertal development	2.4(0.7)	2.4	1–3.8
BMI z-score	0.34(0.93)	0.40	−2.2–2.2
Percentile	59.8(28.2)	66.6	1.5–98.7
BRIEF Inhibit subscale, Percentile (t-scores analyzed)	59.0(23.7)	58	23–99
Go/No-Go Performance			
Hit Rate (% Correct Go)	94.6(6.5)	97.6	71.5–100
False Alarm Rate (% Incorrect No go)	42.4(19.8)	37.0	3.7–77.7
Response time, ms (Correct Go)	320.6(50.6)	317.0	245.1–453.0
Dietary intake			
Total daily energy intake (kcal)	1889(890)	1741	568–5645
Omega-3 Index daily intake (EPA + DHA), mg	125(151)	50	0–640
Omega-3 Index daily intake, energy adjusted (mg/1000 kcal)	68.7(89.4)	30.0	0–437.1

PFC Activity During Successful Response Inhibition

Main effects within the PFC for the contrast of interest (Correct No-Go > Incorrect No-Go) reflect activation in areas recruited during response inhibition (**Supplementary Figure S1** and **Supplementary Table S1**). Using a small volume corrected (SVC) approach limited to the PFC, voxel-wise regression analysis revealed that activation during successful inhibitions (Correct No-Go > Incorrect No-Go) was inversely associated with energy-adjusted Omega-3 Index in five clusters (**Table 2**). **Figure 2** shows activation in the dorsal anterior cingulate cortex (dACC; 307

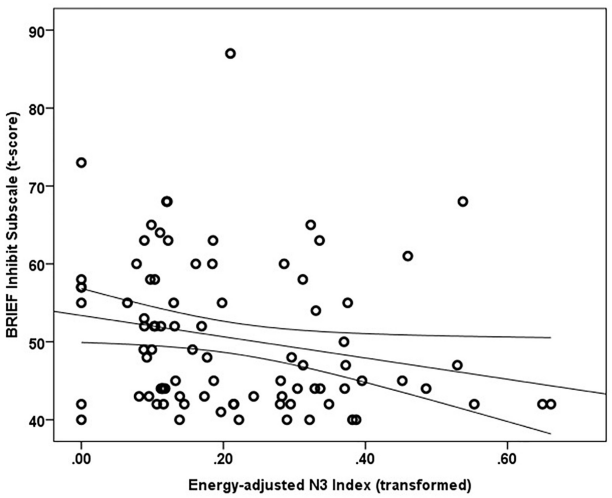

FIGURE 1 | Energy adjusted Omega-3 index intake is inversely related to response inhibition as rated by caregivers based on the BRIEF Inhibit subscale *t*-score (r_s = –0.257, p = 0.017).

TABLE 2 | MNI Coordinates of local maxima for activation during successful inhibitions (Correct No-Go > Incorrect No-Go) inversely associated with dietary Omega-3 Index intake (cluster defining threshold ke = 10, uncorrected p = 0.001, df = 85).

Region	*x*	*y*	*z*	Max *t*	Volume (mm^3)
Inferior frontal gyrus, left	−42	2	24	3.47	12
Insula, right	46	−2	−2	4.05	25
Insula, left	−40	−2	6	3.87	30
	−35	0	−2	3.57	
Anterior cingulate, left	−14	42	−6	3.51	14
Cingulate gyrus, right [BA 32/24]*	8	22	30	4.60	307*
	2	26	22	3.88	

**Group-level statistical maps were small volume corrected (SVC) for multiple comparisons within the PFC. Cluster surviving FWE correction at p < 0.05 denoted.*

voxels; peak MNI 8, 22, 30; max *t* 4.60), which survived correction for multiple comparison (cluster level FWE, p = 0.004; cluster level FDR p = 0.014). Though Omega-3 Index intake was not associated with pubertal status, a subsequent analysis to ensure pubertal status was not of substantial influence revealed little impact of Pubertal Development Score on the present results (**Supplementary Figure S2** and **Supplementary Table S2**). There were no clusters where activation was positively correlated with Omega-3 index. β-weights were extracted from this cluster using MarsBar and plotted against energy-adjusted Omega-3 Index for visualization purposes and to examine heterogeneity of activation (**Figure 3**).

DISCUSSION

The present study examined the extent to which dietary omega-3 fatty acids contribute to PFC function during successful impulse control in typically developing adolescents. We found that response inhibition during a simple Go/No-Go task was not significantly associated with reported energy-adjusted Omega-3 Index. However, caregiver ratings of their child's general ability to inhibit impulses were significantly inversely related to omega-3 intake; adolescents reporting lower omega-3 intake were rated as less able to control impulses in real-world situations. Furthermore, we found significant inverse relationship between energy-adjusted Omega-3 Index and activity in the dACC during successful response inhibition, such that adolescents with lower omega-3 intake exhibit hyper-activation in the dACC in order to achieve similar behavioral performance as their peers reporting higher omega-3 intake. To our knowledge, this is the first study to correlate dietary long chain omega-3 fatty acids with impulse control and prefrontal function in adolescent boys and girls. Together with the extant comparable literature (reviewed below), the current findings suggest that long-chain omega-3 fatty acids may be particularly relevant to function of medial prefrontal cortex, particularly the dorsal region of the anterior cingulate cortex.

The PFC exerts top-down control over behavior and the anterior cingulate is putatively involved in, among many cognitive functions, performance monitoring during situations where the chance of error is high (Carter et al., 1998) or where there is need for heightened vigilance because conflicting responses are possible (Brown and Braver, 2005). Indeed, in adults, engaging in a block-design Go/No-Go task recruits a network of regions including the dACC (Ogg et al., 2008). Among task events, successful inhibitions specifically recruit a network of regions including the rostral portion of superior medial frontal cortex (Simmonds et al., 2008). Though Simmonds et al. (2008) identified recruitment of a region slightly more dorsal than that reported in the present study (pre-supplementary motor area), the slight differences in activation coordinates may be partly due to inclusion of adults and contrasts examined (Correct No-Go > baseline versus Correct No-Go > Incorrect No-Go in the present study). Nevertheless, dACC function during error processing has been found to be critical to improvements in inhibitory control observed over development (Ordaz et al., 2013).

In the present study, consuming lower amounts of long chain omega-3 fatty acids was related to greater activity in the dACC during impulse control, but unrelated to task performance (rate of successful inhibitions). This result may suggest that for adolescents with lower omega-3 intake, greater dACC neural activity is required to accomplish the same level of inhibitory control as their high omega-3 intake peers. Omega-3 fatty acids are involved in functions at the cellular level that may represent a potential mechanism for the cortical inefficiency attributed to low omega-3 adolescents here. Interestingly, omega-3 deficiency has been shown to impair cortical glucose transport and utilization (Ximenes da Silva et al., 2002; Pifferi et al., 2005) and boys with lower omega-3 status exhibit indices of metabolic dysfunction in the ACC compared to their higher omega-3 peers (McNamara et al., 2013). Furthermore, omega-3 supplementation resulted in increased frontocortical efficiency in a rodent ADHD model (Liso Navarro et al., 2014). Relatedly, dACC activity attenuates over the course of engagement with the

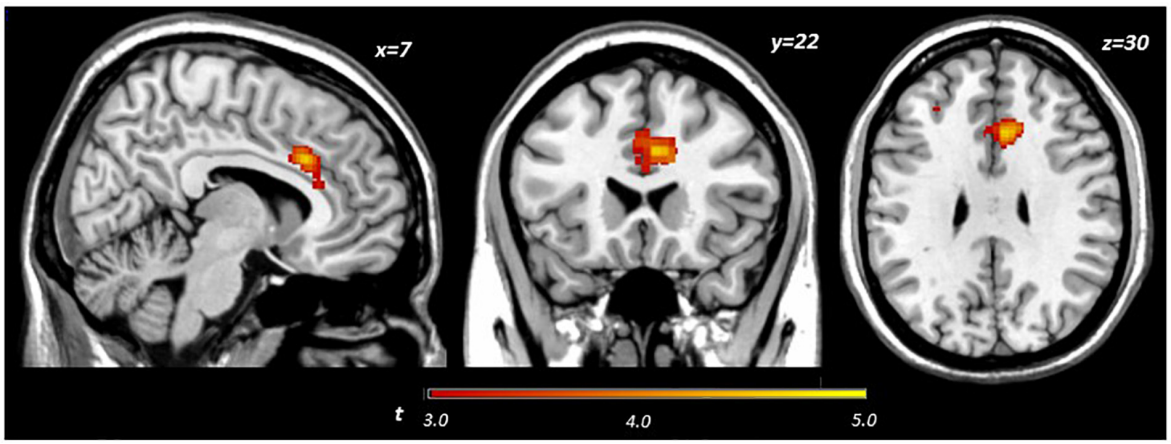

FIGURE 2 | Activation during successful inhibitions (Correct No-Go > Incorrect No-Go) inversely related to dietary Omega-3 index.

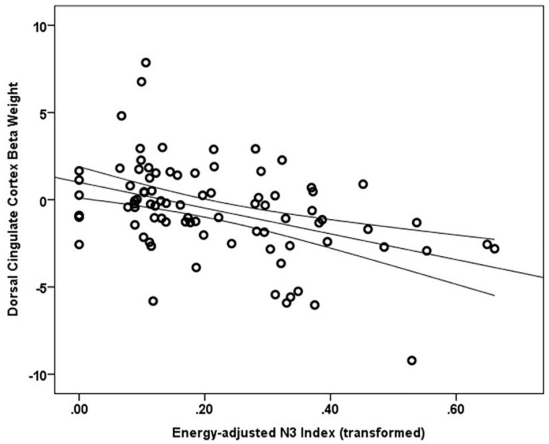

FIGURE 3 | Relationship between dACC activation (regression coefficient β-weights) and energy-adjusted Omega-3 Index intake.

task (Tana et al., 2010). Thus, it is possible that, in adolescents reporting a lower Omega-3 Index, the cingulate experiences inefficient metabolism, or shows protracted habituation to the effort level required to perform the task, potentially signifying decreased efficiency. Additionally, given that DHA restriction leads to impaired pruning of superfluous axonal connections (de Velasco et al., 2012), it is possible that adolescents with lower Omega-3 Index have greater activity in the ACC because this network has not yet undergone pruning of superfluous synapses, a concept proposed by Berl et al. (2006). Indeed, separate analyses conducted revealed that energy adjusted DHA intake was the main driver of the observed BOLD effect in the present study (data not shown). Thus, inefficiency in metabolism and/or impaired cortical pruning may contribute to the greater ACC activity, which compensates for lower intake of long-chain omega-3 fatty acids.

The present results confirm previous reports of a relationship between omega-3 fatty acid status and the anterior cingulate (Conklin et al., 2007a; McNamara et al., 2013; Almeida et al., 2017). Further, since the current study includes both boys and girls, the present results extend the association to adolescent girls' diet, dACC function and caregiver ratings of impulse control. It is notable that the current study also found a relationship between dietary Omega-3 Index and cingulum activation without an *a priori* cingulate ROI. This confirms previous reports utilizing ROIs and suggests that midline structures may be sensitive to omega-3 levels (Conklin et al., 2007a; McNamara et al., 2013; Almeida et al., 2017). It should be noted that the region of interest for both McNamara et al. (2013) and Almeida et al. (2017) was more rostral and anterior to the cluster observed after a voxel-wise PFC analysis in the current study. Location of their ROI and other slight methodological differences may explain why we report a task-related difference while Almeida et al. (2017) did not observe a difference in ACC BOLD signal during task blocks requiring response vigilance/inhibition between boys with low and high blood omega-3 levels. Also, in contrast to Almeida et al. (2017) the current study distinguished between successful and unsuccessful trials in a hybrid design, rather than a block design, specifically to examine activation associated with successful response inhibition rather than attention *per se*. Additionally, the current study examined early adolescents (age 11–16 years) versus children (8–10 years), and included both males and females, where both developmental stage and sex have been demonstrated to have influence on developmental status/trajectory of BOLD signal (Ordaz et al., 2013) and neuroanatomical development (Giedd et al., 1999).

Consistent with our hypothesis, greater reported intake of omega-3 fatty acids was associated with better inhibitory control as rated by caregivers. Given that the BRIEF is not subject to adolescents' self-report bias, our finding is notable in that it lends a degree of external validity to our results. Others have reported associations between omega-3 fatty acid status and self-reported impulsivity in adults (Conklin et al., 2007b) and in children (Gispert-llaurado et al., 2016). Together with previous studies, the present results support a role for omega-3 fatty acid status in generalized impulse regulation.

Contrary to our hypothesis, however, response inhibition as measured by ability to inhibit prepotent responses on the Go/No-Go task was unrelated to dietary Omega-3 Index. Additionally, a *post hoc* analysis did not find any relationship between BOLD activity (β-weights) in the ACC cluster recruited during successfully inhibited events and BRIEF Inhibit subscale *t*-scores (data not shown). Disparity between BRIEF subscales and presumably related task performance has been reported previously (McAuley et al., 2010). It is possible that assessing response inhibition using a task in a laboratory setting is a more circumscribed measure of inhibitory control, compared to caregiver report on general inhibitory control ability in real-world settings over the 6 months prior to the study visit. As acknowledged by Aron (2011), "the stopping of motor responses, no matter how sophisticated the model, will only be relevant for impulse control some of the time" (Aron, 2011). Consistent with our study, McNamara and colleagues also did not find differences between high and low DHA groups on false alarm rate using a similar task measuring response inhibition and sustained attention in boys (McNamara et al., 2010). Given that others have observed improved Go/No-Go performance (accuracy and response time) with supplementation in adults (Fontani et al., 2005), and have seen associations between (posterior) cingulate activation and performance during difficult but not easy task conditions (Boespflug et al., 2016), it is possible that the intake levels reported in this study are too low and/or the current task is too easy (median hit rate 97.6%; median successful response inhibition rate 55.6%) to detect Omega-3 associations with performance. Additionally, given that only a small, but significant, portion of the variance in attention (processing speed and omission errors) was attributable to Omega-3 Index in a large (n = 266) cohort of adolescents (van der Wurff et al., 2016), it is possible that the current sample size (n = 87) was underpowered to detect an association with task metrics of motoric response inhibition.

One of the main limitations of the current study is that the food frequency questionnaire is dependent on recall of usual diet. While other methods (24-h recall, quantitative 7-food records) may increase accuracy, they are often difficult to implement with larger samples and require greater resources in terms of study team time and financial commitment. The food frequency questionnaire is an accepted method for measuring intake of nutrients with very high day-to-day variability, and its output represents the respondent's chronic/habitual nutrient intake over specified periods of time (e.g., preceding 12 months) rather than a reliable calculation of absolute values (Subar et al., 2001). It is worth noting that reported intake has been previously shown to correlate well with serum biomarkers (Sun et al., 2007; Kuratko and Salem, 2009; Vandevijvere et al., 2012), which was also demonstrated in a subset of participants in the current study providing both food frequency data as well as whole blood for essential fatty acid analysis. Furthermore, a review of reported global intake of dietary DHA found that 12–19 year-olds consume 30–50 mg/day in the United States (Flock et al., 2013), which is comparable to our sample (median reported daily intake DHA 30 mg, data not shown). Thus, the present results demonstrate the relative reliability and feasibility of assessing diet in a moderately sized neuroimaging cohort. Another potential limitation is the relatively limited number of No-Go events (27 events) under analysis. While the infrequency of these events (18% of task trials) is a necessary condition to elicit a prepotent response and the number of events is on par with some other studies (e.g., Rubia et al., 2006), future studies would be well served to expand the task duration to accumulate more trials with which to compare BOLD signal. Some strengths of the present study include the inclusion of both male and female adolescents and a relatively restricted age range to minimize the variance of brain development due to chronological age.

Summary and Implications

Dietary Omega-3 Index intake was significantly inversely related to both activity in the dACC during successfully inhibited trials and to a general measure of impulsivity. Our results also reveal that energy-adjusted Omega-3 Index accounts for a similar amount of unique variance in caregivers' ratings of adolescent inhibitory control as accounted for by socioeconomic status, an established environmental factor in brain structure and function (Johnson et al., 2016).

Dietary intake of effective omega-3 fatty acids, compared to omega-6 fatty acids, which are a competitive substrate for metabolism, has been on the decline in the United States over the past century (Blasbalg et al., 2011). While there are no dietary reference intakes for long chain omega-3 fatty acids (EPA and DHA), the consensus among experts is that the current level of intake is far below that desired for optimal health (Flock et al., 2013). A general theme in development is that the central nervous system's DHA requirements are heightened during rapid growth of specific tissues. For example, early in postnatal development the retina has increased requirements for DHA and deficiency during this period reliably results in poor visual acuity (Agostoni, 2008). It is conceivable that the PFC may be comparably sensitive to insufficiency of DHA though over the longer developmental period of adolescence. Post mortem examinations indicate that PFC DHA content increases until 18 years of age (Carver et al., 2001), suggesting protracted accumulation in cortex. Given that cortical DHA levels would be predicted to fall by 5% within a few months of an omega-3 deficient diet in adults (Umhau et al., 2008), the metabolic needs of the developing adolescent brain may render it more sensitive to DHA levels. Thus, reduced DHA intake during adolescence, may delay and/or limit proper development of the PFC during this critical period in development, potentially leading to negative long-term consequences related to executive function though this hypothesis remains to be formally tested. To our knowledge this is the first larger-scale neuroimaging study in a sample of typically developing male and female adolescents to report a relationship between Omega-3 Index and the ability to inhibit responses, a key executive function, as well as associated neural activity. While not evidence of a causal relationship, taken together these results suggest that intake of long chain omega-3 fatty acids is related to caregiver perceptions of their adolescent's ability to control impulses and function of the dACC, a prefrontal region implicated in a number of executive functions including

monitoring errors and performance. Unlike other comparable contributors like socioeconomic status *per se*, diet is a factor that may be more easily modified in the service of catalyzing morphological and functional neurodevelopment, specifically to increase behavioral self-control, which ultimately may have an impact on preventing maladaptive outcomes.

AUTHOR CONTRIBUTIONS

VD was responsible for research question or concept, study design, data analysis, interpretation of results, and writing of the manuscript. GM contributed to data analysis, interpretation of results, and writing of the manuscript. DF contributed to interpretation of results and writing of the manuscript. JV contributed to study design, data analysis and interpretation of results, and writing of the manuscript.

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SUPPLEMENTARY MATERIAL

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Maternal Supplementation With Avocado (*Persea americana* Mill.) Pulp and Oil Alters Reflex Maturation, Physical Development, and Offspring Memory in Rats

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Avocado (*Persea americana* Mill.) is an oleaginous fruit source of fatty acids with high levels of neuroprotective phytocomplexes. The objective of this study was to evaluate the development of reflex and somatic maturation, fatty acid profiles in the brain, and memory in different stages of life in the offspring of dams supplemented with avocado pulp and oil during gestation and lactation. The dams were randomly divided into three groups ($n = 15$ pups/group), and recieved by gavage supplementation: control group (CG)–distilled water; Avocado Oil (AO)−3,000 mg avocado oil/kg animal weight, and Avocado Pulp (AP)−3,000 mg avocado pulp/kg animal weight. We performed the following tests: Analysis of Somatic Development and Ontogeny of Postnatal Reflex (T0 to T21), the Open Field Habituation Test and the Object Recognition Test (ORT) in the adolescent (T45) and adult (T90) phases. The cerebral fatty acids content was evaluated at times T0, T21, T45, and T90. The results were analyzed using the statistical program GraphPad Prism and significant statistics were considered when $p < 0.05$. Acceleration of reflex maturation and reflex ontogeny was observed in the offspring of AO and AP fed dams, with the results being more pronounced in the pulp fed group ($p < 0.05$). All groups presented a decrease in the ambulation parameter in the second exposure to the Open Field Habituation Test, at T45 and T90 ($p < 0.05$). In the ORT, the AO and AP offspring presented memory improvements in the short and long term in the adult and adolescent phases ($p < 0.05$). The results of the brain fatty acid profiles presented higher polyunsaturated fatty acids (PUFA) content in the AO and AP groups at T21, T45, and T90. The docosahexaenoic fatty acid (DHA) content was higher at T21

(AO and AP), at T45 (AO and AP), and at T90 (AP) ($p < 0.05$). The arachidonic acid (ARA) content was higher at T45 (AO and AP), and at T90 (AO) ($p < 0.05$). Maternal supplementation with avocado oil and pulp anticipates reflex maturation and somatic postnatal development, and improves memory during the adolescent and adult phases.

Keywords: avocado, cerebral fatty acids, postnatal development, memory, rats

INTRODUCTION

Adequate fetal and postnatal development is influenced by maternal nutrition (Brenna and Lapillonne, 2009; Mennitti et al., 2015). During this period, considered developmentally critical, lipids are essential to tissue construction and determination of body growth (Morgane et al., 1993; Herrera and Ortega-Senovilla, 2014). Lipids structurally compose the nervous system, stimulate its development and differentiation, and even regulate neuronal cell migration (González and Visentin, 2016; Prado et al., 2018).

The quality of lipids in the diet during gestation and lactation determines the type of fatty acid (FA) that will accumulate in the fetal tissue through placental transfer and through the breast milk after birth (Lauritzen and Carlson, 2011; Innis, 2014). Fatty acids are essential nutrients for the development and maintenance of brain functions and are closely related to learning processes and memory. They demonstrate a positive correlation to neurodevelopment in the offspring through maternal lipid intake (Apryatin et al., 2017; Melo et al., 2017; Pase et al., 2017).

The principal FAs involved in brain development are polyunsaturated fatty acids (PUFAs): linoleic acid (C18: 2 ω-6) (LA), α-linolenic acid (C18: 3 ω-3) (ALA), arachidonic acid (ARA; 20:4 ω-6), docosahexaenoic acid (DHA; 22:6 ω-3), and eicosapentaenoic acid (EPA, 20:5, ω-3) (Makrides et al., 2011; González and Visentin, 2016). Since they are not endogenously synthesized, they are considered essential, and their aquisition occurs only through dietary intake of sources rich in endogenous precursors; ALA and LA (Sinclair, 1975). FA accumulating in brain tissue actively participates in the formation of neuronal membranes (Yehuda, 2012), improving learning, and memory and increasing synaptic and neurogenic plasticity (Dyall, 2017). The influence of maternal PUFAs on the development of reflexes has been evaluated in experimental studies with the offspring (Souza et al., 2012).

Non-essential FA, such as oleic monounsaturated fatty acid (18: 1 ω-9) and palmitic saturated (16: 0 ω-7), can be endogenously synthesized and also transferred through the placenta during gestation; secreted into the maternal milk and accumulate in the brain and other organs during fetal development (Innis, 2004, 2005). Oleic fatty acid is one of the main constituents of myelin (Garbay et al., 2000); it is related to axonal growth and neuronal grouping (Medina and Tabernero, 2002). Palmitic fatty acid participates in the processes of palmitoylation, gliogenesis, synaptogenesis, and myelination (González and Visentin, 2016).

Several sources of fatty acids can be used for maternal supplementation. The avocado (*Persea americana* Mill.) is an oleaginous fruit that has thus aroused scientific interest. Its lipidic composition includes monounsaturated oleic fatty acid (ω-9), saturated palmitic (ω-7), and two linoleic polyunsaturates; (ω- 6), and (ω-3) at lower levels (USDA, 2011; Dreher and Davenport, 2013). Avocado is also a source of neuroprotective antioxidant phytocomplexes (phytosterols, carotenoids, flavonoids) (Ameer, 2016).

Considering associations between maternal lipid consumption and its effects on the neurodevelopment of the offspring and the scarcity of information in the literature on the effect of avocado consumption at this stage, we hypothesized that maternal supplementation with avocado might anticipate the appearance of the reflexes and somatic maturation, and improve the offspring's memory. The objective of this research was to evaluate the offspring of dams supplemented with avocado oil and pulp during gestation and lactation for somatic and reflex development, analyze fatty acid profiles in the brain, and memory function through adulthood.

MATERIALS AND METHODS

Avocado

Avocado (*Persea americana* Mill.) of the Hass variety was obtained from the commercial producer: Fazenda Jaguacy Avocado Brasil®, located in the municipality of Bauru, São Paulo: latitude 22°19′18″S, longitude 49°04′13″W, and 526 m altitude. Part of the fruit was used to extract oil and another part was lyophilized to obtain pulp powder. The lyophilized powder was vacuum packed, and stored at −20°C. The oil and pulp were offered by gavage starting on the seventh day of gestation and throughout the lactation period until the 21st postnatal day.

Analysis of Fatty Acid Composition in Avocado Oil and Pulp

The fatty acid profiles of the oil and pulp were analyzed (Folch et al., 1957; Hartman and Lago, 1973) (**Table 1**).

Lipidic Extraction

Sample were weighed (2 g of each) in a beaker and added to 30 ml of chloroform:methanol mixture (2:1). After this addition, the content was transferred to a deep glass container with the side covered with aluminum foil and stirred for 2 min with the help of grinder. The triturate was filtered through qualitative filter paper into a 100 ml graduated cylinder with a polished mouth. Next, the vessel walls were washed with an additional 10 mL of chloroform:methanol which was also filtered with the previous volume. The volume of the filtered extract of the graduated cylinder was recorded with the graduated cylinder closed. Twenty

TABLE 1 | Fatty acid composition of avocado oil and lyophilized pulp (*Persea americana* Mill.): hass variety.

		Avocado oil	Avocado pulp
Acids Fat		**100 g^{-1} lipids**	
SATURATED			
Palmitic acid	C16:0	22.80	22.41
Stearic acid	C18:0	0.60	0.64
Araquidic acid	C20:0	0.07	0.06
Lignoceric acid	C20:4	0.07	0.08
∑ SFA		23.54	23.19
MONOUNSATURED			
Palmitoleic acid	C16:1ω-7	12.98	13.40
Heptadeacenoic acid	C17:1ω-7	0.10	0.09
Oleic acid	C18:1ω-9	45.92	41.66
Gondoic acid	C20:1ω-9	0.16	0.14
∑ MUFA		59.16	55.29
POLYUNSATURED			
Linoleic acid	C18:2ω-6	12.10	13.11
α-linolenic acid	C18:3ω-3	0.72	0.81
∑ PUFA		12.82	13.93

percentage of the final volume of the filtered extract was added to 1.5% sodium sulfate. The mixture was stirred with the graduated cylinder closed and given time for the phases to separate. It was observed that the upper phase was ∼40% and the bottom 60% of the total volume. The volume of the lower phase was recorded and then the upper phase was discarded by suction with a graduated pipette. For lipid quantification, an extracted aliquot of 5 mL (lower phase) was separated with a volumetric pipette and transferred to a previously weighed beaker. This beaker was placed in an oven at 105°C so the solvent mixture could evaporate, being careful that the fat would not be degraded by heat. After cooling in a desiccator, the beaker was weighed and the fat residue weight was obtained from the difference (Folch et al., 1957).

Transesterification of Fatty Acids

In the sample treatment, methylation of fatty acids present in the lipid extract was carried out following the methodology described by Hartman and Lago (1973). An aliquot of the lipid extract was taken, calculated for each sample according to the fat conte2nt found in the lipid measurement, and performed according to the (Folch et al., 1957), adding 1 ml of internal standard (C19:0) and a saponification (KOH) solution. This solution was subsequently brought to heating under reflux for 4 min. Esterification solution was added immediately after, returning the solution to heating under reflux for 3 more minutes. Next, the sample was allowed to cool before subsequent washings with ether, hexane and distilled water, finally obtaining an extract (with the methyl esters and solvents), which was conditioned into a properly identified amber glass until complete drying of the solvents. After drying, a suspension in 1 ml of hexane was made and packaged into a vial for further chromatographic analysis. The aliquots of saponification and esterification solutions were determined according to the methodology described by Hartman and Lago (1973).

Gas Chromatography Analysis

A gas chromatograph (VARIAN 430-GC, California, EUA), coupled to a capillary column of fused silica (CP WAX 52 CB, VARIAN, California, EUA) with dimensions of 60 m × 0.25 mm and 0.25 mm film thickness was used with helium as carrier gas (Flow rate of 1 ml/min). The initial oven temperature was 100°C programmed to reach 240°C, increasing 2.5°C per minute for 30 min, totaling 86 min. The injector temperature was maintained at 250°C and the detector at 260°C. 1.0 μl aliquots of esterified extract were injected in a Split/Splitless injector. The chromatograms were recorded using Galaxie Chromatography Data System software. The fatty acids results were quantified by integration the areas of the methyl esters and are expressed in percentage by area.

Analysis of Antioxidant Content of Oil and Lyophilized Avocado Pulp

The oil and pulp were analyzed for their total phenolic, flavonoid, and carotenoid components. The antioxidant capacity was also analyzed using the ABTS, FRAP, and IC_{50} methods.

Extraction

Avocado pulp constituents were extracted with both 80:20 EtOH:H_2O v/v and evaluated for ABTS scavenging capacity, ferric reducing activity (FRAP) and total flavonoids. For total phenolic contents 100% MeOH. Oil constituents were extracted with both 80:20 MeOH:H2O v/v and evaluated for FRAP, ABTS, total phenolic and flavonoids contents. All the extractions were performed in triplicate.

Determination of Total Phenolic Compounds (TPC)

In order to estimate the total phenolic compounds, the methodology described by Liu et al. (2002) was used with minor modifications. The absorbance of the extract was compared with a gallic acid standard curve for estimating concentration of TPC in the sample. The TPC was expressed as mg of gallic acid equivalents (GAE) per 100 g of avocado oil and pulp on the basis of dry weight (DW).

Determination of Total Flavonoids

The total flavonoid content was measured using the colorimetric assay developed by Zhishen et al. (1999). The absorbance of the extract was compared with a catechin standard curve for estimating concentration of flavonoids contents in the sample. The flavonoids contents was expressed as mg of catechin equivalents (QE) per 100 g of avocado oil and pulp on the basis of dry weight (DW).

Antioxidant Activity–FRAP Method

The FRAP method was performed according to Benzie and Strain (1999), with modifications proposed by Pulido et al. (2000). The FRAP solution was used as reference reagent, and absorbance was read at 593 nm. The results were expressed in μmol of trolox equivalents per gram of avocado pulp on dry weight (DW) basis (μmol TE/g^{-1}).

Antioxidant Activity–ABTS Method+

The ABTS method was carried out according to the methodology described by Surveswaran et al. (2007), with modifications. The results were expressed in μmol of trolox equivalent per gram of avocado oil and pulp on dry weight (DW) basis (μmol TE/g^{-1}). Where A_0 is the absorbance of the control and as is the absorbance of the sample. The effective concentration had 50% radical inhibition activity (IC_{50}), expressed as mg extract/mL, which was determined from the graph of the free radical scavenging activity (%) against the extract concentration.

The pulp and oil, respectively, presented total phenolic contents of 64.61 and 49.50 mg GAE/100 g, total flavonoids of 39.38 and 33.75 mg CE/100 g, and total carotenoids of 87.00 and 9.87 mg/100 g. For antioxidant activity, the pulp and oil presented respective FRAP values of 0.08 and 0.03 μmol TE/g, ABTS of 2.02 and 0.17 μmol TE/g, and IC_{50} of 59.86 and 443.99 mg/mL.

Animals and Experimental Groups

Females of the Wistar lineage (90 days old/weights 250 ± 50 g) were obtained from the Laboratory of Experimental Nutrition, at the Federal University of Campina Grande–LANEX/UFCG and were breeded to obtain 45 newborn rats. The females were mated while maintained at the ratio of two females to each male. After confirmation of pregnancy, the rats were housed in individual polypropylene maternity cages (60 cm in length, 50 cm wide, and 22 cm in height), under standard laboratory conditions (temperature 22 ± 1°C, humidity 65 ± 5%, light/dark cycle of 12/12 h–artificial light from 6:00 to 18:00).

To obtain the offspring, 24 (Folch et al., 1957) female rats were randomly divided into three groups ($n = 15$ pups for each group): Control (CG)–supplemented with distilled water; Avocado Oil (AO)–supplemented with 3,000 mg of avocado oil/kg of animal weight; and Avocado Pulp (AP)–supplemented with 3,000 mg of avocado pulp/kg of animal weight. Gavage was administered from the 7th day of gestation until the 21st day of lactation: Standard feed (Presence Purina®, São Paulo, Brazil) and water was offered *ad libitum*. After weaning, the offspring received standard ration until adulthood. The research followed an experimental protocol in accordance with the ethical recommendations of the National Institute of Health (Bethesda, USA), and was approved by the ethics research committee of the Federal University of Campina Grande No: 006/2017 and avocado registered in SisGen n°A737D56.

Experimental Procedures

The neonates were weighed and evaluated for reflex ontogenesis and somatic development parameters each day from birth until weaning. For fatty acid content analysis, brains were collected on the first day of life (T0), on weaning day (T21), at adolescence (T45), and as adults (T90). The memory evaluation tests were performed in adolescence and adulthood. The experimental protocol is detailed in **Figure 1**.

Removal of Brains and Fatty Acid Content Analysis

At T0, after sexing and manipulation for litter reduction, surplus puppies were randomly chosen for removal of the brain, which was removed upon decapitation. At T21, T45, and T90 brains were also removed and stored at −20°C until the day of analysis and quantification of fatty acid content ($n = 6$).

The fatty acid profile of the brains was determined using the (Hartman and Lago, 1973) method with transesterification and subsequent identification by gas chromatography (Varian 430GC).

Reflex Ontogeny and Somatic Response

Each day, from the 1st to the 21st day of life at from between 06:00 to 8:00 a.m. in the morning, somatic responses and reflex ontogeny were evaluated. The response was considered consolidated when the expected reaction was repeated for three consecutive days, being the 1st day of the appearance considered as the day of consolidation. The daily observation time for each parameter was 10 s. The reflex study followed the experimental model established by Smart and Dobbing (1971) (**Table 2**). Somatic maturation indicators were also evaluated: Aural "Pavilion" Opening (APO), Auditory Conduit Opening (ACO), Eye Opening (EO), Eruption of Upper Incisive Teeth (EUIT) and Inferior Teeth (EIIT), Appearance of Epidermal Hair (AEH), and Tail Length (TL).

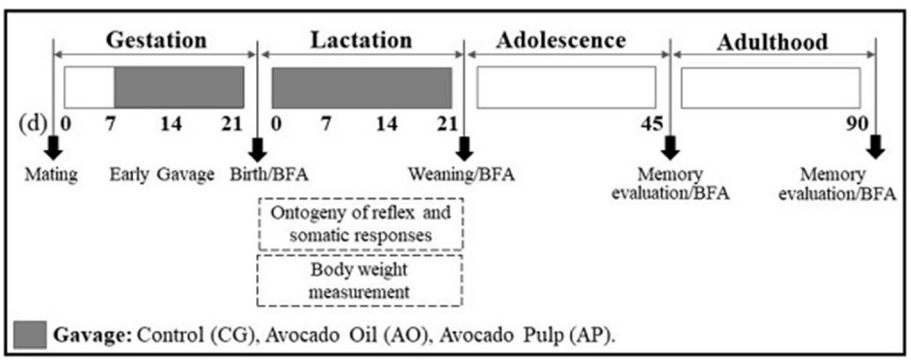

FIGURE 1 | Experimental protocol. Sequence of experimental days conducted with Wistar rats supplemented during gestation and lactation, and of their offspring. (d): day; BFA: brain fatty acids.

TABLE 2 | Description of the reflex test.

Reflex	Stimulus	Response
Palmar grasp (PG)	Light percussion on the palm of the right foreleg.	Quick bending of ankles.
Righting reflex (RR)	The rat is placed in supine position on a surface.	Return to the prone position with all paws in 10 s.
Cliff avoidance (CA)	The rat is placed on a flat and high surface (table), with legs toward the extremity.	Moves to one side and walks in the opposite direction to the edge.
Vibrissa placing (VP)	The animal is suspended by the tail and its vibrissae lightly touch the edge of a flat surface.	Both front legs are placed on the table, performing march movements.
Negative geotaxis (GN)	The rat is placed at the center of an inclined ramp with head facing downwards.	Body spin at an angle of 180°, positioning head upwards.
Auditory startle response (AS)	Intense and sudden sound stimulus.	Retraction of anterior and posterior legs, with rapid and involuntary body immobilization.
Free-fall righting (FFR)	Held by four legs at a height of 30 cm, it is released in free fall on a synthetic foam bed.	Position recovery during freefall on the surface supported by four paws.

Memory Evaluation Tests

Open Field Habituation Test

During adolescent phase and adulthood the animals were submitted to the Open Field Habituation test and the Object Recognition Test (ORT). Each animal was exposed to the open field twice, in the first stage, the habituation test was performed; and after 7 (seven) days, the same test was repeated in order to compare the locomotor activity of the animals for evaluation of non-associative learning (Rachetti et al., 2012). The parameter analyzed through this test is the amount of explorative interactions taken by the animal to the field, considering the locomotion of the four legs toward the interior of each field. The test observation time was 10 min. The procedure was performed between 06:00 and 08:00 a.m., on each test day, and the sessions were filmed with a video camera. For each animal tested, the apparatus was cleaned before starting, and after completion of the test with a 10% alcohol solution.

Object Recognition Test (ORT)

To evaluate the short and long term memory, the Object Recognition Task (ORT) was used. The test was performed in the open field apparatus (60 × 60 × 60 cm), colored black, with six lines crossing forming 6–20 × 20 cm quadrants, uniformly lit, and with black color objects, with different shapes (rectangular or pyramid), and textures (smooth or rough) (Nava-Mesa et al., 2013).

The test consisted of 4 (four) 10 min trials, taking place in 3 (three) steps: (1) Day 1–habituation for 10 min to minimize manipulation stress; (2) Day 2–performed 24 h after the habituation test, where each animal was placed in the open field containing two objects (FO1 and FO2) with identical textures (smooth), but with different forms (triangle and prismatic rectangle), located in two randomly chosen opposite corners. On the same day, yet 1 h later, the animal was placed in the open field again to explore two objects (FO1 in its original location, and a new object–NO1, identical to FO1 but with a different texture, and located in the place where FO2 had been placed during the habituation test; and (3) Day 3–was performed 24 h after the short duration test; each animal was placed in the open field to explore two objects (FO2 in its original place) and a new object (NO2) being identical to FO2 but with different texture (**Figure 2**).

To evaluate short-term memory, the time spent by the animal in exploring the new differently textured object (NO1) was observed. To evaluate the long-term memory, the time spent by the animal in exploring the new differently textured object (NO2) was observed at 24 h after the first exploration, on day 2. The sessions were filmed with a video camera and for each animal tested; the device was cleaned with 10% alcohol before starting and after the test. The results for the exploration times were calculated for each animal and expressed by the ratio TN/(TF + TN) TN = time spent exploring the new object; TF = time spent exploring the familiar object (Gustavsson et al., 2010; D'avila et al., 2017).

Statistical Analysis

The results of the evaluation of reflex ontogeny and somatic development were expressed as median values for the day (Min-Max), and analyzed by Kruskal-Wallis variance analysis followed by Dunn's test ($p < 0.05$). Other results were expressed as mean ± SEM, and analyzed by ANOVA followed by Tukey ($p < 0.05$). The statistical program GraphPad Prism was used.

RESULTS

Composition of Fatty Acids in Brains of Offspring After Birth

The composition of saturated fatty acids in the AO group offspring brains on the first day of life presented reduced myristic, palmitic, and stearic fatty acids as compared to the CG and AP groups ($p < 0.05$); the AP group offspring presented lower levels of myristic and higher levels of palmitic fatty acids as compared to CG offspring ($p < 0.05$) (**Table 3**).

Palmitoleic, vaccenic e oleic (monounsaturates) were also found decreased in the AO group offspring as compared to the CG and AP group offspring ($p < 0.05$). However, the AP groups presented higher values for these fatty acids then the CG and AO ($p < 0.05$) (**Table 3**).

The total PUFA content was 15% lower in the AO group offspring (10% lower in the AP group) as compared to the CG offspring. The AO offspring presented reductions in linoleic, eicosadienoic, arachidonic, docosatetraenoic, and docosahexaenoic polyunsaturated fatty acids as compared to the CG and AP offspring ($p < 0.05$). AP offspring also presented reductions in linoleic, eicosadienoico, docosatetraenoic, and docosahexaenoic acids as compared to the CG offspring ($p < 0.05$). However, eicosatrienoic and

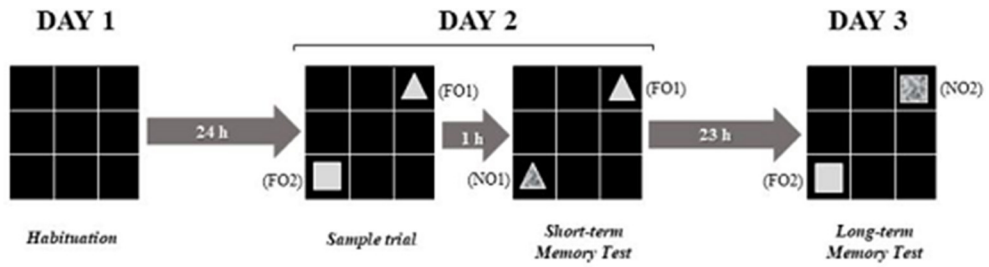

FIGURE 2 | Experimental design adapted from the object recognition test. Source: adaptation of Nava-Mesa et al. (2013).

docosapentaenoic acid levels were higher in the AP and AO offspring brains compared to the CG offspring ($p < 0.05$) (**Table 3**).

Composition of Fatty Acids in Offspring Brains at the end of Lactation (21 Days of Life)

At 21 days of age, myristic, palmitic, stearic, and behenic saturated fatty acids levels were found decreased in the AO and AP group offspring brains when compared to the CG ($p < 0.05$). The AP group presented higher levels of these fatty acids than the AO group ($p < 0.05$) (**Table 3**).

Both AO and AP groups presented lower total MUFA values, with reductions in palmitoleic, vaccenic, oleic, and erucic fatty acids in AO brains compared to the CG. For palmitoleic and oleic fatty acids, the AP group brains also presented lower total values as compared to the CG and ($p < 0.05$). Gondoic acid alone was higher in the AP group as compared to the CG ($p < 0.05$) (**Table 3**).

The polyunsaturates (linoleic, eicosadienoic, arachidonic, and docosatetraenoic acid) were decreased in the AO and AP brains as compared to the CG ($p < 0.05$). However, total PUFAs were, respectively, 13.5 and 28% higher in the AO and AP groups as compared to the CG; due to the increased DHA and docosapentaenoic acid levels. Also the total PUFAs were higher in the AP group when compared to the AO group ($p < 0.05$) (**Table 3**).

Composition of Fatty Acids in Offsprings' Brains in Adolescence (45 Days of Life)

Saturated fatty acid levels in the offspring brains (adolescents) were similar for all groups; except for behenic acid, which was higher in the AO and AP groups as when compared to the CG ($p < 0.05$) (**Table 4**).

There was no difference for monounsaturated acid contents. However, total PUFAs were 48.85% higher in the AO brains and 54.77% in the AP brains than in the CG brains. Compared to the CG brains, increased levels of DHA and docosapentaenoic fatty acid were found in the AO brains; and arachidonic, and docosahexaenoic acids were higher in the PA brains ($p < 0.05$) (**Table 4**).

Composition of Fatty Acids in the Adult Offspring Brain (90 Days of Life)

In adulthood, the content of saturated palmitic, stearic and behenic fatty acids in the AO offspring group brains was higher than the AP or control groups ($p < 0.05$). In relation to monounsaturated fatty acids, vacênic acid was different between the groups, with higher levels in the AO and AP brains as compared to the CG ($p < 0.05$). Monounsaturate erucic acid was higher in the AO brain as compared to the AP group and the controls ($p < 0.05$) (**Table 4**).

Linoleic, eicosadienoic and eicosatrienoic polyunsaturates presented higher levels in the AP offspring than in the AP and CG offspring ($p < 0.05$). Arachidonic acid was higher in the AO groups as compared to the CG, and docosahexaenoic acid presented higher levels in the AP group as compared to the AO and control groups ($p < 0.05$) (**Table 5**). Total PUFAs were higher in the brains of the AP (22%) groups as compared to the controls (**Table 4**).

Body Weight and Tail Length

The body weight results for offspring of mothers treated with avocado oil and pulp during gestation and lactation are shown in **Figure 3**. The weights of the offspring of the pulp group (AP) were significantly lower than the control group (CG) during the first week of lactation (1st and 7th day), and when compared to the oil group (AO), the weights were lower from the 7th to the 21st day ($p < 0.05$). Only on the 14th day of lactation did the AO pups present significantly higher weights as compared to the CG ($p < 0.05$). By the end of lactation, the differences differences did not persist.

The tail lengths presented significant differences only on the first day of life, where the AP pupils presented larger sizes as compared to the AO group ($p < 0.05$) (**Figure 4**). The difference did not remain beyond the 7th day (through the end of lactation).

Ontogenesis of Reflex, and Somatic Maturation

The offspring of mothers supplemented with pulp (AP) compared to the CG presented early disappearance of the PG, and appearance of the following reflexes: VP, CA, GN, AS, and FFR ($p < 0.05$). These same pups also anticipated the VP, GN, and AS reflexes as compared to the AO group ($p < 0.05$). The

TABLE 3 | Composition of fatty acids present in the brain puppies (T0 and T21) of dams supplemented with oil and avocado pulp.

	Groups					
	Brain–T0 day of life			Brain–T21 day of life		
Fatty acids	**CG**	**AO**	**AP**	**CG**	**AO**	**AP**
SATURATED						
Myristic acid **C14:0**	1.51 ± 0.05	1.15 ± 0.04*	1.40 ± 0.05*#	0.40 ± 0.00	0.32 ± 0.01*	0.43 ± 0.11*#
Palmitic acid **C16:0**	25.27 ± 0.30	22.57 ± 0.20*	26.16 ± 0.40*#	20.11 ± 0.05	17.58 ± 0.07*	18.88 ± 1.01*#
Stearic acid **C18:0**	15.63 ± 0.10	13.40 ± 0.04*	15.01 ± 0.09*#	17.90 ± 0.50	15.16 ± 0.64*	15.92 ± 0.84*#
Behenic acid **C22:0**	–	–	–	0.16 ± 0.01	0.13 ± 0.02*	0.12 ± 0.01*
Total	42.41	37.12*	42.57*	38.57	33.19*	35.35*#
MONOUNSATURATED						
Palmitoleic acid **C16:1ω7c**	3.74 ± 0.03	1.48 ± 0.02*	3.93 ± 0.03*#	0.69 ± 0.00	0.47 ± 0.01*	0.39 ± 0.02*#
Vaccenic acid **C18:1ω7c**	2.90 ± 0.02	2.63 ± 0.03*	3.03 ± 0.01*#	2.91 ± 0.10	2.54 ± 0.18*	2.76 ± 0.22#
Oleic acid **C18:1ω9**	11.12 ± 0.10	9.86 ± 0.09*	11.41 ± 0.10*#	12.96 ± 0.20	10.83 ± 0.26*	11.74 ± 1.19*#
Gondoic acid **C20:1ω9**	0.20 ± 0.02	0.22 ± 0.01*	0.20 ± 0.02#	0.52 ± 0.04	0.60 ± 0.06	0.67 ± 0.12*
Erucid acid **C22:1ω9**	–	–	–	0.09 ± 0.01	0.06 ± 0.01*	0.07 ± 0.03
Total	17.96	14.19*	18.57*#	17.17	14.50*	15.56*#
POLYUNSATURATED						
Linoleic acid **C18:2ω6c**	1.04 ± 0.10	0.78 ± 0.07*	0.86 ± 0.09*#	1.37 ± 0.10	0.93 ± 0.08*	0.94 ± 0.16*
Eicosadienoic acid **C20:2ω6**	1.01 ± 0.10	0.15 ± 0.01*	0.16 ± 0.01*#	0.27 ± 0.02	0.20 ± 0.02*	0.20 ± 0.04*
Dihomo-γ-linolenic acid **C20:3 ω6**	0.46 ± 0.20	0.58 ± 0.30*	0.61 ± 0.40*#	0.39 ± 0.01	0.39 ± 0.04	0.39 ± 0.07
Arachidonic acid **C20:4ω6c**	10.15 ± 0.10	8.67 ± 0.09*	10.14 ± 0.12#	10.18 ± 0.20	8.76 ± 0.35*	9.27 ± 0.44*#
Docosatetraenoic acid **C22:4 ω6**	3.27 ± 0.02	2.60 ± 0.28*	2.64 ± 0.20*#	3.48 ± 0.02	2.47 ± 0.09*	3.11 ± 0.56#
Docosapentaenoic acid **C22:5 ω3**	2.71 ± 0.09	4.00 ± 0.12*	3.35 ± 0.10*#	0.78 ± 0.10	4.17 ± 0.02*	6.99 ± 0.04*#
Docosahexaenoic acid **C22:6ω3**	8.19 ± 0.32	6.35 ± 0.23*	6.78 ± 0.40*#	10.74 ± 0.00	12.17 ± 1.69*	13.98 ± 0.9*#
Total	26.83	23.13*	24.27*#	27.31	31.00*	34.88*#
SUMS AND RATIONS						
PUFA/SFA	0.63	0.62*	0.57*#	0.47	0.93*	0.99*#
ω3	11.36	10.93*	10.47*#	12.01	18.64*	21.36*#
ω6	15.47	12.20*	13.80*#	15.30	12.36*	13.52*#
ω9	0.20	0.22*	0.20#	0.61	0.66*	0.67*
ω6/ω3	1.36	1.12*	1.32*	1.27	0.67*	0.63*#

*Data expressed as mean ± standard deviation. CG, Control Group; AO, Avocado Oil Group; AP, Avocado Pulp Group. *vs. CG. #vs. AO. T0, at birth; T21, at weaning (21 days of life). Statistical test used was One way Anova, followed by Tukey with a ($p < 0.05$) level of significance.*

AO offspring, in relation to the CG, presented early PG onset, and the appearance of CA, GN, and FFR ($p < 0.05$) (**Table 5**).

For the somatic indicators, the AP neonates presented anticipation in auditory conduction opening and epidermic hair appearance, yet delayed eruption of inferior incisors as compared to the CG ($p < 0.05$). The same group (AP) when compared to the AO group presented anticipated auditory conduit opening together with superior incisor eruption ($p < 0.05$). The neonates of the AO group presented auditory conduction opening and inferior incisor eruption delays when compared to the CG ($p < 0.05$) (**Table 6**).

Behavioral Testing

Open Field

Open Field Habituation Test ambulatory analysis at 45 days (adolescent stage) presented differences between the first and second exposures, with a decrease in the ambulation parameter during the second exposure for all groups: CG (77. 50 ± 5.75 and 55.36 ± 5.44), AO (129.92 ± 11.16 and 55.75 ± 5.44), and AP (115.56 ± 11.13 and 56.25 ± 5.10) ($p < 0.05$) (**Figure 5A**). In the adult phase (T90) the same differences persisted, yet with ambulation exposure decreases in the CG (112.82 ± 10.57 and 51.67 ± 5.57), AO (95.83 ± 8.77 and 38.50 ± 4.97), and AP (99.71 ± 9.09 and 60.50 ± 5.95) ($p < 0.05$) **(Figure 5B)**.

Object Recognition Test (ORT)

Adolescent phase

In the adolescent phase, the rate of new object exploration in the short term and in the long term tests was higher in the AO and AP groups, presenting higher exploration rates as compared to the CG ($p < 0.05$) (**Figures 6A,B**). The groups AO and AP presented greater time for the new object, relative to the familiar object, in both short and long periods ($p < 0.05$) (**Figures 6C,D**).

Adulthood

Adult offspring in the AP and AO groups also presented higher rates of new object exploration in the short and long term ($p < 0.05$) (**Figures 7A,B**). The groups AO and AP

TABLE 4 | Composition of fatty acids present in the brain offspring (T45 and T90) of dams supplemented with oil and avocado pulp.

	Groups					
	Brain–T45 day of life			Brain–T90 day of life		
Fatty acids	**CG**	**AO**	**AP**	**CG**	**AO**	**AP**
SATURATED						
Myristic acid **C14:0**	0.12 ± 0.04	0.11 ± 0.00	0.12 ± 0.01	0.13 ± 0.02	0.13 ± 0.02	0.11 ± 0.01
Palmitic acid **C16:0**	15.82 ± 2.60	14.89 ± 1.14	16.22 ± 2.11	16.11 ± 0.90	17.83 ± 0.42*	16.28 ± 1.73#
Stearic acid **C18:0**	16.21 ± 2.91	15.29 ± 1.02	15.89 ± 1.11	16.42 ± 0.94	18.65 ± 0.21*	17.04 ± 1.62#
Behenic acid **C22:0**	0.19 ± 0.03	0.24 ± 0.02*	0.22 ± 0.01*	0.25 ± 0.02	0.33 ± 0.01*	0.27 ± 0.05#
Lignoceric acid **C24:0**	–	0.15 ± 0.01	0.16 ± 0.01#	0.19 ± 0.01	0.19 ± 0.01	0.19 ± 0.01
Total SAT	32.34	30.68*	32.61*#	33.10	37.13*	33.89*#
MONOUNSATURATED						
Palmitoleic acid **C16:1ω7c**	0.34 ± 0.02	0.30 ± 0.05	0.45 ± 0.29	0.30 ± 0.03	0.27 ± 0.04	0.25 ± 0.07
Vaccenic acid **C18:1ω7c**	3.22 ± 0.61	3.09 ± 0.07	3.32 ± 0.34	3.65 ± 0.34	4.07 ± 0.06	3.78 ± 0.66
Oleic acid **C18:1ω9**	13.37 ± 2.28	13.05 ± 0.19	13.95 ± 1.39	15.47 ± 1.36	17.24 ± 1.21	16.23 ± 2.52
Gondoic acid **C20:1ω9**	1.66 ± 0.28	1.72 ± 0.16	1.79 ± 0.02	2.72 ± 0.35	3.16 ± 0.11	2.99 ± 0.91
Erucid acid **C22:1ω9**	0.17 ± 0.08	0.20 ± 0.06	0.17 ± 0.07	0.29 ± 0.00	0.38 ± 0.03*	0.27 ± 0.11#
Total Monounsat	18.69	18.36*	19.68*#	22.43	25.11*	23.52*#
POLYUNSATURATED						
Linoleic acid **C18:2ω6c**	0.59 ± 0.13	0.63 ± 0.08	0.61 ± 0.11	0.61 ± 0.00	0.67 ± 0.04*	0.53 ± 0.06*#
Eicosadienoic acid **C20:2ω6**	0.23 ± 0.06	0.22 ± 0.00	0.22 ± 0.02	0.22 ± 0.01	0.25 ± 0.00*	0.12 ± 0.02*#
Dihomo-γ-linolenic acid **C20:3 ω6**	0.35 ± 0.07	0.36 ± 0.03	0.39 ± 0.00	0.28 ± 0.02	0.36 ± 0.01*	0.24 ± 0.02#
Arachidonic acid **C20:4ω6c**	6.41 ± 0.43	7.09 ± 0.52	8.06 ± 0.64*#	7.23 ± 0.61	8.17 ± 0.74*	7.59 ± 0, 10
Docosatetraenoic acid **C22:4 ω6**	3.20 ± 0.70	3.17 ± 1.02	3.90 ± 0.59	2.74 ± 0.23	2.79 ± 0.16	2.80 ± 0.18
Docosapentaenoic acid **C22:5 ω3**	3.28 ± 0, 69	4.60 ± 0.89*	3.28 ± 0.85#	1.43 ± 0.13	1.40 ± 0.02	1.40 ± 0.09
Docosahexaenoic acid **C22:6ω3**	11.39 ± 1.37	15.86 ± 1.03*	16.76 ± 1.52*	11.45 ± 1.24	10.48 ± 0.43	15.80 ± 0.97*#
Total	25.45	31.93*	33.22*#	23.96	24.12*	28.48*#
SUMS AND RATIONS						
PUFA/SFA	0.79	1.04*	1.02*#	0.72	0.65*	0.84*#
ω3	15.02	20.82*	20.43*#	13.16	12.24*	17.44*#
ω6	10.43	11.11*	12.79*#	10.80	11.88*	11.04*#
ω9	1.83	1.92*	1.96*#	3.01	3.54*	3.26*#
ω6/ω3	0.69	0.53*	0.63*#	0.82	0.97*	0.63*#

*Data expressed as mean ± standard deviation. CG, Control Group; AO, Avocado oil Group; AP, Avocado Pulp Group of. *vs. CG. #vs. AO. T45, adolescent phase (45 days of life); T90, adult phase (90 days of life). Statistical test used was One Anova way followed by Tukey with a level of significance of ($p < 0.05$).*

presented greater time for the new object, relative to the familiar object, in both short and long periods ($p < 0.05$) (**Figures 7C,D**).

DISCUSSION

Maternal fatty acids transferred via the placenta, and through the breast milk are considered critical for growth and development (Lauritzen and Carlson, 2011; Innis, 2014). Thus, during gestation and lactation, manipulation of lipids can affect the availability of fatty acids to the fetus and the infant. In the present study, avocado oil and pulp supplementation during gestation and lactation positively influenced the offspring in: (1) reflex development, (2) somatic maturation, (3) and memory acquisition (4) the fatty acid profiles of the brains of the neonates, adolescents, and adult offspring.

Maternal consumption of distinct lipids presents differing consequences for the weight, growth, and somatic parameters in their offspring. At birth, and during the first week of lactation, the results reveal that the offspring of the AP mothers had lower weights than the CG. Several studies have reported a decrease in the body weights of offspring with mothers receiving lipids from differing sources; at times presenting similar lipid profiles as compared to those used in the present research, such as cashew nuts (Melo et al., 2017), which has fiber as Avocado pulp and Buriti oil (*Mauritia flexuosa*) (Medeiros et al., 2015), and olive oil (Sánchez et al., 2012; Priego et al., 2013), which are source of polyunsaturated fatty acids as the lipids used in the present research (pulp and avocado oil). The presence of fiber and polyunsaturated fatty acids in maternal diet can induce reduction of plasma triglycerides (TG). Increased maternal levels have been used as a biochemical marker to increase offspring birth weight (Barbour and Hernandez,

TABLE 5 | Reflex maturation in offspring of mothers supplemented with avocado oil and pulp during gestation and lactation.

Reflexes	Groups		
	CG	AO	AP
Palmar grasp (PG)[a]	8 (6–13)	5 (3–7)*	4 (3–5)*
Righting reflex (RR)	4 (1–9)	4 (2–7)	4 (2–6)
Vibrissa placing (VP)[b]	10 (5–13)	9 (7–10)	7 (7–10)*#
Cliff avoidance (CA)[b]	10 (6–15)	6 (5–8)*	6 (2–10)*
Negative geotaxis (GN)[b]	20 (19–21)	13 (12–14)*	10 (10–12)*#
Auditory tartle response (AS)[b]	12 (11–13)	13 (12–13)	11 (10–12)*#
Free-fall righting (FFR)[b]	12 (8–15)	7 (2–14)*	5 (2–9)*

Data were expressed as mean values of the day (Min-Max) and analyzed by Kruskal-Wallis analysis of variance followed by Dunn's test ($p < 0.05$).
**Compared to control group.*
#Compared to the avocado oil group.
Considering: [a]Day of response disappearance and [b]Day of response appearance. CG (Control Group-n = 15), AO (Avocado Oil Group-n = 15), AP (Avocado Pulp Group-n = 15).

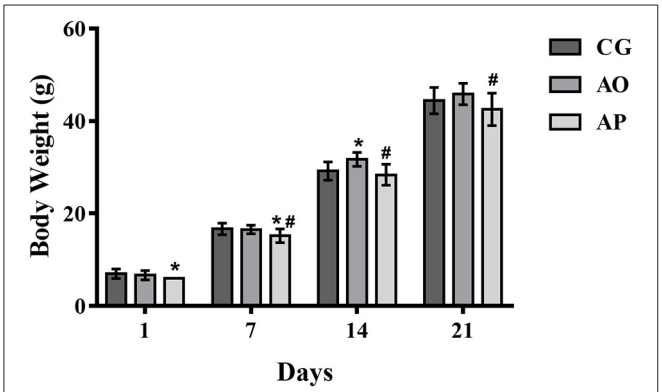

FIGURE 3 | Mean body weight in grams (g) (±SEM) of neonatal rats whose mothers received supplementation with avocado oil and pulp (3,000 mg/kg body weight) during gestation and lactation. ANOVA followed by Tukey ($p < 0.05$). (*) statistically different as compared to CG; (#) statistically different as compared to AO. CG (Control Group-$n = 15$), AO (Avocado Oil Group-$n = 15$), AP (Avocado Pulp Group-$n = 15$).

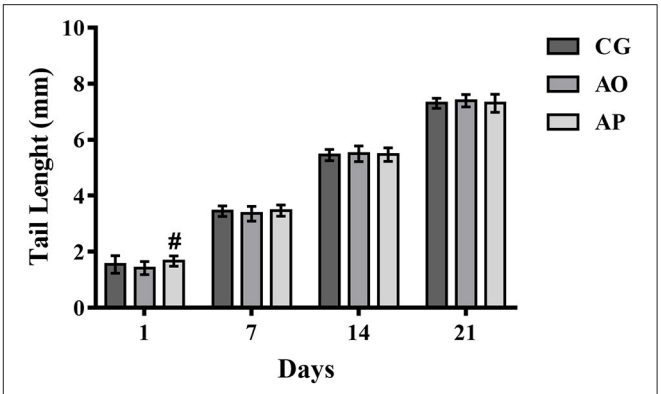

FIGURE 4 | Tail lengths of the offspring of mothers supplemented with avocado oil and pulp (3,000 mg/kg body weight) during gestation and lactation. Data expressed as mean ± SEM and analyzed by ANOVA followed by Tukey ($p < 0.05$). (#) statistically different as compared to the AO group. CG (Control Group-$n = 15$), AO (Avocado Oil Group-$n = 15$), AP (Avocado Pulp Group-$n = 15$).

TABLE 6 | Somatic development in offspring of mothers supplemented with avocado oil and pulp during gestation and lactation.

Physical characteristics	Groups		
	CG	AO	AP
Ear unfolding	3 (2–4)	3 (2–4)	3 (2–4)
Auditory conduit opening	14 (13–15)	13 (12–13)*	11 (10–12)*#
Eye opening	14 (12–15)	13 (12–15)	14 (12–15)
Eruption of superior incisors	10 (8–12)	11 (9–12)	9 (8–11)#
Eruption of inferior incisors	4 (2–5)	8 (8–9)*	7 (7–8)*
Epidermic hair appearance	3 (2–4)	3 (3–3)	3 (2–3)*

Data were expressed as mean values of the day (Min-Max), analyzed by Kruskal-Wallis analysis of variance, followed by Dunn's test ($p < 0.05$).
**Compared to the control group.*
#Compared to the avocado oil group.
CG (Control Group-n = 15), AO (Avocado Oil Group-n = 15), AP (Avocado Pulp Group-n = 15).

2018) and their lower plasma levels have been associated with improved insulin sensitivity and lower caloric influx (Nolan et al., 1995). In contrast, diets with high levels of SFA induce an increase in plasma triglycerides, consequently, they also can induce increase in the offspring weight (Ferro Cavalcante et al., 2013; Soares et al., 2013; Cadena-Burbano et al., 2017). Therefore, it was observed in the present research a reduction in TG at the end of the lactation of the mothers fed with the pulp, when compared to the others groups (data not shown). These findings are in agreement with Barbour and Hernandez (2018).

Maternal supplementation with avocado promoted acceleration in the postnatal appearance of several somatic parameters. Lipids are recognized for promoting somatic growth in the offspring (Del Prado et al., 1997). Both avocado oil and pulp, despite having high amounts of oleic and palmitoleic acids in their composition, have ω-6 and ω-3 fatty acids, which have been associated with physical growth in rat progeny (Santillán et al., 2010; Ferro Cavalcante et al., 2013). These results are consistent with experiments that used PUFA and MUFA in the source foods (Ferro Cavalcante et al., 2013; Melo et al., 2017), and the same for avocado lipids, yet results diverge for SFA-source diets (Soares et al., 2009). The findings confirm that both the quality and amount of lipids in the maternal diet directly influence physical development in the offspring (Hausman et al., 1991). DHA and ARA (in combination) are essential for optimal growth and development early in life (Harauma et al., 2017).

During the critical developmental phase, essential fatty acids are needed for physical growth and good brain development. The brain goes through processes that include neural network organization; accumulation of DHA and ARA occurs to support active neurogenesis and neuronal growth (Lauritzen and Carlson,

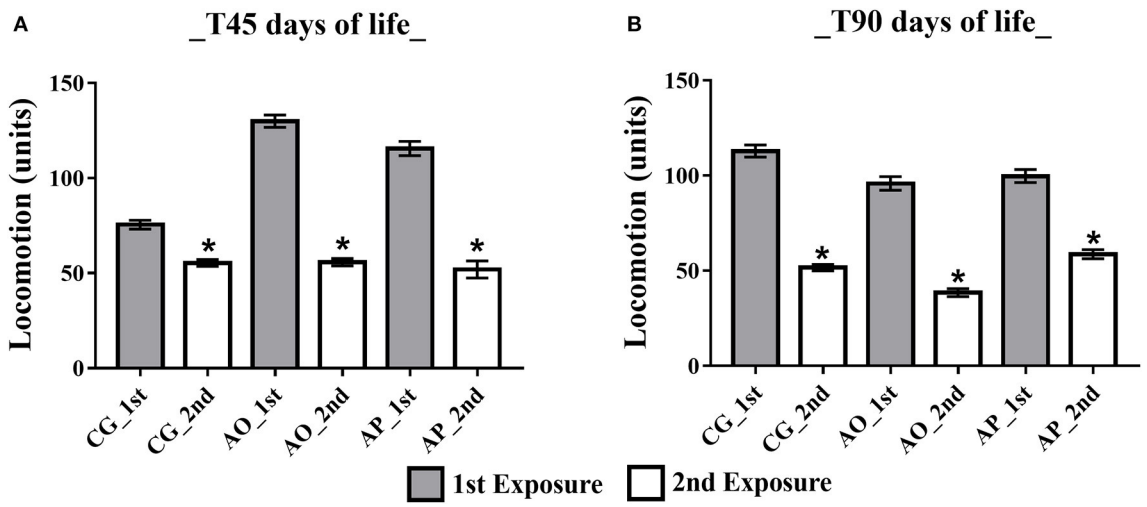

FIGURE 5 | Influence of maternal supplementation with avocado oil and pulp on total offspring ambulation. Data expressed as mean and standard error (±SEM), analyzed by ANOVA, and followed by Tukey ($p < 0.05$). **(A)** Adolescent phase offspring (T45); **(B)** Adult offspring (T90). 1st: first exposure; 2nd: second exposure. CG (Control Group-$n = 15$), AO (Avocado Oil Group-$n = 15$), AP (Avocado Pulp Group-$n = 15$). *$p < 0.05$ vs. 1st exposure in the open field.

2011; Innis, 2014), while modifying the fluidity and signaling of neuronal membranes (Bazinet and Layé, 2014). In this period, specific brain regions, including the hippocampus, striatum, visual and auditory cortices respond similarly to nutritional insults (Kretchmer et al., 1996), leading to long-term effects (Morgane et al., 1993; Arcego et al., 2017).

Our results showed that at the end of gestation, or at the beginning of the postnatal phase (T0), there was less incorporation of DHA (C22: 6n3) in the brains of the AO and AP offspring. However, by the end of lactation, levels of DHA had increased in brains of the AO and AP offspring as compared to the control groups. In rodents, fetal demand for fatty acid incorporation occurs from the last week of gestation to the end of lactation (Morgane et al., 2002). This explains the observed increase in DHA incorporation in the T21 brain levels, as compared to T0 levels. Accumulation of fatty acids in the offsprings' brains is influenced by pre-fetal and post-fetal maternal supply (Innis, 2011). Avocado oil and pulp present low linolenic acid content (ALA); a DHA precursor. The increases observed in the brain levels for this fatty acid in the offspring of mothers who consumed avocado oil and pulp oppose studies that have found a positive relation between low ALA content and low proportions of DHA in offspring brain tissue (Amusquivar et al., 2000; Melo et al., 2017; Lopez-Soldado et al., 2018). However, avocado presents high phospholipid (PL) content, present in the lipid fraction of its pulp (Cowan and Wolstenholme, 2016; Pacetti et al., 2017) and oil (Takenaga et al., 2008). Increases in DHA uptake in the brains of the AO and AP offspring can be explained by the presence of phospholipids in avocado. DHA is synthesized by ALA desaturation and stretching reactions (Pereira et al., 2003; Novak et al., 2008), and when esterified into PLs, is more efficiently incorporated into brain tissue (Murru et al., 2013; Kitson et al., 2016; Destaillats et al., 2018). Of the phospholipids, lyso-phosphatidylcholine (LPC) as esterified to DHA (LPC-DHA) is the most efficient way to cross the blood-brain barrier inducing a greater deposition of DHA in the brain (Nguyen et al., 2014). In the fetal brain formation and postnatal development periods, LPC-DHA is associated with an increase in exogenous PUFA uptake and deposition in the membranes of brain tissue, which promotes higher DHA deposition (Chan et al., 2018). One study reveals that offspring of mothers fed LPC from DHA-enriched eggs present higher levels of this FA in certain brain regions (Valenzuela et al., 2010). These findings are similar to the data found in the present study.

Reflex ontogeny is another parameter used to evaluate development because it measures maturation and central nervous system function early in life (Fox, 1965; Smart and Dobbing, 1971). It also reflects the integrity of cerebellar and sensorimotor development, and of vibrissae integration (Zhang et al., 2010). Adequate reflex development depends on myelination and synapse processes, and the action of neurotransmitters (Bourre et al., 1987; Morgane et al., 1993). The anticipation of the negative geotaxis demonstrates positive evolution in labyrinth and/or vestibule function, while anticipation of cliff avoidance reflects sensorimotor function maturity (Santillán et al., 2010). Righting reflex involves both motor and visual functions (Boyle, 2001) and confirms the nervous system's maturation. Our results showed that avocado oil and pulp promoted acceleration of neonate reflex maturation. A number of experimental studies support the results of the present study for animals treated with cashew nuts (Melo et al., 2017), soybean and fish oil (Santillán et al., 2010), and goat's milk fat (Soares et al., 2013). However, our results verify that consumption of avocado pulp promotes a more pronounced acceleration in reflex; by anticipating six of the seven observed parameters. At the end of lactation, SFA and MUFA levels were lower in the brains of the animals treated with oil and pulp than in the control group; while PUFA levels were higher in the

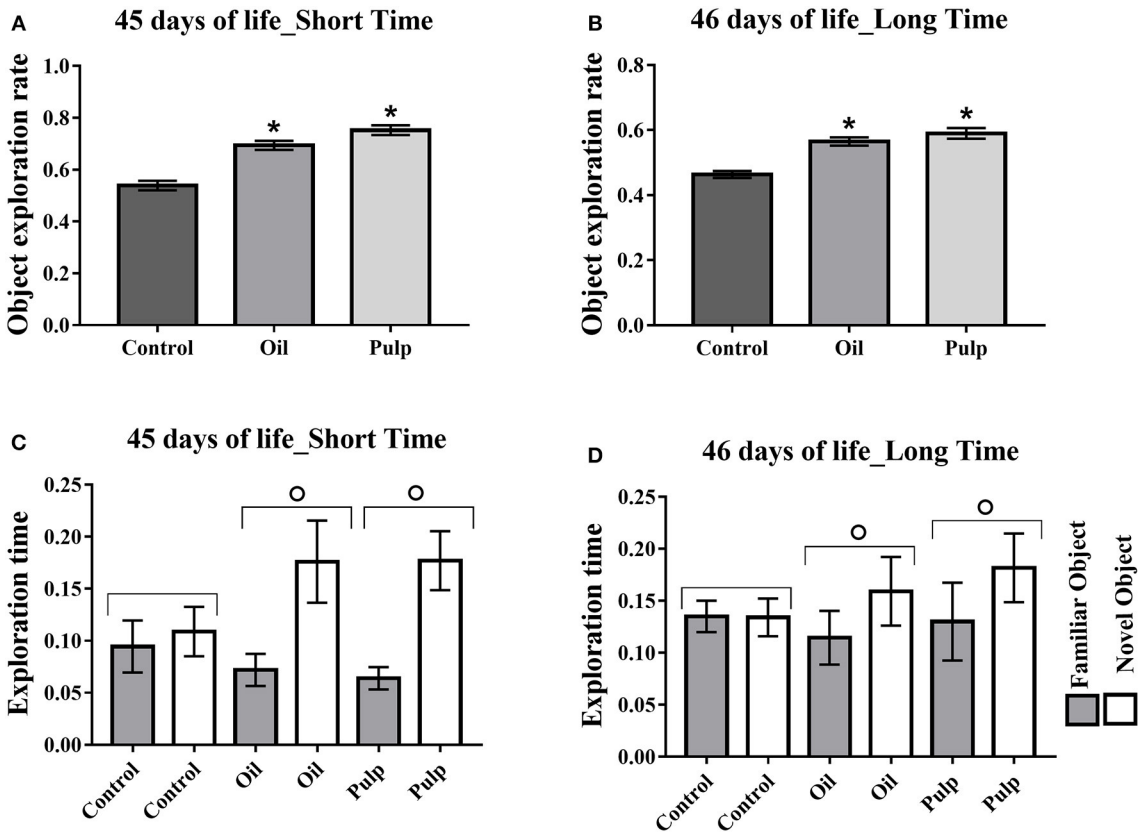

FIGURE 6 | Adolescent offspring; short and long term memory test; preference in the object exploration. Data expressed as mean and standard error (±SEM) **(A,B)** and mean and standard deviation (±SD) **(C,D)** Analyzed by ANOVA, and followed by Tukey ($p < 0.05$). **(A)** Object exploration rate short term. **(B)** Object exploration rate long term. **(C)** Time of exploration of the family object and new object in the short term. **(D)** Time of exploration of the familiar object and new object in the long term. *Indicates a significant difference between the AO and AP groups vs. the CG in new object exploration time ($p < 0.05$). °Indicates a significant difference for the same group, in the time of exploration of the familiar object and the new object. Control (Control Group-$n = 15$), Oil (Avocado Oil Group-$n = 15$), Pulp (Avocado Pulp Group-$n = 15$).

brains of the animals treated with oil (13.5%) and pulp (28%). These results suggest that high levels of PUFA may be directly related to the offspring's reflex development and the higher consumption of pulp justifies the better result observed in these groups.

The avocado used in the present research is a source of bioactive components such as phenolics, flavonoids, and carotenoids (Ameer, 2016), and the pulp has more of these compounds than the oil. These substances cross the placental barrier reaching the fetal tissue (Todaka et al., 2005), accumulating in the retina (carotenoids) and playing an important role in the development of vision and the nervous system (Hammond, 2015; Zielinska et al., 2017). Thus, neonate neuroprotection (polyphenols) (Loren et al., 2005) can induce acceleration of somatic development and reflex in the offspring (phenolics and flavonoids) (Ajarem et al., 2017). As well was observed in the present work, where both avocado oil and pulp promoted such acceleration in the development of the offspring; the results for pulp being more pronounced. An opposing result was found by Medeiros et al. (2015) D'avila et al. (2017), where the offspring of mothers supplemented with Buriti oil (rich in carotenoids), presented delayed onset for palm grasp, righting reflex and cliff avoidance reflexes.

In addition, we investigated long-lasting effects of maternal supplementation on adolescent (T45) and adult (T90) offspring, evaluating the influence of avocado consumption on animal memory. At different stages of the cycle, neurons are continuously produced in the dentate gyrus of the hippocampus, but the ontogenetic stage in which the neurogenesis occurs is crucial for memory processing. Neurons in the neonatal phase are activated through different memory processes (Tronel et al., 2015). Learning and memory processes are performed in the hippocampus dentate gyrus in cooperation with the cerebral cortex (Eichenbaum and Lipton, 2008; Coutureau and Di Scala, 2009), and PUFAs, through metabolic imprinting mechanisms affect brain functions during the development phase and promote permanent effects (van Dijk et al., 2011; Yehuda, 2012).

ARA and DHA are important constituents of membranes, especially brain tissue (Martinez, 1992; Innis, 2007) and are involved in different mechanisms that affect animal memory.

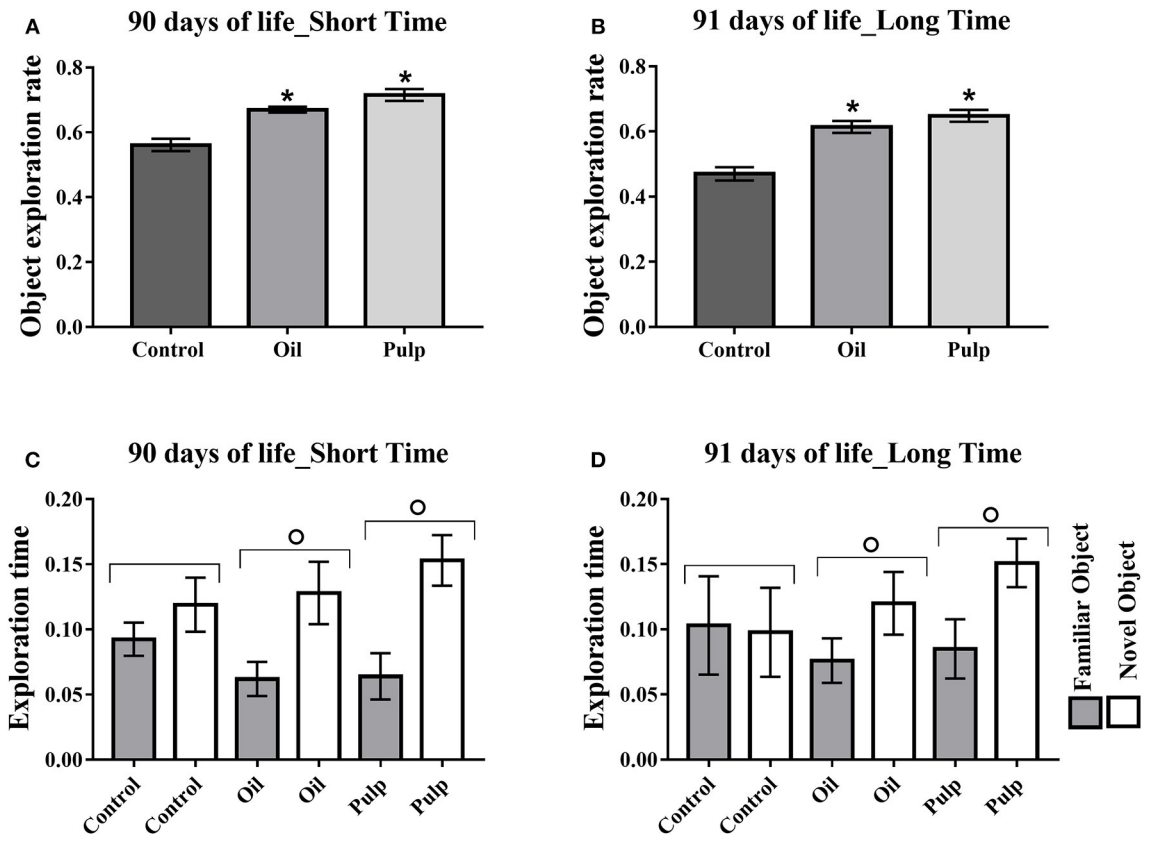

FIGURE 7 | Exploration of test objects (short and long-term memory) in adult offspring. Data expressed as mean and standard error of the mean (±SEM), **(A,B)** and mean and standard deviation (±SD) **(C,D)** analyzed by ANOVA, followed by Tukey ($p < 0.05$). **(A)** Short term memory object exploration test. **(B)** Long-term memory object exploration test. **(C)** Time of exploration of the family object and new object in the short term. **(D)** Time of exploration of the familiar object and new object in the long term. *Indicates a significant difference between the AP and AO groups vs. the CG in new object exploration time. °Indicates a significant difference for the same group, in the time of exploration of the familiar object and the new object. Control (Control Group-$n = 15$), Oil (Avocado Oil Group-$n = 15$), Pulp (Avocado Pulp Group-$n = 15$).

DHA is involved in the expression of BNDF (brain derived neurotrophic factor), NMDA receptor (N-methyl-D-aspartate) synthesis, induction of LTP (long-term potential), and liberating glutamate in glutamatergic functions. Deficiency of ω-3 PUFA alters the fatty acid composition of the fetal brain with repercussions in the adult phase, increases fetal inflammatory processes, and induces deficits in development and memory (Labrousse et al., 2018). ARA is involved in the regulation of the cholinergic neurotransmission system and in the GABA/Glu regulatory system decreasing oxidative damage, and cellular apoptosis (Li et al., 2015). These two PUFAs were incorporated into the offsprings' brains through maternal supplementation with avocado, and ARA presented higher levels in the brains of the AO group in adolescence and the AP group as adults, while DHA presented higher levels in the AP and AO groups in adolescence and only in AP animals in adulthood.

In the present study we used the Open Field Habituation test and the Object Recognition Test (ORT) for evaluation of non-associative learning of the adolescent and adult offspring. In the Open Field Habituation test, repeated exposure to the same environment tends to cause a decrease in locomotion, recognized as a form of non-associative learning (Rachetti et al., 2012). Our results showed that in the adolescent and adult offspring, maternal supplementation with avocado oil and pulp reduced locomotion in the second exposure. The same effect has also observed in the animals fed a diet containing cashew nuts (Melo et al., 2017) and fish oil (Rachetti et al., 2012). Increased habituation, yet with memory impairment has occurred with peanut oil, containing little LA (Frances et al., 1996), but with an excess in saturated fat (Page et al., 2014).

The Object Recognition Test (ORT) involves an acquisition phase, where the rodent explores a chamber containing two similar objects and a recall phase, which occurs after a time interval in which one object is replaced by a new one. From the time interval used between the exposures, and from the ratio of time spent on the exploration of the new vs. the familiar object, and from the greater interaction with the new object, we may observe facilitation of short and long term memory (Cordner and Tamashiro, 2015); recognition of place, that involves the hippocampus (Barker and Warburton, 2011) and preference for

the new object, which involves the prefrontal cortex (Mumby and Pinel, 1994; Bussey et al., 2000). Our results demonstrated that maternal supplementation with avocado oil and pulp facilitated acquisition of recognition memory in the adolescent and adult offspring, evidenced by a higher exploration rate (of the new object), both short and long term. Melo et al. (2017) has demonstrated that a maternal diet containing cashew nuts yields good short-term memory performance in the offspring. The offspring of mothers supplemented with fish oil has been shown to present good long-term memory performance (Rachetti et al., 2012). Other studies have demonstrated improvements in cognitive performance in offspring in relation to maternal consumption of olive (Pase et al., 2015) and linseed oils (Fernandes et al., 2011). However, maternal consumption of high ω-6/ω-3 ratio (Lépinay et al., 2015); saturated fats (Frances et al., 1996; Souza et al., 2012; Arcego et al., 2017), hydrogenated vegetable fat (Pase et al., 2017) and interesterified fat (D'avila et al., 2017) caused damage to the animals' memory. The results obtained in our study demonstrate that increased ARA and DHA levels in the brains of the offspring of the supplemented groups interfered directly in memory development. Yet both DHA and ARA are responsible for maintaining optimal growth and functional behavior of the offspring (Harauma et al., 2017). DHA, in particular, is capable of protecting the hippocampus against oxidative stress and apoptosis; preventing memory deficits (Gao et al., 2016).

The positive effects of maternal supplementation with avocado oil and pulp on the memory of from adolescents to adult offspring can also be explained by its antioxidant potential. Experimentally, the antioxidant action of this fruit has been proven in diabetic rats supplemented with its oil (Ortiz-Avila et al., 2015). Studies have shown that the effect of maternal consumption of flavonoids on offspring memory is associated with decreased oxidative brain damage; due to reductions in lipid peroxidation levels, and generation of reactive species, and an increase in the antioxidant defense system as well as BDNF in the adult rat pre-frontal cortex (Bussey et al., 2000), and modulation of hippocampal signaling (Corona et al., 2013).

In the present study, the fatty acid profile in the brains of the offspring of mothers supplemented with avocado oil and pulp during gestation and lactation was measured at different stages of the life cycle. The animals of the two experimental groups, oil and pulp showed better somatic maturation, an anticipation of reflexes and improvement in memory. These findings demonstrate the benefits that maternal supplementation with a source of monounsaturated fatty acids and antioxidant compounds can bring to the development of the brain, persisting into adulthood.

As a limitation of this study, the groups treated with avocado were not compared with animals treated with lipid source deficient in essential fatty acids. On the other hand, we objected with the present study to define whether maternal supplementation with pulp and avocado oil could have a distinct effect on neurodevelopment of the offspring.

CONCLUSION

Maternal supplementation with avocado oil and pulp influences the development of the nervous system of the offspring in the short and long term, accelerating somatic development and reflex maturation while improving memory in the adolescent and adult phases.

AUTHOR CONTRIBUTIONS

This research was carried out by all authors. JS, MP, and MM designed the theme of the study. MM, RM, ES, DP, and MC performed the experimental methods designed. SS and CD performed fatty acid analysis and VV carried out analysis of the antioxidant components. JS, MO, FM, and MM analyzed the data. JS and MM interpreted the results and wrote the article.

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REVIEW
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Glycolysis-Derived Compounds From Astrocytes That Modulate Synaptic Communication

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Based on the concept of the tripartite synapse, we have reviewed the role of glucose-derived compounds in glycolytic pathways in astroglial cells. Glucose provides energy and substrate replenishment for brain activity, such as glutamate and lipid synthesis. In addition, glucose metabolism in the astroglial cytoplasm results in products such as lactate, methylglyoxal, and glutathione, which modulate receptors and channels in neurons. Glucose has four potential destinations in neural cells, and it is possible to propose a crossroads in "X" that can be used to describe these four destinations. Glucose-6P can be used either for glycogen synthesis or the pentose phosphate pathway on the left and right arms of the X, respectively. Fructose-6P continues through the glycolysis pathway until pyruvate is formed but can also act as the initial compound in the hexosamine pathway, representing the left and right legs of the X, respectively. We describe each glucose destination and its regulation, indicating the products of these pathways and how they can affect synaptic communication. Extracellular L-lactate, either generated from glucose or from glycogen, binds to HCAR1, a specific receptor that is abundantly localized in perivascular and post-synaptic membranes and regulates synaptic plasticity. Methylglyoxal, a product of a deviation of glycolysis, and its derivative D-lactate are also released by astrocytes and bind to $GABA_A$ receptors and HCAR1, respectively. Glutathione, in addition to its antioxidant role, also binds to ionotropic glutamate receptors in the synaptic cleft. Finally, we examined the hexosamine pathway and evaluated the effect of GlcNAc-modification on key proteins that regulate the other glucose destinations.

Keywords: astrocyte, glycolysis, GSH, lactate, methylglyoxal, neurotransmission

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AIM

Glucose is the major energetic source of neural cells. In addition to providing ATP via the glycolytic pathway, glucose provides metabolites that are key to brain activity, such as glutamate and NADPH for lipid and glutathione (GSH) synthesis, as well as recycling of ascorbic acid. This short review will focus on glucose-derived compounds from astrocytes that modulate neurotransmission, in addition to providing energetic and substrate replenishment for brain activity. Glutamate, for example, is the main excitatory neurotransmitter and originates from astroglial glucose, as it is synthetized *de novo* from alpha-ketoglutarate. Two specific astrocyte enzymes, pyruvate carboxylase and glutamine synthetase, are necessary for this process. We will restrict this review to the role of glucose-derived compounds (arising directly from the glycolytic pathway in the cytoplasm of astroglial cells) that modulate synaptic receptors or transporters

by binding to them, such as lactate, methylglyoxal, and GSH. Moreover, we will review the regulatory role of uridine diphosphate-*N*-Acetylglucosamine (UDP-GlcNAc), which covalently regulates several astrocyte proteins, including glucose metabolism enzymes and related transcription factors, which in turn modulate synaptic communication.

INTRODUCTION

Preliminarily, it is important to highlight the importance of astrocytes in the synapse, particularly in glucose metabolism. Although there is no doubt about the significance of blood glucose for brain activity, the mode of entry of glucose to the brain and its cell distribution are still debated (e.g., Lundgaard et al., 2015; Barros et al., 2017). Once inside the cell, glucose is phosphorylated on carbon 6 by hexokinase (HK), generating glucose-6P, which is converted to fructose-6P via the action of an isomerase. These two compounds (glucose-6P and fructose-6P) are used in at least two different pathways. It is possible to propose a metaphorical "X" intersection of these reactions, as illustrated in **Figure 1**, to describe the destinations of glucose in the neural cells. Glucose-6P can be converted to fructose-6P, but can also be used for glycogen synthesis or in the pentose phosphate pathway (PPP) (left and right arm of the X, respectively). On the other hand, fructose-6P continues through the glycolysis pathway until pyruvate is formed, but can also act as the initial compound in the hexosamine pathway, as represented by the left and right legs of the X, respectively.

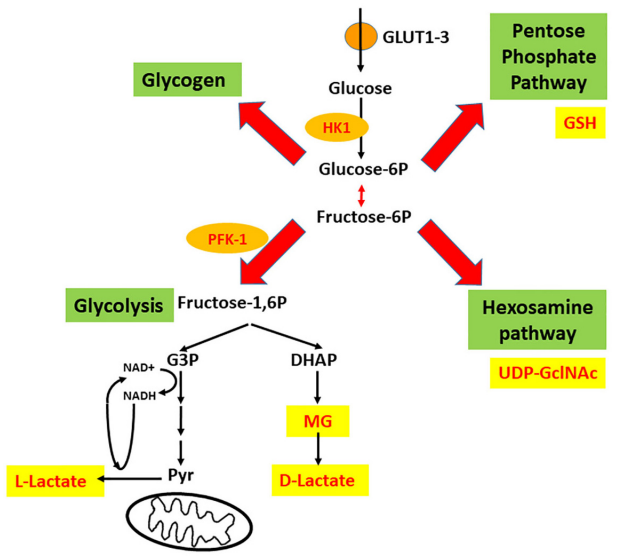

FIGURE 1 | Four intracellular destinations of glucose that suggest an intersection in "X." Glucose enters astrocytes mainly via GLUT1, and neurons mainly via GLUT3 and is trapped by phosphorylation (catalyzed by hexokinase 1, HK1). Afterward, four destinations are possible; these form a crossroads in the shape of an X, where glycogen synthesis and the pentose phosphate pathway (PPP) are the left and right arms of the X, and glycolysis (until pyruvate) and the hexosamine pathway (HP) are the left and right legs of the X. The deviation of glycolysis that generates methylglyoxal (MG) is also indicated. PFK-1, phosphofructokinase-1; G-3P, glyceraldehyde 3-phosphate; DHAP, dihydroxyacetone phosphate.

Glucose Transport and Phosphorylation

Before discussing the destinations of glucose, it is important to understand how it enters the neural cells, via the glucose transporter (GLUT) and sodium-glucose co-transporter (SGLT), which are blocked by phloretin and phlorizin, respectively (Shah et al., 2012). GLUTs are passive and bidirectional transporters. GLUT1 is the main isoform found in cells forming the blood-tissue barriers (endothelial and choroid plexus), astrocytes and the ependymal cells lining the cerebral ventricles. In addition to glucose, GLUT1 transports dehydroascorbic acid (the oxidized form of vitamin C) (Rivas et al., 2008) and glucosamine (Chopra, 2004). At the blood-brain barrier (BBB), GLUT1 works at about one-third of maximal capacity under basal conditions (Leybaert et al., 2007). Neurons express mainly GLUT3, although some neurons in the rodent brain also express the insulin-sensitive GLUT4 (in the hippocampus, the cerebellum and the hypothalamus) (Choeiri et al., 2002) and SGLT1 (in the hippocampus and the cerebral cortices) (Yu et al., 2010). However, glucose transportation by these carriers is not considered a rate-limiting step in brain energy metabolism. In contrast, the next step, glucose phosphorylation by hexokinase, represents the rate-limiting step.

Notably, more than 90% of non-fenestrated capillary brain vessels are covered by astrocytic end-feet (Jukkola and Gu, 2015). Moreover, the tight junctions between endothelial cells (responsible for non-fenestration) are actively regulated by astrocyte signals (Ballabh et al., 2004). These aspects indicate the importance of astrocytes in glucose distribution. However, this does not mean that glucose needs to pass through astrocytes to reach neurons. In fact, after crossing endothelial cells via GLUT 1, glucose can reach neurons directly via GLUT3, because there is room for molecular diffusion, since astrocytes form gap junctions among themselves instead of tight junctions.

Glucose phosphorylation on C_6, catalyzed by HK, is the first rate-limiting step of glycolysis. All three isoforms of HK (of low Km) are present in brain tissue, but HK1 is the most abundant isoform in neurons and astrocytes and it is assumed to be "the brain hexokinase." HKs 1 and 2 bind to the outer mitochondrial membrane by a hydrophobic sequence at their N-terminal, close to the pore that allows ATP output (Pastorino and Hoek, 2008). The activation of Akt kinase (or inhibition of glycogen synthase kinase 3, GSK-3) favors the binding of HK to the mitochondria by a mechanism that is still unclear. Glucose-6P induces a conformational change of the HK, displacing it from the mitochondria and decreasing its activity. Glucose-6P acts as a non-competitive inhibitor of HK, which under basal conditions is predominantly inhibited (DiNuzzo et al., 2015).

PFK-1 Catalyzes the Other Rate-Limiting Step of Glycolysis

Glucose-6P is isomerized to fructose-6P, which in turn is converted to fructose-1,6-bis-phosphate (F1,6BP, see **Figure 1**).

This reaction is the second rate-limiting step of glycolysis and is catalyzed by PFK-1. HK has been reported to be higher expressed in neurons than in astrocytes (Lundgaard et al., 2015); however, the activity and regulation of PFK-1 in astrocytes suggest a higher glycolytic activity in these cells (Bolaños et al., 2010). PFK-1 is allosterically downregulated by metabolites ATP, citrate and long-chain fatty acids (Jenkins et al., 2011) and upregulated by fructose 2,6 bisphosphate (F2,6BP) (Mor et al., 2011). Moreover, lactate, at least in muscle cells, is able to disarrange the tetrameric structure and reduce the enzymatic activity of PFK-1 (Costa Leite et al., 2007). F2,6BP, the main allosteric activator, is present in astrocytes at higher concentrations than in neurons. In fact, the enzyme responsible for the generation of F2,6BP from fructose-6P in brain tissue, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3 (PFKFB-3), has lower expression levels in neurons due to elevated proteosomal degradation (Herrero-Mendez et al., 2009). PFKFB-3 is the target of several kinases, including Akt and AMP-activated protein kinase (AMPK; Marsin et al., 2000). AMPK is able to phosphorylate/activate PFKFB-3 as well as PFK-1 (Bartrons et al., 2018), indicating a direct regulatory role of AMP.

The lower activities of PFK-1 and PFKFB-3 in neurons suggest that glucose uptake could be conducted to the PPP to generate NADPH, which is required for the regeneration of GSH in these cells (Bolaños et al., 2010). Neurons have low concentrations of GSH (Dringen et al., 2005) and activity of γ-glutamyl cysteine ligase (GCL; Makar et al., 1994) when compared to astrocytes. Interestingly, oxidative stress-mediated S-glutathionylation of PFKFB-3 decreases its catalytic activity in cancer cells, redirecting the glycolytic flux to the PPP (Seo and Lee, 2014). Understanding the metabolic regulation of these three enzymes (HK1, PFK-1, and PFKFB-3) is important for comprehending the journey of glucose to pyruvate (including the passage through glycogen) or to ribulose-5 (PPP), as well as the effects of products of these pathways (lactate, methylglyoxal, and GSH) on synaptic communication. All these regulatory enzymes of glucose flow are direct or indirect targets of GlnNAcylation, which in turn depends on glucose flow itself, as we will discuss below.

THE LEFT ARM AND LEG OF GLUCOSE METABOLISM MODULATE SYNAPTIC TRANSMISSION VIA LACTATE

Lactate, directly derived from glucose or glycogen (in astrocytes), performs functions beyond energy supply. These functions are mediated by different mechanisms and newly presented pathways (already verified or still proposed), including a specific receptor and its signaling transduction pathways.

The Lactate Receptor: For Every Orphan, a Family

The lactate receptor, initially known as GPR81, belongs to a family of G protein-coupled receptors (GPRs). It was first mapped via a genomic sequence database and then identified in the human pituitary gland (Lee et al., 2001). At that time, in the absence of a specific ligand, the receptor was considered an orphan. Later, the receptor was shown to be highly expressed in adipose tissue (Liu et al., 2009). The subsequent pharmacological characterization of L-lactate as a ligand for GPR81 was initiated, taking into account the similarity of GPR81 with other receptors from the GPR family, GPR109a and GPR109b, which also have β-hydroxybutyrate as a ligand. L-lactate inhibits lipolysis via GPR81 in adipocytes from human, mouse, and rat adipose tissue (Cai et al., 2008; Liu et al., 2009). The suggestion that lactate may act in a hormone-like manner comes from the demonstration of an insulin-dependent inhibition of lipolysis via GPR81 by Ahmed et al. (2010).

As all GPR ligands are hydroxy-carboxylic acids, the GPRs are now HCA receptors (HCARs; Ahmed et al., 2009; Blad et al., 2011; Offermanns, 2017). GPR89/HCAR1 has received more attention during recent years and has been implicated in inflammation and cancer signaling [for reviews, see (Haas et al., 2016; Offermanns, 2017)]. A compound present in fruits (Liu et al., 2012; Bergersen, 2015), 3,5-dihydroxybenzoic acid (DHBA), was identified as an agonist for HCAR1, inhibiting lipolysis in wild-type mouse adipocytes, but not in HCAR1 knocked-down adipocytes (Liu et al., 2012). The HCAR2 ligand 3-hydroxy-butyrate has been considered as an antagonist for the HCA1 receptor and has been used experimentally as such (Shen et al., 2015).

To characterize HCAR1 signaling pathways, a study using a cell line designed to express human HCAR1 (CHO-K1) showed activation of extracellular signal-regulated kinases (ERK1/2) via HCAR1 in response to lactate and DHBA and sensitivity to the G_i protein inhibitor pertussis toxin (Li et al., 2014). Moreover, the Gαγ subunit dissociated from the activated G_i protein was central in the regulation of HCAR1-activated ERK1/2 phosphorylation via extracellular Ca^{2+}, protein kinase C (PKC), and insulin-like growth factor-1 receptor (IGF-1R) activation. Arrestin-2 and 3 had no effect on ERK1/2 activation, whereas HCAR1 internalization was dependent on arrestin-3 (Li et al., 2014). Supposed non-canonical actions of the HCAR1 receptor, i.e., without involving cyclic AMP (cAMP) reduction, have been proposed based on β-arrestin actions but still await future confirmation (Bergersen, 2015; Morland et al., 2015) and a different yet unknown receptor has also been suggested (Tang et al., 2014).

D-lactate, the stereoisomer of L-lactate, is produced at very low concentrations under physiological conditions from methylglyoxal (MG, see below). It is considered to be a partial agonist of the HCAR1 receptor with maximal stimulation significantly lower than that by L-lactate (Cai et al., 2008). As reported throughout this review, although some studies have shown D-lactate as a positive control for L-lactate HCAR1 signaling in neurons (Bozzo et al., 2013), many other studies have shown antagonistic (Tang et al., 2014), absent (Herrera-López and Galván, 2018), and controversial actions of the lactate enantiomer on neuroprotection and

cognitive functions (Gibbs and Hertz, 2008; Castillo et al., 2015).

Synaptic and Vascular Modulation by Lactate

The presence of the – at that time – orphan GPR81 was first demonstrated in brain tissue in 2001 (Lee et al., 2001). HCAR1 is located at synaptic membranes of excitatory synapses in the hippocampus and the cerebellum. It is located predominantly at postsynaptic sites, but it is also present in astroglial end-feet processes and endothelial membranes, indicating that energy metabolism is associated with synaptic function (Lauritzen et al., 2014). The vascular endothelial density of the receptor is twice the density at the astrocytic end-feet, suggesting a lactate control of cerebral blood flow (see **Figure 2**). The effect of physical exercise on the density of capillaries via HCAR1 in the sensorimotor cortex and more markedly in the hippocampus, was reproduced by daily subcutaneous injections of L-lactate (about 10 mM in the blood). The regulation of angiogenesis via HCAR1 and downstream Erk1/2 and Akt signaling resulting in vascular endothelial growth factor (VEGF) production were confirmed by the extensive expression of the receptor at perivascular pial and pericyte-like cells (Morland et al., 2017).

Even before the deorphanization of the GPR81, several non-metabolic actions of lactate have been reported in neural cells. Lactate increases the action potential frequency of glucose-sensing neurons at the ventromedial hypothalamic nucleus via K_{ATP} and chloride channels (Song and Routh, 2005). Another study using cortical astrocyte cultures and SH-SY5Y neuronal cells incubated with lactate showed increased brain-derived neurotrophic factor (BDNF) and inducible nitric oxide synthase (iNOS) expression in astrocytes but not in SH-SY5Y cells. However, the authors centered their discussion only on the energetic aspect of lactate (Coco et al., 2013).

The first demonstration of a signaling pathway involving lactate and receptor interaction in the brain was demonstrated by Bozzo et al. (2013), who showed that L-lactate modulated the calcium spike frequency in primary mouse neuron cultures. The authors showed, for the first time, a brain non-energetic effect of L-lactate via HCAR1, since other metabolic substrates, such as pyruvate or glucose, could not mimic this effect of L-lactate. In addition, they used the agonist DHBA, reproducing the effects of lactate, and D-lactate, which has a poor affinity for the monocarboxylate transporter 2 (MCT2) and works as a partial agonist. On the other hand, a non-metabolic astrocyte-neuron signaling modulation by lactate, through a different

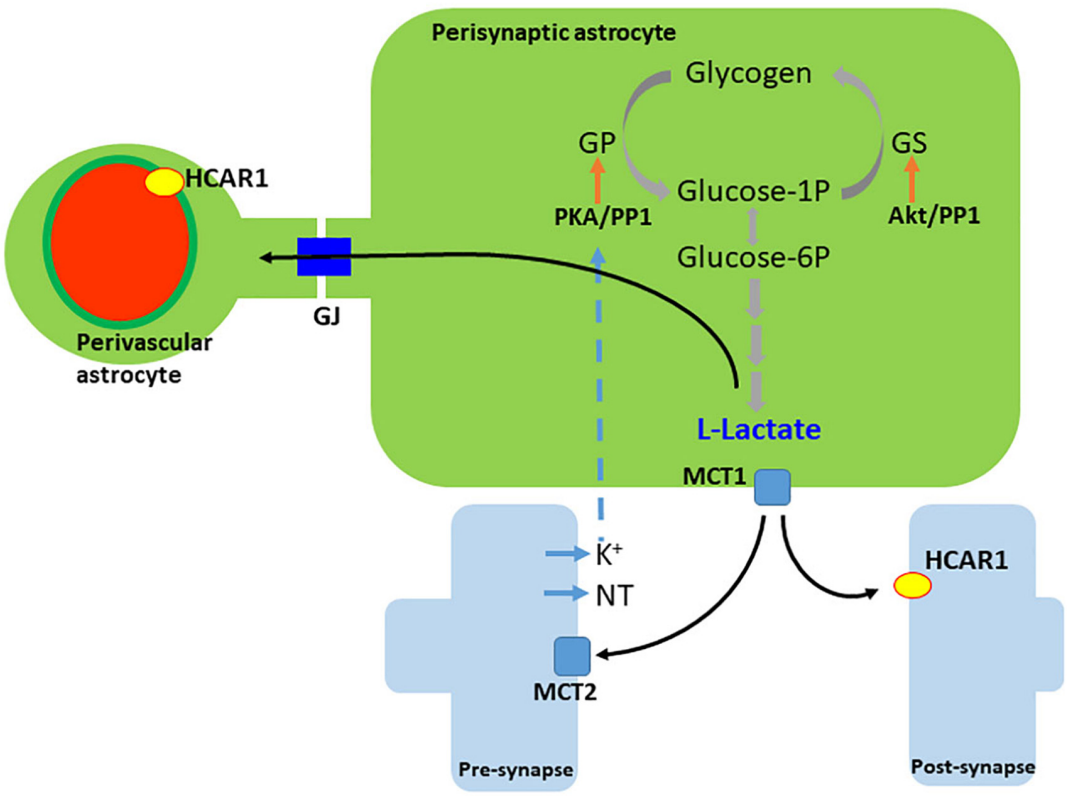

FIGURE 2 | Generation and release of lactate in astrocytes. L-lactate is generated either from recently uptaken glucose or from glycogen. Neurotransmitters (NT), and/or extracellular K^+, trigger glycogen breakdown until lactate, via cAMP/PKA signaling. Lactate leaves the cell by the monocarboxylate transporter 1 (MCT1) and enters neurons via the monocarboxylate transporter 2 (MCT2). Extracellular lactate also binds to hydroxy-carboxylic acid receptor 1 (HCAR1), which is found more abundantly in perivascular and post-synaptic membranes. Lactate travels among astrocytes through gap junctions (GJ). PKA, protein kinase A; GP, glycogen phosphorylase; GS, glycogen synthase; PP1, protein phosphatase 1.

and unknown mechanism, was demonstrated by Tang et al. (2014). This investigation elegantly showed the release of L-lactate by astrocytes employing *in vitro* optogenetics. They also demonstrated exogenous lactate in cultured and acute brain slices and showed that *in vivo* lactate administration modulates the excitability of noradrenergic neurons from the locus coeruleus. The authors suggested a possible receptor, other than HCAR1, since D-lactate acted as an inverse agonist and lactate concentrations used were about ten times lower than the IC50 for the G_i-coupled receptor.

A study revealed L-lactate upregulation of immediate early genes associated with *N*-methyl-D-aspartate (NMDA) transmission in neuronal cultures from the mouse neocortex and *in vivo* administration of L-lactate (Yang et al., 2014). Genes such as Arc, c-Fos, and Zif268 had an increased expression after lactate treatment in a range between 2.5 and 20 mM in a time-dependent manner, with a one-hour peak. An energetic effect was excluded after the ineffectiveness of D-lactate, pyruvate, and glucose in an equicaloric concentration at the same experimental conditions. Moreover, signaling of lactate on these plasticity-related genes was intracellular, since the MCT blocker UK5099 abolished this effect. Interestingly, after longer treatment periods, lactate also stimulated an increase in BDNF expression and the phosphorylation of Erk1/2. The authors showed lactate action via NMDA receptors, but not a specific lactate receptor.

More recently, one study showed the modulation by lactate of the action potential frequency in pyramidal cells from the CA1 region of the hippocampus, under stable energetic conditions. Both lactate and its agonist, DHBA, induced a biphasic modulation in neuronal excitability, inducing reduced excitability at lower concentrations (lactate at 5 mM and DHBA at 0.56 mM), while higher concentrations (lactate at 30 mM and DHBA at 3.1 mM) increased firing frequencies. Use of a neuronal MCT2 blocker did not abolish the lactate effect and neither did D-lactate alter the firing frequency of the cells; however, G_i protein inhibition via pertussis toxin confirmed the effect of lactate via HCA1R (Herrera-López and Galván, 2018).

Lactate release in response to glutamate uptake was described 20 years ago (Pellerin and Magistretti, 1994); however, an alternative molecular pathway for lactate efflux, induced by neuronal depolarization has been proposed (Choi et al., 2012). A soluble adenylyl cyclase (sAC) sensitive to HCO_3^- is found abundantly expressed in astrocytes and responds to extracellular K^+ elevation. An increase in cAMP secondary to HCO_3^-influx (via HCO_3^-/Na^+ transporter) was observed in cultured astrocytes and in brain slices; furthermore, sAC was found to be responsible for the production and release of lactate as a consequence of the glycogen breakdown coupled with K^+ increase in astrocytes (see **Figure 2**). As cAMP levels stimulate glycogen breakdown (Pellerin et al., 2007), HCA1 receptor (coupled to G_i protein) activation via lactate could mediate glycogenolysis feedback control by lactate in astrocytes.

The lactate response during neurotransmission is fast and independent of the metabolic status or oxygen availability, leading to the observation by Sotelo-Hitschfeld et al. (2015) of a steady-state reservoir of lactate (Sotelo-Hitschfeld et al., 2015). The synaptic activity and consequent depolarization caused by extracellular K^+ have been reported to elicit glycogen mobilization (Choi et al., 2012) and lactate generation (Sotelo-Hitschfeld et al., 2012). On the other hand, using a FRET lactate sensor, the group demonstrated that depolarization, via depletion of the astrocytic lactate reservoir in cultured astrocytes, may occur via a non-identified anion channel (Sotelo-Hitschfeld et al., 2015).

HCAR1-mediated lactate effects have been suggested to be neuroprotective in depression (Carrard et al., 2018) and cerebral ischemia (Berthet et al., 2009, 2012). More recently, in a middle cerebral artery occlusion stroke model, HCAR1 receptor expression was increased in the hippocampus, the cortex, and the striatum after ischemia (Castillo et al., 2015). Moreover, in hippocampal slices in an oxygen and glucose deprivation model, DHBA and D-lactate protected the CA1 region from the insult (Castillo et al., 2015). Although it is not possible to determine the definitive role for HCAR1 in the synaptic communication at this moment, the astrocyte lactate released during glycogenolysis is reportedly crucial for memory consolidation (see Hertz and Chen, 2018 for review). However, recent data indicate that an aging-associated shift of glycogen metabolism enzyme concentrations, and their localization in astrocytes and neurons, may occur (Drulis-Fajdasz et al., 2018). In addition to its role in synaptic signaling modulation, the astrocytic steady-state reservoir, and the rapid response to depolarization, lactate may act as a gliotransmitter molecule (Tang et al., 2014; Sotelo-Hitschfeld et al., 2015). However, synthesis rather than release should be considered as the limiting step for lactate signaling in the brain (Mosienko et al., 2015).

Methylglyoxal and D-Lactate Also Affect Neurotransmission

Methylglyoxal is a dicarbonyl compound (formula $CH_3C(O)CHO$) derived from endogenous and exogenous sources and responsible for most of the glycation reactions in cell metabolism. The endogenous source of MG comes from enzymatic or non-enzymatic reactions of reducing sugars, lipids and amino acids in the cell. The main source of MG synthesis is from aldehydes, which are intermediates of the glycolysis pathway, such as glyceraldehyde 3-phosphate and dihydroxyacetone-phosphate (see **Figures 1**, **3**). At physiological or pathological conditions, MG is produced through spontaneous dephosphorylation of dihydroxyacetone-phosphate (Angeloni et al., 2014; Muronetz et al., 2017). Coffee, alcoholic beverages, cigarette smoke and food are all exogenous sources of MG (Nemet et al., 2006; Angeloni et al., 2014).

Methylglyoxal is also present in different biological materials (tissues, urine, plasma and the cerebrospinal fluid) and its concentration is related to the status of glucose metabolism (Nemet et al., 2006; Angeloni et al., 2014). It has been suggested that about 0.1–0.4% of the glycolysis pathway results in the formation of MG (Kalapos, 2008). More recently, it has been proposed that MG, at physiological levels (μM), acts as an agonist of the γ-aminobutyric acid type A ($GABA_A$) receptor and is associated with anxiolytic behavior (Distler et al., 2012) and induction of sleep (Jakubcakova et al., 2013). However, due to the

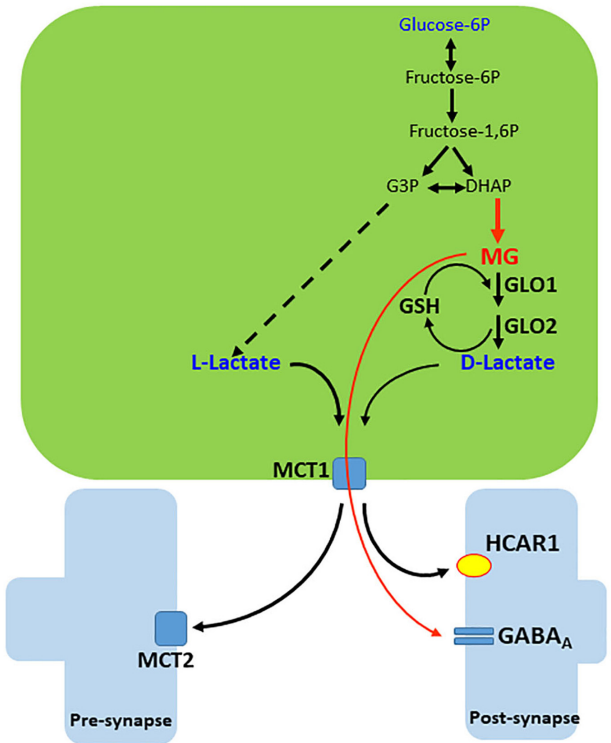

FIGURE 3 | Generation and release of MG and D-lactate in astrocytes. Methylglyoxal (MG) is produced from dihydroxyacetone phosphate (DHAP) by a deviation of the glycolytic pathway. MG is condensed with glutathione (GSH) and then, by sequential action of glyoxalases 1 and 2 (GLO1 and 2), generates D-lactate and recycles GSH. D-lactate leaves the cell via monocarboxylate transporter 1 (MCT1) but disturbs lactate and pyruvate flows to mitochondria (not illustrated). MG and D-lactate leave the cell and act on the $GABA_A$ receptor and hydroxy-carboxylic acid receptor 1 (HCAR1), respectively. Extrasynaptic $GABA_A$ receptors of MG are not illustrated.

lower levels of MG in the synaptic cleft (compared to those of GABA), it has been proposed that MG has a modulatory effect on $GABA_A$ receptors in the extrasynaptic space (Tao et al., 2018).

On the other hand, the relationship between $GABA_A$ and glucose metabolism (more precisely, glucose uptake) has been investigated over the last 30 years, but findings are not clear and sometimes conflicting (Ito et al., 1994; Peyron et al., 1994; Parthoens et al., 2015). Currently, measurement of glucose metabolism (based on deoxyglucose uptake) is associated with glutamatergic activity, mainly because glutamate is the predominant neurotransmitter. However, other neurotransmitters such as noradrenaline and adenosine, as well as K^+ itself, released by neuronal activity, modulate energetic metabolism (Hertz et al., 2015; Waitt et al., 2017). In this scenario, MG is a glucose-derivative molecule that putatively connects energetic metabolism and the GABAergic system.

Methylglyoxal is metabolized predominantly by the cytoplasmic glyoxalase system, formed by two enzymes, glyoxalases 1 and 2 (GLO1 and 2), which act sequentially. GLO1 depends on GSH. GSH reacts directly with MG and produces hemithioacetal and GLO1 converts this metabolite to S-Lactoylglutathione. Subsequently, this compound is hydrolyzed by GLO2 into D-lactate and GSH is regenerated (**Figure 3**). Notice that, due to the lower activity of the left leg of the destination of glucose in neurons, it may be assumed that extracellular MG and D-lactate, as well as L-lactate, originate predominantly from astroglial cells.

D-lactate is oxidized to pyruvate by a D-isomer-specific lactate dehydrogenase (D-LDH; Cristescu et al., 2008). Mitochondrial D-LDH activity in rat brain tissue is about 60% lower than in liver (Ling et al., 2012). High levels of D-lactate inhibit the membrane L-lactate transport and pyruvate transport to mitochondria in astrocytes (Gibbs and Hertz, 2008) and neurons (Ros et al., 2001), which explains the neurotoxicity of this compound. However, a neuroprotective effect of D-lactate has been proposed in seizures (Angamo et al., 2017), possibly due to energy impairment. More recently, in addition to confirming the presence D-LDH activity in mouse brain tissue, it was shown that D-lactate is a partial agonist of HCAR1 (Castillo et al., 2015).

Astrocyte Dysfunction, MG-Induced Protein Glycation, and Neurodegenerative Diseases

It is well known that in aging, diabetes mellitus, and neurodegenerative diseases, MG is elevated to sub-millimolar levels, working as a potent glycant agent (Srikanth et al., 2013; Maessen et al., 2015). Elevated D-lactate levels from liver metabolism are observed in diabetic animals (Kondoh et al., 1994) and the accumulation of this compound could contribute to memory impairment, dependent on lactate flow (Suzuki et al., 2011). However, when the detoxifying system fails due to a reduction in glyoxalase activity or GSH deficiency, MG and advanced glycation end-products (AGEs) formation increases, but D-lactate levels can be reduced, as has been observed in endothelial cells (Li et al., 2013).

Methylglyoxal promotes glycation on lipids, nucleic acid and proteins (Allaman et al., 2015). MG mainly promotes post-translation modifications on proteins by the Maillard reaction on amino acid residues. The most common glycated amino acids are arginine and lysine and consequently the formation of AGEs such as argpyrimidine, hydroimidazolone (MG-H1), Nε-(1-carboxyethyl)-L-lysine (CEL), and Nε-(1-carboxymethyl)-L-lysine (CML) adducts, respectively (Rabbani and Thornalley, 2012). AGEs are ligands of the receptor for advanced glycation end products (RAGE) and induce inflammation by activation of the nuclear factor κB (NF-κB) pathway in all neural cells (Chavakis et al., 2004; Donato et al., 2009).

Elevated MG seems to increase the expression of astrocyte markers (glial fibrillary acidic protein – GFAP and S100B) and cytokines in astrocyte culture and *in vivo*, leading to astrogliosis and neuroinflammation (Chu et al., 2016). However, cognitive impairment has also been reported without changes in classical parameters of astrogliosis (Hansen et al., 2016b). Impairment in glucose flow and/or dysfunction of the glyoxalase system is a common and early event in neurodegenerative diseases, such as Alzheimer's (AD) and Parkinson's diseases (PD).

Elevated MG and AGEs levels play a key role in protein misfolding and oxidative stress, and are also involved in AD (Angeloni et al., 2014). The increases in glycation reactions and MG levels are suggested as a possible link in diabetic individuals that develop AD (Janson et al., 2004). In different *in vitro* models, MG induced glutamatergic excitotoxicity by promoting glutamate release (Arriba et al., 2006) and glutamate uptake disturbance (Hansen et al., 2016a, 2017). Elevated MG and AGEs stimulate apoptosis and reduce neurogenesis as well as neuronal survival in the hippocampus by downregulation of BDNF expression and its signaling pathways (Di Loreto et al., 2008; Falone et al., 2012; Chun et al., 2016). MG is involved in tau hyperphosphorylation through activation of GSK-3β and p38 mitogen-activated protein kinases (p38 MAPK) (Li et al., 2012). Moreover, AGEs and hyperphosphorylated tau are co-localized in the cytoplasm of neurons, possibly contributing to neurofibrillary tangle formation (Lüth et al., 2005; Fawver et al., 2012). AGE immunoreactivity has also been observed in amyloid plaques (Münch et al., 1997; Krautwald and Münch, 2010); recently, MG was shown to glycate Lys-16 and Arg-5 residues on β-amyloid, resulting in glycated Aβ (Fica-Contreras et al., 2017). The Aβ-AGE form is more insoluble, neurotoxic and resistant to protease reactions (Angeloni et al., 2014).

In PD, a predictor event is the low activity of neuronal PPP enzymes (Dunn et al., 2014) and mitochondrial dysfunction (Dranka et al., 2012; Hipkiss, 2014), which changes the redox cell status and increases the anaerobic glycolysis pathway and MG formation. In addition, dysregulation of the glyoxalase system (Joe et al., 2018) also leads to high MG levels and an increase in glycation reactions. MG reacts directly with dopamine, reducing its concentration and generating the salsolinol-like compound, 1-acetyl-6.7- dihydroxy-1,2,3,4-tetrahydroisoquinoline, which promotes mitochondrial dysfunction (Hipkiss, 2014). Moreover, the glycation of the N-terminal region of α-synuclein reduces its ability to remain attached to the plasma membrane. In fact, the accumulation of aggregated glycated-α-synuclein in the cytoplasm results in neurotoxic effects on dopaminergic neurons (Vicente Miranda et al., 2016).

THE RIGHT ARM MODULATES GLUTATHIONE HOMEOSTASIS AND GLUTAMATERGIC NEUROTRANSMISSION

Glucose metabolism, in addition to producing energy, supports other important functions, for example the generation of reducing equivalents for antioxidant defenses and biosynthetic pathways, in both astrocytes and neurons (Magistretti and Allaman, 2018). The PPP, which constitutes the right arm of the glucose destinations (**Figure 1**), is the main cytosolic source of NADPH and is essential for regeneration of GSH. This pathway decarboxylates glucose-6-phosphate into ribulose-5-phosphate, a precursor for the nucleotide biosynthesis, conserving the redox energy as NADPH. Glucose-6-phosphate dehydrogenase (G6PD) is the rate-limiting enzyme for the PPP (see **Figures 4**, **6**), which in the resting brain represents a minor pathway for glucose metabolism (Gaitonde et al., 1983; Wamelink et al., 2008; Bouzier-Sore and Bolaños, 2015). However, under several conditions, for example in response to injury, the PPP can be markedly upregulated (Bartnik et al., 2005; Jalloh et al., 2015; Rosa et al., 2015) and has demonstrated protective roles because it provides precursors for tissue repair, as well as increases GSH to avoid oxidative stress and neuroinflammation.

In astrocytes, the PPP is essential for maintaining an adequate pool of reduced GSH, since these cells export this antioxidant molecule to neurons. Activation of the PPP in astrocytes protects neurons from oxidative stress by increasing astrocytic GSH levels (Takahashi et al., 2012). In neurons, the low expression of PFKFB-3 (**Figure 6**) results in a low glycolytic rate, and glucose is diverted to the PPP (Almeida et al., 2004; Herrero-Mendez et al., 2009). This is particularly important for generating GSH, since neuronal cells have a lower synthesis and, consequently, GSH concentrations, compared to astrocytes (Makar et al., 1994; Dringen et al., 2005).

Glutathione is an essential molecule for cellular antioxidant defense and detoxification processes, conferring neuroprotection (Dringen, 2000b; Dringen et al., 2015; Kinoshita et al., 2018). Impaired GSH metabolism is associated with oxidative stress and inflammatory responses, which have been linked to cerebral diseases and neurodegeneration (Dringen, 2000a; Lee et al., 2010; Aoyama and Nakaki, 2013; Arús et al., 2017). An age-dependent reduction in resting NADPH concentration, accompanied by a decrease in GSH levels, has been reported in cultured neurons, making them more susceptible to glutamate exposure (Parihar et al., 2008). This impaired reducing power can lead to pathological aging, since the inability of neurons to regenerate GSH is a hallmark of AD and PD (Currais and Maher, 2013).

Glutathione is a tripeptide, consisting of glutamate, cysteine and glycine, that is synthesized by two enzymatic steps. First, GCL catalyzes the reaction between glutamate and cysteine to form the dipeptide γ-glutamylcysteine. The second step is the reaction of GSH synthase, which mediates the addition of glycine to γ-glutamylcysteine to form GSH (Lu, 2013). While the first step catalyzed by GCL is considered the rate-limiting reaction, the intracellular content of cysteine is the rate-limiting precursor for GSH biosynthesis (Griffith, 1999; **Figure 4**).

Glutathione in the brain is present in both intra- and extracellular compartments and acts as a dynamic buffer of the redox state. Non-enzymatically, GSH is able to react directly with free radicals including superoxide, hydroxyl radical, nitric oxide, peroxynitrite and MG (Clancy et al., 1994; Winterbourn and Metodiewa, 1994; Aoyama and Nakaki, 2015). Moreover, GSH can react with protein thiol groups, leading to a reversible formation of mixed disulfides (S-glutathionylation), which are important for preventing protein oxidation, thus preserving and modulating its functions (Giustarini et al., 2004). GSH also participates in enzymatic reactions, such as those catalyzed by GSH peroxidase (GPx) and glutathione-S-transferase (GST). GPx detoxifies hydrogen peroxides and other endogenous hydroperoxides using GSH as an electron donor. In this reaction, GSH is oxidized to glutathione disulfide (GSSG; Dringen et al., 2015); GSSG is then reduced back to GSH via glutathione

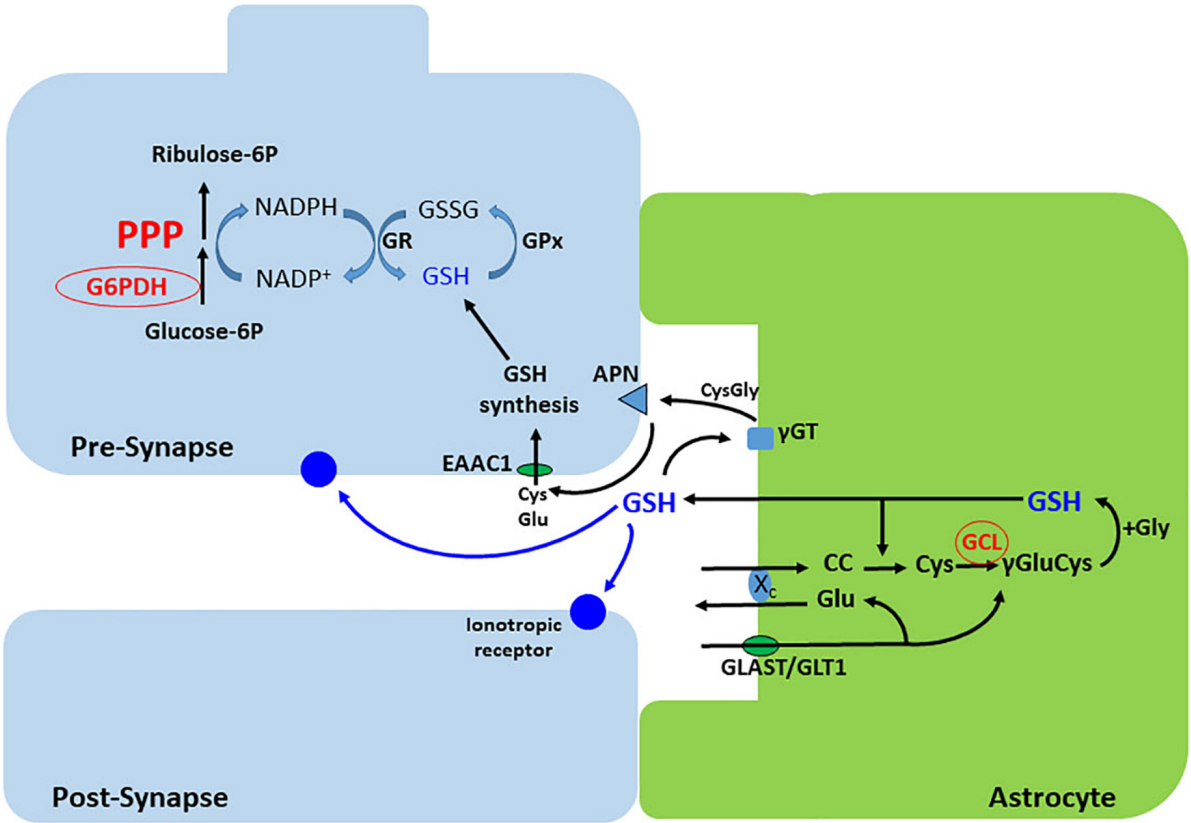

FIGURE 4 | Synthesis and release of GSH in astrocytes. Cystin (cisteinyl-cystein, CC) is uptaken via the xc$^-$ exchanger, which releases glutamate. This exchanger is functionally coupled to the Na^+- dependent glutamate transporters, GLAST or GLT-1. Cystin is reduced by GSH to two cysteines. The gamma acid group of glutamate then condenses with the amine group of cysteine, forming gamma-glutamyl-cysteine (γ Glu-Cys), by the action of the glutamate cysteine ligase (GCL). Addition of glycine completes the synthesis of GSH that, in part, is exported to modulate ionotropic receptors and as a source of cysteine for GSH synthesis in neurons. The extracellular cysteine from astroglial GSH is generated after sequential action of the extracellular peptidases, γGT and APN. Neurons synthesize low levels of GSH compared with astrocytes, but exhibit a high capacity of GSH regeneration, which depends on NADPH synthesis in the PPP. γGT, gamma peptidyl transpeptidase; APN, aminopeptidase neuronal; GPx, glutathione peroxidase; GR, glutathione reductase; EAAC, excitatory amino acid carrier.

reductase (GR) using NADPH (Dringen and Gutterer, 2002; Ren et al., 2017).

The high oxidative metabolic rate can increase mitochondrial reactive oxygen species (ROS) production, rendering the brain vulnerable to oxidative stress. Astrocytes and neurons exhibit differences in GSH metabolism and different mechanisms maintain GSH homeostasis between these cell types. In astroglial cells, the basal levels of GSH are higher than in neuronal cells, showing their pivotal antioxidant role in the central nervous system (Dringen et al., 2005). Neurons, in turn, depend on astrocytic GSH release for providing extracellular cysteine for their synthesis of GSH (**Figure 4**). As previously mentioned, cysteine is the limiting precursor for synthesis of GSH.

Astrocytes have a Cys-Glu exchanger (system xc$^-$) that mediates the uptake of cystine, the bioavailable form of cysteine, in exchange for glutamate (Bridges R.J. et al., 2012; Ottestad-Hansen et al., 2018). Moreover, they express the glutamate/aspartate transporter GLAST (also known as excitatory amino acid transporter 1, EAAT1 in humans) and glutamate transporter 1 (GLT1, or EAAT2 in humans) (Lehre and Danbolt, 1998), which provide intracellular glutamate for GSH synthesis and for system xc$^-$ operation (Reichelt et al., 1997). Importantly, these glutamate transporters also account for the majority of glutamate removal from the synaptic cleft, maintaining extracellular glutamate concentrations low to avoid excitotoxicity (Anderson and Swanson, 2000; Rose et al., 2017). In this regard, system xc$^-$ and glutamate transporters are associated with both GSH biosynthesis and modulation of glutamatergic neurotransmission, as xc$^-$ mediates glutamate release. Altered function of these transporters can result in GSH depletion and/or, consequently, glutamate excitotoxicity in pathological conditions (Ré et al., 2003; Yi and Hazell, 2006; Bridges R. et al., 2012; Thorn et al., 2015). At the same time, GLAST/GLT1 are also related to glucose metabolism; as they are sodium-dependent, their activity increases the intracellular sodium concentration, consequently activating the Na^+/K^+ ATPase pump, which consumes ATP in astrocytes. ATP, in turn, can be supplied by the glycolytic pathway (for a review, see Bélanger et al., 2011).

Glutamate is also involved in the mechanism by which astrocytes are able to readily release GSH in response to neural

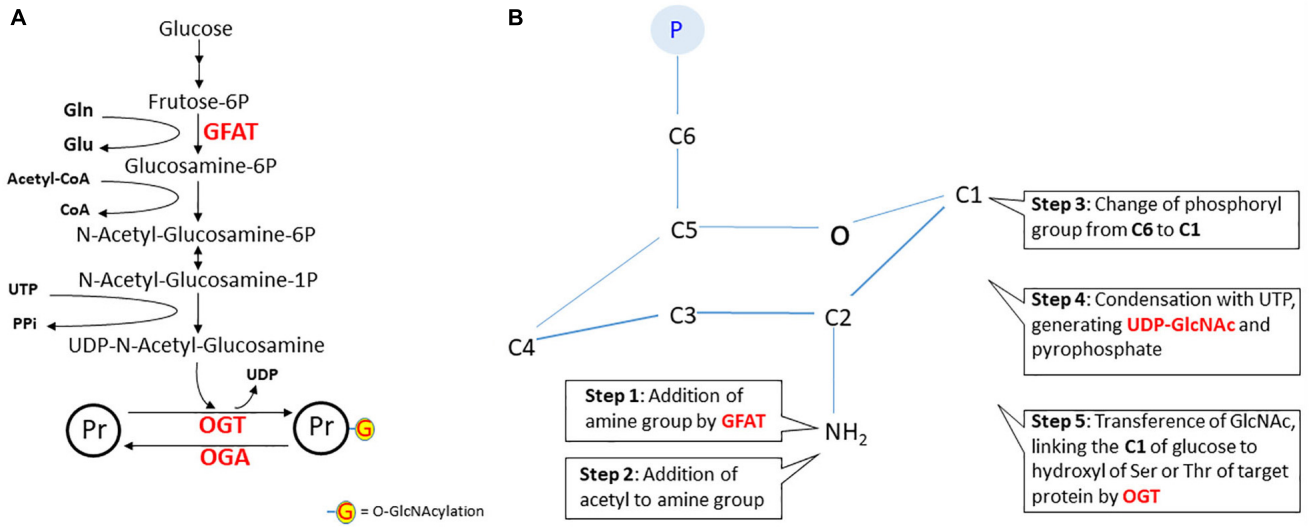

FIGURE 5 | The hexosamine pathway. In panel **A**, the steps of the hexosamine pathway (HP) from frutose-6P to UDP-*N*-acetyl-glucosamine. Notice that glutamine (Gln), Acetyl-CoA and UTP are key substrates in this pathway. The rate-limiting step is catalyzed by glutamine:fructose-6P aminotransferase (GFAT). In panel **B**, a schematic representation of the structural changes during *N*-acetyl-glucosamine synthesis. The first reaction is the amination of fructose-6P (fructofuranose) to glucosamine-6P (glucopyranose). It is worth noting that, in step six, carbon 1 of glucopyranose binds to the hydroxyl of serine or threonine of the protein target. In fact, this is a reaction of *O*-linked β-*N*-acetylglucosaminylation, but for simplification, the nomenclature widely used is O-GlcNAcylation. However, this gives the wrong idea that linking occurs at the acyl group. It would be better to use NAGylation, since NAG is another (and the simplest) abbreviation of *N*-AcetylGlucosamine, found in some polymers. Herein, we will maintain the use of the term "O-GlcNAcylation." Abbreviations: OGT, O-GlcNAc transferase; OGA, O-GlcNAcase; Pr, protein.

activity, to maintain neuronal GSH levels via the astrocyte-neuronal GSH shuttle. In astrocytes, glutamate triggers a cascade of signals that promote the expression of antioxidant genes through activation of the nuclear factor (erythroid-derived 2)-like 2 (Nrf2), leading to the biosynthesis of GSH (Frade et al., 2008; Jimenez-Blasco et al., 2015; McGann and Mandel, 2018). In the extracellular space, GSH can be hydrolyzed by γ-glutamyl transpeptidase forming γ-glutamyl and the dipeptide CysGly. CysGly is, in turn, cleaved by the neuronal aminopeptidase N into cysteine and glycine, which serve as precursors for neuronal GSH synthesis (Dringen et al., 1999, 2001; Hertz and Zielke, 2004; **Figure 4**). Neuronal cells are less capable of importing cystine, but the sodium-dependent excitatory amino acid carrier 1 (EAAC1, also known as EAAT3 in humans) is able to uptake cysteine in addition to glutamate (Zerangue and Kavanaugh, 1996; Shanker et al., 2001). EAAC1/EAAT3 supplies neurons with the rate-limiting precursor for GSH synthesis, directly influencing their redox homeostasis (Paul et al., 2018). Furthermore, EAAC1/EAAT3 acts as a bridge between astrocytic and neuronal GSH metabolism by importing cysteine released from the extracellular breakdown of astrocytic GSH.

Glucose metabolism, GSH synthesis and glutamatergic homeostasis are closely associated processes and share extracellular and intracellular regulatory mechanisms. Among these, the neurotrophic factor BDNF has recently been demonstrated as a key regulator of central energy homeostasis (Marosi and Mattson, 2014). BDNF increases glucose transport in neurons by inducing the expression of GLUT3 through phosphatidylinositol-3 kinase (PI3K) and Akt kinase (Burkhalter et al., 2003). Because of the low neuronal content of PFKFB-3, glucose can be used for the generation of GSH through the PPP. Additionally, BDNF can activate hypoxia-inducible factor-1 (HIF-1) and Nrf2, which are related to the induction of enzymes that participate in glucose metabolism in both astrocytes and neurons. Importantly, Nrf2 regulates G6PD, GCL, GSH synthase, system xc^-, and EAAC1/EAAT3 (Thimmulappa et al., 2002; Shih et al., 2003; Escartin et al., 2011; Niture et al., 2014), thus facilitating both regeneration and synthesis of GSH.

Experimental data have suggested a role for GSH as a neuromodulator. As a thiol-containing compound, GSH may regulate the redox sites of several ionotropic receptors and ion channels, altering their functional characteristics (Gozlan and Ben-Ari, 1995; Pan et al., 1995). In this regard, both GSH and GSSG have been demonstrated to modulate neuronal depolarization, calcium ion influx and second messenger activity (Janáky et al., 1993; Varga et al., 1997) through the glutamatergic receptors NMDA and α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA). Such effects appear to be dependent on GSH concentrations. At micromolar levels, GSH is inhibitory via its interaction with glutamate binding sites. In contrast, at millimolar concentrations, GSH activates NMDA receptors by reducing functional thiol groups (Janáky et al., 1993). Interestingly, as a consequence, GSH becomes oxidized to GSSG, which triggers an increase in the PPP to generate GSH (Delgado-Esteban et al., 2000).

In retinal glial cells, GSH induces calcium shifts in a P2X7 (a purinergic receptor subtype), but not ionotropic glutamate receptor dependent manner. In contrast, GSSG

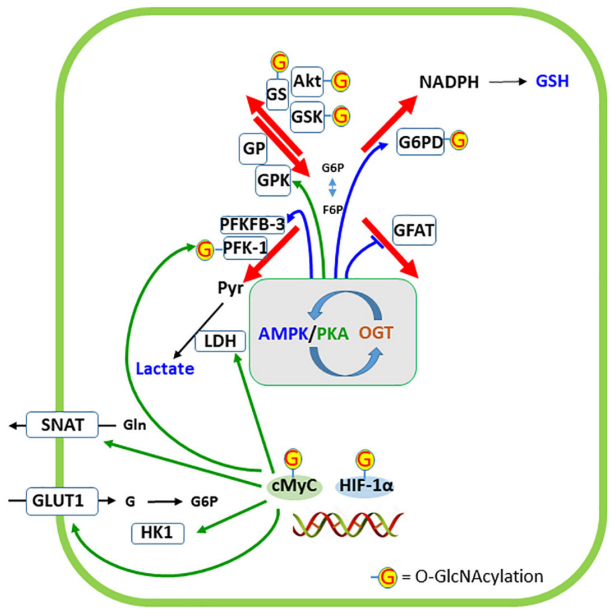

FIGURE 6 | Regulation of cellular destinations of glucose by O-GlcNAc modification of proteins. AMP-activated protein kinase (AMPK) and protein kinase A (PKA) have a narrow interaction in the regulation of glucose metabolism as well as phosphorylate and are targets of O-GlcNAc transferase (OGT). The phosphorylation of specific targets of AMPK or PKA in the four cellular destinations of glucose are indicated by the respective colored arrows. Targets of OGT are indicated by G. Two transcription factors closely related to glucose metabolism (cMyc and HIF-1α) are represented at the double-strand DNA and five proteins whose expressions are regulated by these transcription factors are indicated. G6PD, glucose-6-phosphate dehydrogenase; GFAT, glutamine:fructose-6P aminotransferase; GLUT1, glucose transporter 1; GP, glycogen phosphorylase; GPK, glycogen phosphorylase kinase; GS, glycogen synthase; GSK, glycogen synthase kinase; HIF-1α, hypoxia-inducible factor-1α; HK1, hexokinase 1; LDH, lactate dehydrogenase; PFK-1, phosphofructokinase 1; PFKFB-3, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3; SNAT, sodium-neutral amino acid transporter (type 3 or 5, in astrocytes).

did not reproduce this effect, indicating that the antioxidant and/or structural features of GSH are essential to promote elevations in cytoplasmic calcium levels (Freitas et al., 2016). In cortical brain slices, GSH was able to evoke a depolarizing potential that appears to be mediated by sodium ions. As this potential was not blocked by antagonists of glutamate receptors, GSH may act through its own receptor-mediated channels (Shaw et al., 1996). In this regard, radioligand binding assays have shown the presence of binding sites for GSH in different neural cell preparations, including brain synaptosomal membranes (Janáky et al., 2000) and astrocytes (Guo and Shaw, 1992). GSH also seems to be released upon calcium-dependent depolarization in brain slices (Zängerle et al., 1992) and is able to modulate the release of neurotransmitters, including GABA (Janáky et al., 1994; Freitas et al., 2016) and dopamine (Janáky et al., 2007). Interestingly, GSH can reverse aging—associated hippocampal synaptic plasticity deficits (Yang et al., 2010). Together, these data support an emerging role of GSH in signal transduction and synaptic transmission.

THE RIGHT LEG CONTROLS THE OTHER DESTINATIONS OF GLUCOSE IN NEURAL CELLS BY PROTEIN GLCNACYLATION

The Protein GlcNAcylation and Hexosamine Pathway

The post-translational modification of proteins by *O*-linked-*N*-acetyl-D-glucosamine (O-GlcNAc) is assumed to be a glucose-responsive mechanism that modulates cellular signaling (see Nagel and Ball, 2014 for a review). O-GlcNAc rapidly cycles onto the serine or threonine residues of target proteins. This process is equivalent to phosphorylation and occurs via the activity of two enzymes: O-GlcNAc transferase (OGT) and O-GlcNAcase (OGA), which add and remove GlcNAcetyl, respectively (see **Figure 5**). GlcNAcetyl is derived from the hexosamine biosynthetic pathway (HP), the right leg in our X of metabolic glucose destination. It is estimated that 2–5% of incoming glucose goes to the HP, which has UDP-GlcNAc as its final product, the donor of GlcNAc (Ozcan et al., 2010). In the liver, the levels of O-GlcNAc-modified proteins fluctuate according to the nutrient status, i.e., they are regulated by intracellular concentrations of UDP-GlcNAc that rise with feeding and are increased in diabetes mellitus patients (Hanover et al., 2010; Nagel and Ball, 2014). In the brain, under conditions of hypometabolism of glucose, as observed in AD, the levels of O-GlcNAc-modified proteins are reduced (Dos Santos et al., 2018). However, it is necessary to identify specific changes in protein GlcNAcylation to understand particular protein alterations in physiological and pathological conditions. Herein, we will discuss some aspects of HP regulation and specific targets of O-GlcNAcylation in astrocytes, which modulate glucose metabolism and synaptic communication.

The first reaction of HP is catalyzed by the glutamine:fructose-6P aminotransferase (GFAT) enzyme. The glutamine transfers the amine group to carbon 2 of fructose-6P, converting it to glucosamine-6P (Yuzwa and Vocadlo, 2014). Note that neurons depend on astroglial glutamine, since glutamine synthetase is a glial enzyme. In the next step, acetyl-CoA transfers acetyl to the amine group and then the phosphate from carbon 6 is transferred to carbon 1. This compound, *N*-acetyl-glucosamine-1P, reacts with UTP to release the end products UDP-GlcNAc and PPi. The rate-limiting step of HP is the reaction catalyzed by GFAT, which is negatively modulated by AMPK (Eguchi et al., 2009), like other glycolytic key enzymes such as PFK-1 and PFKFB-3. Therefore, besides glucose flow, GlcNAcylation of proteins is regulated by GFAT, and also by the activities of OGT and OGA (Worth et al., 2017).

The OGT and OGA enzymes are evolutionarily well conserved and have many targets involved in signal transduction, transcription, translation, cell cycle control and apoptosis. However, since these are just two enzymes (in contrast to the

hundreds of protein kinases and phosphatases), there is still little understanding of how their targets are recognized (Yang and Qian, 2017). Moreover, Ser and Thr sites for GlcNAcylation and phosphorylation co-exist in the same protein and these modifications often share the same sites, establishing a complex functional relationship (Wang et al., 2007; Hu et al., 2010).

Glucose Flow Regulates Its Own Fate and Its Derivatives

The main protein targets of O-GlcNAcylation, which modulate glucose flow and/or destination, are indicated in **Figure 6**. GFAT, the regulatory enzyme of HP, is inhibited by phosphorylation, catalyzed by AMPK, at Ser 243 (Eguchi et al., 2009). Therefore, activated AMPK decreases UDP-GlcNAc levels. However, AMPK is also able to phosphorylate (activate in this case) the OGT at Thr 444, which in turn could lead to O-GlcNAcylation of AMPK, resulting in a complex regulation that involves changes in the activities of enzymes as well as their cellular localizations (Bullen et al., 2014). It is possible, but has not yet been determined, that this triangular interaction among AMPK, GFAT, and OGT occurs in neural cells, particularly in astrocytes, where AMPK has a crucial role (Bolaños, 2016).

Glutathione, as discussed above, has an important antioxidant role in neurons. However, at higher concentrations in astrocytes, it is exported not only to support neuronal synthesis, but also to modulate ionotropic synaptic receptors. Glucose generates NADPH in the PPP to recycle GSH in neurons and astrocytes. The G6PD enzyme is the rate-limiting step of this pathway. G6PD is modulated by O-GlcNAcylation, as demonstrated in several cell lines (Rao et al., 2015). In contrast to O-GlcNAcylation of GFAT, this modification of G6PD activates the enzyme, increasing the activity of the PPP and NADPH formation. Although this is of importance in neurons to regenerate GSH, the effect of O-GlcNAcylation on neuronal G6PD has not yet been analyzed. However, it is possible to realize the importance of glutamine from astrocytes to neuronal synthesis of glucosamine-6P, the precursor of UDP-GlcNAc. Another important aspect of GSH synthesis, particularly in astrocytes, is its modulation by AMPK (Guo et al., 2018). AMPK positively regulates the expression of the modulatory subunit of enzyme GCL through the transcriptional co-activator peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), (**Figure 4**), which catalyzes the first step (and regulatory step) of GSH synthesis.

Glycogen synthase (GS) is covalently modified by phosphorylation (induced by catecholamines and insulin) and O-GlcNAcylation, at least in adipocytes (Parker et al., 2004). Phosphorylated GS is less sensitive to the allosteric activator, glucose-6P. Insulin phosphorylates both GSK3 and the glycogen-targeting protein through the PI3K/Akt pathway, in turn activating protein phosphatase 1, PP1. Phosphorylation of both GSK-3 and PP1 activates GS, which leads to glycogen formation (Obel et al., 2012). Similarly, glucose flow in the HP leads to O-GlcNAcylation and activation of the GS. This is an example of mutual exclusivity, where O-GlcNAcylation acts similarly to dephosphorylation, which cannot be generalized to other conditions. Moreover, this relationship is more complex because the upstream enzymes, Akt and GSK3, are also targets of O-GlcNAcylation (e.g., Park et al., 2005).

Glycogen breakdown is also regulated by phosphorylation of glycogen-targeting protein by protein kinase A (PKA) at a different site of Akt (Bak et al., 2018). This PKA-induced phosphorylation is triggered by neurotransmitters. The resulting phosphorylation of glycogen-targeting protein at the glycogen granule dissociates PP1, glycogen phosphorylase (GP), glycogen phosphorylase kinase (GPK), and GS. PKA phosphorylates/activates GPK, which in turn phosphorylates/activates GP. This dissociation is an important step because both GP and GPK are targets of PP1. Moreover, PKA phosphorylates/activates the inhibitor 1 of PP1. It was recently reported that the catalytic subunit of PKA is a target of O-GlcNAcylation (Xie et al., 2016), but the direct effect on glycogen breakdown remains unclear. Interestingly, O-GlcNAc protein modification increases in tumor cells, in response to glucose deprivation, through glycogen degradation (Kang et al., 2009), contradicting the idea that an increase in O-GlcNAcylation acts as a negative feed-back signal to ATP generation from glucose. This phenomenon may involve changes in GS/GP balance, due to changes in OGT and/or OGA, and not UDP-GlcNAc levels (Taylor et al., 2008).

Lactate generation is strongly regulated by O-GlcNAcylation because PFK-1 and PFKFB-3 are direct and indirect targets of OGT, respectively. O-GlcNAc modification of PFK-1 at Ser529 is induced by hypoxia in cancer cells and this modification inhibits enzyme activity and redirects the flux of glucose to PPP (Yi et al., 2012). The authors also observed a modest O-GlcNAcylation of HK. PFKFB-3, which regulates PFK-1, is phosphorylated by AMPK and Akt, which are targets of OGT, as mentioned above. Therefore, it is possible to conceive an interaction between AMPK, PFKFB-3, and OGT, that is just as complex or even more than the interaction between AMPK-GFAT-OGT (Bullen et al., 2014). Moreover, at least two transcription factors that have been well studied in tumor cells and are connected to glycolysis are modified by O-GlcNAcylation: c-Myc and HIF-1α. Akt/c-Myc activation induces expression of GLUT-1, HK 1 and 2, PFK-1, lactate dehydrogenase A and glutamine transporters (Miller et al., 2012; Swamy et al., 2016; Zhang et al., 2017). HIF-1α induces GLUT-1 and 3, hexokinases, and PFK-1 (Chen and Russo, 2012). Interestingly, also in cancer cells, lactate is able to trigger changes in glutamine uptake and metabolism (Pérez-Escuredo et al., 2016), which are necessary not only for cell proliferation but also for protein O-GlcNAcylation. Considering the importance of glutamine/glutamate in brain tissue and lactate in neuron/astrocyte communication, it would seem that this mechanism is worthy of investigation in the nervous system.

Finally, the interplay between AMPK and PKA in glucose metabolism should be considered. Microdomains of cAMP have been characterized to explain the localized action of this intracellular messenger, at the membrane or on soluble adenylyl cyclase. cAMP acts on PKA and is inactivated by phosphodiesterase. Both enzymes (PKA and sAC) bind to A-kinase anchoring protein (AKAP; Zippin et al., 2004; Oliveira et al., 2010). A physical connection between PKA and AMPK, via AKAP, has been proposed in muscle cells, where AMPK

phosphorylates AKAP, releasing PKA (Hoffman et al., 2015). In hepatocytes, PKA phosphorylates and reduces AMPK activity (Hurley et al., 2006). Again, although most data regarding protein O-GlcNAcylation derive from tumor and/or non-neural cells, protein O-GlcNAcylation may also occur in the brain. Therefore, it is possible that a general cross-talk occurs between PKA and AMPK (involving O-GlcNAcylation, because both are targets of OGT) with a role in the metabolic regulation of glucose destination and synaptic plasticity.

SUMMARY

The importance of glucose for brain activity is very clear, since glucose provides ATP and replenishment of substrates, such as glutamate and cholesterol. Additionally, glucose metabolism provides derivatives such as lactate, MG and GSH, which regulate synaptic communication. Herein, we propose an intersection in an "X" that defines the four destinations of glucose in neural cells, where astrocytes work as integrative and modulatory elements in the synaptic communication. Such destinations depend on the metabolic arrangement in each cell type, which in turn depends on the glucose supply and neural activity. Extracellular L-lactate released by astrocytes, either generated from recently captured glucose or from glycogen, binds to HCAR1, a specific perivascular and post-synaptic receptor, regulating synaptic plasticity. Currently, lactate is being considered as a putative gliotransmitter. MG results from a deviation of the glycolytic pathway and is metabolized to D-lactate. Both MG and D-lactate are released and modulate neuronal activity, possibly through $GABA_A$ and HCAR1, respectively. The main cellular antioxidant GSH, whose regeneration depends on the PPP, is also released by astrocytes and alters the synaptic response by modulating the redox and non-redox sites of ionotropic receptors and ion channels. O-GlcNAcylation is an important post-translational protein modification for cell signaling, and the glucose flow through the HP regulates the content of UDP-GlcNAc. The levels of UDP-GlcNAc, in part, are determinant for the GlcNAc-modification of proteins, including the proteins that modulate the glucose destinations. As discussed above, glutamine from astrocytes is essential, literally, for the neuronal synthesis of UDP-GlcNAc. Although the coupling between astrocytes and neurons most often addresses the relationship of glutamate or GABA with the glycolytic pathway, we should not forget that other neurotransmitters also modulate glucose metabolism, which then regulates neurotransmission through glycolysis-derived products.

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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Matured Hop Bitter Acids in Beer Improve Lipopolysaccharide-Induced Depression-Like Behavior

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Recent studies have demonstrated a close association between neural inflammation and development of mental illnesses, such as depression. Clinical trials have reported that treatment with non-steroidal anti-inflammatory drugs is associated with reduced risk of depression. Moreover, nutritional approaches for the prevention and management of depression have garnered significant attention in recent years. We have previously demonstrated that iso-α-acids (IAAs)—the bitter components in beer—suppress hippocampal microglial inflammation, thereby improving cognitive decline. However, effects of hop-derived components other than IAAs on inflammation have not been elucidated. In the present study, we demonstrated that consumption of matured hop bitter acids (MHBAs) generated from α- and β-acids, which show a high similarity with the chemical structure of IAAs, suppress lipopolysaccharide (LPS)-induced cytokine productions in the brain. MHBAs administration increased norepinephrine (NE) secretion and reduced immobility time which represents depression-like behavior in the tail suspension test. Moreover, MHBAs components, including hydroxyallohumulinones and hydroxyalloisohumulones, reduced LPS-induced immobility time. Although further researches are needed to clarify the underlying mechanisms, these findings suggest that MHBAs reduce inflammatory cytokine productions and increase NE secretion, thereby improving depression-like behavior. Similarly, inoculation with LPS induced loss of dendritic spines, which was improved upon MHBAs administration. Additionally, vagotomized mice showed attenuated improvement of immobility time, increase in NE level, and improvement of dendrite spine density following MHBAs administration. Therefore, MHBAs activate the vagus nerve and suppress neuronal damage and depression-like behavior induced by inflammation.

Keywords: matured hop bitter acids, depression, inflammation, vagus nerve, norepinephrine, dendritic spine, hippocampus, brain-gut axis

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INTRODUCTION

The incidence of mental illnesses, particularly depression, has increased in recent years, which has become a major social problem. The World Health Organization (WHO) has reported that over 300 million people worldwide suffered from depression in 2015. Medical costs related to mental illnesses represent an economic burden, and according to the Organization for Economic Co-operation and

Development, they can account for up to 4% of the gross domestic product. Unfortunately, the existing anti-depressant therapies show therapeutic efficacy in as few as one-third of the patients suffering from depressive disorders (Trivedi et al., 2006). Thus, effective treatment for depression remains an unmet clinical need. Recent studies have shown that brain inflammation plays a role in the pathophysiology of major depressive disorders (Dantzer et al., 2008; Hashimoto, 2015; Setiawan et al., 2015; Miller and Raison, 2016). Moreover, anti-inflammatory agents, such as celecoxib and minocycline, decrease depressive symptoms (Kohler et al., 2014; Dean et al., 2017; Husain et al., 2017). Microglia—one of the sites of pro-inflammatory cytokine production—are the key players in the neuronal immune system and are most enriched in the hippocampus. They play critical roles in the development of depression-like phenotype in rodents (Lawson et al., 1990; Zhang et al., 2014). Brain imaging has revealed that microglia in patients suffering from depression are more activated than in healthy subjects (Setiawan et al., 2015). Therefore, anti-inflammatory treatments may be applicable as anti-depressant treatments.

WHO has reported that the proportion of patients suffering from depression who receive appropriate diagnosis and treatment is <10%, particularly in developing countries (World Health Organization, 2017). Nutritional intervention in daily life and nutrition-based approaches for the prevention and management of depression have garnered much attention globally. Recent studies have shown that some nutrients suppress inflammation, leading to the prevention of depression and depression-like behavior. Quercetin (a type of flavonoid) prevented chronic unpredictable stress-induced behavioral dysfunction in mice by alleviating hippocampal oxidative and inflammatory stress (Mehta et al., 2017). Previously, we have demonstrated that iso-α-acids (IAAs)—the hop-derived bitter components in beer—prevented hippocampal inflammation and cognitive impairment by acting on microglia in a mouse model of Alzheimer's disease and in high-fat diet-induced obese mice (Ano et al., 2017; Ayabe et al., 2018a). However, effects of hop-derived components other than IAAs on inflammation remain unknown.

Matured hop bitter acids (MHBAs), generated by oxidizing α- and β-acids that comprise the bitter components in beer, show chemical structures similar to IAAs derived from α-acids. We have recently reported that MHBAs administration improved hippocampus-dependent memory in mice through increased norepinephrine (NE) secretion in the hippocampus (Ayabe et al., 2018b); however, effects of MHBAs on brain inflammation and mental disorders were not elucidated in that study.

In the present study, we examined whether MHBAs supplementation suppressed inflammation in the hippocampus and evaluated the ability of MHBAs and their components to prevent inflammation-induced depression-like behavior. To this end, we used the acute neural inflammatory animal model induced by lipopolysaccharide (LPS) that demonstrates depression-like behavior (Frenois et al., 2007; Bay-Richter et al., 2011; Lawson et al., 2013; Chen et al., 2017; Li et al., 2017).

MATERIALS AND METHODS

Materials

Matured hop bitter acids and each of its major components [4′-hydroxyallohumulinones (HAH); 4′-hydroxyalloisohumulones (HAIH); tricyclooxyisohumulones A (TCOIH-A); hulupones; and humulinones] were prepared from hop pellets as described elsewhere (Taniguchi et al., 2015a,b). NE was purchased from Sigma-Aldrich Co. (St. Louis, MO, United States) for the analysis of monoamines.

Animals

Five-week-old male Crlj:CD1 (ICR) mice and vagotomized male ICR mice were purchased from Charles River Laboratories Japan, Inc. (Tokyo, Japan). In the vagotomized mice, vagus nerves were cut under the diaphragm. Vagotomy was performed on 5-week-old ICR mice at Charles River Laboratories Japan, Inc. The control group underwent sham operation. The vagotomized and sham operated mice were used for experimental procedures only after they turned 6–8 week old. Mice were maintained at room temperature (23 ± 1°C) under constant 12/12-h light/dark cycle (light period from 8:00 a.m. to 8:00 p.m.). All mice were acclimatized by feeding a standard rodent diet (CE-2; Clea Japan, Tokyo, Japan) for 7 days before experimental procedures. All animal care and experimental procedures were conducted by July in 2018 in accordance with the guidelines of the Animal Experiment Committee of Kirin Company, Ltd. All efforts were made to minimize animal suffering. All studies were approved by the Animal Experiment Committee of Kirin Company, Ltd.

Drug Treatment

Test compounds [MHBAs; 1, 10, or 50 mg/kg, HAIH; 10 mg/kg, TCOIHA; 1 mg/kg, HAH; 1 mg/kg] or distilled water (DW) were orally administered once a day for 6 consecutive days after habituation. To induce brain inflammation after 60 min of the last administration, mice were deeply anesthetized with sodium pentobarbital (Kyoritsu Seiyaku, Tokyo, Japan), and LPS (15 μg/mouse, from *Escherichia coli* O111:B4; Sigma Aldrich, St. Louis, MO, United States) or saline (for sham-operated controls) was then injected by hand into the cerebral ventricle in a volume of 5 μL/hemisphere in accordance with a previous study (Ano et al., 2015). At 23 h after the injection of LPS or saline (experimental day 7), mice were orally administered with test compounds or DW and subjected to behavioral evaluation (**Figure 1A**); cytokine and NE content were analyzed. In experiments using vagotomized mice, animals were subjected to Golgi–Cox staining after behavioral evaluation.

Tail Suspension Test (TST)

Tail suspension test (TST) was performed 60 min after the final administration of test compounds or DW to evaluate the effect of test compounds on depression-like behavior. One end of an adhesive tape (width 1 cm, length 15 cm) was fixed to the upper surface of a box such that the head of the mouse,

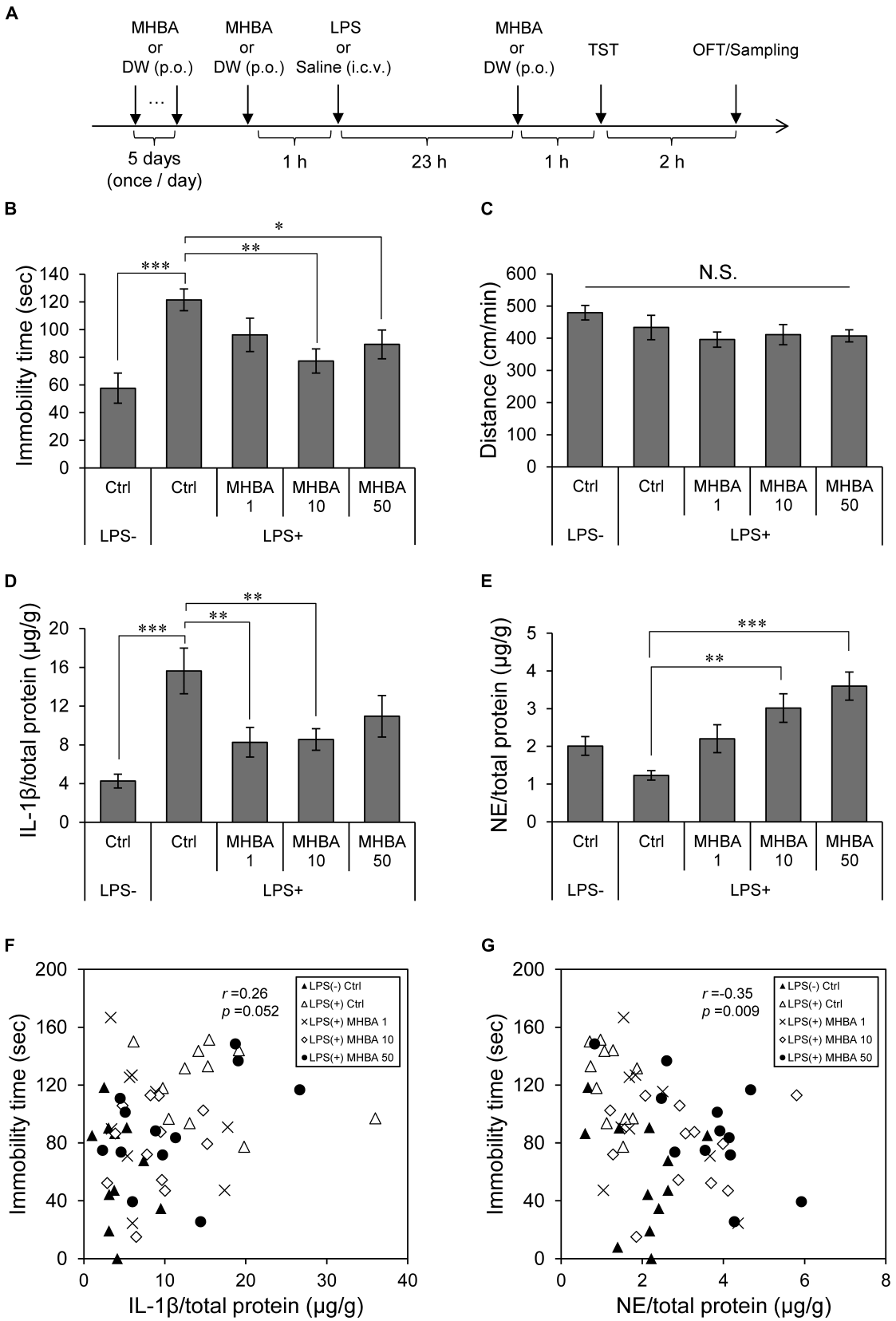

FIGURE 1 | Continued

FIGURE 1 | Repeated MHBAs administration improves depression-like behavior induced by LPS and increases norepinephrine (NE) levels in the hippocampus. **(A)** At 6 days after oral treatment with distilled water (DW) or matured hop bitter acids (MHBAs; 1, 10, or 50 mg/kg), saline or lipopolysaccharide (LPS; 15 μg/mouse) was injected intracerebroventricularly to induce depression-like behavior. Additional MHBAs or DW was orally administered 23 h after treatment with LPS or saline. Tail suspension test (TST) was performed 60 min after the final administration of test compounds or DW, and open-field locomotor test (OFT) was performed 120 min after performing TST. **(B)** Immobility time, which represented the depression-like behavior, was evaluated by TST for 6 min. Ctrl indicates control; LPS- and LPS+ indicate the absence and presence of LPS, respectively, and MHBAs 1, 10, and 50 indicate MHBAs concentrations in mg/kg. **(C)** Distance moved in an open field was evaluated for 6 min by OFT following TST. Ctrl indicates control; LPS- and LPS+ indicate the absence and presence of LPS, respectively, and MHBA 1, 10, and 50 indicate MHBAs concentrations in mg/kg. **(D)** Interleukin-1β (IL-1β) levels in hippocampus were measured using ELISA. Ctrl indicates control; LPS- and LPS+ indicate the absence and presence of LPS, respectively, and MHBA 1, 10, and 50 indicate MHBAs concentrations in mg/kg. **(E)** Following OFT, hippocampus samples containing monoamines were prepared. NE levels were determined using HPLC–ECD. Ctrl indicates control; LPS- and LPS+ indicate the absence and presence of LPS, respectively, and MHBA 1, 10, and 50 indicate MHBAs concentrations in mg/kg. **(F)** Correlation between immobility time and hippocampal IL-1β levels is shown (Pearson correlation coefficient $r = 0.26$, $P = 0.052$). **(G)** Correlation between immobility time and hippocampal NE levels is shown (Pearson correlation coefficient $r = 0.35$, $P = 0.009$). All values are expressed as means and SEM (n = 9–12 mice per group). Experimental data were analyzed by Dunnet's test; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared with LPS-treated group administered DW.

in an inverted state, would be at a height of 45–50 cm from the bottom of the box. The other end (2 cm) of the tape was firmly wrapped around the tail (1 cm from the tip) and the mouse was suspended from its tail for 6 min. The mice were visually inspected from a separate room using a video camera and monitor, and the immobility time within 6 min was evaluated. Mice were considered immobile only when they hung passively and completely motionless.

Open-Field Locomotor Test (OFT)

To measure locomotor activities, open-field locomotor test (OFT) was performed 120 min after TST. Mice were individually placed at the centre of a cubic chamber (40 cm × 40 cm × 40 cm) made of gray polyvinyl chloride. Horizontal movements of mice were measured using automatic actography (SMART Video Tracking System, Harvard Apparatus, Holliston, MA, United States). The test lasted for 6 min.

Brain Sample Preparation for Measuring of Cytokine and NE Levels

The hippocampus was collected from each hemisphere and homogenized using a multi-bead shocker (Yasui Kikai, Osaka, Japan) in RIPA buffer (Wako, Osaka, Japan) containing a protease inhibitor cocktail (BioVision, Mountain View, CA, United States). Supernatants were collected after centrifugation at 14,000 rpm for 30 min.

Analysis of Inflammation in the Hippocampus

Supernatant obtained after processing of the left hippocampus was used for cytokine evaluation. Total protein concentration of each supernatant was measured using the BCA Protein Assay Kit (Thermo-Scientific, Yokohama, Japan). For quantifying cytokines, supernatant was evaluated using enzyme-linked immunosorbent assay (ELISA) kit (eBiosciences, San Diego, CA, United States) in accordance with manufacturer's instructions.

Analysis of NE in the Hippocampus

Supernatant obtained after the processing of the right hippocampus was used for monoamine analysis after treatment with 0.2 M perchloric acid and filtration using 0.22 μM membrane filter (Millipore, Bedford, MA, United States). Monoamines were quantified using high-performance liquid chromatography coupled with electrochemical detection (HPLC–ECD; Eicom, Kyoto, Japan) using Eicompak SC-5ODS and PrePak columns (Eicom, Kyoto, Japan). The mobile phase comprised 83% 0.1 M acetic acid in citric acid buffer (pH 3.5), 17% methanol (Wako, Osaka, Japan), 190 mg/mL of sodium 1-octanesulfonate sodium (Wako, Osaka, Japan), and 5 mg/mL EDTA-Na_2. For ECD, the applied voltage was 750 mV vs. an Ag/AgCl reference electrode.

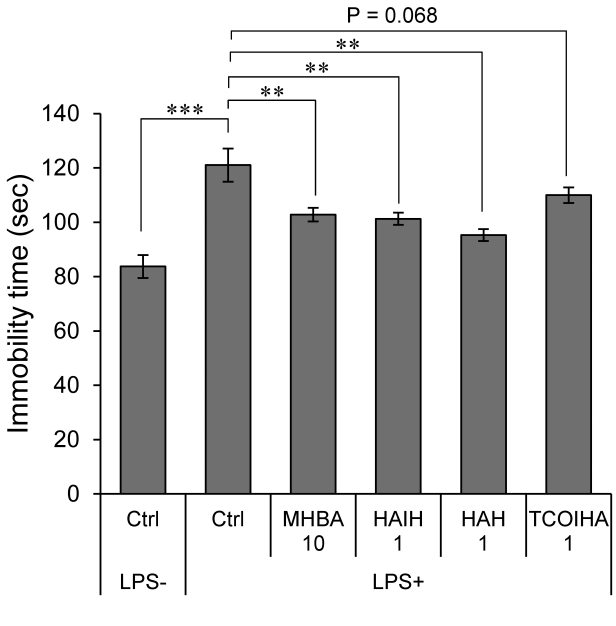

FIGURE 2 | Major compounds in MHBAs exert anti-depressant effect. Effects of MHBAs (10 mg/kg) and each of its major compounds [HAIH (1 mg/kg), HAH (1 mg/kg), and TCOIHA (1 mg/kg)] on depression-like behavior were evaluated with TST using an identical procedure reported in **Figure 1A**. Ctrl indicates DW-treated control group; LPS- and LPS+ indicate the absence and presence of LPS, respectively and MHBA 10, and HAIH, HAH, and TCOIHA indicate concentrations of each compound in mg/kg. All values are expressed as means and SEM (n = 5 mice per group). Experimental data were analyzed by Dunnet's test; $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with LPS-treated group administered DW.

Golgi–Cox Staining and Evaluation of Dendritic Spine Density

To evaluate dendritic spine density in the hippocampus, the left hemispheres were stained using commercially available FD Rapid GolgiStain Kit (FD Neurotechnologies, Columbia, MD, United States) following manufacturer's instructions. Brains were immersed in the impregnation solution, which comprised equal volumes of Solutions A and B, and stored at room temperature for 2 weeks in the dark. The impregnation solution was replaced with fresh solution after the first 6 h of immersion. The brains were then transferred into Solution C and stored at room temperature for 72 h in the dark. The solution was replaced with fresh solution on the next day. Stained brains were cryosectioned at -2.16 mm relative to Bregma in horizontal section planes at a thickness of 100 μm. Hippocampal CA3 dendrites were microscopically imaged using a UPlanApo 20× objective (Olympus, Tokyo, Japan), and spines were counted starting from their point of origin from the primary dendrite to the secondary branch of dendrites. For the measurement of spine densities, only those spines that emerged perpendicular to the dendritic shaft were counted. Regions of 20–30 μm were randomly and individually selected from all the neurons where the image was clearly captured, and spine density was evaluated. Consequentially, three to five neurons were measured per mouse, and average value was calculated as the individual value for the mouse.

Statistical Analysis

All values are expressed as means and SEM. Experimental data were analyzed by Dunnett's test or Tukey–Kramer's test. $P < 0.05$ was considered statistically significant. All statistical analyses were performed using the BellCurve for Excel (Social Survey Research Information Co., Ltd., Tokyo, Japan).

RESULTS

Effects of MHBAs on LPS-Induced Depressive Behavior

To evaluate effects of MHBAs on depression-like behavior induced by inflammation, we conducted TST using mice that were intracerebroventricularly inoculated with LPS. TST test revealed that immobility time in LPS-treated mice was significantly longer than that in sham-treated mice, indicating LPS-induced depression-like behavior in mice (**Figure 1B**). In contrast, the immobility time in LPS-inoculated mice treated with MHBAs at 10 or 50 mg/kg was significantly shorter than that in LPS-inoculated sham-treated mice. Locomotor activities, as evaluated by OFT, did not differ among the groups (**Figure 1C**). These results indicate that MHBAs improved LPS-induced depression-like behavior. Moreover, we measured interleukin (IL)-1β levels in the hippocampus to evaluate inflammation level (**Figure 1D**). IL-1β level in the hippocampus of LPS-inoculated mice was significantly increased compared with that in the hippocampus of sham-treated mice but significantly decreased in the hippocampus of LPS-inoculated mice treated with either 1 or 10 mg/kg MHBAs. These results suggest that MHBAs prevent inflammation in the hippocampus. Previous studies have shown that NE might play important roles in depression-like behavior (Liu et al., 2012; Barua et al., 2018). Thus, we quantified NE level in the hippocampus of mice using HPLC (**Figure 1E**). NE levels in the hippocampus of mice treated with 10 and 50 mg/kg MHBAs were significantly increased. IL-1β ($r = 0.26$, $P = 0.052$) (**Figure 1F**) and NE ($r = -0.35$, $P = 0.009$) (**Figure 1G**) levels in the hippocampus of mice were weakly correlated with immobility time observed in TST.

Effects of Major Compounds in MHBAs on LPS-Induced Depression-Like Behavior

Matured hop bitter acids constitute both α- and β-acid oxidants with β-tricarbonyl structures (Taniguchi et al., 2015a,b). Effects of three major constituents (HAH, HAIH, and TCOIHA) of MHBAs on depression-like behavior were examined in TST. Immobility time in mice treated with 1 mg/kg HAH and HAIH was significantly lower than that in the sham-treated mice and was equivalent to the effect of MHBAs at 10 mg/kg (**Figure 2**). Moreover, mice treated with 1 mg/kg TCOIHA showed a reduced immobility time ($P = 0.068$). These results indicate that major compounds in MHBAs contribute to the suppression the concomitant depression-like behavior in TST induced by inflammation.

Involvement of the Vagus Nerve in Effects of MHBAs on Depression-Like Behavior

To elucidate whether MHBAs act through the blood–brain barrier (BBB) or through the vagus nerve, vagotomized or sham-treated mice were administered oral MHBAs and subjected to TST. Immobility time in LPS-inoculated vagotomized mice was significantly longer than that in saline-inoculated vagotomized mice (**Figure 3A**). In contrast, immobility time remained unchanged in vagotomized mice administered MHBAs or DW. Locomotor activities in OFT remained unchanged among all vagotomized groups (**Figure 3B**). These results indicate that the vagus nerve was involved in effects of MHBAs on the improvement of LPS-induced depression-like behavior.

In sham mice, NE level in the hippocampus increased upon MHBAs administration [vs. LPS (+) Ctrl, $P = 0.060$] (**Figure 3C**). However, in vagotomized mice, NE levels did not change upon MHBAs administration. We used Golgi–Cox staining to evaluate effects of MHBAs on dendritic changes in LPS-inoculated mice. The density of dendritic spines in the hippocampus significantly decreased after LPS treatment (**Figures 3D,E**), which was consistent with previous reports (Zhang et al., 2014; Ma et al., 2017). MHBAs administration improved reduction in the density of dendritic spines in LPS-inoculated mice. Conversely, no such improvement was observed upon MHBAs administration in vagotomized mice. These results suggest that effects of MHBAs on depression-like

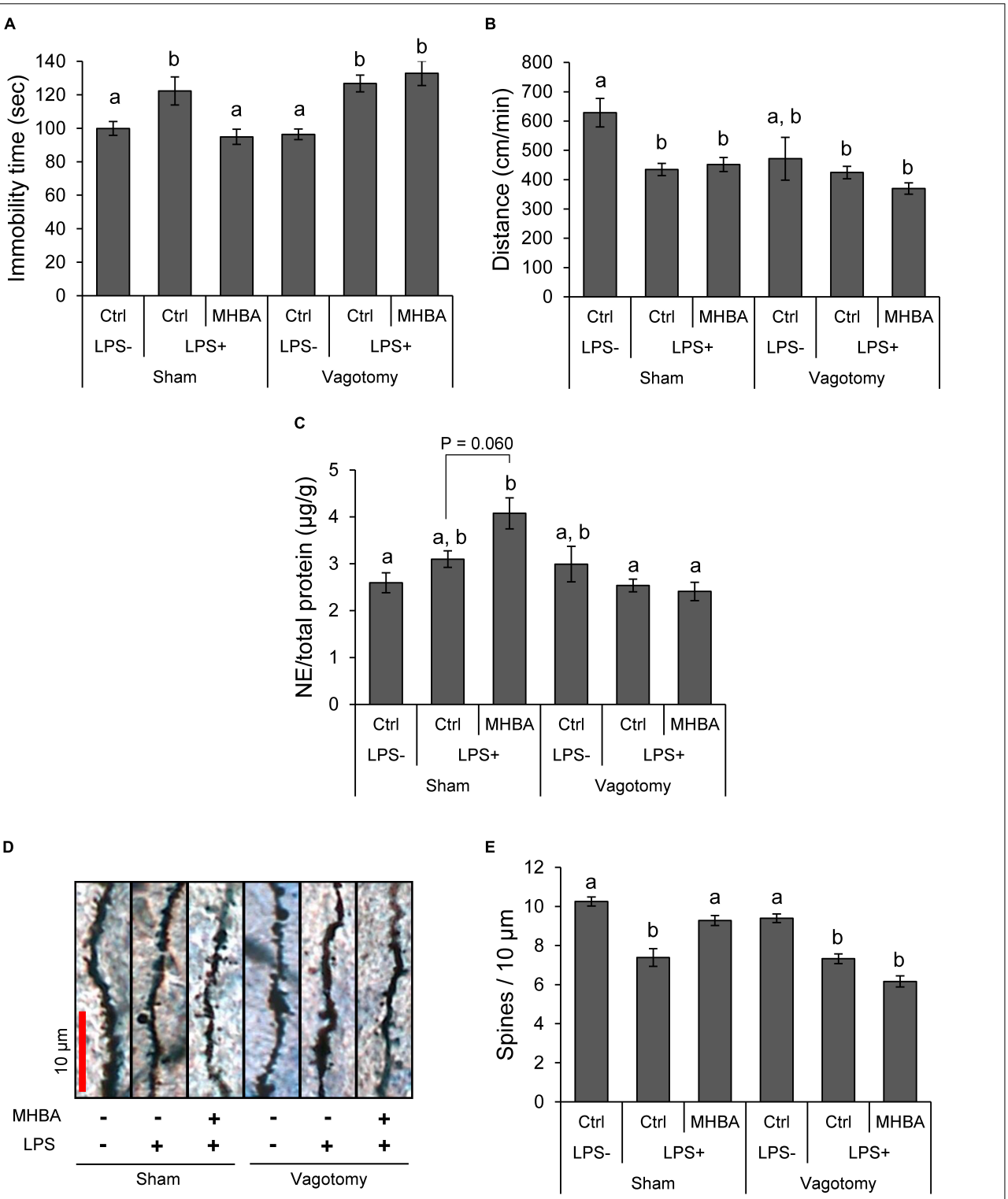

FIGURE 3 | Effects of MHBAs on depression-like behavior were mediated by vagus nerve stimulation. Five-week-old mice are vagotomized and used for experimental procedures at 6–8 weeks of age. The drug treatment procedure was essentially similar to that reported in **Figure 1A**. **(A)** Anti-depressant effects of MHBAs (10 mg/kg) on sham or vagotomized mice were evaluated using TST. **(B)** Distance moved in open field was evaluated for 6 min using OFT following TST. **(C)** After OFT, mice were sacrificed and hippocampal samples containing monoamines were prepared. Norepinephrine (NE) levels were determined using HPLC–ECD. **(D,E)** Density of dendritic spines in the hippocampus was evaluated by Golgi–Cox staining. Ctrl indicates control, and LPS- and LPS+ indicate the absence and presence of LPS, respectively. Experimental data were analyzed using Tukey–Kramer's test (n = 5–7 mice per group). $P < 0.05$ was considered significant. Different letters indicate significant differences ($P < 0.05$) between the groups.

behavior and hippocampal NE levels are mediated by the vagus nerve.

DISCUSSION

Inflammation can elicit severe behavioral changes, including the onset of depressive symptoms characterised by feelings of sadness and fatigue and withdrawal of social behavior (Slavich and Irwin, 2014). To the best of our knowledge, the present study is the first report to demonstrate that the administration of MHBAs derived from hops improves LPS-induced depression-like behavior. In addition, elevated NE levels and suppressed IL-1β levels were confirmed. Previous studies have indicated that chronic stress significantly increases hippocampal IL-1β levels (Liu et al., 2014; Zhang et al., 2016), which plays a key role in the development of depression owing to the high density of hippocampal microglia (Bluthe et al., 2000; Tsai, 2017; Yamawaki et al., 2018). In the present study, IL-1β levels in the hippocampus were correlated with immobility time in TST (**Figure 1F**). Therefore, although further studies are required to prove a direct causal relationship between the attenuation of depression-like behavior and the suppression of inflammatory responses by MHBAs.

Moreover, depression is characterized by dendritic changes (Duman and Duman, 2015). MHBAs administration prevented dendritic loss induced by LPS (**Figures 3D,E**). Decrease in dendritic spine density due to inflammation is mediated by decrease in levels of neural protective factors, such as BDNF (Zhang et al., 2014). Therefore, anti-inflammatory effects of MHBAs are certainly one of the protective mechanisms against changes in dendritic spine density.

MHBAs are a mixture of α- and β-acid oxidants, which are characterized by a common β-tricarbonyl moiety (Taniguchi et al., 2015a,b). We demonstrated that HAIH and HAH significantly improved LPS-induced depression-like behavior and TCOIHA tended to improve symptoms (**Figure 2**). Thus, the common β-tricarbonyl moiety of HAIH, HAH, TCOIHA, and IAAs might contribute to the improvement of depression-like behavior.

Consistent with a previous report, our results indicated that MHBAs administration increased NE levels in the hippocampus (Ayabe et al., 2018b). Hippocampal NE levels and immobility time in TST were weakly correlated (**Figure 1G**). Increased hippocampal NE levels reportedly contribute to the improvement of depression-like behavior (Liu et al., 2012; Barua et al., 2018). This might partly explain the mechanism underlying the suppression of inflammation-induced depression-like behavior in TST by MHBAs. However, decrease in immobility time after MHBAs administration to non-LPS-treated mice was not significant (data not shown), suggesting that the suppression of depression-like behavior in TST was not mediated by NE alone; moreover, the concentration of MHBAs (at 50 mg/kg) that mediated the highest increase in NE level did not induced the shortest immobility time. Previous studies performed under normal conditions have suggested that NE aids microglia in brain function regulation (Kaczmarczyk et al., 2017). Adrenergic β1- and β2-receptors are the only functionally significant adrenergic receptors in the microglia, which are activated primarily by NE (O'Donnell et al., 2012). Thus, increase in NE levels mediated by MHBAs might suppress increase in inflammatory cytokine levels in the microglia. However, since anti-inflammatory effects of MHBAs and NE levels were not significantly correlated (r = -0.12, P = 0.37, data not shown), the existence of several mechanisms underlying these anti-inflammatory effects are plausible. Further studies are warranted to elucidate the association between the suppression of inflammation and increase in NE production in the hippocampus.

Furthermore, the anti-depressant effects of MHBAs were mediated by the vagus nerve (**Figure 3**), suggesting that vagus nerve stimulation (VNS) from the intestinal tract is involved in the effect of MHBAs on depression-like behavior rather than the direct absorption of MHBAs into the brain through BBB. In 2005, the Food and Drug Administration has approved VNS therapy using a small device, which elicits electrical stimulation, for managing treatment-resistant depression (Sackeim et al., 2001). Despite its effectiveness in treating depression, the underlying mechanisms through which VNS mediates depression remain unclear (Nemeroff et al., 2006; Vonck et al., 2014). Previous studies have indicated that VNS increases hippocampal NE levels via the locus coeruleus (Hassert et al., 2004; Roosevelt et al., 2006). Taken together, VNS may exert its positive effects against depression by increasing NE levels in the brain and suppressing of inflammation.

CONCLUSION

MHBAs administration suppressed neural inflammatory responses, increased hippocampal NE levels, and attenuated LPS-induced depression-like behavior. Furthermore, these effects of MHBAs were mediated by VNS. However, detailed mechanisms underlying anti-depressant effects of MHBAs warrant further research. Activation of the vagus nerves through specific diet, which can in turn improve depression-like behavior, is a safe and novel approach for treating depression.

AUTHOR CONTRIBUTIONS

TF and YA designed and performed the experiments, analyzed the data, and wrote the paper. RO and KK performed the experiments.

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frontiers in Neuroscience

REVIEW
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Ketogenic Diet and Epilepsy: What We Know So Far

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The Ketogenic Diet (KD) is a modality of treatment used since the 1920s as a treatment for intractable epilepsy. It has been proposed as a dietary treatment that would produce similar benefits to fasting, which is already recorded in the Hippocratic collection. The KD has a high fat content (90%) and low protein and carbohydrate. Evidence shows that KD and its variants are a good alternative for non-surgical pharmacoresistant patients with epilepsy of any age, taking into account that the type of diet should be designed individually and that less-restrictive and more-palatable diets are usually better options for adults and adolescents. This review discusses the KD, including the possible mechanisms of action, applicability, side effects, and evidence for its efficacy, and for the more-palatable diets such as the Modified Atkins Diet (MAD) and the Low Glycemic Index Diet (LGID) in children and adults.

Keywords: refractory epilepsy, ketogenic diet, modified Atkins diet, low glycemic index, diet therapy

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INTRODUCTION

Epilepsy is a disabling and common neurological disease, which can be controlled successfully in most patients with one or more antiepileptic drugs. Approximately 30% of patients with epilepsy have refractory epilepsy, that is, have a failure of adequate trials of two tolerated, appropriately chosen and used antiepileptic drug schedules to achieve sustained relief of seizures (Picot et al., 2008; Kwan et al., 2009). Some of these patients are not surgery candidates, so it is necessary to search for alternative treatments for epilepsy such as palliative surgery, neuromodulation, and a ketogenic diet (KD).

The classic ketogenic diet (CKD) consists of a high-fat and low-protein and carbohydrate diet, with restricted calories and fluids. The diet mimics the fasting state, altering the metabolism to use fats as a primary fuel source; catabolism of fatty acids in the liver produces ketone bodies (KB), which induces urinary ketosis (Rho, 2017).

Recent studies have found a significantly positive outcome with the use of the KD for treatment of refractory epilepsy in children and adults (Barborka, 1928; Neal et al., 2008; Kverneland et al., 2015; Liu et al., 2018).

Regardless of the efficacy of the KD, most patients discontinue the diet because of its unpalatable and restrictive features. In the last 20 years, new variants of the KD diet have emerged, including the Modified Atkins diet (MAD), a low-glycemic-index diet, which although it has a high fat content, allows more protein and does not restrict calories and fluids. Several studies have shown that the new variants of the KD have a similar efficacy to the CKD (Kossoff et al., 2006; Tonekaboni et al., 2010; Coppola et al., 2011; Miranda et al., 2012; El-Rashidy et al., 2013).

As presently understood, the KD is involved in multiple mechanisms responsible for biochemical alterations, including cellular substrates and mediators responsible for neuronal hyperexcitability. However, it is not yet known with certainty whether the success of the KD is due to a single or several mechanisms (Bough and Rho, 2007; Lutas and Yellen, 2013; Rho, 2017; Youngson et al., 2017).

Because epilepsy is a metabolic disease (Clanton et al., 2017), interest in studies of alterations of metabolism by anticonvulsants such as the KD has increased, as has their importance for the treatment of drug-resistant epilepsy. This contribution reviews the use and effects of the KD and its variants for the treatment of adults and children with intractable epilepsy.

KETOGENIC DIET PAST TO PRESENT

Dietary treatments for diseases have probably been used for over 2000 years (Yuen and Sander, 2014). Fasting is the only therapeutic measure against epilepsy recorded in the Hippocratic collection. Two Parisian physicians, G Guelpa, and A Marie, recorded the first modern use of starvation as a treatment for epilepsy in 1911 (Wheless, 2008). The modern use of this form of therapy began in the early 1920s (Lima et al., 2014; Yuen and Sander, 2014), when Drs. Stanley Cobb and W.G. Lennox of Harvard at Harvard Medical School observed the effects of starvation as a treatment for epilepsy, noting that seizure improvement typically occurred after 2–3 days (Wheless, 2008). In the same period, Dr. Russel M. Wilder a physician at the Mayo Clinic in Minnesota, suggested that a specific diet could produce similar benefits to fasting, and proposed a diet that produced ketonemia. He studied a series of patients with epilepsy and demonstrated a result equivalent to fasting and that was maintained for a much longer period. This new concept of diet was designated the "KD." Peterman, also at the Mayo Clinic, described a composition of the KD similar to that used today (Wilder, 1921).

In 1970, Robert C. Atkins developed a weight-loss diet that restricted the intake of carbohydrates (Sharma and Jain, 2014), and this diet was later evaluated for seizure treatment. The first patient was a 7-year-old girl with intractable epilepsy due to a left parietal cortical dysplasia, who used the Atkins diet for a week in order to acclimate to the CKD. After 3 days, her seizures stopped, and she remained seizure-free for 3 years with continued dietary treatment (Kossoff et al., 2013). In 2006, this diet was first formally referred to as the "MAD" to distinguish it from the Atkins diet (Kossoff et al., 2013). The MAD has three significant differences from the first version: the induction phase of limiting carbohydrates is maintained indefinitely; high-fat foods are not only allowed, but encouraged; and the primary goal of the diet is seizure control (Atkins, 2002; Sharma and Jain, 2014).

Nowadays, despite the new generation of anti-epileptic drugs, 35% of patients remain refractory. Interest in dietary therapy continues as a means of treatment for this group, even more with advances in knowledge regarding the association of gut microbiota and neurological diseases.

CLASSIC KETOGENIC DIET

What Is Classic KD?

The CKD is rich in lipids (90%) and low in carbohydrates and protein, in order to produce ketosis, and simulates a starvation state. It is a rigid diet, mathematically and individually calculated, and medically monitored (Armeno et al., 2014). It must also provide adequate vitamins and minerals. The shift in the energy metabolism from glycolytic energy production to energy generation through oxidative phosphorylation (fatty acid b-oxidation and ketone-body production) is part of the anticonvulsant mechanism of the KD (Bough, 2008; Liu et al., 2018). This is discussed in more detail in the section on the mechanism of action.

Indication and Contraindications

Traditionally, the KD has been considered the gold standard for the treatment of metabolic diseases such as Glucose Transporter Protein 1 (GLUT-1) deficiency syndrome and Pyruvate Dehydrogenase Deficiency. At present, the KD has been consistently reported as more beneficial, with more than 70% patients showing positive responses, as opposed to the average 50% response in several conditions such as infantile spasms (**Table 1**). The KD has also been used in other conditions with less evidence, but possible benefits (**Table 2**) (Kossoff et al., 2018). Additionally, the KD is an important alternative treatment for patients with refractory epilepsy (Rho, 2017) that are not surgery candidates.

Kossoff et al. (2018) proposed that dietary therapy should be considered earlier as an option for treatment of intractable epilepsy, because of its proven efficacy, the poor chance of improvement with further anticonvulsant administration, and the possibility of using the MAD (Kossoff et al., 2006) and low-glycemic-index treatment (LGIT) (Pfeifer and Thiele, 2005), which are easier to manage in adults.

In contrast, some pathologies are considered contra-indicated for KD. Absolute contraindications have been described and

TABLE 1 | Epilepsy syndromes and some conditions in which the KD therapies has been reported probable benefit*.

Angelman syndrome
Complex 1 mitochondrial disorders
Dravet syndrome
Epilepsy with myoclonic–atonic seizures (Doose syndrome)
Glucose transporter protein 1 (Glut-1) deficiency syndrome (Glut1DS)
Febrile infection–related epilepsy syndrome (FIRES)
Formula-fed (solely) children or infants
Infantile spasms
Ohtahara syndrome
Pyruvate dehydrogenase deficiency (PDHD)
Super-refractory status epilepticus
Tuberous sclerosis complex

**Adapted from Kossoff et al. (2018). Optimal clinical management of children receiving dietary therapies for epilepsy: Updated recommendations of the International Ketogenic Diet Study Group (Kossoff et al., 2018).*

TABLE 2 | Epilepsy syndromes and some conditions in which the KD therapies has been reported possible benefit (one case report or series)*.

Adenylosuccinate lyase deficiency
CDKL5 encephalopathy
Childhood absence epilepsy
Cortical malformations
Epilepsy of infancy with migrating focal seizures
Epileptic encephalopathy with continuous spike-and-wave during sleep
Glycogenosis type V65 Juvenile myoclonic epilepsy
Lafora body disease
Landau-Kleffner syndrome
Lennox-Gastaut syndrome
Phosphofructokinase deficiency
Rett syndrome
Subacute sclerosing panencephalitis (SSPE)

**Adapted from Kossoff et al. (2018). Optimal clinical management of children receiving dietary therapies for epilepsy: Updated recommendations of the International Ketogenic Diet Study Group (Kossoff et al., 2018).*

TABLE 3 | Absolute contraindications for the use of KD therapies*.

Carnitine deficiency (primary)
Carnitine palmitoyltransferase (CPT) I or II deficiency
Carnitine translocase deficiency
β-oxidation defects
Medium-chain acyl dehydrogenase deficiency (MCAD)
Long-chain acyl dehydrogenase deficiency (LCAD)
Short-chain acyl dehydrogenase deficiency (SCAD)
Long-chain 3-hydroxyacyl-CoA deficiency
Medium-chain 3-hydroxyacyl-CoA deficiency
Pyruvate carboxylase deficiency
Porphyria

**Adapted from Kossoff et al. (2018). Optimal clinical management of children receiving dietary therapies for epilepsy: Updated recommendations of the International Ketogenic Diet Study Group (Kossoff et al., 2018).*

summarized by Kossoff et al. (2018) (**Table 3**). The surgical epilepsies, whenever the patient or caregivers are having difficulty maintaining compliance with the diet, are relative contraindications for KD (**Table 4**) (Kossoff et al., 2018).

Pre-KD Counseling and Evaluation

To obtain the optimum engagement of the family and the patients, providing information and training is essential because the diet is difficult to maintain. Counselors should talk with the family about their expectations and make clear the efficacy rate and adverse events (AE), to reduce the abandonment of the diet. Websites, videos and publications, especially from support groups, can be very helpful and should be encouraged. It is also important to review the medications and change from oral solutions (carbohydrate content) to tablets (Armeno et al., 2014). The KD counseling, evaluation and follow-up should be done by a multidisciplinary team. A pediatric neurologist or neurologist and a nutritionist are the minimum team requirements.

Before starting the diet, the patient should maintain a seizure diary to establish a frequency parameter. Also needed are a laboratory evaluation including selenium and carnitine

TABLE 4 | Relative contraindications for the use of KD therapies*.

Inability to maintain adequate nutrition
Surgical focus identified by neuroimaging and video-EEG monitoring
Parent or caregiver noncompliance
Propofol concurrent use (risk of propofol infusion syndrome may be higher)

**Adapted from Kossoff et al. (2018). Optimal clinical management of children receiving dietary therapies for epilepsy: Updated recommendations of the International Ketogenic Diet Study Group (Kossoff et al., 2018).*

TABLE 5 | Laboratory evaluation*.

Complete blood count with platelets
Electrolytes to include serum bicarbonate, total protein, calcium, zinc, selenium, magnesium, and phosphate serum
Liver and kidney tests (including albumin, blood urea nitrogen and creatinine
Fasting lipid profile
Serum acylcarnitine profile
Urinalysis
Urine calcium and creatinine
Anticonvulsant drug levels [a]
Urine organic acids [b]
Serum amino acids [b]
Vitamin D level

**Adapted from Kossoff et al. (2018). Optimal clinical management of children receiving dietary therapies for epilepsy: Updated recommendations of the International Ketogenic Diet Study Group (Kossoff et al., 2018). [a]if applicable; [b]if diagnosis unclear.*

levels (**Table 5**), electroencephalogram (EEG), and a magnetic resonance image (MRI) of the brain. A renal ultrasound should be done in case of kidney stones; an electrocardiogram and carotid ultrasound are considered optional (Kossoff et al., 2018). The nutritional evaluation includes a nutritional anamnesis including a 3-day food report, food habits, allergies, aversions, and intolerances. Baseline weight, height, and the ideal weight for stature and body mass index (BMI) are needed to calculate the ketogenic ratio, calories, and fluid intake. The diet formulation should be established according to the patient's age and the administration route (Kossoff et al., 2009).

Diet Initiation

The goal is to reach a ratio of four portion of fat to one portion of protein plus carbohydrate, described as "4:1." To achieve this level, one of two approaches, with or without fasting, may be used. In the former approach, the patient must be hospitalized for 12–48 h, or when ketones are present in the urine (Rubenstein, 2008), to prevent the development of hypoglycemia and dehydration. This method tends to accelerate the development of ketosis although it can generate more stress on the patient (Armeno et al., 2014). When ketosis is reached, the meals are calculated to maintain a constant KD ratio, while calories are added until full-calorie meals are tolerated (Kossoff et al., 2009). The latter approach requires no hospitalization and the KD ratio increases weekly, from 1:1, 2:1 and 3:1 to 4:1 (Bergqvist et al., 2005). Most of the literature suggests that there is no significant difference between the two approaches in terms of the time needed to reach ketosis and the occurrence of

hypoglycemia (Kim et al., 2004), so nowadays patients tend to not fast.

Taking into account that the KD provides only small amounts of fruits, vegetables, grains, milk and cheese, supplementation is essential. Low-carbohydrate multivitamin and mineral supplements should be taken daily.

Follow Up

Patients on the KD should be seen regularly every 3 months, and the family should be able to easily contact the diet team to resolve possible doubts and discuss adverse effects. In each evaluation, the seizure dairy and the child's cognitive development and behavior should be observed (Auvin and Nabbout, 2011). It has been noted that it is possible to improve the cognitive development and behavior even without a change in the seizure frequency. Although some authors have reported no relationship between the efficacy and the level of ketosis, it is still recommended to measure the urine ketosis several times a week (Kossoff et al., 2009).

For efficacy, the KD requires a period of at least 3 months from the time that the patient reaches ketosis, so it is important to encourage the patient and the family to continue with the diet for this period without changing the medication.

Adverse Effects

Because KD is not a physiological diet, it is necessary to recognize and closely manage AE (Kossoff et al., 2009). Acute AE include dehydration, hypoglycemia, lethargy, metabolic acidosis, and gastrointestinal symptoms. However, most of the side effects involve weight loss, high levels of low-density lipoprotein, and elevated total cholesterol (Liu et al., 2018). Other important AE are gastrointestinal symptoms, which include constipation, diarrhea, vomiting, and abdominal pain.

The family should also be informed about how to recognize the symptoms of hypoglycemia and be advised to administer a small amount of juice or other forms of dextrose (Kossoff et al., 2018). Nephrolithiasis may also develop, and an abdominal ultrasonography should be requested.

MECHANISM OF ACTION

The understanding of the mechanisms of action of KD is incomplete; however, some theories have been advanced about how it modifies the neuronal metabolism and excitability in order to reduce the seizure frequency. Possibly, the real mechanism of reduction of cortical hyperexcitability involves multiple factors. Some of the systems involved in seizure reduction are related to metabolic changes in the blood and cerebrospinal fluid (CSF), including a decrease in glucose levels and an increase in KB. The mitochondria function and energy reserve may also play a role in the KD mechanisms, resulting in synapse stabilization and excitatory decrease.

Ketone Bodies: Anticonvulsant Effects

Ketone bodies, acetoacetate, and β-hydroxybutyrate (βOHB), are byproducts of fatty acid oxidation in the mitochondrial matrix of the hepatocytes. There are many theories about the role of KB, but the existence of an anticonvulsant effect is controversial. Some authors have found no relationship between KB and synaptic transmission and seizure control.

Experimental studies in an animal model showed that in rats exposed to KD there was no change in synaptic plasticity, using paired-pulse modulation and long-term potentiation (Thio et al., 2010). Similarly, Likhodii et al. (2003) did not detect any anticonvulsant effects in either ketone body (Likhodii et al., 2003). In spontaneously epileptic *Kcna1*-null mice, KB supplementation resulted in attenuation of electrographic seizure-like events (Kim et al., 2015). These authors also observed an inhibitory effect of KB on mitochondrial permeability transition related to apoptotic and necrotic death. Moreover, in experimental models, acetoacetate exerted a broad-spectrum anticonvulsant effect (Rho et al., 2002). In another study, Rho (2017) described a relationship among KB, neurotransmitter release and ATP-sensitive potassium channels (Rho, 2017). Similarly, to these studies, injection of KB led to the reduction of seizure susceptibility (Gasior et al., 2008). Ma et al. (2007) found a decrease of the spontaneous firing rate in sections of mouse tissue, which was eliminated in the absence of ATP-sensitive potassium channels (KATP). In addition, KB can exert a direct inhibitory effect on the vesicular glutamate transport (Juge et al., 2010). It is possible that these divergent results are related to the different concentrations of KB used in these studies and the diverse seizure thresholds of the animal models. These conflicting results can be also explained by differences in diet composition.

Neuronal Metabolism and Synaptic Function

Another hypothesis regarding the function of the KD is related to changes in neuronal metabolism, mitochondrial function and energy reserve, and the environment. In normal conditions, the usual substrate for the neurons is glucose. To facilitate its diffusion through the brain-blood barrier, glucose transports are present in the brain capillary endothelial layer (Greene et al., 2003). The glucose metabolism produces the rapidly available energy that is necessary for seizure activity. Therefore, in patients on the KD, the blood glucose energy levels are low, and the brain begins to use KB for energy. This anaerobic metabolism slows the energy availability, which reduces seizures. The anticonvulsant propriety of a decrease in glucose metabolism has been shown in experimental models in which the administration of 2-Deoxy-D-glucose elevates the seizure threshold (Garriga-Canut et al., 2006). The anticonvulsant effect of the KD can be quickly reversed after glucose infusion (Huttenlocher, 1976). Based on these data, we can postulate the influences not only of the KB, as discussed above, but also the reduction in glucose levels as a mechanism of action of the KD.

Chronic ketosis may play a role in the KD anticonvulsant properties, since it has been shown that chronic ketosis elevates

the brain energy reserve via stabilization and reduction of excitability of synapses (Devivo et al., 1978). The energy reserve is directly associated with mitochondria, which is an important element to consider in the antiepileptic effect of KD. Bough et al. (2006) demonstrated an increase in mitochondria biogenesis in an experimental model of rats fed with KD, indicating an increase in the energy stores (Bough et al., 2006). The increase in mitochondrial metabolism leads to an increase in ATP production, which activates KATP, in turn attenuating neuronal excitability. This activation may be associated with adenosine A1 receptors (Li et al., 2010) and GABAB receptors (Mironov and Richter, 2000).

In this process, we can postulate that modifications of the metabolism are associated with an increase of ATP, and improve mitochondrial capacity and cell energy, with an increase in metabolic resilience.

Neurotransmitter Function

The KD-induced synaptic stabilization is additionally related to changes in critical amino acids as a result of ketone metabolism. It has been proposed that KD interferes with the concentration of gamma-aminobutyric acid (GABA), the major inhibitory neurotransmitter. There is evidence in clinical practice of increased GABA levels in the CSF of patients on the KD diet (Wang et al., 2003). The decrease in aspartate levels promoted by KB lead to the synthesis of GABA. This occurs because of the inhibitory effect of aspartate on glutamate decarboxylase and the facilitation of the conversation of glutamate to glutamine in the astrocytes (Yudkoff et al., 2008). Not only can GABA be increased, but also other neurotransmitters such as adenosine A1 can be implicated in the anti-seizure effect of the KD (Szot et al., 2001). However, more evidence is needed.

Gut Microbiota, Inflammation, and Genetic

The role of gut microbiota has recently been studied for its effect on several diseases, especially those with some inflammatory involvement. Several metabolic pathways are known to be modulated by the gut microbiota. Olson et al. (2018) demonstrated the impact of gut microbiota on the anti-seizure effect of KD. She found that KD modifies the gut microbiota, with a decrease in alpha-diversity and increases in the putatively beneficial bacteria *Akkermansia muciniphila* and *Parabacteroides* spp. This microbiota transformation leads to changes in the colonic luminal metabolome, with a decrease in gamma-glutamyl amino acids. This increases the GABA/glutamate content in the brain by decreasing gamma-glutamyl amino acids in the blood (Olson et al., 2018). In an acute electroshock model, it is reported that KD confers protection against seizures. Moreover, KD decreases the frequency of spontaneous seizures in Kcna1 knockout mice (Kim et al., 2015). In summary, changes in the gut microbiota seem to be important for the KD-mediated seizure protection.

The role of inflammatory cytokines in epilepsy is well known, and there is evidence that KD also interferes with pro-inflammatory cytokines. Dupuis et al. (2015) showed a peripheral and brain reduction of interleukin 1β and other pro-inflammatory cytokines in rats treated with KD in the LPS model.

Notably, there is a relationship between metabolic and epigenetic modifications. Shimazu et al. (2013) observed that βOHB inhibits class I histone deacetylases. During the KD, the elevation of βOHB causes changes in large-scale gene transcription but particularly those linked to oxidative-stress resistance factors. This result emphasizes that the KD has a potential role as a disease-modifying treatment in epilepsy.

In conclusion, all the mechanisms described above lead to systemic modifications and a dynamic metabolic homeostasis, in which the interplay among KB, glucose levels, mitochondrial function, synaptic neurotransmitters, and channel modifications can lead to changes in the seizure threshold and hyperexcitability. These changes contribute to the final antiseizure mechanism of KD.

Multiple mechanisms of action may explain why the modification of the KD can be effective even without ketosis. Importantly, the KD systemic action can have a broad spectrum of effects that may be beneficial in the treatment of different types of epilepsy and associated comorbidities such as cognition impairment, psychiatric disturbance, and sudden unexplained death.

MODIFIED ATKINS DIET IN PATIENTS WITH REFRACTORY EPILEPSY

Definition and Diet Composition

The MAD aims to provide increased flexibility and palatability, with a 1:1 ratio of fat to carbohydrates and protein, and contains around 65% fat, 25% protein, and 10% carbohydrate (Payne et al., 2018). Fat is encouraged and the carbohydrate intake is limited to 10–20 g/day in children and 15–20 g/day in adults (Kossoff, 2004; Kossoff and Dorward, 2008). Because of carbohydrate restriction, the MAD can also produce urinary ketones (Carrette et al., 2008). The MAD does not require weighing food on a gram scale, or restriction of calories, protein or liquids, and may be a good option for patients who are unable to tolerate a more restrictive diet such as the classical ketogenic diet (KD) (Cervenka et al., 2012). Low-carbohydrate multivitamin and calcium carbonate supplementation is recommended in the MAD (Kossoff et al., 2009).

Efficacy in Children

Several studies have shown that the MAD, besides being more palatable, is as effective as the KD in the treatment of drug-resistant epilepsy in children (Miranda et al., 2011; Martin et al., 2016). A study performed using 20 children receiving 10 g of carbohydrates daily showed that 65% of the children had a >50% seizure reduction, 35% of the children had >90% improvement, and four children were seizure-free at 6 months (Kossoff et al., 2006). In a study in South Korea, 36% of 14 children treated with the MAD showed improvement of >50% in seizures and 12% were seizure-free (Kang et al., 2007). A recent meta-analysis performed using 70 studies concluded that the MAD and classical

KD do not differ in reduction of seizure frequency at month 3 and month 6, with ≥50% and ≥90% reductions, respectively (Rezaei et al., 2017). A retrospective study showed >50% of seizure reduction in 65% of the 10 children who remained on the diet for up to 6 months, and 20% of them were seizure-free (Park et al., 2018).

Treatment with MAD was shown to be more effective in seizure control when the MAD was started with lower carbohydrate limits (Kossoff et al., 2010). In a randomized study with 20 children with drug-resistant epilepsy, 60% of them showed fewer seizures in the first 3 months on the MAD, with 10 g/day of carbohydrate intake against 10% of reduction with 20 g/day (p = 0.03). In the same study, after 3 months, an increase in carbohydrate intake to 20 g/day, maintained seizure control and improved tolerability, suggesting that a lower carbohydrate limit is important only in the first 3 months (Kossoff et al., 2007; Kossoff and Dorward, 2008).

Efficacy in Adolescents and Adults

The efficacy of the MAD is also proven for the treatment of drug-resistant epilepsy in adults and adolescents. In this patient group, carbohydrate intake is generally around 15–20 g/day and the rates of seizure reduction and adherence are lower compared to those of the child population (Kossoff et al., 2008; Zare et al., 2017; Payne et al., 2018).

In a recent meta-analysis, eight studies were identified that used the MAD in adult patients with refractory epilepsy, aged between 15 and 86 years, with treatment times ranging from 3 to 36 months. In these studies, the proportion of patients who showed >50% seizure reduction ranged from 20 to 70% and the rate of seizure freedom ranged from 7 to 30%. The rate of abandonment of the diet varied between 12.5 and 82% of the patients (Liu et al., 2018).

Side Effects

The MAD has been shown to be better tolerated than the classical KD, but some typical side effects such as gastrointestinal complaints, dyslipidemia and weight loss are reported (Zare et al., 2017). Beneficial effects have also been reported, such as mood improvement (Carrette et al., 2008).

LOW GLYCEMIC INDEX DIET IN PATIENTS WITH REFRACTORY EPILEPSY

Definition and Diet Composition

The use of the LGIT in the treatment of drug-resistant epilepsy was initially reported in 2005 by Pfeifer and Thiele (2005). This alternative diet treatment is based on a ratio 0.6:1 of fat to carbohydrates and protein, containing 60% fats, 30% protein, and 10% carbohydrates with a low glycemic index (GI) (GI<50) (Pfeifer and Thiele, 2005; Payne et al., 2018). The GI measures the tendency of a food to raise the blood glucose levels, compared to an equivalent amount of the reference carbohydrate, usually glucose (Pfeifer et al., 2008). Compared to classic the KD, the LGIT produces a smaller increase in ketone body levels, but has comparable efficacy, better tolerability and easier implementation (Pfeifer and Thiele, 2005; Pfeifer et al., 2008).

Efficacy

The LGIT has proven to be effective in the treatment of focal and generalized epilepsies, with a reduction in seizure frequency occurring at 3–14 months and seizure control continuing for at least 1 year after the end of treatment (Pfeifer et al., 2008; Kim et al., 2017; Rezaei et al., 2018). Pfeifer and Thiele (2005) reported the use of LGIT in 20 drug-resistant epilepsy patients aged 5 to 34 years. After an average of 20 weeks of treatment, 50% of the patients had a >90% reduction in seizures. Coppola et al. (2011) studied 15 children, adolescents and young adults with refractory epileptic encephalopathies treated with LGIT. After 12 months they found 75–90% seizure reduction in 6 patients (40%), 50% reduction in 2 patients (13.3%), and the seizure frequency unchanged in 7 (46.7%). In a retrospective review of LGIT in 76 children, Muzykewicz et al. (2009) found an overall >50% reduction in seizure frequency in 50% of the patients at 3 months, which reached 66% at 12 months.

However, according to a recent systematic review, the positive results for LGIT efficacy in epileptic patients are doubtful because of the low number of high-quality studies. In this review, which included all electronic literature databases until July 2017, the authors found only eight studies with good or fair quality (69).

Side Effects

Constipation and vomiting are the most common adverse effects reported in patients on the LGIT (Rezaei et al., 2018).

CONCLUSION

The CKD and its variants should be considered as an alternative for non-surgical pharmacoresistant patients with epilepsy, of any age. Each patient must have an individually designed diet; however, adult patients have more difficulty in maintaining the CKD. It is essential to inform the patient and the family about the efficacy and AE related to the KD, and the use of websites and videos may help in this education. Although several theories exist regarding the mechanisms of action, further study is needed nevertheless the positive results are probably due to several mechanisms.

AUTHOR CONTRIBUTIONS

All the authors contributed substantially to the writing and revising of the manuscript. ID'A, HP, TR, MP, PC, and LK participated in the conception and design of the study, collected the literature, prepared the tables, and wrote the manuscript. ID'A, TR, and HP reviewed and edited the manuscript and approved the final version.

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Prenatal Protein Malnutrition Produces Resistance to Distraction Similar to Noradrenergic Deafferentation of the Prelimbic Cortex in a Sustained Attention Task

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Exposure to malnutrition early in development increases likelihood of neuropsychiatric disorders, affective processing disorders, and attentional problems later in life. Many of these impairments are hypothesized to arise from impaired development of the prefrontal cortex. The current experiments examine the impact of prenatal malnutrition on the noradrenergic and cholinergic axons in the prefrontal cortex to determine if these changes contribute to the attentional deficits seen in prenatal protein malnourished rats (6% casein vs. 25% casein). Because prenatally malnourished animals had significant decreases in noradrenergic fibers in the prelimbic cortex with spared innervation in the anterior cingulate cortex and showed no changes in acetylcholine innervation of the prefrontal cortex, we compared deficits produced by malnutrition to those produced in adult rats by noradrenergic lesions of the prelimbic cortex. All animals were able to perform the baseline sustained attention task accurately. However, with the addition of visual distractors to the sustained attention task, animals that were prenatally malnourished and those that were noradrenergically lesioned showed cognitive rigidity, i.e., were less distractible than control animals. All groups showed similar changes in behavior when exposed to withholding reinforcement, suggesting specific attentional impairments rather than global difficulties in understanding response rules, bottom-up perceptual problems, or cognitive impairments secondary to dysfunction in sensitivity to reinforcement contingencies. These data suggest that prenatal protein malnutrition leads to deficits in noradrenergic innervation of the prelimbic cortex associated with cognitive rigidity.

Keywords: cognition, prefrontal cortex, distractibility, norepinephrine, rat model of malnutrition

INTRODUCTION

Malnutrition impacts approximately one in four children worldwide (WHO, 2012). Longitudinal studies of adults with exposure to prenatal food restriction during the Dutch famine and the Chinese famine of 1959–1961 (St. Clair et al., 2005) have shown an increased prevalence of neuropsychiatric disorders (Brown et al., 1995; Brown and Susser, 2008) and attentional impairments (De Rooij et al., 2010; Li et al., 2015). Similarly, a longitudinal study of Barbadian adults exposed to protein-calorie malnutrition limited to the first year of life has documented impaired attention (Galler et al., 2012), and affective processing (Waber et al., 2014). Attentional problems were evident in this cohort across the life span, as confirmed by parent, and teacher ratings (Galler et al., 1983, 1990; Galler and Ramsey, 1989) as well as self-reports in middle adulthood (Galler et al., 2012). Neuropsychological testing showed that deficits in attention, as assessed using the Behavioral Rating Inventory of Executive Function (BRIEF), the Wisconsin Card Sorting Task (WCST; Waber et al., 2014), and a continuous performance task (Galler et al., 2012), persisted well into middle adulthood. This study also confirmed that exposure to early childhood malnutrition entailed long-lasting epigenetic signatures in the Barbados cohort that were closely associated with the attention problems, even after adjusting for socioeconomic and ecologic conditions in the household (Peter et al., 2016). However, consistent and compelling data from human studies are often complicated by a multitude of other factors that coincide with childhood malnutrition, e.g., poverty, infection, stress, and maternal depression (Salt et al., 1988; Galler et al., 2000; Walker et al., 2011). While these long-term effects are hypothesized to result from dysregulation of the prefrontal cortex, it is difficult to ascertain the relationship between prenatal malnutrition and the prefrontal cortex in human studies. Animal models that reproduce these conditions are therefore better suited to elucidate causal relationships among malnutrition, cognition, and changes in the brain (Tonkiss et al., 1993; Galler et al., 1996). Animal models have shown impaired attentional processing as a result of prenatal iron deficiency (Mohamed et al., 2011), vitamin D (Turner et al., 2013), and protein levels (McGaughy et al., 2014). Moreover, these nutritional deficits are also known to impair prefrontal circuits hypothesized to be critical to attention (Groves et al., 2013; Grissom et al., 2014; McGaughy et al., 2014).

The present studies investigated the impact of prenatal malnutrition on sustained attention and distractibility using a previously validated task (McGaughy and Sarter, 1995; Demeter et al., 2008; Newman et al., 2008). Prior studies using a well-defined animal model of prenatal protein malnutrition revealed cognitive rigidity in a test of attentional set shifting (McGaughy et al., 2014). We hypothesized another outcome of this rigidity would be that prenatally malnourished rats would be less sensitive to the detrimental effects of a distractor than controls. Cognitive rigidity can result from decreased functioning of prefrontal noradrenergic systems (Tait et al., 2007; Newman et al., 2008; McGaughy et al., 2014), while increased distractibility may result from hypofunctioning of the cholinergic systems (Newman et al., 2008; Berry et al., 2014). Because of the critical role of these two neuromodulatory systems in attentional processing, we performed histological analyses on these systems upon completion of behavioral testing. Rats exposed to prenatal malnutrition had fewer noradrenergic afferents in the prelimbic cortex relative to control subjects, but cholinergic afferents were unchanged by prenatal malnutrition. Based on this finding, we assessed the effects of selective noradrenergic deafferentation of the prelimbic cortex in adult rats on distractibility to compare with the effects of prenatal protein malnutrition. We hypothesized that noradrenergic deafferentation would reproduce cognitive rigidity found in subjects exposed to prenatal malnutrition.

MATERIALS AND METHODS

Prenatal Nutritional Treatment

For the prenatal malnutrition studies, viral-free virgin, female, Long-Evans hooded rats (175–200 g) (obtained from Charles River, Wilmington, MA, United States) were randomly assigned to one of two nutritional conditions. As described in detail previously (Galler and Tonkiss, 1991), one group was placed on an adequate protein diet, 25% casein (Teklad Laboratories, Madison, WI, United States); 5 weeks prior to mating and throughout pregnancy (Fischer et al., 2015), while the other group received an isocaloric, low protein diet, 6% casein (Teklad Laboratories, Madison, WI, United States) during the same period. Beginning the experimental diets prior to pregnancy ensured that there was no impact on food intake during pregnancy as a result of the diet change and was more representative of human malnutrition which is most often a chronic state (Galler and Tonkiss, 1991). All females were mated with males that had been acclimated to these respective diets for 1 week. Throughout pregnancy, dams were singly housed in individual polysulfone breeding cages (39.5 × 34.6 × 21.3 cm; Tecniplast USA Inc., Exton, PA, United States). Following parturition, litters from both nutritional groups were culled to eight pups (two females and six males) and fostered as whole litters to well-nourished lactating foster dams receiving the 25% casein diet (**Table 1**). Each foster dam had given birth within the same 24 h period. At birth, pups born to mothers on the 6% casein diet that were fostered to mothers on the 25% casein diet were designated as members of the 6/25 (prenatally malnourished) group, while pups born to mothers on a 25% casein diet that were also fostered to mothers on a 25% casein diet were designated as members of the 25/25 (prenatally well-nourished or control) group. All dams and litters were provided with *ad libitum* access to the 25% casein diet during the litter period. At postnatal day (PND) 21, all rats were weaned, placed on a standard laboratory chow diet containing 23% protein (Purina Mills Inc., Richmond, IN, United States; Formula 5001) and pair housed with littermates in polysulfone breeding cages (Tecniplast USA Inc., Exton, PA, United States). One week prior to behavioral assessment, subjects were single-housed and began food restriction.

Subjects

For the prenatal nutrition animals, the vivarium was maintained at 22–24°C with 40–60% humidity and kept on a 12:12 h reverse

TABLE 1 | Schematic of prenatal nutritional treatment groups.

Animal model of prenatal malnutrition				
Diet	**Before mating (5 weeks)**	**Pregnancy (3 weeks)**	**(PND 0)**	**After birth (PND 0–PND 21)**
Low protein (6/25)	6% casein	6% casein	→	
Adequate protein (25/25)	25% casein	25% casein	→	**25% casein**
Foster mothers	25% casein	25% casein	→	**25% casein**

All pups are fostered (as whole litters, culled to eight pups) at birth (P0) to well-nourished mothers, as indicated by the underlining and bold font. Female, Long-Evans rats were randomly assigned to low protein diet (6% casein) or an isocaloric adequate protein diet (25% casein) 5 weeks prior to mating and throughout pregnancy. After birth, all pups were fostered by dams that had an adequate protein diet (25% casein) and litter size was uniform (two females and six males).

light/dark cycle with lights on at 19:00 h to accommodate to the waking state of the rats. During the dark cycle, red florescent lighting provided continuous dim illumination. Behavioral testing started at PND 90 and occurred during the dark phase of the cycle between the hours of 9:00 and 13:00 h, 6 days per week. One male rat from each of 10 6/25 prenatally malnourished litters and 17 25/25 control litters served as subjects and were singly housed in polycarbonate cages. In no instance were littermates tested. The norepinephrine (NE) lesion study used 24 adult male Long Evans rats (Harlan, Indianapolis, IN, United States) housed separately, kept on a 12:12 h light/dark cycle (lights on at 6 am) in a climate-controlled environment, and only tested during the light hours.

All subjects received ∼18 g of standard rat chow daily to allow them to maintain weights that were approximately >90% of age-matched controls. Water was available *ad libitum*. All animals were weighed weekly to assure healthy weights relative to age-matched controls. All personnel involved in collecting behavioral and weight data were blind to condition during data collection. Procedures were approved by the University of New Hampshire Institutional Animal Care and Use Committee and the University of New England Institutional Animal Care and Use Committee in accordance with guidelines outlined in *Guide for the Care and Use of Laboratory Animal (Approval No. 20101005MOK)*.

Apparatus and Materials

Operant chambers (Med Associates, St. Albans, VT, United States) equipped with two retractable levers, a houselight (2.8 W), a 45 mg pellet dispenser, a 2,900-Hz sonalert tone generator, and three panel lights (2.8 W) were used. Each chamber was outfitted with two retractable response levers mounted 11 cm apart with associated stimulus light. The third panel light was centered between the two other lights above the food hopper. A houselight, located at the top of the back panel of each operant chamber, provided ambient illumination during a test session. A pellet dispenser delivered reinforcers (Bioserv, 45 mg; Research Diets, Frenchtown, NJ, United States or Noyes Precision Pellets, 45 mg; Research Diets, New Brunswick, NJ, United States) into a food hopper, located halfway between both response levers. The food hopper, panel lights, tone generator and retractable levers were all located on the same wall, whereas the houselight was located on the opposite wall. Signal presentation, lever operation, and food pellet delivery were recorded using a PC with Windows XP and the Med-PC IV software (Med Associates, St. Albans, VT, United States).

Behavioral Training

Rats were initially trained to bar press for food in the operant chamber in accordance with an FR1 schedule of reinforcement with the houselight illuminated. Reinforcement was suspended when the rat pressed one lever over five times more than the other lever to prevent the development of a side bias. Once the animals made at least 50 responses for two consecutive days, training in the sustained attention task (SAT) was begun.

Sustained Attention Task (SAT): Shaping

After learning to bar press for food, the animals were trained to discriminate between signal and non-signal trials as described in previous studies (Newman et al., 2008). Training sessions consisted of a total of 162 trials. Rats were placed into the operant chambers for 1 min prior to the onset of training. The houselight remained illuminated for the duration of the session. Signal and non-signal trials were presented in a pseudo-randomized sequence so that each block of 54 trials consisted of an equal number of signal and non-signal events. Signal trials consisted of illuminating the central and left panel lights for 1 s, whereas the lights were not illuminated for non-signal trials. Animals were cued to respond by the extension of both levers into the box two seconds after the signal or non-signal event. Levers remained extended for 4 s or until a lever press occurred. Animals were reinforced for responding to the light stimuli by pressing the left lever (hit) and by pressing the right lever in the absence of the light (correct rejection). Incorrect lever presses were defined as misses when they occurred on a signal trial and false alarms when they occurred on a non-signal trial. If the animal failed to respond or responded incorrectly, the levers were retracted and the inter-trial interval (ITI; 12 ± 3 s) was reinstated. After an incorrect response, the trial was repeated up to three times (correction trials). If the animal failed to respond correctly after three correction trials, a forced-choice trial was initiated. In forced-choice trials, the event (signal or non-signal) was repeated but only the correct lever was extended and remained active for 90 s. On forced-choice, signal trials, the lights remained illuminated for 90 s. These trials facilitated discriminative conditioning and prevented the development of a side bias. After the animals responded correctly to ≥70% of both the signal and non-signal events for at least two consecutive testing days, they participated in a second shaping task. During this task, the central panel light was only illuminated for 1 s during signal trials. All other aspects of the task were the same as the previous shaping task. After the animals responded correctly to ≥70% of both the signal and non-signal events for at least two consecutive testing days in this phase of shaping, they entered the final

baseline task that served as the comparator for all tests of altered attentional demand.

Baseline Sustained Attention Task (SAT)

In the final version of the SAT, the length of the signal duration was changed from 1 s to pseudorandom presentation of 25, 100, and 500 ms. Sessions consisted of 27 trials of each of the three signal lengths and 81 trials of the non-signal trials, yielding a total of 162 trials per session. As performance changes were analyzed across three blocks of 54 trials each, the sequence of signal and non-signal trials was pseudo-randomized so that one block consisted of 27 signal and 27 non-signal trials with each signal length being presented nine times. In addition, both correction and forced-choice trials were discontinued. Animals were trained to a criterion of >70% hits to the 500 ms signals and >70% correct rejections to non-signal trials for at least two consecutive sessions, at which point they were considered ready to undergo tests of altered attentional demand in the prenatal malnutrition study or ready for surgery (see the section "Surgery") in the noradrenergic prelimbic lesion study. Tests of varied attentional demand were counterbalanced across subjects to control for possible practice and order effects.

Effects of Distracting Visual Stimuli

To allow comparison with previously published studies (McGaughy and Sarter, 1995; McGaughy et al., 1997; Newman et al., 2008), we assessed the effects of flashing the houselight in a predictable pattern for one session (0.5 Hz, Predictable Distractor, dSAT) or an unpredictable pattern with an average on/off cycle similar to the 0.5 Hz (0.25, 0.5, 1.0, 1.5, 2.0, or 3.0 s on/off; Unpredictable Distractor, uSAT). Additionally, as previous work in our laboratory has shown that lesions to the posterior parietal cortex increase susceptibility to task irrelevant stimuli that are identical in duration to those of the target stimuli (Newman and McGaughy, unpublished data), we also assessed the effects of this type of distractor in the present study (Overlapping Distractor; 0.025, 0.1, 0.5 s on/off, oSAT).

Effects of Withholding Reinforcement

As prenatal protein restriction has been shown to influence sensitivity to reward (Morgane et al., 1993; Tonkiss et al., 1993), we directly assessed the effect of withholding reinforcement on attentional performance in the SAT (SATwr) by omitting reinforcement after correct responses.

Behavioral Measures

For each test session, the number of hits, misses, correct rejections, false alarms and errors of omission were recorded. The relative number of hits (% hits = hits/hits + misses) was computed for each signal length along with the relative number of correct rejections (% CR = correct rejections/correct rejections + false alarms). In addition, we calculated the relative number of left lever presses (hits + false alarms/all responses) as a measure of side bias. This was done when the initial analyses of hits and correct rejections suggested such a bias could explain the pattern of results (e.g., hits were significantly increased while correct rejections were significantly decreased).

Surgery

After learning the SAT and prior to the testing of task variants, rats in the noradrenergic lesions study underwent intracranial surgery. Subjects were anesthetized with an intramuscular (i.m.) injection of ketamine (85 mg/kg/ml) and xylazine (8.5 mg/kg/ml) then placed in a stereotaxic frame using atraumatic ear bars. Rats received either lesions of the noradrenergic afferents to the prefrontal cortex using a solution of 0.01 μg/μl dopamine beta-hydroxylase saporin (DBH-SAP) in a sterile phosphate buffer or sham-lesions produced by infusing sterile phosphate buffer into medial, prefrontal cortex (Newman et al., 2008). All infusions (0.5 μl/hemisphere) were made at the following coordinates: toothbar: −3.3; anteroposterior (AP): Bregma +2.8; mediolateral (ML): Bregma ±0.6; dorsoventral (DV): Skull −5.2 using a 26 gauge, 10 μl microsyringe attached to an electronic infusion pump (Micro 4^{TM} Microsyringe Pump Controller, World Precision Instruments, Sarasota, FL, United States). To prevent unwanted diffusion, the toxin or its vehicle was infused at a rate of 125 nl/min with the needle left in place for 4 min before and after infusion. Post-surgery animals were given 7 days of recovery time to allow for retrograde transport of the toxin and apoptotic cell death to occur. During recovery, rats were given *ad libitum* food and water.

Postoperative Training

Rats in the noradrenergic lesion study received 2 weeks of *ad libitum* food and water prior to the reinstatement of food restriction and the onset of post-operative behavioral testing. When rats performed at criterion performance (>75% hits 500; >75% correct rejections) for two consecutive days, variations of attentional demands began. After the completion of a testing session, rats were returned to training in the SAT and again required to perform at criterion levels in the SAT for 2 days prior to the next test of altered cognitive demand.

Histology

Following the completion of behavioral testing, rats were deeply anesthetized with Euthasol (Virbac USA, Fort Worth, TX, United States), ex-sanguinated with 0.9% saline and then 4% paraformaldehyde in 0.1 M phosphate buffer. Perfused brains were then placed in 30% sucrose to provide cryoprotection. Sections (50 μm) were collected using a microtome (Leica, Buffalo Grove, IL, United States) attached to a freezing stage (Physitemp, Clifton, NJ, United States). Alternate sections were stained for DBH positive fibers, acetylcholinesterase positive fibers (AChE+) or Nissl bodies using thionin. To prevent uneven staining, all rinses and incubations were performed using an orbital shaker.

Dopamine β-Hydroxylase (DBH) Immunohistochemistry

Sections were initially placed into a solution of 1% hydrogen peroxide and 3% normal goat serum in phosphate-buffered saline (PBS). Without rinsing, sections were then transferred to a solution of 1:2000 mouse anti-DBH (EMD Millipore, Billerica, MA, United States) in 0.2% Triton X-100 in PBS and left overnight. Subsequent to 3 × 10 min rinses in PBS, sections

were incubated in biotinylated secondary antibody (Goat anti-mouse, Santa Cruz Biotechnology, Dallas, TX, United States) for 2 h. After rinsing 3 × 10 min in PBS, sections were incubated in the avidin biotin complex solution (ABC; Vector Labs, Burlingame, CA, United States) for 1.5 h. Subsequent to rinsing with PBS (3 × 10 min), visualization was accomplished with a solution of nickel enhanced 3,3-diaminobenzidine (Vector Labs, Burlingame, CA, United States) until cortical layers became visible (1–5 min). Finally, sections were rinsed with PBS (3 × 10 min) prior to mounting on gelatin coated slides. Sections were dried overnight in a 37°C oven prior to dehydration, defatting, and cover-slipping.

Acetylcholinesterase Staining

The staining procedure used for acetylcholinesterase was modified from a protocol described previously by Tago et al. (1986). Sections were placed in phosphate buffer (pH 7.4) with 0.1% hydrogen peroxide for 30 min and then were washed in 0.1M maleate buffer (three rinses, 3 min each) in order to modify the pH to 6.0. An incubation solution of 5 mg of acetylthiocholine iodide, 0.174 g of sodium citrate, 0.075 g of copper sulfate, and 0.0164 g of potassium ferricyanide in 0.1 maleate buffer was then used to soak sections for 60 min. Sections were next washed for a total of three rinses in 50 mM Tris buffer (pH 7.6) for 3 min each. They were then soaked for 10 min in a second incubation solution of 0.05 g of diaminobenzidine and 0.375 g of nickel ammonium sulfate in 125 ml of 50 mM Tris buffer. Twelve drops of hydrogen peroxide were then added to the solution until the details of the sections became apparent. Finally, the sections were thoroughly rinsed three times in 5 mM Tris buffer (3 min each) and mounted on gelatin-coated slides after which they were dried overnight in a 37°C oven prior to dehydrating, defatting, and cover-slipping.

Microscopic Analyses

Brain sections were analyzed using an Olympus Bx51 microscope (Optical Analysis Corporation, Nashua, NH, United States) at 400× magnification in conjunction with a Nikon DXM 1200 camera at 10× magnification. Image Pro Plus software v.6.0 (Media Cybernetics, Silver Springs, MD, United States) was used to superimpose a grid over the brain images. The number of fibers that definitively crossed the perimeter of the grid were counted and recorded. Counts were taken in the prelimbic cortex (PL) at Bregma +4.7, +3.7, and +2.7 mm; and anterior cingulate cortex (ACC) at Bregma +3.7, +2.5, and +0.7 mm anterior.

Statistical Analyses

All statistical analyses were performed using SPSS 25.0 (IBM, Armonk, NY, United States). The degrees of freedom in all analyses were corrected using the Huynh-Feldt correction in the case of a violation of sphericity. Epsilon (ε) values not equal to 1 are reported below. The prenatal nutrition study and the NE lesion study had separate controls (25/25 casein and SHAM-LX) and therefore analyses from each study were run separately.

Histological Analyses

Histological data were analyzed using a mixed factor ANOVA for each cortical subregion with Nutrition (two levels) or Lesion (two levels) as the between-subjects factor and the within-subjects factor of Rostral to Caudal (three levels in counts from PL; three levels in counts from ACC). Poor fixation on one subject in the prenatal malnutrition study precluded histological processing, resulting in final n's of 9 and 17 for the 6/25 and 25/25 groups, respectively. Staining from one NE-LX rat was lost to an error in tissue processing.

Baseline Sessions

All dependent measures were analyzed using separate mixed factor ANOVAs. In order to determine if there was any difference based on prenatal nutritional treatment or lesioning, on performance on the SAT, baseline days prior to each test of attentional variation was compared to the other days. For the analysis of the effects of time on task (vigilance decrement), test sessions were divided into three blocks of 54 trials each (see above). The effects of signal length and block over the days of baseline on hit accuracy were analyzed using a mixed factor ANOVA with one between-subjects factor [e.g., Nutrition (2)] and three within subject factors [Day (4), Block (3), and Signal (3)]. The effects of block on correct rejection accuracy were analyzed using a mixed factors ANOVA with one between-subjects factor [e.g., Lesion (2)] and two within-subject factors [Day (4) and Block (3)].

Sessions With Varied Attentional Demand

Baseline performance for each dependent measure was calculated using session in the standard task (ITI: 12 ± 3 s) immediately prior to the test of altered attentional demand (e.g., 0.5 Hz Distractor), so that each ANOVA had two levels that allowed a comparison of performance in the standard SAT to the test session. The effects of varied attentional demand were assessed in independent, mixed factors ANOVAs with one between-subjects factor [Nutrition (2)] and three within subject factors [Task Variation (2), Block (3), and Signal (3)]. The effects on correct rejection accuracy were analyzed using a mixed-factors ANOVA with one between-subjects factor [Nutrition (2)] and two within-subject factors [Task Variation (2) and Block (3)]. A summary of the effects of prenatal malnutrition or noradrenergic lesions on % hits and % correct rejections can be found in **Table 2**.

Prenatal Protein Malnutrition

The average number of days to achieve criterion performance in the SAT was compared using an independent samples *t*-test to determine if there was any difference in the rate of acquisition based on prenatal nutritional treatment.

RESULTS

Histological Analyses

Prenatal Protein Malnutrition

Prenatal protein malnutrition resulted in significantly fewer DBH positive axons in the PL relative to control values [$F(1,24) = 5.61$, $p = 0.03$; **Figures 1A,B**, **2A**] but did not alter axon density in ACC [$F(1,24) = 0.26$, $p = 0.61$; **Figure 2A**]. When compared to 25/25 control subjects, the prenatally malnourished 6/25 rats had

TABLE 2 | A summary of the main effects of prenatal protein malnutrition and noradrenergic lesions on the primary dependent measures from the SAT.

	Prenatal malnutrition				Noradrenergic lesion			
	% Hits		% CR		% Hits		% CR	
Test	25/25	6/25	25/25	6/25	SHAM-LX	NE-LX	SHAM-LX	NE-LX
dSAT	42.9 ± 4.2	47.4 ± 5.5	82.1 ± 1.6	85.4 ± 2.2	35.9 ± 3.6	55.9 + 3.5	83.9 + 1.8	74.8 + 2.8
uSAT	45.5 ± 3.0	57.1 ± 4.1	71.9 ± 2.6	68.0 ± 3.5	53.2 ± 4.9	54.6 ± 3.2	67.8 + 3.0	67.8 + 1.9
oSAT	45.9 ± 2.7	45.8 ± 3.5	82.1 ± 1.6	78.9 ± 2.2	n.a.	n.a.	n.a.	n.a.
SATwr	63.7 ± 3.1	63.3 ± 4.0	68.8 ± 4.8	80.4 ± 2.2	54.1 ± 3.0	62.0 ± 3.5	80.5 ± 1.7	76.8 ± 3.0

Shading is used to indicate significant group differences within each study.

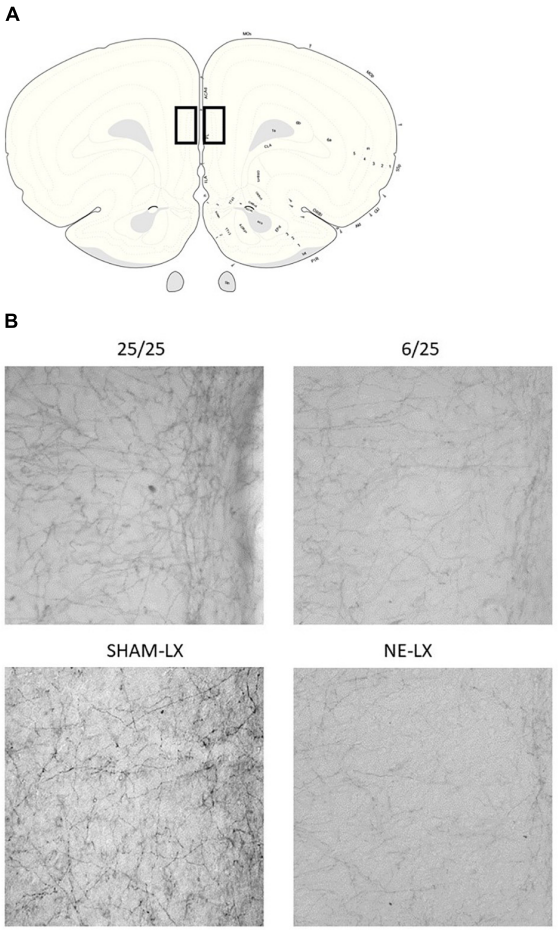

FIGURE 1 | (A) A schematic diagram of a coronal slice from the rat brain approximately 3.7 mm anterior to Bregma. Black squares indicate the location of the prelimbic cortex (PL) (Paxinos and Watson, 2007). **(B)** Photomicrographs of the PL showing noradrenergic axons stained for dopamine beta hydroxylase (DBH). The top left image is taken from well-nourished control rats (25/25) and is compared to subjects exposed to prenatal malnutrition (6/25) shown on the right. Subjects from the second study are shown in the row below with SHAM-LX rats shown on the left and NE-LX shown on the right.

21.7 ± 7.0 % fewer DBH positive axons in the PL. These findings did not differ along the rostro-caudal axis in the PL and the ACC showed no significant differences between groups (all $p > 0.12$). There was no difference in cholinergic fiber density as a result of prenatal malnutrition in either PL (25/25: 355.7 ± 30.0; 6/25: 352.0 ± 38.9) or ACC (25/25: 354.0 ± 20.0; 6/25: 370.0 ± 28.0; all $p > 0.65$; data not shown).

Noradrenergic Lesions of PL

Damage that resulted from infusion of DBH-saporin in the medial prefrontal cortex produced noradrenergic deafferentation in the PL [$F(1,20) = 18.13$, $p < 0.001$; **Figures 1A,B**, **2B**] but not in the anterior cingulate or orbitofrontal cortices (all $p > 0.45$; **Figure 2B**). There was no difference in the extent of damage along the rostro-caudal axis or between hemispheres (all $p > 0.05$). On average, the immunotoxin produced a 38.8% ± 2.3% loss of DBH positive fibers at all rostro-caudal levels assessed in PL cortex.

Prenatal Protein Malnutrition: Effects of Prenatal Protein on Body Weights

Weights prior to the onset of behavioral testing were compared between the two nutrition groups. No differences were found in weight based on the prenatal nutrition group [$t(25) = 1.37$, $p = 0.19$; 6/25: 415.6 ± 11.8 g; 25/25: 438.9.5 ± 11.1 g]. Our aim was to allow subjects to maintain a body weight ≥90% of pre-restriction during behavioral testing. We calculated the lowest post-restriction body weight/pre-restriction body weight for each rat to determine how well they maintained body weight after dietary restriction. After the implementation of food restriction, all rats maintained body weights nearly identical to their pre-restricted weights (6/25 = 100% ± 2.9%; 25/25 = 102% ± 2.0%), and there was no difference between the nutrition groups [$t(25) = 0.59$, $p = 0.55$]. Therefore, weight was not included in further analyses of behavioral performance.

Baseline SAT

Prenatal Protein Malnutrition

Prenatal malnutrition did not impair acquisition of performance in the standard version of the SAT. The number of days required to achieve criterion performance in the SAT did not differ as a result of prenatal nutritional treatment [$t(25) = 1.69$, $p = 0.10$; range 14–40 days; mean ± SEM; 25.15 ± 1.39]. Rats from the 6/25 group were better at detecting signals than 25/25 rats [$F(1,25) = 4.78$, $p = 0.04$]. Regardless of prenatal nutritional treatment, subjects showed signal length dependent performance on hits [$F(2,50) = 319.96$, $p < 0.0001$; **Figure 3A**] that was consistent across blocks of testing trials ($p > 0.7$; **Figure 3B**).

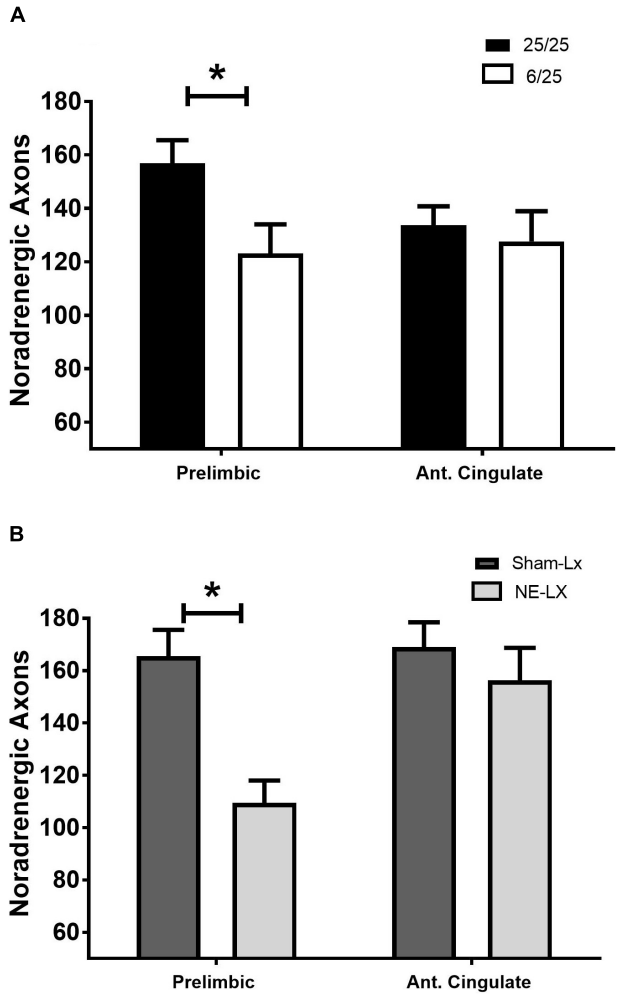

FIGURE 2 | (A) Rats exposed to prenatal malnutrition (6/25; white bars) had significantly fewer noradrenergic axons in PL than well-nourished control subjects (25/25; black bars). There was no difference between the number of noradrenergic axons in the ACC between control and malnourished rats. **(B)** Data from experiment two is shown with control subjects indicated by dark gray bars (SHAM-LX) and lesioned rats indicated by light gray bars (NE-LX). Infusions of DBH-saporin produces noradrenergic deafferentation in the PL cortex while sparing fibers in the nearby ACC. Though the extent of noradrenergic damage produced by lesioning the PL was greater than the extent of damage found to result from malnutrition (38% reduction in axons due to lesioning vs. 21.7% after malnutritions), damage following both treatments was limited to the PL. * indicates $p < 0.05$.

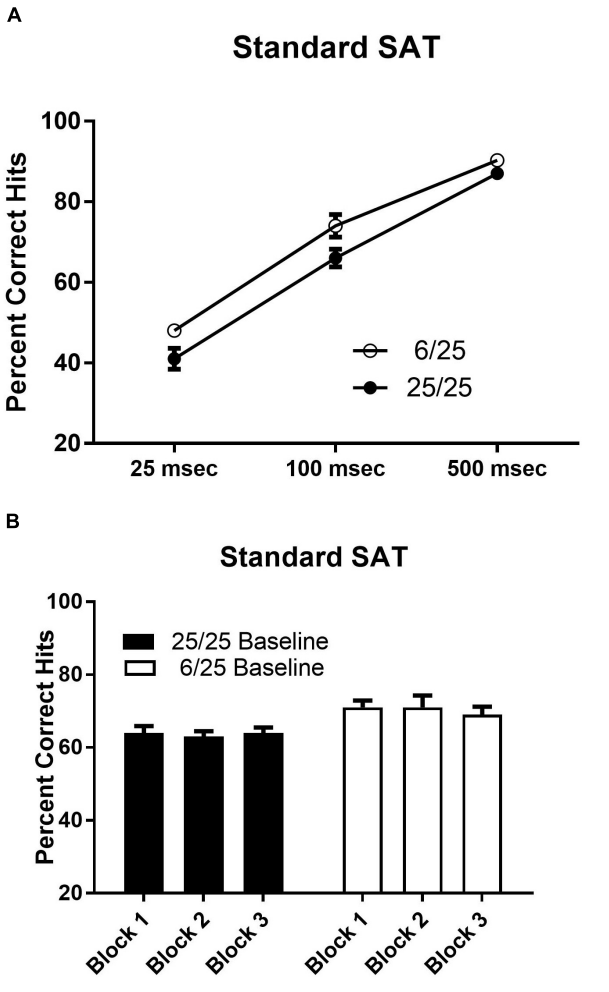

FIGURE 3 | Performance on the baseline SAT task in prenatally well-nourished and malnourished rats. **(A)** The ordinate shows the percent correct responding to signal trials. The abscissa depicts performance at each stimulus length (25, 100, and 500 ms). When accuracy was averaged across all stimulus lengths, 6/25 (empty circles) rats showed statistically significant higher accuracy on signal trials than controls (25/25; filled circles). Means and SEMs averaged across signal lengths are provided in **Table 2**. **(B)** Blocks of 54 trials are shown on the *x*-axis. Each block contained 27 signal trials and 27 non-signal trials presented in a pseudorandom order so that each target duration was presented on nine trials. There was no change in performance over the course of the testing session in the SAT for either 6/25 (white bars) or 25/25 rats (black bars).

Noradrenergic Lesions of PL

One SHAM-LX animal failed to complete post-surgical training so data from that subject were excluded from statistical analyses. For both groups, $n = 11$. Accuracy on signal trials varied by signal duration [$F(3,60) = 371.95$, $p < 0.0001$; data not shown], in both sham- and NE-lesioned rats ($p > 0.2$). Signal detection was unchanged over the course of the testing session and did not differ after NE lesions (all $p > 0.5$). Hits were similar for all days prior to a test of varying attentional demand in both groups (all $p > 0.17$). Noradrenergic lesions did not change the ability of rats to correctly reject non-signals (all $p > 0.44$). There were no other significant main effects of treatment or interactions in the analyses of hits and correct rejections. Both SHAM-LX and NE-LX had similar side biases that were approximately neutral (mean ± SEM; SHAM-LX: 0.43 ± 0.01; NE-LX: 0.44 ± 0.01).

Task Irrelevant Lights

Predictable Distractor

Prenatal protein malnutrition

Though the presence of the 0.5 Hz houselight impaired performance regardless of treatment [$F(1,25) = 59.51$, $p < 0.0001$]

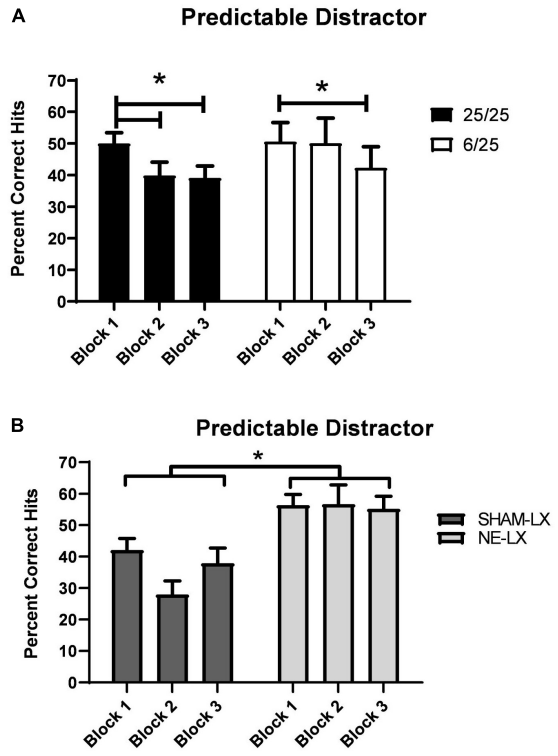

FIGURE 4 | (A) 6/25 rats (white bars) were more resistant to cognitive fatigue produced by the presence of a 0.5 Hz visual distractor than 25/25 rats (black bars). Prenatally protein malnourished rats did not show a significant decline in performance under these conditions until the last block of trials. In contrast, 25/25 rats were significantly impaired in detecting signals by Block 2 and their signal detection remained impaired throughout the rest of the testing session. **(B)** Rats with selective noradrenergic lesions of the PL cortex (light gray bars) were more resistant to the effects of a 0.5 Hz visual distractor than controls (dark gray bars) as shown by the higher number of hits (ordinate) over the course of the testing session. * indicates $p < 0.05$.

prenatally malnourished rats were less susceptible to the detrimental effects of the predictable distractor (**Table 2**). Specifically, well-nourished rats showed more rapid cognitive fatigue in the face of this distractor than did the prenatally malnourished rats [$F(2,50) = 5.61$, $p < 0.006$, see **Figure 4A**; black bars]. Planned comparisons revealed that 25/25 rats showed significant decreases in performance in Block 2 relative to Block 1 of the distractor session [$t(16) = 3.14$; $p = 0.006$]. This impairment was also observed during the third and final block of testing [Block 1 vs. 3: $t(16) = 3.88$, $p = 0.001$; Block 2 vs. 3: $t(16) = 0.24$, $p = 0.81$]. In contrast to these findings, prenatally protein malnourished rats performed at similar levels of accuracy in Blocks 1 and 2 ($p = 0.82$; **Figure 4A**; white bars). However, they were significantly impaired by Block 3 relative to the first block of the distractor session [$t(9) = 4.05$, $p = 0.003$] with a trend for poorer performance in Block 3 versus Block 2 [$t(9) = 2.18$; $p = 0.06$]. There were no other significant main effects or interactions found in the hits analyses.

Rats from both nutritional groups emitted more false alarms and fewer correct rejections in the dSAT than the SAT [$F(1,25) = 8.66$, $p = 0.007$; data not shown]. This impairment was largest in the first block of the 0.5 Hz session relative to the baseline session for all rats [Day × Block: $F(2,50) = 9.25$, $p < 0.001$, $\varepsilon = 0.86$; Baseline vs. 0.5 Hz Block 1: $t(26) = 5.41$, $p < 0.001$]. The rats' performance did not differ in subsequent blocks (all $p > 0.05$). Prenatal malnutrition did not significantly alter non-signal accuracy or interact with the effects of the distractor (**Table 2**; all $p > 0.05$).

Noradrenergic lesions of PL

Though the presence of the 0.5 Hz houselight (dSAT) impaired performance in both groups [$F(1,20) = 102.96$, $p < 0.001$], noradrenergically lesioned rats were better able to detect signals during this session than SHAM-LX rats [Lesion: $F(1,20) = 15.52$, $p < 0.001$; Lesion × Day: $F(1,20) = 5.71$, $p = 0.03$; **Table 2** and **Figure 4B**]. All subjects maintained signal length dependent performance in both the baseline and dSAT session [$F(2,40) = 147.86$, $p < 0.001$]. There were no other significant main effects or interactions found in the hits analyses.

Rats from both groups emitted more false alarms and fewer correct rejections [$F(1,20) = 33.07$, $p < 0.001$] during the dSAT than the SAT. This impairment was largest in the first block of the 0.5 Hz session relative to the baseline session for all rats [Day × Block: $F(2,40) = 12.27$, $p < 0.001$, $\varepsilon = 0.91$; SAT vs. dSAT Block 1: $t(21) = 7.84$, $p = 0.001$]. Noradrenergically lesioned rats emitted more false alarms and fewer correct rejections than SHAM-LX rats [Lesion: $F(1,20) = 5.91$, $p = 0.03$, $\varepsilon = 0.84$]. The presence of the flashing houselight exacerbated this difference [$F(1,20) = 4.67$, $p = 0.04$, $\varepsilon = 0.91$; **Table 2**]. The rats tended to respond more on the non-signal lever during the flashing houselight session [Day: $F(1,20) = 28.47$, $p < 0.001$], which was more pronounced in SHAM-LX versus NE-LX rats during the 0.5 Hz distractor (Baseline SHAM-LX: 0.42 ± 0.01; NE-LX: 0.46 ± 0.01; 0.5 Hz: SHAM-LX: 0.26 ± 0.03; NE-LX: 0.40 ± 0.03). This increased non-signal lever responding was confirmed by *t*-tests comparing side bias during the SAT and the dSAT session for both groups [SHAM-LX: $t(10) = 5.37$, $p = 0.001$; NE-LX: $t(10) = 2.03$, $p = 0.07$].

Unpredictable Distractor

Prenatal protein malnutrition

The temporally unpredictable distractor (uSAT) impaired performance of all subjects regardless of prenatal nutritional treatment [Day: $F(1,25) = 33.42$, $p < 0.001$]. Subjects were less impaired in the first block of testing during the uSAT than during subsequent blocks [Day × Block: $F(2,50) = 7.17$, $p < 0.002$; Block 2: $t(26) = 6.78$; Block 3: $t(26) = 4.77$, both $p < 0.001$]. This effect did not vary based on signal duration [Day × Signal × Block: $F(4,100) = 0.44$, $p = 0.78$]. Although there were no significant main effects of prenatal diet (Nutrition) or time on task (Block), a significant interaction was found [Block × Nutrition: $F(2,50) = 3.71$, $p = 0.03$; **Figure 5A**]. When the unpredictable distractor was first introduced, 25/25 rats and 6/25 rats showed similar performance (Block 1 6/25 vs. 25/25; $p = 0.38$), but 6/25 rats (white bars) were more resistant to the detrimental effects of this distractor over the course of the testing session than 25/25 rats (black bars) in Block 2 of the

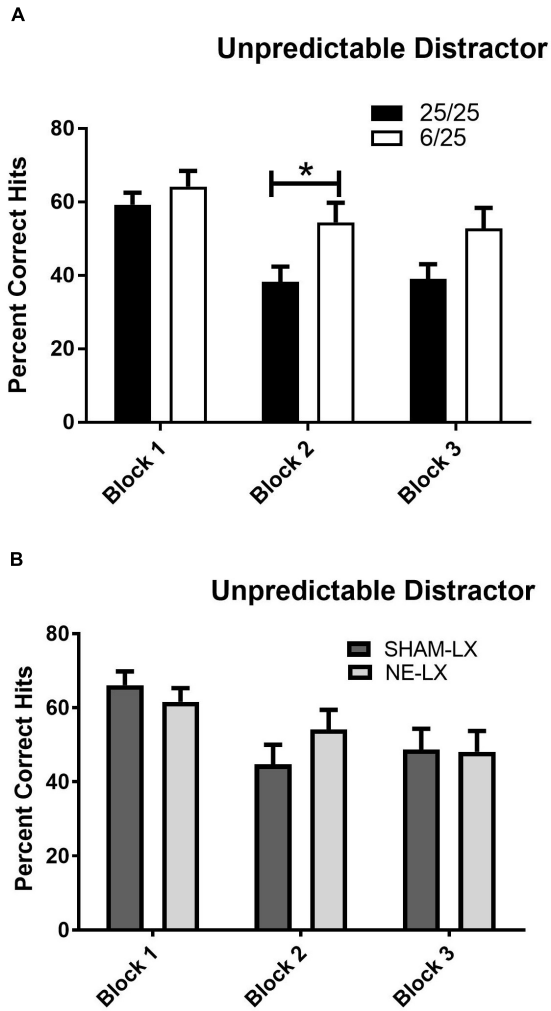

FIGURE 5 | (A) 25/25 rats (black bars) were more impaired than 6/25 (white bars) at discriminating visual target stimuli from the unpredictable distractor (0.25, 0.5, 1.0, 1.5, 2.0, or 3.0 s on/off) during the second block of testing. While 6/25 rats were better able than 25/25 rats to maintain signal detection in the presence of this distractor, both groups of rats were impaired when performance in this session was compared to performance in the prior day's SAT performance as reported in the results. For comparison, see data in **Figure 3B**. **(B)** Performance in the uSAT was unchanged by noradrenergic lesions with no differences found in the % hits of sham-lesioned (dark gray bars) and noradrenergically lesioned rats (light gray bars). *indicates $p < 0.05$.

session [$t(25) = 2.41$, $p = 0.02$], with a similar trend in Block 3 [$t(25) = 1.93$, $p = 0.06$; **Figure 5A**].

All subjects emitted more false alarms and fewer correct rejections during the uSAT than SAT [Day: $F(1,25) = 45.91$; $p < 0.001$]. All subjects showed an improvement in non-signal performance in later blocks of the uSAT relative to the first block [Block: $F(2,5) = 27.36$; $p < 00.1$; Block 1 vs. Block 2: $t(26) = 7.37$; Block 1 vs. 3: $t(26) = 7.43$; both $p < 0.001$; Block 1: 56.5 ± 2.9; Block 2: 76.3 ± 1.2 Block 3: 78.1 ± 1.5]. There was no difference in correct rejection accuracy based on prior nutritional treatment (all $p > 0.32$; **Table 2**).

Noradrenergic lesion study

Both sham and NE-lesioned rats emitted fewer hits during the uSAT than the SAT [$F(1,20) = 70.61$, $p < 0.001$]. All subjects were less impaired in the first block of testing during the uSAT than during subsequent blocks [Day × Block: $F(2,40) = 12.79$, $p < 0.001$; Block 1 vs. 2: $t(21) = 4.17$; Block 1 vs. 3: $t(21) = 4.61$; both $p < 0.001$; Block 2 vs. 3 $t(21) = 0.32$, $p = 0.75$; **Figure 5B**]. There was no effect of noradrenergic lesions on uSAT performance (all main effects and interactions, $p > 0.12$; **Table 2**).

All subjects also emitted more false alarms and fewer correct rejections during the uSAT than the SAT [NE lesion study: $F(1,20) = 109.48$; $p < 0.001$]. All subjects showed an improvement in non-signal performance in the last block of the uSAT [Block 1 vs. 3: $t(21) = 8.42$, $p < 0.001$; Block 2 vs. 3: $t(21) = 2.58$, $p = 0.017$; data not shown]. Noradrenergic lesions did not impair non-signal performance (all main effects and interactions $p > 0.67$; **Table 2**). No other effects or significant interactions between treatments and any other factor were found in the analyses of signal, non-signal accuracy or side bias.

Overlapping Distractor

Prenatal protein malnutrition

Distracting stimuli with durations equivalent to target stimuli (oSAT) were only tested in the prenatal malnutrition study. Signal detection of all rats was impaired in the oSAT relative to baseline performance [Day: $F(1,25) = 61.00$, $p = 0.001$]. These effects did not differ between the two prenatal nutritional groups (all $p > 0.08$; **Table 2**).

All subjects were less able to correctly reject non-signal stimuli in the presence of the overlapping distractor [Day: $F(1,25) = 12.54$, $p = 0.002$] despite shifting responding toward the non-signal lever during this session [$F(1,25) = 19.81$, $p < 0.001$; Side Bias oSAT: 0.33 ± 0.014]. In contrast to the effects of the other distracting stimuli, the presence of the overlapping light decreased accurate, non-signal responding in 6/25 rats but not in 25/25 rats (**Table 2**). When performance in the oSAT was compared to the SAT, rats with prenatal protein malnutrition (6/25; white bars) showed a significant increase in false alarms [Day × Nutrition: $F(1,25) = 5.82$, $p = 0.02$; $t(9) = 4.47$, $p = 0.002$; **Figure 6**], while well-nourished rats did not [25/25; $t(16) = 0.86$, $p = 0.40$; **Figure 6**]. There was no difference based on prenatal nutrition in side bias during the overlapping session [$F(1,25) = 0.08$, $p = 0.78$].

SAT Without Reinforcement (SATwr)

Prenatal protein malnutrition

To test the possibility that nutritional treatment altered subjects' responses to food reward, we tested SAT performance without food reinforcement. There was no difference based on prenatal malnutrition to effects of withholding reinforcement in the SAT (% hits: all $p > 0.45$; % CR: all $p > 0.1$; **Table 2**). For all animals, accuracy on both signal [$F(1,25) = 4.50$, $p = 0.04$] and non-signal trials [$F(1,25) = 15.32$, $p = 0.001$] was decreased in this session. The largest drop in accuracy during the SATwr was in response to the 500 and 100 ms signal [Day × Signal Length: $F(2,50) = 11.84$, $p = 0.0001$, $\varepsilon = 0.91$; Baseline vs. SATwr: 500 ms: $t(26) = 5.27$, $p = 0.001$; 100 ms: $t(26) = 2.21$, $p = 0.04$; data not shown].

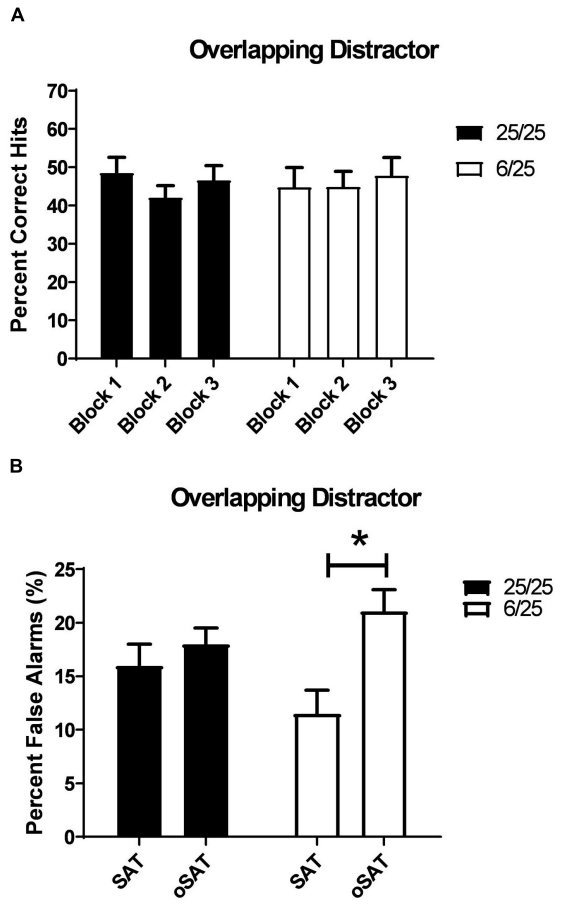

FIGURE 6 | (A) The effects of a distracting, visual stimulus that overlapped in duration with targets (500, 100, or 25 ms on/off; oSAT) is shown. All rats were impaired in signal detection during this session when performance was compared to the baseline. For comparison, see data in **Figure 3B**. This distractor produced similar deficits in all rats regardless of prenatal protein levels. **(B)** Prenatally malnourished rats (6/25, white bars) emitted a greater number of false alarms when the houselight was flashed at durations equivalent to that of target stimuli (oSAT) than when the houselight was constantly illuminated (SAT). Rats from well-nourished mothers (black bars) did not show significantly higher numbers of false alarms during the overlapping distractor session (oSAT) than during than baseline SAT with the houselight constantly illuminated (SAT). These data support the hypothesis that malnutrition contributes to impairments in response inhibition. *indicates $p < 0.05$.

The effects of increased time on task exacerbated the effects of withholding reinforcement on signal detection [$F(2,50) = 8.06$, $p = 0.001$, $\varepsilon = 0.81$]. Though there was no difference in signal detection when the first block of the test session was compared to the same block in the baseline session, performance did differ between sessions in the third block [$F(2,50) = 8.06$, $p = 0.001$, $\varepsilon = 0.81$; Baseline vs. SATwr: Block 3: $t(26) = 3.28$, $p = 0.003$; data not shown]. The impairments in accuracy were not a result of rats adopting a side bias as all subjects regardless of dietary treatment maintained a neutral side bias during this session. Additionally, while omissions during signal and non-signal trials were significantly higher during the session without reinforcement, they remained extremely low [Signal Trials: $F(1,25) = 18.89$, $p < 0.001$; Baseline: 0.1 ± 0.09; SATwr: 1.16 ± 0.23; Non-signal Trials: $F(1,25) = 19.73$; $p < 0.001$; Baseline: 0.80 ± 0.25; SATwr: 4.46 ± 0.81]. There was no difference in omission rate on signal trials based on prenatal nutrition group [$F(1,25) = 0.099$, $p = 0.76$].

Noradrenergic lesion study

There was no difference between NE-LX and SHAM-LX rats during the SATwr (% hits: all $p > 0.36$; % CR: all $p > 0.13$; **Table 2**), Accuracy on signal trials [$F(1,20) = 22.19$, $p < 0.001$] was decreased during the SATwr relative to SAT for both lesioned and sham-lesioned subjects. The largest drop in accuracy during this session was in response to the 500 and 100 ms signal [Day × Signal Length: $F(2,40) = 8.05$, $p < 0.001$; SAT vs. SATwr: 500 ms: $t(21) = 7.14$, $p < 0.001$; 100 ms: $t(21) = 5.82$, $p < 0.001$; 25 ms: $t(21) = 1.57$, $p = 0.13$]. Though there was no difference in signal detection when the first block of the SATwr session was compared to the same block in the baseline session, performance did differ between sessions in Block 2 and 3 [Day × Block: $F(2,40) = 14.01$, $p < 0.001$; $\varepsilon = 0.76$; SAT vs. SATwr: Block 1: $t(21) = 0.62$, $p = 0.54$; Block 2: $t(21) = 4.88$, $p < 0.001$; Block 3: $t(21) = 4.50$, $p < 0.001$]. The impairments in accuracy were not a result of rats adopting a side bias as all subjects maintained a neutral side bias during this session (SATwr = 0.44 ± 0.02). Additionally, while omissions during signal trials were significantly higher during the session without reinforcement, they remained extremely low [$F(1,20) = 92.27$, $p < 0.001$; Baseline: 0.13; No reinforcement: 1.74 ± 0.18]. Noradrenergic lesions did not alter the response to withholding of reinforcement on hits or omissions (all main effects and interactions $p > 0.15$; **Table 2**).

Withholding reinforcement decreased non-signal accuracy [$F(1,20) = 43.71$, $p < 0.001$; **Table 2**] in all subjects. Non-signal performance declined significantly over the course of the SATwr session [$F(2,40) = 4.28$, $p = 0.02$] with a large decrease in accuracy when Block 2 was compared with Block 1 [$t(21) = 3.25$, $p = 0.004$] but no further decline in performance in Block 3 relative to Block 2 [$t(21) = 0.98$, $p = 0.34$]. Omissions on non-signal trials were higher when reinforcement was withheld but remained low [Day: $F(1,20) = 102.07$, $p < 0.001$; Baseline: 0.54 ± 0.13; No reinforcement: 6.62 ± 0.62]. There were no other main effects or interactions in the analyses of the effects of noradrenergic lesions on performance in the SATwr.

DISCUSSION

The present study is the first, to our knowledge, to directly compare the effects of prenatal protein malnutrition to selective noradrenergic deafferentation of the PL cortex. Few studies have assessed the effects of noradrenergic damage on sustained attention in the rat, and published results have been inconclusive. Prior work by McGaughy et al. (1997) showed that lesions to the dorsal noradrenergic bundle failed to impair performance on the SAT or dSAT. Assessments of lesions in this study were based on homogenates of the entire frontal cortex and failed to differentiate damage within prefrontal sub-regions so the extent

of damage to PL is unknown. It is possible the failure to dissociate the attentional performance of lesioned and sham-lesioned rats results from the relative sparing of PL. Previous studies by Carli et al. (1983) showed that lesions to the dorsal noradrenergic bundle sufficient to deplete >80% of cortical norepinephrine did not impair baseline performance in a visual search task. These subjects were, however, impaired by the interpolation of a loud, white noise distractor (Carli et al., 1983) which was interpreted as an increased sensitivity to the stress-related effects of this distractor. The present study did not directly examine the effects of distraction in conjunction with stress, but prior studies have shown that prenatal malnutrition confers a vulnerability to stress concomitant to alterations in cortical monoamines (Mokler et al., 2007). Future studies will be aimed at determining how stress interacts with distractibility in both noradrenergic lesioned and malnourished subjects.

Prenatal malnutrition and noradrenergic deafferentation of the PL did not affect performance significantly in the baseline SAT. This is consistent with prior work in lesioned rats (Carli et al., 1983; McGaughy et al., 1997). Additionally, neither group of experimental animals was found to be more sensitive to withholding reinforcement. This preservation of function confirms prior reports that malnutrition produces specific attentional impairments rather than global difficulties in understanding response rules, bottom-up problems in perception or cognition secondary to primary dysfunction in sensitivity to reinforcement contingencies (Morgane et al., 1993; Tonkiss et al., 1993; McGaughy et al., 2014).

Prenatally malnourished rats were less susceptible to the effects of a predictable and unpredictable visual distractor than well-nourished control subjects. Prenatally malnourished rats maintained higher target detection rates for a greater portion of the testing session than control subjects in tests of distractibility. These findings may seem counterintuitive as they suggest that prenatally malnourished rats perform better in the face of distraction. However, these data, in conjunction with previous data (Tonkiss et al., 1993; Strupp and Levitsky, 1995), support the hypothesis that prenatal malnutrition produces cognitive rigidity and this confers resistance to distraction. While behavioral inflexibility may be beneficial when disregarding a distractor, it was found to be detrimental when a subject was required to change its strategy and to attend a novel stimulus dimension in a test of attentional set shifting (McGaughy et al., 2014), demonstrating that normal attention requires adaptation to current cognitive demands.

The results of the NE lesion study show that acute noradrenergic deafferentation of the PL cortex produces similar, but distinctive, results to prenatal protein malnutrition. Noradrenergic lesioned rats were more resistant to the effects of the predictable distractor than controls, but the groups did not differ in their responses to the unpredictable distractor. The present study also shows that prenatal protein malnutrition results in fewer noradrenergic afferents to PL cortex but does not impact afferents to the ACC or cholinergic afferents to any prefrontal sub-region sampled. These findings are in line with recent data showing noradrenergic innervation of the cortex is topographically discrete with less overlap in cortical innervation than previously assumed (Chandler et al., 2013). It is perhaps unsurprising that adult lesions restricted to PL cortex failed to fully replicate the neurodevelopmental insult. Though the extent of noradrenergic damage produced by lesioning the PL cortex was greater than the extent of damage found to result from malnutrition (38% vs. 21.7% reduction in fibers), prenatally malnourished rats were resistant to both predictable and unpredictable distractors, while lesioned rats were resistant only to the predictable distractor suggesting malnutrition produces a more severe cognitive rigidity than acute noradrenergic lesioning. However, a closer look at these data, show remarkable similarities between the performance of prenatally malnourished and lesioned rats in the presence of the unpredictable distractor (uSAT 6/25: 57.1 $\pm$ 4.1; NE-LX: 54.6 $\pm$ 3.2). *Post hoc* comparison of the well-nourished and sham-lesioned rats failed to reveal a statistically significant difference in the performance of these groups during the distractor (uSAT: 25/25: 45.5 $\pm$ 3.0; SHAM-LX: 53.2 $\pm$ 4.9), but the sham-operated controls in Experiment 2 had more variable performance in the presence of the unpredictable distractor. It is therefore possible that the differences in interpretation of the data may be due to differences between control groups rather than a critical difference in the effect of prenatal malnutrition and noradrenergically lesioned rats.

Previous research has documented increased levels of brain catecholamines after perinatal food restriction and protein malnutrition (Burns and Brown, 1977; Ramanamurthy, 1977; Molendi-Coste et al., 2006). However, adults that had previously been fostered to well-nourished dams *did not* show increased cortical norepinephrine (Soto-Moyano et al., 1999). Additionally, when found these higher than normal levels of cortical norepinephrine have been shown to decrease by adulthood (Stern et al., 1975; Chen et al., 1997; Soto-Moyano et al., 1999). Previous research has also shown that these changes were regionally specific (Stern et al., 1975; Chen et al., 1997). An *in vivo* microdialysis study of the ventral mPFC, including the PL, has revealed decreased levels of both norepinephrine and dopamine in the right hemisphere of the PL (Mokler et al., 2019). At present, we are not able to reconcile the unilateral extent of decreased cortical efflux with decreased noradrenergic axons occurring bilaterally. The changes in cortical norepinephrine do not seem to result from changes in the locus coeruleus which is unchanged by prenatal protein malnutrition in male rats (King et al., 1999). However, it is important to note that the locus coeruleus is sexually dimorphic (Pinos et al., 2001; Bangasser et al., 2016). Because data from human studies of early life malnutrition have found the attentional and emotional impairments (Galler and Ramsey, 1989; Galler et al., 1990, 2012; Waber et al., 2014) are equally prevalent in males and females, we have focused our pre-clinical studies on male rats. However, the effects of prenatal malnutrition on female subjects in this animal model remains an underexplored and critical question. From these studies and others, it is apparent that prenatal protein malnutrition alters the development of the noradrenergic systems centrally and peripherally, and these effects can vary depending on the severity of malnutrition, the timing of the insult, the age of the animal at testing, and the brain region

studied. Though the precise mechanism of these changes remains unresolved, prenatal malnutrition has been shown to produce epigenetic changes in both humans and a rodent model (Peter et al., 2016). Specifically malnutrition lowers transcription of the catechol-*O*-methyltransferase gene in the humans and the prefrontal cortex of male rats which may contribute to altered catecholamine signaling in the cortex of malnourished subjects (Peter et al., 2016).

To our knowledge, the present experiment is the first-time noradrenergic fiber density has been studied in the prefrontal cortex after prenatal malnutrition, and, as these regions are more topographically restricted than previously assumed (Chandler and Waterhouse, 2012), it may not be unexpected that the fiber density varies across prefrontal subdivisions. The assessment of axons in the present study found that the density of axons is similar across prefrontal sub-regions. This is similar to prior studies of the number of axons (Cerpa et al., 2019). In contrast, the density of noradrenergic varicosities has been shown to be higher in the ACC than the more ventral regions of the medial prefrontal cortex, i.e., PL combined with IL (Agster et al., 2013). Interestingly the study by Cerpa et al. (2019) founds a substantial overlap between measurements of axon density and varicosities. This suggests the difference is not related to the dependent measures but additional studies where both axons and varicosities are assessed are needed to resolve this point. Another source of the discrepancy between our study and Agster's (Agster et al., 2013) may be due to a nearly 2 mm difference in sampling of ACC between the studies. The current experiment and other work has focused on pre-genu portions of the ACC in the rat based on functional homologies of this region to the dorsal ACC in humans (Milham and Banich, 2005; Orr and Weissman, 2009; Newman and McGaughy, 2011; Newman et al., 2015), but future studies will be aimed at assessing caudal regions of the ACC as well to determine how malnutrition impacts them.

In contrast to the resistance to distraction found with other distractors, the overlapping distractor, revealed a unique susceptibility of the prenatally malnourished rats. Malnourished rats were more likely than control subjects to emit false alarms during the session with the overlapping distractor while target detection did not differ between the groups. The increase in false alarms may reflect additional problems in response inhibition caused by the prenatal insult that are revealed only under conditions of high perceptual overlap. These data are in line with previous research by this group showing mild, but consistent, impairments in response inhibition resulting from prenatal protein restriction (Tonkiss et al., 1993; McGaughy et al., 2014). The difference between the malnourished subjects' response to the overlapping distractor and other distracting stimuli is likely to reflect the impact of prenatal protein malnutrition on cortical regions beyond the PL. Though the integrity of the prefrontal cortex is required to disregard distracting stimuli (Newman et al., 2008; Berry et al., 2014), the ability to differentiate perceptually identical distractors from targets relies on the parietal cortex (Buschman and Miller, 2007). Though assessments of parietal cortex were not undertaken in the present study, future work will address the impact of prenatal protein malnutrition on this region critical to attentional control. Unfortunately, this variant was not tested in the assessment of PL lesioned rats, so direct comparisons on this particular measure cannot be made between the two studies.

Neither prenatally malnourished nor noradrenergically lesioned rats were impaired in the baseline version of the task. These data are consistent with findings in the prenatally malnourished rats that cholinergic afferents to prefrontal cortex were unaffected by malnutrition. Phasic activity of the cholinergic system in the prefrontal cortex is critical to allowing subjects to shift between internally and externally driven attention (Howe et al., 2013), while tonic levels are hypothesized to allow target detection (Sarter and Paolone, 2011). Though our histochemical analyses provide only a static measure of the integrity of the prefrontal cholinergic system, the preservation of baseline performance on the SAT is consistent with our finding of unaltered cholinergic afferents.

The current data show that rats with fewer noradrenergic axons in PL resulting from lesioning or prenatal malnutrition are less distractible than controls. Early-life malnutrition produces cognitive rigidity in our rodent model in the present study and in prior work (McGaughy et al., 2014; Tonkiss et al., 1993) that parallel reports of cognitive rigidity and reduced response inhibition in a human population with histories of early childhood malnutrition (Galler et al., 2012; Waber et al., 2014; Peter et al., 2016). Drugs that increase cortical norepinephrine, e.g., selective reuptake inhibitors, have consistently been shown to improve cognitive flexibility and response inhibition in humans (Chamberlain et al., 2007) and in rodent models that assess the effects of noradrenergic lesions (Robinson et al., 2007; Newman et al., 2008; Cain et al., 2011; Harvey et al., 2013). Increased levels of cortical norepinephrine have been hypothesized to be necessary to broaden attention when response strategies are no longer successful (Aston-Jones and Cohen, 2005; Bouret and Sara, 2005; Berridge et al., 2012). Because malnutrition impacts noradrenergic afferents in PL cortex and alters cognitive flexibility as well as response inhibition (McGaughy et al., 2014), future studies should be aimed at assessing the efficacy of drugs and other interventions that augment noradrenergic function in vulnerable populations. The current study is limited by the focus on maternal malnutrition. Because our aim is to translate our findings to human populations where paternity may be difficult to determine, we have focused on understanding how maternal malnutrition impacts attention and cognition. It is clear that nutritional status of the father is also an important question to address in future studies. An additional limitation is the use of only male subjects. Human studies have shown that prenatal malnutrition impacts attentional performance males and females equally (Galler et al., 2012; Waber et al., 2014) but future studies in our animal model will include females to directly address this in the current animal model. In summary, prenatal malnutrition produces selective impairments in attention and response inhibition that are hypothesized to result, at least in part, from the lower levels of noradrenergic afferents in PL cortex. These deficits are not secondary to basic perceptual or learning impairments and

reflect the unique vulnerability of the prefrontal cortex to this neurodevelopmental insult.

AUTHOR CONTRIBUTIONS

LN contributed to the acquisition and analyses of the data. JB contributed to the acquisition of the data. DM, JG, and JM contributed to the conception, design, analyses, and interpretation of work. AR contributed to the analyses of the data and manuscript preparation.

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Prenatal Protein Malnutrition Leads to Hemispheric Differences in the Extracellular Concentrations of Norepinephrine, Dopamine and Serotonin in the Medial Prefrontal Cortex of Adult Rats

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Exposure to prenatal protein malnutrition (PPM) leads to a reprogramming of the brain, altering executive functions involving the prefrontal cortex (PFC). In this study we used *in vivo* microdialysis to assess the effects of PPM on extracellular concentrations of norepinephrine (NE), dopamine (DA) and serotonin (5-HT) bilaterally in the ventral portion of the medial prefrontal cortex (vmPFC; ventral prelimbic and infralimbic cortices) of adult Long-Evans rats. Female Long-Evans rats were fed either a low protein (6%) or adequate protein diet (25%) prior to mating and throughout pregnancy. At birth, all litters were culled and fostered to dams fed a 25% (adequate) protein diet. At 120 days of age, 2 mm microdialysis probes were placed into left and right vmPFC. Basal extracellular concentrations of NE, DA, and 5-HT were determined over a 1-h period using HPLC. In rats exposed to PPM there was a decrease in extracellular concentrations of NE and DA in the right vmPFC and an increase in the extracellular concentration of 5-HT in the left vmPFC compared to controls (prenatally malnourished: $N = 10$, well-nourished: $N = 20$). Assessment of the cerebral laterality of extracellular neurotransmitters in the vmPFC showed that prenatally malnourished animals had a significant shift in laterality from the right to the left hemisphere for NE and DA but not for serotonin. In a related study, these animals showed cognitive inflexibility in an attentional task. In animals in the current study, NE levels in the right vmPFC of well-nourished animals correlated positively with performance in an attention task, while 5-HT in the left vmPFC of well-nourished rats correlated negatively with performance. These data, in addition to previously published studies, suggest a long-term reprogramming of the vmPFC in rats exposed to PPM which may contribute to attention deficits observed in adult animals exposed to PPM.

Keywords: 5-hydroxytryptamine, *in vivo* microdialysis, neurotransmission, 5-HT, infralimbic – prelimbic cortex

INTRODUCTION

Protein malnutrition is one of the most prevalent forms of malnutrition in the world. Studies in human populations exposed to prenatal protein malnutrition (PPM) during the Dutch Famine have shown an increased lifetime risk of depression and schizophrenia (Susser et al., 1998; St Clair et al., 2005). The 45+ year Barbados Nutrition Study has documented cognitive and emotional development across the lifespan associated in a cohort that suffered from moderate-severe malnutrition limited to the first year of life and healthy comparison cases (Galler et al., 2012; Waber et al., 2014). Although they caught up completely in physical growth, the previously malnourished cohort displayed reductions in IQ, cognitive flexibility, impaired visuospatial processing, as well as problems with impulsivity and attentional dysregulation (Galler et al., 2012; Waber et al., 2014, 2018). Cognitive and attentional problems (Galler et al., 2012), including cognitive rigidity and poor cognitive control, led to poor performance on a national high school entrance exam (Galler et al., 1990); persisted at least to 40 years of age and were are also seen in the offspring of the original study participants (Waber et al., 2018). These attentional problems were closely associated with epigenetic changes in both the parent and offspring generation and may represent a mechanism underlying the long-term effects of early protein malnutrition (Peter et al., 2016).

Depression, schizophrenia, cognitive impairment, and attentional problems involve executive functions modulated by the prefrontal cortex (PFC) (Fuster, 2001; Robbins and Arnsten, 2009; Bagot et al., 2016). Furthermore, these disorders have been linked to imbalances in neurotransmitter systems in the PFC [See Robbins (2000) and Robbins and Arnsten (2009) for reviews]. In previous studies we have shown in a rat model of PPM that there are extensive changes in the serotonergic (5-HT) systems of the brain including an increase in 5-HT in the hippocampus of both 30-day old and adult rats (Mokler et al., 1999, 2003). Other studies using this model have shown that animals exposed to PPM have decreases in the dendritic arborization of serotonergic raphé cells and decreases in serotonergic terminal densities in the hippocampus as reflected by decreases in serotonin transporters (SERT) and 5-HT1A receptors (Blatt et al., 1994). We have also examined the effects of PPM on dopamine (DA) and shown, using *in vivo* microdialysis, that there is a decrease in the extracellular concentration of DA in the ventral medial prefrontal cortex (vmPFC) of adult rats following PPM (Mokler et al., 2007). Other laboratories have also reported on changes in 5-HT and DA in the brains of prenatally malnourished animals [see Almeida et al. (1996a) and Alamy and Bengelloun (2012) for reviews].

Although cognitive rigidity associated with malnutrition has been reported in humans (Waber et al., 2014) and in preclinical studies (McGaughy et al., 2014), which has been consistently linked to hypofunction of prefrontal norepinephrine (NE) (Tait et al., 2007; Newman et al., 2008; Mokler et al., 2017); the role of prefrontal NE in the cognitive deficits resulting from PPM are less clear. In the present study we used *in vivo* microdialysis to assess the effects of PPM on NE as well as 5HT and DA in the ventral portion of the medial prefrontal cortex. We focused on the ventral portion of the mPFC which consists of the ventral prelimbic and infralimbic PFC as defined in the atlas of Paxinos and Watson (2005). This distinction of the ventral mPFC is based on a functional view of the PFC discussed in more detail in Morgane and Mokler (2006). Based on our previous work showing differences in extracellular levels of these monoamines in the left and right cerebral hemispheres (Staiti et al., 2011), here we assessed laterality of concentration for all three monoamines using dual probe microdialysis. Our hypothesis is that exposure to PPM alters the pattern of 5-HT, NE, and DA levels in the left and right hemispheres of the medial PFC. In a subset of animals, we compare these monoaminergic changes to prior performance in an attentional task designed to assess cognitive flexibility as an initial step in linking changes in cortical neuromodulators to cognition.

MATERIALS AND METHODS

Subjects and Housing Conditions

Long-Evan hooded rats were obtained from Charles River (Wilmington, MA, United States). They were housed in animal quarters maintained at a temperature of 23°C (±2°) and at 45–55% humidity with a reverse 12 h night (0700–1900) 12 h day (1900–0700) light cycle. Microdialysis experiments occurred during the dark phase of the cycle between 0900 and 1300 h, enabling observations during the active waking period of the rat and allowing for measurement of neurotransmitters during the same diurnal period as behavioral testing [as reported by McGaughy et al. (2014)]. Red fluorescent lighting in the animal room during the dark phase of the cycle provided continuous dim illumination for animal care and testing. All procedures described in this paper were approved by the University of New England Institutional Animal Care and Use Committee (protocol 20101005MOK) in accordance with guidelines outlined in the NIH Guide for the Care and Use of Laboratory Animals and the Society for Neuroscience Policies on the Use of Animals and Humans in Neuroscience Research.

Nutritional Treatment and Breeding

This model of PPM implements dietary restriction prior to and during pregnancy with nutritional rehabilitation commencing at birth. Virgin female Long-Evan hooded rats were randomly assigned to one of two nutritional conditions. One group of females was fed an adequate protein diet (25% casein, Teklad Laboratories, Madison, WI, United States) beginning 5 weeks prior to mating and continuing throughout pregnancy, while the second group received an isocaloric, low protein diet (6% casein, Teklad Laboratories, Madison, WI, United States). These diets have been described in detail elsewhere (Tonkiss and Galler, 1990; Tonkiss et al., 1990) and in this issue (McGaughy et al., this issue). All females were mated with males that had been acclimated to the same diet for 1 week. Throughout pregnancy, dams were singly housed in individual polysulfone breeding cages, 39.5 cm × 34.6 cm × 21.3 cm (l × w × h; Tecniplast,

Maywood, NJ, United States). Following parturition, litters from both nutritional groups were culled to eight pups (two females and six males) and were fostered as whole litters within 24 h of birth to foster dams receiving the 25% casein diet that had given birth within the same 24 h period. Pups born to mothers provided with the 6% casein diet were fostered to mothers on the 25% casein diet and designated as members of the 6/25 (prenatally malnourished) group, while pups born to mothers provided with the 25% casein diet that were also fostered to other mothers on a 25% casein diet were designated as members of the 25/25 (prenatally well-nourished) group and served as control subjects. This model was designed to investigate the effects of PPM during gestation with nutritional rehabilitation beginning at birth. On postnatal day (PND) 21, all rats were weaned and provided with a standard laboratory chow diet (Purina Mills Inc., Richmond, IN, United States; Formula 5001). Subjects were then pair-housed with same-sexed littermates and given *ad libitum* access to food and water. Research personnel were blind to dietary condition until the completion of data collection.

In vivo Microdialysis

Stereotaxic Surgery

Male adult Long-Evans rats (90–120 days of age) were included in the present experiments. Only one animal from each litter was used here while littermates were assigned to other experiments [as reported, in part, by McGaughy et al. (2014)]. For surgery, rats were anesthetized with 2% isoflurane with oxygen (0.6 L/min). Lidocaine with epinephrine was injected subcutaneously at the site of the incision. Surgeries were done under aseptic conditions. During surgery, guide cannulae (CMA 12, CMA/Microdialysis AB, Acton, MA, United States) were implanted bilaterally into the vmPFC such that the tip of the guide cannula was located at the coordinates of A 3.2 mm; L ± 0.8 mm; DV 2 mm with reference to bregma according to the atlas of Paxinos and Watson (2005). After the holes for the guide cannulae were drilled, the guide cannulae were slowly lowered into place over a 3-min period. Guide cannula were affixed to the skull using three stainless steel screws with dental acrylic covering the screws and the guides.

Microdialysis Procedure

After 3 days of recovery following implantation of the guide cannulae, animals were placed in a large Plexiglas bowl with a collar attached by a guide wire to a suspension arm (CMA Microdialysis). All experiments took place between 0830 and 1200 h. On the day of the experiment, a 2 mm CMA-12 probe (CMA Microdialysis, Inc., N. Chelmsford, MA, United States) was placed into each cannula while the animal moved freely around the bowl. Artificial cerebrospinal fluid (artCSF; 147 mM NaCl, 1.26 mM $CaCl_2$, 2.5 mM KCl, and 1.18 mM MgCl in sterile water) was perfused through the probe using a CMA/Microdialysis Syringe pump and a 1.0 ml gastight Hamilton syringe at a rate of 1.0 μl/min. After allowing 3 h for equilibration of the probes, samples (20 μl) were collected every 20 min for another 3 h. Animals were only used in one microdialysis experiment.

Analysis of Monoamines

Samples were analyzed by high performance liquid chromatography with electrochemical detection (HPLC-ECD). The analytical system was an ESA Coulochem II (ESA Inc., Chelmsford, MA, United States) using a 3 μM 3 mm × 150 mm C-18 column (MD150, ESA Inc.). The mobile phase consisted of 90 mM sodium dihydrogen phosphate, 50 mM citric acid, 10% acetonitrile, 50 μM EDTA, pH of 3.0. This allowed for the measurement of 5-HT, DA, and NE with a sensitivity of 0.5 fmol/20 μl sample. The area under the curve (AUC) for samples were compared using computer software Chromperfect (Justice Laboratory Software, Palo Alto, CA, United States) with a regression analysis of AUC for three authentic standards (10^{-8}, 5×10^{-9}, 10^{-9} M) injected onto the column at the beginning of each experimental day. 5-HT, DA, and NE were verified by examining the voltammogram for standards against that determined using microdialysis samples.

Histology

At the completion of each study, rats were perfused transcardially with 10% formalin under pentobarbital anesthesia and brains were removed. Perfused brains were then placed in 30% sucrose to provide cryoprotection. Brains were frozen in Tissue-Tek O.T.C. compound, sectioned in the coronal plane on a Leica CM1900 cryostat at 40 μm. Sections were then mounted and stained with cresyl violet for verification of the probe placement.

Laterality

Previous work by Sullivan and coworkers (Sullivan and Dufresne, 2006; Sullivan et al., 2009a,b) and our lab (Staiti et al., 2011) have shown differences in levels of neurotransmitters in the left and right hemispheres dependent on age and sex of the rat. In order to determine the relationship between neurotransmitter levels in the two hemispheres we calculated a laterality index for each neurotransmitter. This index was calculated for each subject by determining the difference in the extracellular concentration of a specific neurotransmitter in left and right vmPFC and dividing by the sum of left and right concentrations, thus giving a value ranging between +1.0 and −1.0 (Staiti et al., 2011). Thus an index of zero indicates equal levels in each hemisphere, an index from 0 to +1 shows laterality to right and a level from 0 to −1 laterality on the left.

Attentional Set Shifting Task (ASST)

In the present study, we report on correlations between neurotransmitter levels in the mPFC and behavior in an attentional task. The full report of the behavioral data has been published (McGaughy et al., 2014) but we summarize it as follows. Adult rats were trained to dig in pots for food reward. Animals were tested on a series of conditional discriminations with two stimuli per trial. The number of trials needed to reach criterion in the task was recorded. For each test, subjects were required to emit six correct consecutive responses. Trials to reach this level of criterion performance was the dependent measure at each stage. On the first discrimination, the stimuli varied on

one dimension (simple discrimination). For all subsequent tests, stimuli differed in multiple dimensions; digging material, scent and texture of the pot. For the first five testing stages, subjects were reinforced for focusing on one attribute, e.g., odor, of a complex stimulus and to disregard other stimulus attributes, e.g., digging media. This same dimension was rewarded during reinforcement reversals and when a novel set of stimuli were introduced to facilitate formation of an attentional set. Once the set was formed, subjects were required to inhibit responding to this set to learn a previously irrelevant attribute of the complex stimuli now predicted reward (extra-dimensional shift). Previous work by McGaughy and co-workers has shown that NE in the PFC is important to the set-shifting portion of the task (McGaughy et al., 2008; Newman et al., 2008) so we determined how post-testing levels of NE, DA, or 5HT correlated with performance in the set-shifting portion of the task. Additionally, these neuromodulators have been shown to be critical to learning changes in reinforcement contingencies, so we also investigated the relationship of NE, DA, and 5HT to reversal learning (Clarke et al., 2005; den Ouden et al., 2013).

TABLE 2 | Correlations (r^2-values) between performance on the ASST and basal 5-HT, DA, and NE levels determined by microdialysis in a sub-set of animals that were first assessed in the ASST (total reversals and extradimensional shift) and then assessed in the microdialysis experiment.

	25/25 (*N* = 6)		6/25 (*N* = 6)	
	Total reversals	**Extra-dimensional shift**	**Total reversals**	**Extra-dimensional shift**
ID/ED trials to criterion	46.7 ± 2.7	15.8 ± 2.3	54.7 ± 4.36	25 ± 3.9
5-HT Left hemisphere	0.649	0.821*	−0.482	0.514
5-HT Right hemisphere	0.666	−0.366	−0.403	0.093
Dopamine L	−0.087	0.511	0.033	0.804
Dopamine R	0.250	−0.798	−0.439	0.794
Norepinephrine L	0.031	0.443	0.309	−0.071
Norepinephrine R	−0.846*	−0.392	−0.252	−0.129

*Values for ID/ED trials to criterion are mean ± SEM [as reported in McGaughy et al. (2014)]. Values for correlations are r-squared values; *$p < 0.05$.*

Statistical Analysis

Extracellular concentrations of neurotransmitter in dialysate were converted to femtomoles per 20 μl sample. Analysis of variance (ANOVA) with day as a repeated measure was used to compare weights between the two nutritional groups (SigmaPlot[1]). Neurotransmitter levels across groups were analyzed using a three-way (ANOVA) with nutrition group, and cortical hemisphere as independent variables and time as a within subject variable. *Post-hoc* comparisons were performed between groups using Student-Newman-Keuls tests. The level of significance was set at $p < 0.05$ for all tests. Correlations between neurotransmitter levels and behavior in the ASST were done using linear correlational analysis (SPSS see text footnote[1]). Two-tailed tests were used for Pearson correlation analysis. **Tables 1**, **2** give F-values and significance between groups and analysis of correlation.

RESULTS

Changes in Basal Levels of Monoamines

Following post-mortem histology, data from two animals in the 6/25 group and three animals in the 25/25 group were eliminated as probe placement was outside the vmPFC. All animals that were included in the present study showed microdialysis probe placement within the vmPFC.

Rats exposed prenatally to a 6% protein diet (6/25 animals) did not differ significantly from the 25% protein control rats (25/25 animals) in initial litter size or postnatal mortality. However, they weighed significantly less than controls between PND 40 and PND 90, after which the weight difference was no longer present [**Supplementary Figure S1**; McGaughy et al. (2014)].

Rats exposed to PPM differed in the basal concentrations of NE, 5-HT and DA, and these differences were lateralized. Basal levels of NE were significantly decreased in the right vmPFC of 6/25 animals but not in the left vmPFC which was reflected by a significant interaction of nutrition group x hemisphere (**Figure 1** and **Table 1**). Basal extracellular serotonin levels were increased in the left vmPFC in 6/25 rats, but unchanged in the right hemisphere relative to 25/25 animals (**Figure 2** and **Table 1**). In regard to serotonin levels, there was a significant nutrition group effect, and a significant interaction between nutrition group and hemisphere (**Table 1**), but no difference in levels between the two hemispheres. DA levels in the vmPFC were significantly decreased in the right hemisphere in 6/25 rats compared to 25/25 rats (**Figure 3** and **Table 1**). DA levels differed between the hemispheres but there was not an effect of diet or interaction between diet and hemisphere. Both NE and DA levels in the left hemisphere were similar regardless of nutrition group. Since each microdialysis sample contained the extracellular concentration of the three neurotransmitters during one 20-min epoch, we also looked at the correlations between the concentrations of DA, 5-HT, and NE in the vmPFC during each 20-min period in each animal in both the left and right hemisphere of the vmPFC. We did not find any significant correlations between the changes in the levels of these neurotransmitters over the time course of the experiment. Thus, for instance, the changes in 5-HT levels that we

[1] systatsoftware.com

TABLE 1 | *F*-values for two-way ANOVAs comparing neurotransmitter levels in 6/25 and 25/25 groups.

	Diet	**Hemisphere**	**Diet × Hemisphere**
NE	1.874	0.009	4.998*
DA	0.032	7.433*	1.906
5-HT	4.928*	3.459	7.249*

$p < 0.05$.

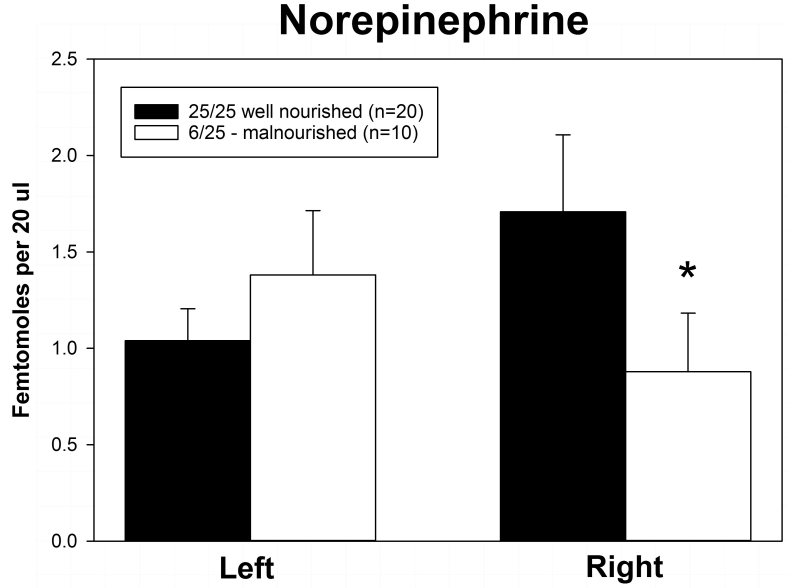

FIGURE 1 | Basal levels of norepinephrine in left and right ventral medial prefrontal cortex of adult male rats exposed to prenatal protein malnutrition (6/25 malnourished) compared to well-nourished controls (25/25 well nourished). NE levels in the right vmPFC of malnourished rats were significantly lower than in well-nourished animals (*$p < 0.05$) but did not differ significantly on the left.

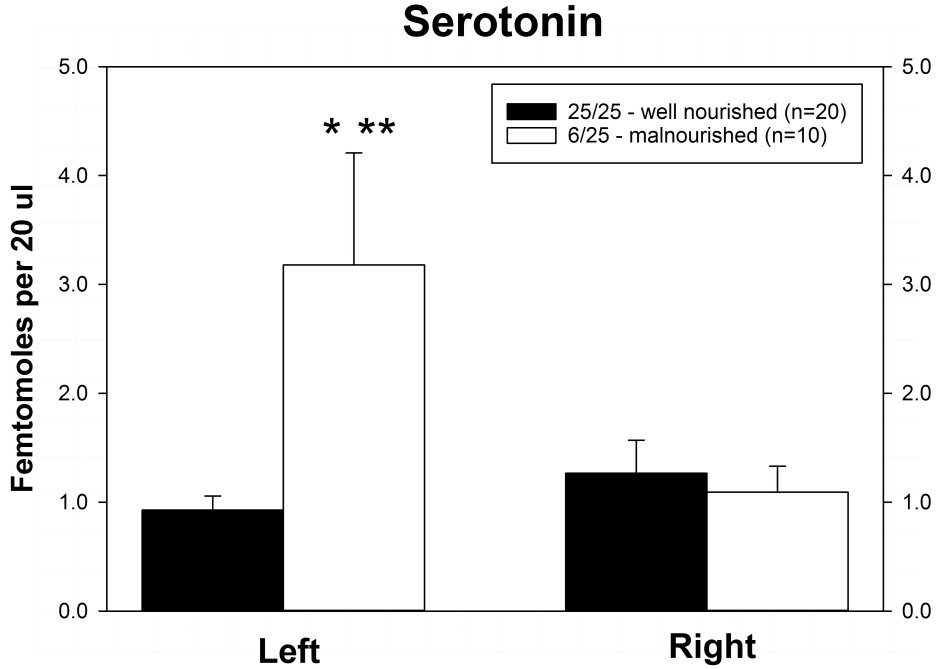

FIGURE 2 | Basal levels of serotonin (5-HT) in left and right ventral medial prefrontal cortex of adult male rats exposed to prenatal protein malnutrition (6/25 malnourished) compared to well-nourished controls (25/25 well nourished). 5-HT levels in the left hemisphere were significantly greater in 6/25 animals than in well-nourished 25/25 controls (*$p < 0.05$) and also greater than 6/25 values in the right vmPFC (**$p < 0.01$).

observed during the experiment did not correlate with changes in either NE or DA.

As shown in **Figure 4**, PPM significantly altered the laterality index for NE and DA. Although the index for serotonin was altered in a similar direction (toward the left hemisphere) as the other two neurotransmitters, this difference did not achieve significance. Interestingly, the balance of all three neurotransmitters was shifted from the right to the left vmPFC.

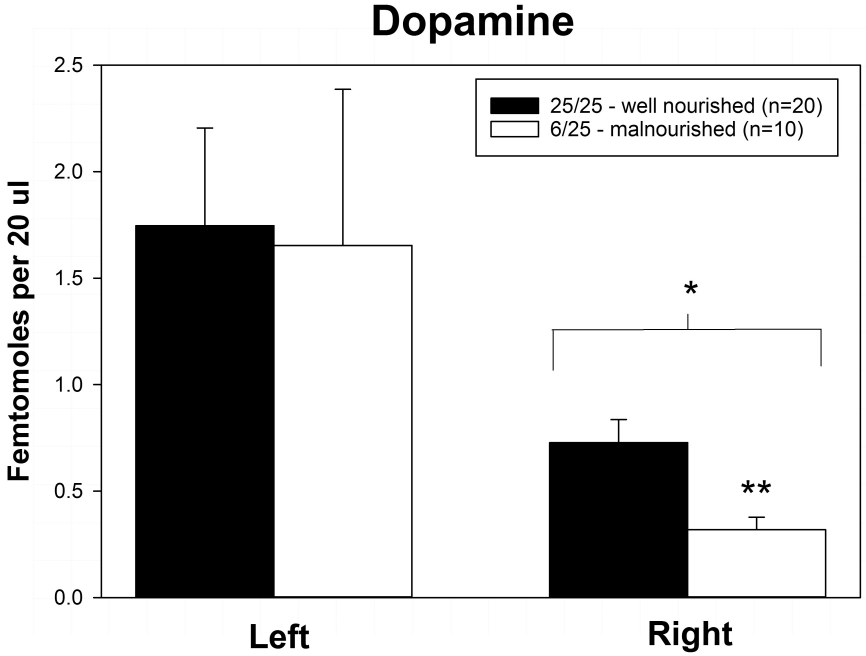

FIGURE 3 | Basal levels of dopamine in left and right ventral medial prefrontal cortex of adult male rats exposed to prenatal protein malnutrition (6/25 malnourished) compared to well-nourished controls (25/25 well nourished). DA levels in the right vmPFC of malnourished rats were significantly lower than in well-nourished animals ($p < 0.05$). Furthermore, DA levels in the right vmPFC of malnourished rats were lower than DA levels in the left vmPFC. *Significantly different from left hemisphere, **Significantly different from well nourished group.

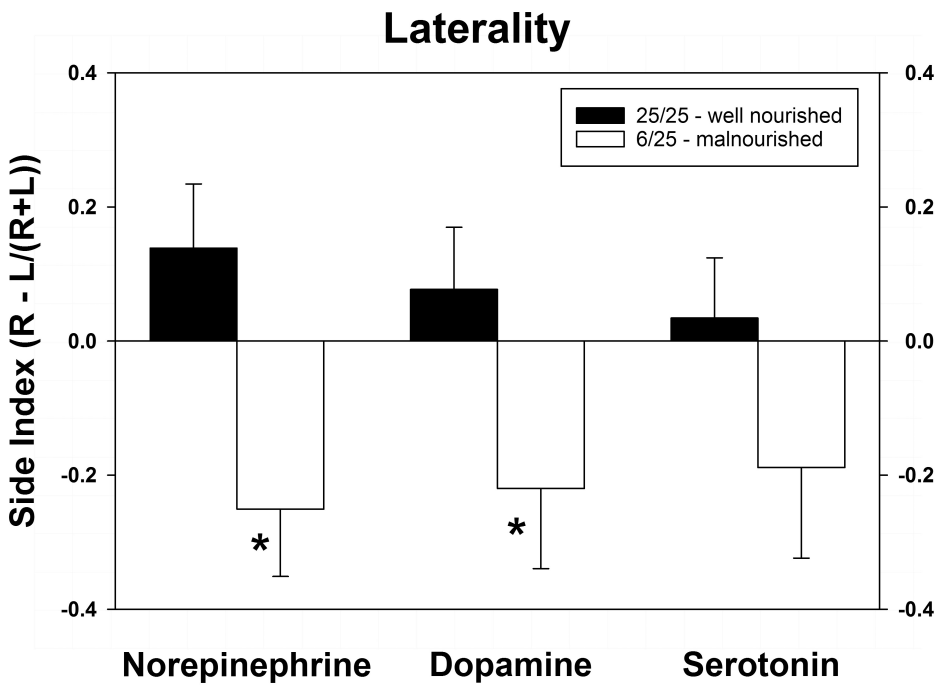

FIGURE 4 | Changes in cerebral laterality in extracellular concentrations of NE, DA, and 5-HT following exposure to prenatal protein malnutrition comparing 6/25 malnourished rats with 25/25 well-nourished adult rats. NE and DA laterality shifted from the right hemisphere to the left hemisphere in 6/25 animals,*$p < 0.05$.

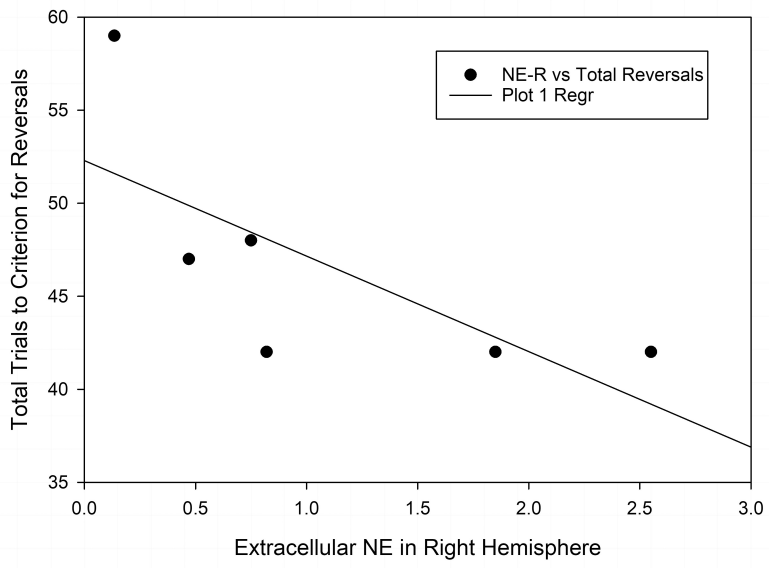

FIGURE 5 | Correlation of extracellular NE in the right vmPFC and total reversals in the ASST. The r-squared for the Pearson correlation is –0.846, $p < 0.05$.

Correlations of Basal Efflux and Prior Cognitive Testing

Finally, we also compared the changes in neurotransmitter levels with the results of the test of set-shifting using a subset of animals ($n = 6$ in each nutrition group) that were tested in an ASST [as reported by McGaughy et al. (2014)] before being assigned to the microdialysis experiment. **Table 2** shows results of correlational analyses comparing the trials to criteria for reversals and the extradimensional shift in the ASST with basal concentrations of NE, DA, and 5-HT in each hemisphere. The number of trials to criteria for reversals had a significant negative correlation with extracellular NE in the right hemisphere of well-nourished animals (r-squared = −0.846, $p < 0.05$, **Figure 5** and **Table 2**). There was also a significant correlation between 5-HT in the left vmPFC and trials to criteria for the extra-dimensional shift of well-nourished animals (r-squared = 0.821, $p < 0.05$). However, there was no significant correlations between performance on the ASST and neurotransmitter levels in the prenatally malnourished 6/25 animals. Due to the low numbers in the sample this association is only tentative and needs to be replicated but is of interest given the role of NE and the mPFC in the ASST task (McGaughy et al., 2014; Mokler et al., 2017).

DISCUSSION

Changes in Dopamine

Here we found a decrease in DA in the right vmPFC of 6/25 animals compared to well-nourished controls (25/25), while there was no difference in left vmPFC. This confirms a similar decrease in extracellular DA in the right vmPFC reported in an earlier study in this laboratory (Mokler et al., 2007). Additionally, Kehoe et al. (2001) reported a decreased DA in hippocampus of malnourished animals in a similar model of PPM, suggesting that there may be widespread decrease of dopaminergic function after PPM.

The role of prefrontal DA in mediating a response to acute stress has been shown to be lateralized. Non-specific lesions to the right infralimbic cortex (IL) suppress the stress response. In contrast, when the lesions are selective for DA in the right IL PFC there is an enhanced stress response (Sullivan and Gratton, 2002; Sullivan, 2004). A number of studies by our group have documented an altered stress response in animals exposed to PPM (Trzcinska et al., 1999; Mokler et al., 2003, 2007) which may therefore be a result of the perturbations in cortical DA. Rats exposed to PPM (Mokler et al., 2007) showed a lack of effect of a restraint stress on DA in the right vmPFC. In contrast, well-nourished controls show a 50% increase in DA efflux after acute stress. Kehoe et al. (2001) have also reported a decreased corticosterone response to stress in animals exposed to prenatal protein dietary restriction. Similar findings have been reported in human populations. For example, in their study of stunted children (growth retardation) in Nepal, Fernald et al. (2003) reported a blunted cortisol response in these children. Overall, these results point to DA in the vmPFC as a potentially important mediator of behavioral responses in the context of stress.

Changes in Norepinephrine

In the present study, we examined changes in extracellular NE in the vmPFC of prenatally malnourished animals due to the important role of NE in the PFC in attention (Mokler et al., 2017) (Newman et al., 2019). We report a decrease in extracellular NE in the right but not the left vmPFC in PPM animals, a finding that

parallels the effects of PPM on DA. In their study of animals who were chronically exposed to an 8% protein diet both prenatally and after birth, Stern et al. (1975) reported an increase in NE in gross brain dissection of telencephalon and diencephalon at some ages but not at day 60, the closest time point to the present study. However, using a model of PPM similar to ours, Soto-Moyano et al. (1999, 2005) reported increased neocortical NE and α2a – adrenoreceptors in rat pups exposed to an 8% protein diet prior to and during pregnancy. These investigators also reported a decrease in NE release as well as increased α2a – adrenoreceptors in the neocortex of adult PPM rats. The decreased extracellular NE in the right PFC reported in the current study may be important for understanding the cognitive rigidity that we have seen in prenatally malnourished animals (McGaughy et al., 2014; Peter et al., 2016; Newman et al., 2019). McGaughy and co-workers (Newman et al., 2019), however, report that in this same model of prenatal malnutrition, there was a bilateral decrease in noradrenergic axons in vmPFC. It is unclear how this finding will be reconciled with our current finding of a decrease in NE in only the right vmPFC. Future studies are needed to determine if other characteristics of noradrenergic axons are changed, e.g., density of varicosities and if this effect is lateralized. It should be pointed out that in earlier studies by our group, we have also seen an increase in extracellular hippocampal 5-HT (Mokler et al., 1999, 2003) despite a decrease in serotonergic fibers innervating the hippocampal formation suggesting more complex effects on metabolism and/or release (Blatt et al., 1994). More work needs to be done to clarify the mechanisms involved in an increase in extracellular neurotransmitter in the face of a decreased in axon density.

To assess the behavioral significance of our findings on NE, we examined the association between extracellular neurotransmitter levels and performance on the ASST, an attentional set shifting task sensitive to PFC manipulations. There was a significant correlation between performance on total reversal trials and cortical NE in the right hemisphere of the vmPFC in well-nourished but not malnourished subjects. Higher levels of NE in the right vmPFC of well-nourished animals were correlated with lower trials to criteria in the ASST task. In malnourished animals the correlation was low (r-squared = −0.252), which together with the significant reduction in basal levels of NE in the right vmPFC further indicates the importance of NE in the ASST behavioral task. Thus, even in the absence of developmental changes induced by PPM, well-nourished animals also show a relationship between cortical NE and reversal learning. In the full cohort of animals tested in the ASST task, malnourished animals had significantly higher total reversal trials than well-nourished animals (McGaughy et al., 2014) Because reversal learning relies on the orbitofrontal cortex and the mPFC, this may reflect impaired cortico-cortical connections in malnourished animals. We are planning to investigate the connectedness of prefrontal circuits after malnutrition and to directly interrogate the orbitofrontal cortex. It also should be noted that there was a low correlation between extracellular NE in the left vmPFC and total reversals, suggesting that the right vmPFC may be more involved in reversal learning. Further research in this area is also underway. It should also be pointed out that microdialysis was not performed in animals during the performance of the task, a critical next step in assessing the dynamic role of NE during attentional performance. These data and the work of Newman et al., 2019 suggest an important role of the PFC and NE in the attentional problems of malnourished animals.

Changes in Serotonin

One of the most consistent neurochemical changes which we have observed in adult male rats exposed to PPM is the alteration of the serotonergic system of the forebrain. Neurochemical analysis of whole brain (Chen et al., 1992, 1995), hippocampus (Mokler et al., 1999, 2003), and PFC (Mokler et al., 2007) of animals exposed to PPM have demonstrated elevations in 5-HT levels. In the present study we also found an increase in the extracellular levels of 5-HT in the left vmPFC in malnourished animals, while the levels of 5-HT in the right vmPFC were unaffected by PPM. Serotonin has been linked to impulsivity, specifically in cortico-striatal circuits involving the PFC (Dalley and Roiser, 2012). 5-HT2A receptor antagonists such as M100907 reduce impulsivity in rats selected for this trait (Anastasio et al., 2015). Thus, the increase in 5-HT observed in animals exposed to PPM may explain our observation of increased impulsivity in our model. Almeida et al. (1996b) reported in this model of malnutrition that malnourished animals showed heightened impulsivity or reduced anxiety on the elevated plus maze. This, in addition to the cognitive rigidity that we have reported in this model (McGaughy et al., 2014; Newman et al., 2019), adds to the phenotype of the prenatally malnourished animals.

There was also a significant positive correlation between 5-HT levels in the left vmPFC and trials to criterion for the extradimensional shift in well-nourished animals. This correlation was not significant in malnourished animals. Thus, in well-nourished animals increased 5-HT in the vmPFC is associated with increased trials to criterion in the extra-dimensional shift. It has been reported that antagonists at the 5-HT_6 receptor improve performance in the ASST (Hatcher et al., 2005). However, Lapiz-Bluhm et al. (2010) showed that global 5-HT depletion did not affect ED in the ASST. While much of the work on 5-HT role in reversal learning and performance in the ASST is focused on the orbital frontal cortex, more research is needed to determine the role of 5-HT in the vmPFC in this task.

Hemisphere Asymmetries in Monoamines and Interaction With PPM

Of particular interest are the hemispheric differences in extracellular concentrations of the neurotransmitters. In each case the differences between 6/25 (prenatally malnourished) and 25/25 (well nourished) animals were limited to one side of the cortex. In the case of DA and NE the changes seen in 6/25 animals were limited to the right hemisphere whereas with 5-HT the changes were seen in the left hemisphere. In our previous observations we also found a decrease in basal extracellular DA in the right mPFC (Mokler et al., 2007). Although our previous study used Sprague-Dawley rats rather than the

Long-Evans rats used in the present study, the degree of DA decrease was similar demonstrating the robustness of the nutrition effect across different rat strains. Furthermore, in agreement with our current findings of a lack of 5-HT changes in 6/25 animals in the right mPFC, in that earlier study which looked only at the right hemisphere, we did not see changes in 5-HT. In this same model of prenatal malnutrition, Lister et al. (2006) have also looked at hemispheric differences in the hippocampal formation. They reported that there is a smaller number of neurons in the right CA1 and CA2/CA3 subfields of the hippocampus; PPM decreases those numbers in both hemispheres.

Interestingly, deficits in right medial PFC function associated with attention have also been reported in a normative animal model. In the five choice serial reaction time test (5-CSRTT), a test of attention and impulsivity, adult rats with attention deficits had higher 5-HT in left PFC and lower DA in right PFC (Davids et al., 2003), similar to our current findings. The findings of an increase in 5-HT in the left vmPFC are in line with our findings in the current study and may be related to the findings of increased impulsivity (or decreased anxiety) in the elevated plus maze and elevated *T*-maze in animals exposed to PPM (Almeida et al., 1996b,c). Overall, more research is needed focused on the significance of hemispheric differences in the rat PFC, but the present results point to the importance of assessing such differences in PPM animals.

Finally, while little is known about lateralization of neuromodulatory systems in human brain, there have been indications of differences in the two hemispheres with regard to attentional problems. Patients with attentional deficit disorder have low extracellular DA in the right basal ganglia compared to matched controls (Davids et al., 2003). Hence in addition to the importance of comparing both hemispheres future studies should be aimed at determining how the neurochemical lateralization reported here in rats pertains to behavior, as well as, hemispheric asymmetries in human brain function.

SUMMARY AND CONCLUSION

In summary, we report significant changes in the balance of the monoaminergic neurotransmitters (serotonin, DA and NE) in the vmPFC of adult rats exposed to PPM. The changes in right vmPFC DA and NE may, in part, play a role in the cognitive inflexibility reported in this model of PPM (McGaughy et al., 2014) (Newman et al., 2019). Furthermore, the increased extracellular 5-HT in the left vmPFC may also be associated with the increased impulsivity (or less anxiety) in this animal model. The association of performance in the attentional set shifting task and levels of NE in the right vmPFC and 5-HT in the left vmPFC of well-nourished animals suggest the need for further studies on the hemispheric roles of these neurotransmitters in learning. While the functional significance and underlying alterations in brain circuits suggested by the hemispheric difference reported here is puzzling, these observations make it imperative that future studies compare neurobiological markers in both the right and left hemispheres using methods like dual probe microdialysis during behavioral assessment so that firm correlations can be evaluated. Further work is needed to assess the anatomical changes which lead to the differences in extracellular neurotransmitter seen in this study.

The present data, as well as previous studies of the effects of PPM in altering the developing and adult brain of the rat (Stern et al., 1975; Chen et al., 1992, 1995; Blatt et al., 1994; Mokler et al., 1999, 2003, 2007; Soto-Moyano et al., 1999, 2005; McGaughy et al., 2014; Newman et al., 2019), suggest a reprogramming of the brain. This reprogramming may be adaptive in allowing the animal to focus more on the need to find nutrition in a nutritionally sparse environment, which is reflected in the reported cognitive inflexibility. More work is needed to determine if, in fact, these changes in brain function may benefit the individual when food scarcity occurs. However, these behavioral changes may not be adaptive after nutrition privation is reversed.

DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

All authors contributed to the design of the experiments and breeding, and were involved in manuscript writing and review. DM, DB, and PM were responsible for the surgeries and microdialysis experiments. DM, DB, and JM were responsible for the behavioral experiments. DM, JM, PM, and JRG were responsible for data analysis.

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SUPPLEMENTARY MATERIAL

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FIGURE S1 | The average weight of all pups after weaning, by diet group, excluding litters with less than four pups and animals on food restriction for behavioral testing. *$p < 0.05$ different compared to 6/25 (malnourished) animals, ANOVA.

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frontiers in Neuroscience

ORIGINAL RESEARCH
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Psychophysical Evaluation of Visual Functions of Ex-Alcoholic Subjects After Prolonged Abstinence

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Chronic alcohol abuse can lead to a brain damages, and the health status of alcoholics even after a long-term alcohol abstinence is a public health concern. The present study investigated the color vision and spatial luminance contrast sensitivity of a group of 17 ex-alcoholics (46.3 ± 6.7 years old) in long-term alcohol abstinence after having been previously under alcohol dependence for many years. We also investigated the association of impaired psychophysical performance in different tests we applied. The mean time of alcohol consumption was 16.9 ± 5.1 years and the mean abstinence period was 12.4 ± 8.5 years. Achromatic vision of all subjects was evaluated using spatial luminance contrast sensitivity function (CSF) test and color vision was evaluated using Mollon–Reffin color discrimination test (MR) and the Farnsworth–Munsell 100 hue arrangement test (FM100). Relative to controls, the spatial luminance contrast sensitivity was lower in 10/17 of the ex-alcoholic subjects. In the color vision tests, 11/16 ex-alcoholic subjects had impaired results compared to controls in the FM100 test and 13/14 subjects had color vision deficits measured in the MR test. Fourteen subjects performed all visual tests, three subjects had impaired results for all tests, seven subjects had impaired results in two tests, three subjects had visual deficit in one test, and one had normal results for all tests. The results showed the existence of functional deficits in achromatic and chromatic vision of subjects with history of chronic alcoholism after long abstinence. Most subjects had altered result in more than one test, especially in the color vision tests. The present investigation suggests that the damage in visual functions produced by abusive alcohol consumption is not reversed after long term alcohol abstinence.

Keywords: alcoholism, contrast sensitivity, color vision, abstinence period, psychophysics

INTRODUCTION

The abusive alcohol consumption results in death of 2.5 million people per year around the world. Approximately 60 different types of pathologies have significant association with alcohol consumption (World Health Organization [WHO], 2000). In the central nervous system alterations involving the thalamus, hypothalamus, cerebellum, frontal lobe, corpus callosum that develop with tissue atrophy and altered regulation of the neurotransmitter functions have been reported (Estruch et al., 1997; Moselhy et al., 2001; Zahr et al., 2011; Zhao et al., 2011). In the visual system, many diseases can be associated with alcoholic behavior, such as cataract, age-related macular degeneration, diabetic retinopathy and glaucoma (Chong et al., 2008; Kanthan et al., 2010; Lee et al., 2010).

Alcohol causes transient and permanent effects on the visual system. The effects seem to be time dependent. Acute alcohol exposure causes transitory effects in the visual system that show partial or total functional recovery in the scale of minutes to hours (Zulauf et al., 1988). Chronic alcohol consumption leads to effects in the visual system with longer duration or even permanent damage (Verriest et al., 1980; Kapitany et al., 1993).

Luminance contrast sensitivity and color vision have been extensively investigated in subjects after acute and chronic alcohol consumption. Previous studies show that alcohol intake induces significant impairment in the alcohol intake impaired the spatial luminance contrast sensitivity. In addition, some reports described luminance contrast sensitivity impairment at a wide range of spatial frequencies (Roquelaure et al., 1995), while other investigations found visual losses at intermediate to high spatial frequencies (Andre et al., 1994; Nicholson et al., 1995; Cavalcanti and Santos, 2008).

The first descriptions of color vision losses associated with alcoholism were made in patients with liver cirrhosis (Cruz-Coke, 1964; Cruz-Coke and Varela, 1965; Fialkow et al., 1966). Selective color vision losses affecting the blue-yellow color contrast mechanisms were reported by several authors (Cruz-Coke and Varela, 1965; Thuline, 1967; Varela et al., 1969; Sassoon et al., 1970; Cruz-Coke, 1972; Adams, 1978; Russell et al., 1980; Verriest et al., 1980; Zrenner et al., 1986), while others have reported a predominant loss of red-green color vision (Sarraux et al., 1966; Sakuma, 1973; Kapitany et al., 1993) or a diffuse color vision loss (Smith and Layden, 1971; Rothstein et al., 1973; Mergler et al., 1988; Castro et al., 2009; Brasil et al., 2015).

Visual tests are designed to evaluate specific visual properties such as hue ordering (for example, Farnsworth Munsell 100 hue test), chromatic discrimination (for example, Cambridge Colour Test), and luminance contrast sensitivity. As the alcohol consumption seems to affect differently the putative contribution of these visual pathways to visual perception (Zrenner et al., 1986; Zhuang et al., 2012). However, it is not clear if the visual losses occurring in a test will also be present in other tests. There is no description regard association of color vision and luminance vision impairments caused by alcohol intake. We investigated color vision and spatial luminance contrast sensitivity of a group of subjects in long-term alcohol abstinence after having suffered from alcohol dependence for many years in the past. We compared visual performance in the different tests to determine if losses are revealed by all tests to find out if they are equally sensitive to the affected mechanisms. We also evaluated the association between visual tests results and the history of alcoholism, smoking and abstinence.

MATERIALS AND METHODS

Subjects

We evaluated 17 subjects, 13 male e 4 female (between 31 and 60 years, 46.3 ± 6.7-years-old), all volunteers and members of the Alcoholics Anonymous, with history of chronic alcoholism. The CAGE test was used to identify alcohol dependence (Ewing, 1984). The study was approved by the Research Ethics Committee (report #28/2003) of the Núcleo de Medicina Tropical, Universidade Federal do Pará, Brazil. All subjects gave written and informed consent for participation in the study.

Exclusion criteria of ex-alcoholic subjects were presence of eye diseases, neurological and systemic pathologies, exposure to neurotoxic chemical substances such as mercury and organic solvents and use of medical drugs which affect the visual system such as chloroquine, hidroxicloroquina, ethambutol, vigabatrin. An inquiry about frequency of alcohol consumption, the symptoms shown in period of alcohol use and in the abstinence period, time of chronic alcoholism, of alcohol abstinence, of smoking and of smoking abstinence was performed. All subjects were evaluated by an ophthalmologist who examined biomicroscopy, fundoscopy, refractometry, and ocular motility. All subjects had normal visual acuity or corrected to 20/20. The subjects were tested monocularly in a dark room.

Procedures and Equipments

Three visual psychophysical tests were used to evaluate the visual system of the ex-alcoholic subjects. To evaluate luminance vision, we estimated the spatial luminance contrast sensitivity (n = 17), and to evaluate color vision, we estimated the color discrimination ellipses using the Mollon–Reffin (MR) test (n = 15) and we also quantified the total error in the Farnsworth–Munsell 100 hue arrangement (FM100) test (n = 16).

Spatial Luminance Contrast Sensitivity

For spatial luminance contrast sensitivity test, we used an IBM model Pentium IV 1.7 GHz, with ANNIHILATOR 2 by CREATIVE and color palette of 24 bits/8 bits per gun to program the test in C++ language. The stimulus was displayed in a color monitor ("21," SONY Multiscan G420 model, spatial resolution of 1024 × 768 pixels, temporal resolution of 75 Hz, Japan). The monitor calibration was performed with a CS-100A chromameter (Konica Minolta, Mahwah, NJ, United States).

The stimulus was composed by achromatic sine-wave stationary gratings, 6.5° x 5° of visual angle, at eleven spatial frequencies (0.2, 0.5, 0.8, 1, 2, 4, 6, 10, 15, 20 and 30 cpd). The mean chromaticity of the stimulus in the CIE 1976 color space was u' = 0.182, v' = 0.474, and the mean luminance of the screen was 43.5 cd/m^2. The test was performed using the method of adjustment to control the Michelson contrast of the

stimulus. Initially, the stimulus was shown in low contrast to the tested subject. The experimenter gradually increased the stimulus contrast until the subject detected the stimulus. The stimulus contrast was then decreased until the subject just ceased to detect the stimulus, to again increase the stimulus contrast. The adjustment to the just perceptible contrast (contrast threshold) was done 6 times. The contrast threshold was the average of 6 measurements.

Mollon–Reffin Test

Mollon–Reffin test (MR) was written using C++ programming language. The software was developed for an IBM POWERStation RISC 6000 (IBM Corporation, New York, NY, United States). The stimuli were generated using *IBM POWER GT4-24bits-3D*. The stimuli were displayed on *IBM 6091 19i*, with spatial resolution of 1280 × 1024 pixels. The calibration was performed with a CS-100A chromameter (Konica Minolta, Mahwah, NJ, United States).

We estimated color discrimination ellipses around 5 central coordinates in the CIE1976 color space as following: Ellipse 1, $u' = 0.215$; $v' = 0.531$; Ellipse 2, $u' = 0.219$; $v' = 0.481$; Ellipse 3, $u' = 0.225$; $v' = 0.415$; Ellipse 4, $u' = 0.175$; $v' = 0.485$; and Ellipse 5, $u' = 0.278$; $v' = 0.472$. The stimuli were composed by mosaic of circles that had different sizes (0.2° and 0.6° of diameter) and luminance (from 12 to 20 cd/m^2). A target differed of the surrounded field by the chromaticity content. The chromaticity of the field was the central coordinate, while the chromaticity of the target was modulated in one out of eight chromatic axes that radiated from the central coordinate.

The target was C-shaped with outer diameter of 4.4°, inner diameter of 2.2° and gap of 1° visual angle. The subject's task was to identify the C gap orientation among four alternatives (up, left, down, right) during 1.5 s. A staircase was used to control the distance between chromaticity of the field and of the target. One hit decreased the length of the vector that linked both chromaticities, and the chromaticity of the target came closer to the chromaticity of the field. One wrong response increased the vector length between the field and target chromaticities, and the chromaticity of the target became more far from the field chromaticity. One hit followed by a mistake or one mistake followed by a hit was considered as one reversal of the staircase. The test ended when 12 reversals were completed. The color discrimination threshold was the mean value of the last 6 reversals of the staircase. The color discrimination thresholds were fitted by an ellipse function using the least square method. The diameter of the circle with equivalent area of the ellipse was the indicator of the color discrimination of each subject.

Farnsworth–Munsell 100 Hue Arrangement Test

For the Farnsworth–Munsell 100 hue arrangement test (FM100), the equipment used was the same used for the spatial luminance contrast sensitivity spatial test. The stimulus was composed by 85 circles varying in hue with same color saturation. The presentation of the stimulus was separated in 3 moments with 21 hues and 1 moment with 22 hues. Each circle had a size of 1° of visual angle and luminance of 42 cd/m^2.

At the beginning of each test all caps were shown ordered in hue during 1 min. After this period, the circles were randomly mixed up. The tested subject had begun by choosing the circle whose hue that was closest to that of a reference circle. The procedure was repeated for the succeeding choices by always choosing the circle with the hue most like the last one chosen. After the complete ordering of the circles, the number of errors in hue sequence was quantified by the software (Farnsworth, 1957). The test was done 4 times for each stimulus presentation. The result was the averaged total error of the 4 trials.

Statistics

The data from ex-alcoholic subjects were compared to data (tolerance intervals) from age matched control subjects for each visual test as follows: spatial luminance contrast sensitivity test (control group; $n = 44$, 43.8 ± 9.4 years), FM100 test (control group; $n = 52$; 42.9 ± 8.8 years), MR test ($n = 33$; 46.2 ± 6.6 years). We quantified the number of ex-alcoholic subjects whose thresholds fell outside the control group tolerance interval for each visual test. The comparison of ex-alcoholic group and control group was done using Kruskal–Wallis test for spatial luminance contrast sensitivity test and using an independent samples *t*-test to compare both group results for FM100 test and for MR test. We use a multivariate Fisher linear discriminant analysis to test which group of tests (color vision or contrast sensitivity) better separate the data from controls and alcoholics. For all statistics was used the Biostat 5.0 software and considered α of 5%.

RESULTS

History of Chronic Alcoholism, Smoking and Abstinence

Table 1 shows the age, alcoholism period and smoking profile of the subjects used in the current study. The mean age was 46 years old ± 7, the mean number of years of alcoholism was 17 ± 5 years and of abstinence was 12 ± 9 years. The mean time of smoking was 17 ± 7.2 years and smoking abstinence was 12 ± 9 years. Three subjects never smoked, four are still smokers, one did not declare and 9 quit smoking.

Spatial Luminance Contrast Sensitivity Function

Figure 1A shows the spatial luminance contrast sensitivity function estimated from the ex-chronic alcohol consumers ($n = 17$) compared to the tolerance interval of the control group (dotted lines). **Figure 1B** compares the mean contrast sensitivity function estimated from the control group and from the ex-alcoholics. We observed that the contrast sensitivity of the ex-alcoholics was significantly lower than the control group at 0.5 and 0.8 cpd ($H = 528.7$, $p < 0.05$).

TABLE 1 | History of chronic alcoholism, smoking and abstinence.

Code	Age range (years)	Duration of alcoholism (years)	Time of alcohol abstinence (years)	Duration of smoking (years)	Time of smoking abstinence (years)	CSF	MR test	FM-100
ACS101112	46–50	22	9	24	0	Yes	Yes	Yes
AVO100403	41–45	14	13	15	0	Yes	Yes	Yes
EJM101220	36–40	20	4	20	4	Yes	Yes	Yes
EWS100303	51–55	20	6	**	**	Yes	Yes	Yes
FMC090703	56–60	10	20	20	18	Yes	Yes	Yes
FMR090707	51–55	8	23	16	25	Yes	Yes	Yes
FPS100305	36–40	12	9	3	22	Yes	Yes	Yes
HNS101019	41–45	21	6	5	6	Yes	Yes	Yes
#ILR110211	36–40	20	3	23	0	Yes	No	Yes
LFS101201	51–55	16	20	23	17	Yes	Yes	Yes
MES100219	46–50	26	13	26	5	Yes	Yes	Yes
MSS100223	46–50	13	36	16	0	Yes	Yes	Yes
OAC101013	46–50	15	15	**	**	Yes	Yes	Yes
#RSC101103	51–55	18	10	10	5	Yes	No	Yes
SSR101110	41–45	21	6	20	2	Yes	Yes	Yes
VMN101223	46–50	22	4	*	*	Yes	Yes	Yes
□VNR090806	36–40	10	15	**	**	No	Yes	No
Mean	46	17	12	17.0	12			
SD	7	5	9	7.2	9			

*SD, standard deviation. *, Not declared. **, Never used.*

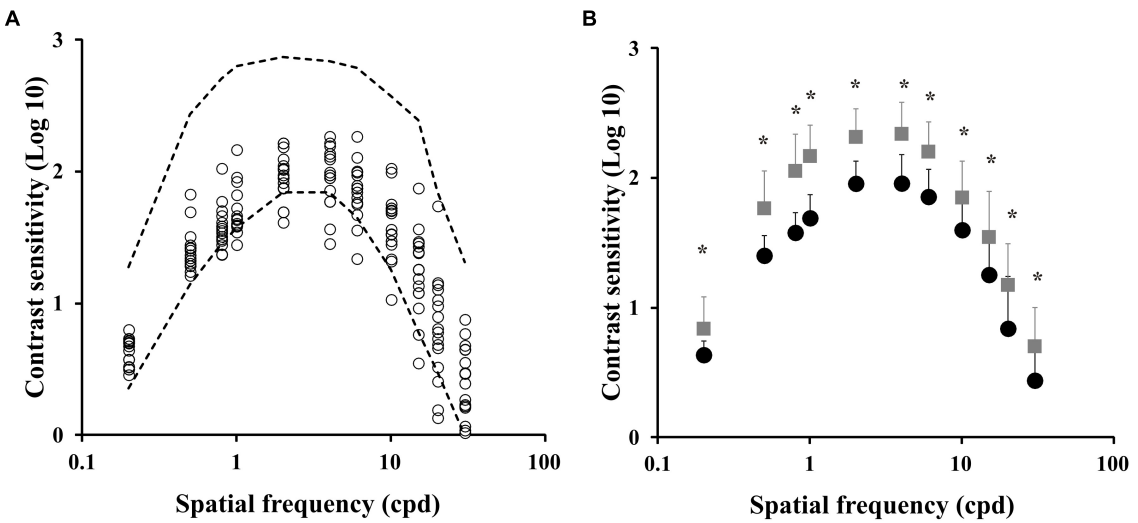

FIGURE 1 | Spatial luminance contrast sensitivity functions of ex-alcoholic subjects. **(A)** Results of the ex-alcoholic subjects (circles) compared to the tolerance interval of the control group (interval between dotted lines). **(B)** Comparison of the mean contrast sensitivity function obtained from control group (gray squares) and ex-alcoholic subjects (black circles). Error bars represent the standard deviation of the mean. *$p < 0.05$.

Farnsworth-Munsell 100 Hue Arrangement Test

Sixteen ex-alcoholics performed the Farnsworth-Munsell 100 hue arrangement test. **Figures 2A,B** shows the individual map of errors from a control subject (**Figure 2A**) and an ex-alcohol consumer (**Figure 2B**). **Figure 2C** shows the scattering of the error values of the ex-alcoholics compared to the normative range of the control group (dotted lines). Eleven out of sixteen ex-alcoholics had higher number of errors than the upper tolerance limit of the control group. The mean error of the alcohol consumer group was higher than the mean error of the control group [$t(66) = 8.85$, $p < 0.05$], as shown in the **Figure 2D**.

Mollon–Reffin Color Discrimination Test

Fourteen ex-alcoholics performed the Mollon–Reffin color discrimination test. **Figures 3A,B** shows the color discrimination ellipses in the CIE1976 color space estimated from a control

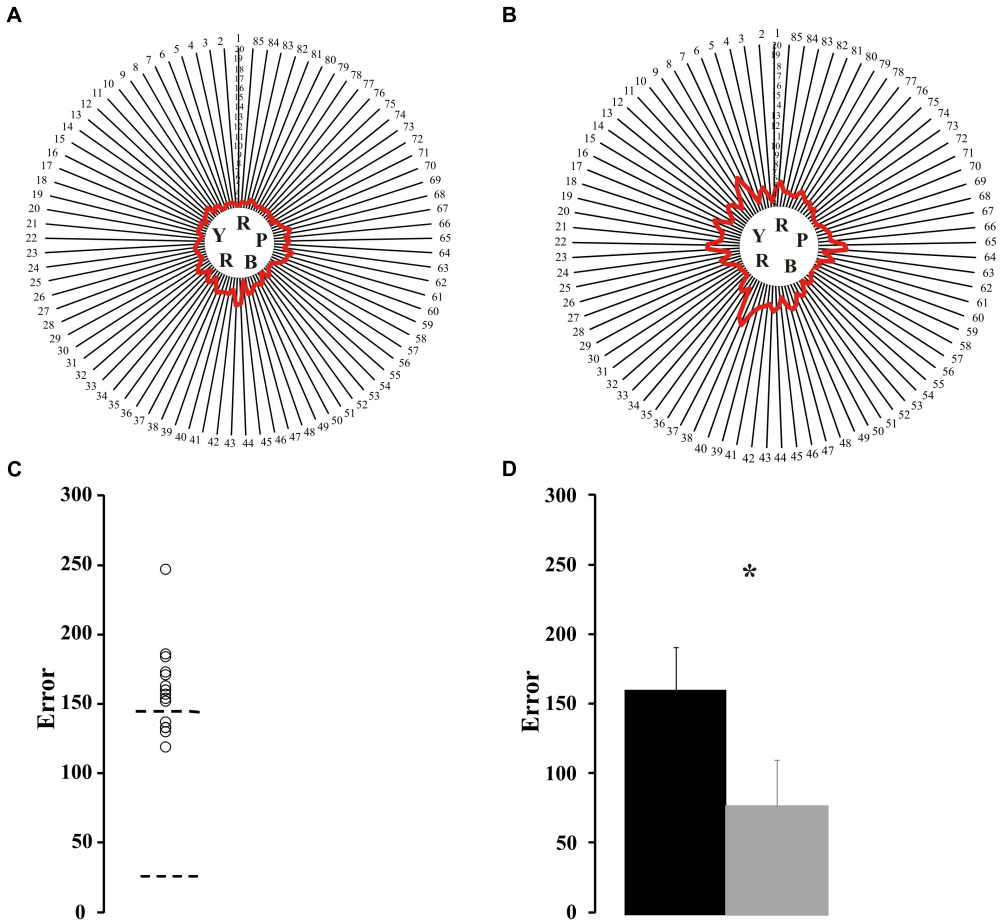

FIGURE 2 | Results of Farnsworth-Munsell 100 hue arrangement test. **(A,B)** Maps of results for the FM100 test from a control subject and an ex-alcoholic. Red lines represent the averaged error for the four trials of each subject. **(C)** Scatter plot for FM100 score estimated from ex-alcoholic subjects (circles). Dotted lines are tolerance interval for the control group. **(D)** Comparison of mean values for the FM100 scores estimated from the control group (gray bar) and ex-alcoholic group (black bar). Error bars represent the standard deviation of the mean. $^*p < 0.05$.

subject (**Figure 3A**) and an ex-alcoholic subject (**Figure 3B**). **Figure 3C** presents the scatter plot of the ellipse area estimated from the ex-alcoholics for each reference chromaticity of the stimulus background. The data from ex-alcoholics are compared to the normative range of the control group for the same stimulus condition (dotted lines). For all central coordinates, the mean ellipse area of the ex-alcoholic group was significantly larger than the controls [**Figure 3D**, C1: $t(45) = 5.07$, $p < 0.05$; C2: $t(45) = 5.79$, $p < 0.05$; C3: $t(45) = 3.89$, $p < 0.05$; C4: $t(45) = 4.49$, $p < 0.05$; C5: $t(45) = 4.05$; $p < 0.05$].

Association Between the Results of Different Psychophysical Tests

Table 1 shows a binary individual result from the ex-alcoholics we evaluated in the present study.

Association Between Contrast Sensitivity Function and Mollon–Reffin Color Discrimination Test

We found that two ex-alcoholics had normal color vision evaluated by MR test and 13 ex-alcoholic subjects had impaired color vision evaluated using MR test. We did not compare the contrast sensitivity between them due the low number of subjects in the group with normal color vision.

We grouped the results from MR test from ex-alcoholic subjects with normal ($n = 9$) or impaired ($n = 5$) contrast sensitivity at any spatial frequency. It resulted in no significant difference between the two groups at all reference coordinates ($p > 0.05$). However, when both groups were compared to the control group, the ex-alcoholic subjects with normal contrast sensitivity had larger ellipses than the controls for all reference coordinates ($p < 0.05$), while the ex-alcoholic subjects with impaired contrast sensitivity had larger ellipses for the reference coordinates C1, C2, C3, and C5 compared to the control group ($p < 0.05$).

Contrast Sensitivity Function and Farnsworth–Munsell 100 Hue Arrangement Test

We compared the contrast sensitivity function from a group of ex-alcoholics with normal ($n = 5$) or impaired ($n = 11$) color vision evaluated by Farnsworth-Munsell 100 hue arrangement

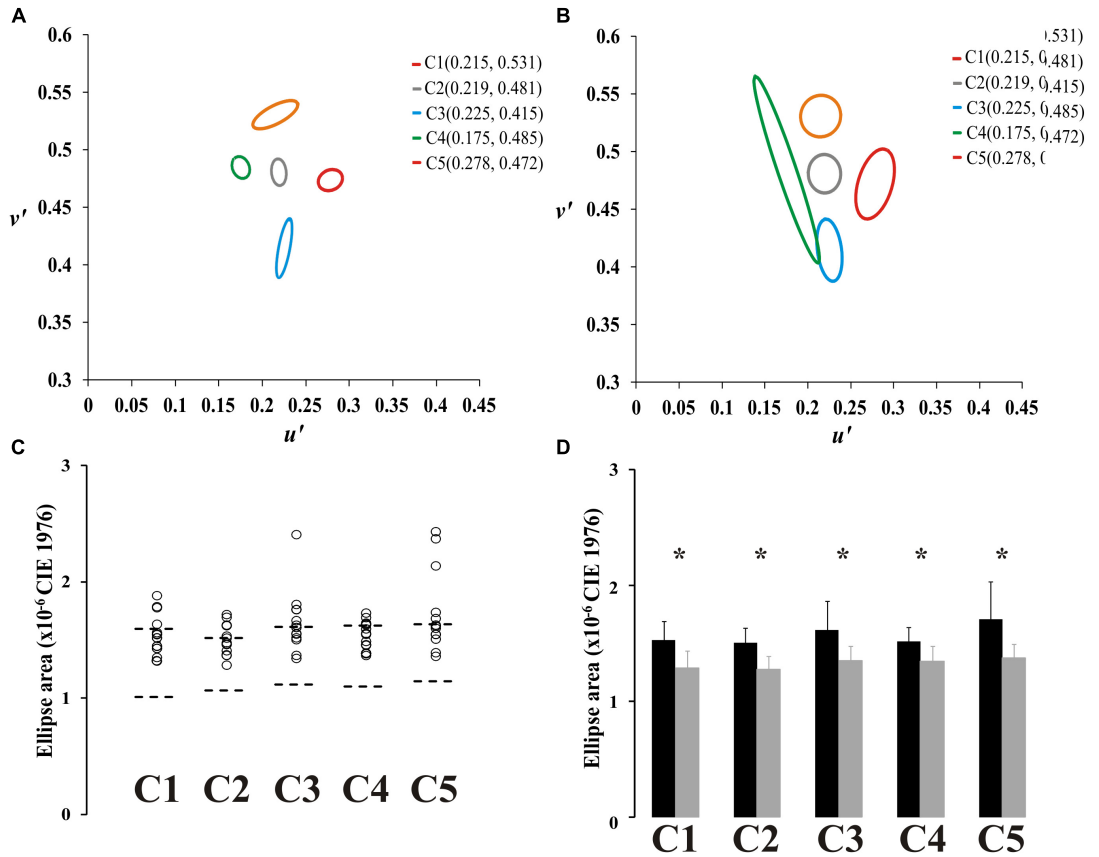

FIGURE 3 | Results of Mollon-Reffin color discrimination test. **(A,B)** Color discrimination ellipses in the CIE1976 color space estimated from a control subject and an ex-alcoholic subject. **(C)** Scatter plots of the diameter of the circle with same area of the ellipse for five reference coordinates in the color space estimated from ex-alcoholic subjects. Dotted lines represent the tolerance interval of the control group. **(D)** Comparison of the mean diameter of the circle with same area of the ellipse for control group (gray bar) and ex-alcoholic group (black bar). Error bars represent the standard deviation. *$p < 0.05$.

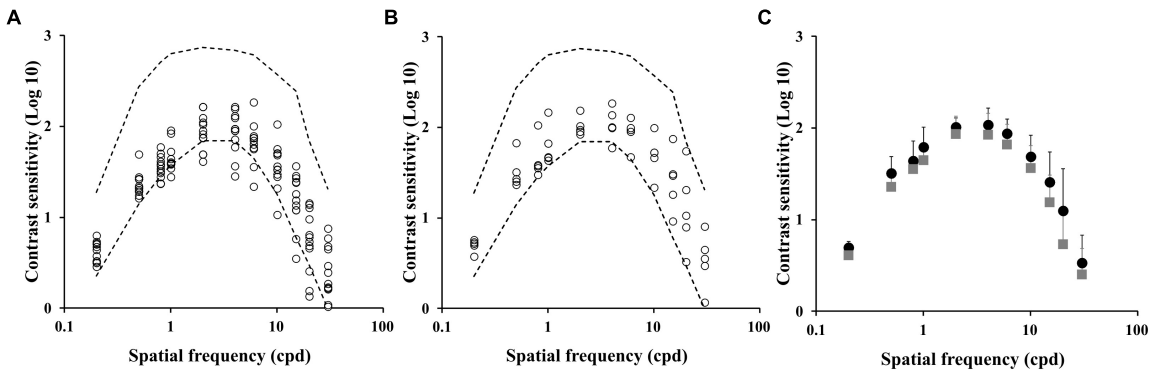

FIGURE 4 | Spatial luminance contrast sensitivity functions from subjects with impaired and normal FM100 test results. **(A,B)** Scatter plots of the contrast sensitivity estimated from ex-alcoholic subjects with impaired and normal FM100 test results, respectively. Dotted lines represent the tolerance interval of the control group. **(C)** Comparison of the mean contrast sensitivity function of the subjects with impaired FM100 test result (gray squares) and normal FM100 test result (black circles). Error bars represent the standard deviation of the mean.

test (FM100). **Figures 4A,B** shows the contrast sensitivity values estimated from the impaired color vision group and normal color vision group, respectively. There was no difference between the contrast sensitivity estimated from both groups of alcoholic subjects (**Figure 4C**, $p > 0.05$). However, the comparison between the control group and ex-alcoholics with normal color vision estimated using FM100 showed contrast sensitivity impairment of the ex-alcoholics from 0.2 to 4 cpd, and 20 cpd ($p < 0.05$),

while the same comparison between the controls and ex-alcoholics with impaired color vision evaluated by FM100 showed impaired contrast sensitivity at the ranges 0.2–4 cpd, and 15–30 cpd ($p < 0.05$).

We also grouped the FM100 results from ex-alcoholic subjects with normal ($n = 10$) or impaired ($n = 6$) contrast sensitivity at any spatial frequency. No difference was found between both ex-alcoholic groups (**Figures 5A–C**, $p > 0.05$), but both groups had higher FM100 errors compared to the control group ($p < 0.05$).

Mollon–Reffin Color Discrimination Test and Farnsworth–Munsell 100 Hue Arrangement Test

We compared the color discrimination ellipse estimated from a group of ex-alcoholics with normal ($n = 5$) or impaired ($n = 9$) color vision evaluated by Farnsworth-Munsell 100 hue arrangement test. **Figures 6A,B** shows ellipse areas estimated from the impaired color vision group and normal color vision group evaluated by FM100 test, respectively. No difference was found between both alcoholic groups (**Figure 6C**, $p > 0.05$). The alcoholic group with normal FM100 results had larger ellipses than the controls for the central chromaticities C2 and C5, while the ex-alcoholic group with impaired FM100 results had larger ellipses than the controls for all central chromaticities ($p < 0.05$).

The mean data from FM100 test estimated from two ex-alcoholic subjects who had normal color vision evaluated by MR test and from 14 ex-alcoholic subjects with impaired results in the MR test. Here, we also did not perform statistical comparisons between the groups due the number of subjects in the group of normal results for MR test.

Multivariate Fisher Linear Discriminant Analysis

Table 2 shows in which visual test the participants had normal or altered results. As we found that contrast sensitivity and

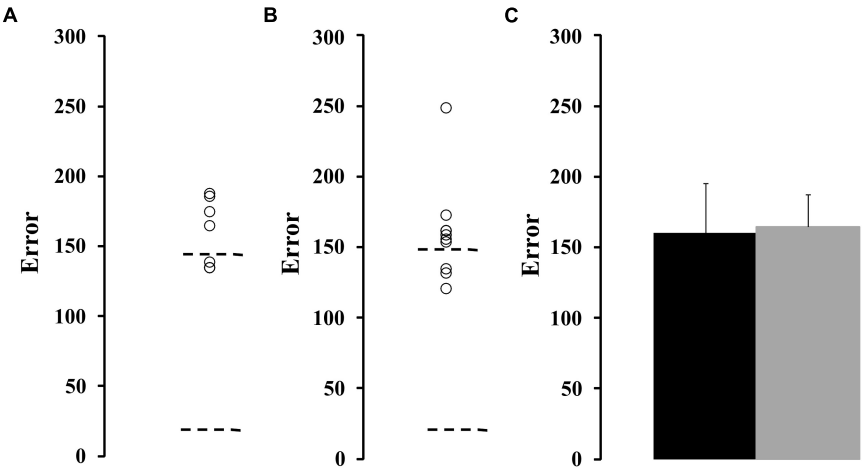

FIGURE 5 | FM100 test results from subjects with normal and impaired contrast sensitivity spatial of luminance. **(A)** Results for subjects with impaired spatial luminance contrast sensitivity in, at least, one spatial frequency. **(B)** Results for subjects with normal contrast sensitivity. Dotted lines represent the tolerance interval of the control group. **(C)** Comparison of mean error value in subjects with impaired contrast sensitivity (gray squares) and normal contrast sensitivity (black circles). Error bars represent the standard deviation of the mean.

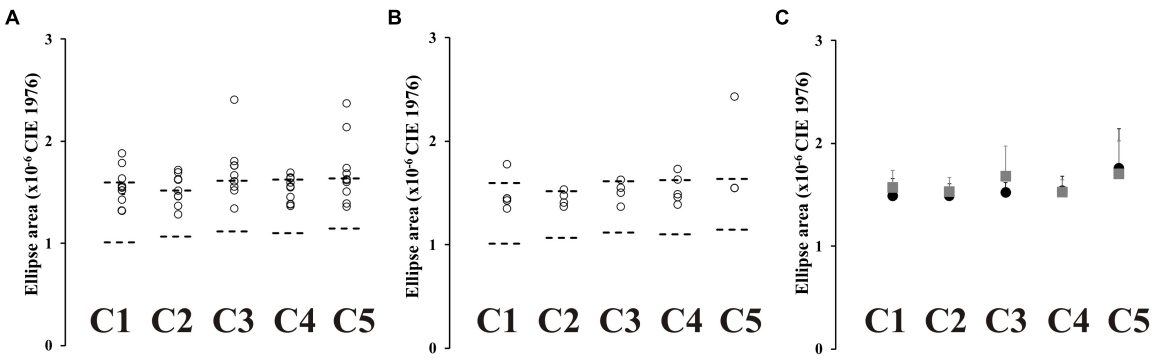

FIGURE 6 | MR test results from subjects with normal and impaired FM100 test. **(A)** Scatter plots for the diameter of the circle with same area of the ellipse estimated from subjects with impaired FM100 results. **(B)** Scatter plots for the diameter of the circle with same area of the ellipse estimated from subjects with normal FM100 results. **(C)** Comparison of mean value of diameter of circle with same area of the ellipse in subjects with impaired FM100 test (gray squares) and normal FM100 test (black circles). Error bars represent the standard deviation of the mean.

TABLE 2 | Individual results of the ex-alcoholics for each test.

Participant	CSF	MR	FM100
MES100219D			
EJM101220 D			
OAC101013D			
SSR101110D			
AVO100403 D			
HNS101019 E			
ACS101112 D			
FMC09073 D			
FMR090707 D			
FPS100305 D			
VMN101223 D			
EWS100303 D			
LFS101201D			
MSS100223D			

Red cells indicate altered results; green cells indicate normal results. CSF, luminance contrast sensitivity function; MR, Mollon–Reffin test; FM 100, Farnsworth–Munsell test.

color vision evaluations were able to detect visual disturbances in alcoholics, we proceeded a multivariate linear discriminant analysis to identify which visual test could be better to separate the data from controls and alcoholics. **Figure 7** shows the two-dimensional spaces of discriminants functions extracted from the analysis that used color vision (5 Mollon-Reffin test and FM100 score, **Figure 7A**) and contrast sensitivity results (11 spatial frequencies, **Figure 7B**), respectively. We observed that both results could separate the database from alcoholics and controls, and that the between-centroids distance from the analysis using color vision database was higher than that measured using the contrast sensitivity database (0.977 vs. 0.424, respectively).

DISCUSSION

Abusive alcohol consumption can cause physical, mental and social damage in humans (World Health Organization [WHO], 2000). In the current study, we have shown visual impairment in chronic alcoholic subjects during their abstinence period. These alterations were diagnosed even when they had no ophthalmologic clinic alterations. We also observed that most subjects present combined impairment of the color vision tests. The subjects presenting contrast sensitivity impairment also showed color vision impairment (in MR test). The subject who had only one impaired result had impairment in color vision test (EJM101220 D). A multivariate analysis showed that the color vision evaluation was better to separate the results from controls and alcoholics.

Color vision has been used to identify earlier effects of different chemicals on the visual system (Ventura et al., 2004; da Costa et al., 2008; Barboni et al., 2009; Feitosa-Santana et al., 2010; Lacerda et al., 2012; Brasil et al., 2015). Visual vulnerability to chemical exposure can be explained by the fact that while the fovea is the main region of the human retina to process color information (Roorda and Williams, 1999; Brainard et al., 2000), this area has limited vascularization in order to provide good optical media to the light reaching on the retina. In conditions of cellular or metabolic aggression, the foveal region becomes more susceptible to oxidative stress than the retinal periphery (Izzotti et al., 2006; Izuta et al., 2010). In addition, the number

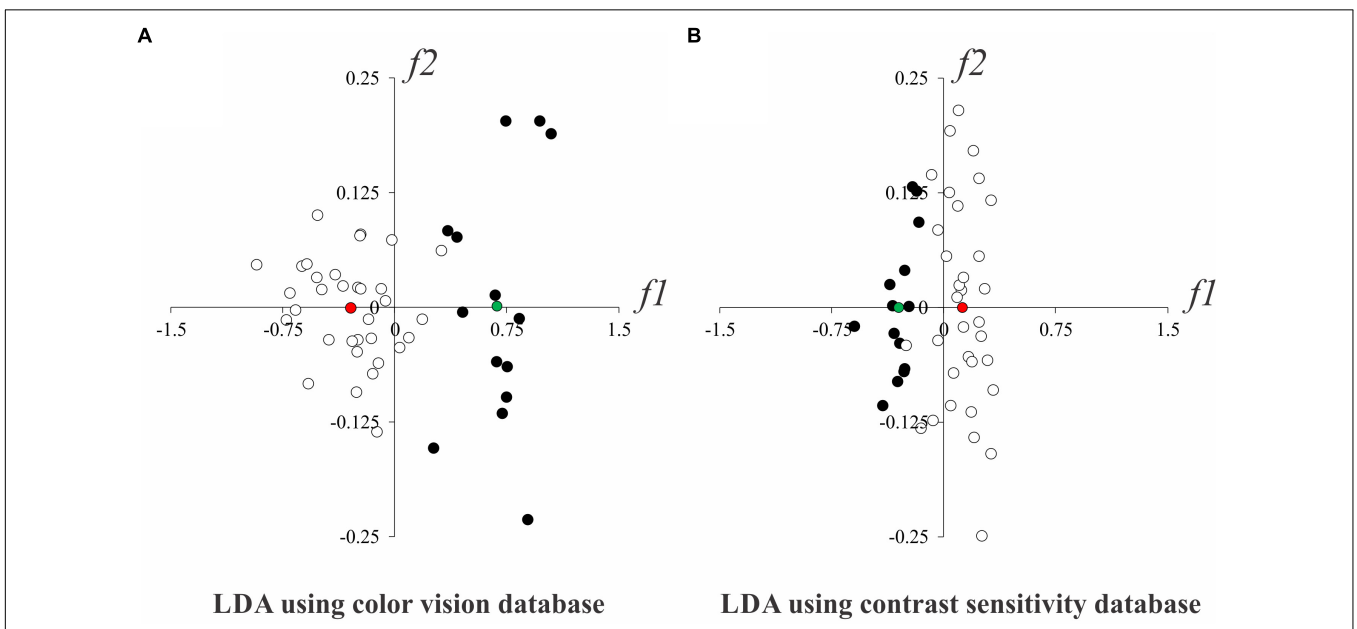

FIGURE 7 | Linear separability of the control (white circles) and alcoholic (black circles) groups in LDA subspace. **(A)** LDA results using color vision evaluation results. **(B)** LDA results using contrast sensitivity evaluation results. Red and green circles represent the centroids from controls and alcoholic subjects, respectively. *f*1 and *f*2 represent the first two discriminant functions from LDA.

of color opponent cells (red-green and blue-yellow opponency) is lower than the number of cells that process luminance opponency along the visual pathways (Brainard et al., 2000). We do not discard the explaining hypothesis that in primary visual cortex the color information is processed in regions named "blobs," that have a high activity of cytochrome oxidase, an enzyme involved in the cellular mechanisms of energy production (Boyd and Casagrande, 1999). Experimental studies which evaluated the effect of alcohol on the visual system of rat described an increase in optic nerve oxidative stress (Aviñó et al., 2002). In monkeys (*Macaca mulatta*) ethanol metabolism in neural tissue and retina was found to change tissue fatty acid and to increase lipid oxidation, contributing to oxidative stress associated with retinal function impairment (Pawlosky et al., 2001).

Initially, the studies that investigated involvement of color vision in the neural damage caused by alcoholism showed selective losses in blue-yellow mechanisms (Cruz-Coke and Varela, 1965; Thuline, 1967; Varela et al., 1969; Sassoon et al., 1970; Cruz-Coke, 1972; Adams et al., 1975; Russell et al., 1980; Verriest et al., 1980; Zrenner et al., 1986). Some other investigations reported red-green or diffuse losses (Sarraux et al., 1966; Smith and Layden, 1971; Rothstein et al., 1973; Sakuma, 1973; Mergler et al., 1988; Kapitany et al., 1993; Castro et al., 2009). We found no preference for any chromatic mechanisms even using two kinds of color tests. FM100 is a task with suprathreshold stimulus, while MR estimates threshold measurements of the color vision. For both tests the visual losses were similar across the chromatic axes.

It has been well-established that changes in neurocognitive networks and concomitant deficits in cognitive function are common in former alcoholics (Pandey et al., 2018). It is reasonable to think that both evaluations of color processing would seem to have a higher cognitive demand than the evaluation of spatial contrast sensitivity, since they require more complex instructions and judgements (i.e., choose the hue closest to the reference hue, which of four orientations is the gap in the C facing, versus "do you see the stimulus or not"). We did not evaluate cognitive functions of our ex-alcoholic sample, but we consider that our results represent the consequence of sensory impairment. Considering the example of the Mollon-Reffin test, the ex-alcoholics could perform the behavioral task in stimulus condition with highly saturated chromaticities and failed in less saturated chromaticity condition (but above the normative thresholds). So, we concluded that they understood the task and their main limitation was perceptual. The same rationale we can extrapolate to the hue ordering test.

A limitation of the present study is the sample size of the ex-alcoholic group, although with this sample size we could find significant differences compared to the controls. Other thing that is important to emphasize is that this sample has no other important clinical complication associated to the abuse of alcohol consume in the past.

The literature diverges as to the possibility of abstinence from alcohol being able to reduce the damage caused by the chronic alcoholism in specific functions of the central nervous system. There is the suggestion that after a prolonged time of abstinence from alcohol, the brain may reorganize to compensate behavioral and structural deficits (Oscar-Berman et al., 2014) including increase in cortical thickness in the brain's extended reward and oversight system (Durazzo et al., 2011), recovery in brain volume, microstructure, and neurochemistry (Oscar-Berman et al., 2014) improvement of gait, balance and neuropsychological functions (Fein et al., 2006), but some deficits have been found to persist, such as problems with executive functioning, motor functions and visuospatial cognition (Smith and Fein, 2011; Oscar-Berman et al., 2014). The color vision functions might be one of these persistent alterations induced by chronic alcoholism even after long abstinence, such as found in the present study.

AUTHOR CONTRIBUTIONS

All authors contributed to the conception of the work, and in drafting and revising the manuscript. All authors have approved the final version and agree to be accountable for all aspects of the work. AOC and LCLS designed the experiments. The psychophysical experiments were carried out by ICVSM, AB, EMCBL, and ARR. The data were analyzed by GSS, AB, and ICVSM. Components of the manuscript were written by GSS, DV, and AH.

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Environmental Impoverishment, Aging, and Reduction in Mastication Affect Mouse Innate Repertoire to Explore Novel Environments and to Assess Risk

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Studies indicate that inhibition of adequate masticatory function, due to soft diet, occlusal disharmony, or molar losses affects the cognitive behavior of rodents. However, no study has tested the effects on new environments exploration and risk assessment coupled with a combination of masticatory function rehabilitation and environmental enrichment. In the present report, we tested the hypothesis that age, environment, and masticatory changes may interact and alter exploratory patterns of locomotor activity and mice preferences in an open field (OF) arena. As OF arenas are widely used to measure anxiety-like behavior in rats and mice. We examined in an open arena, the exploratory and locomotor activities of mature (6-month-old; 6M), late mature (12-month-old; 12M), and aged (18-month-old; 18M) mice, subjected to distinct masticatory regimens and environments. Three different regimens of masticatory activity were used: continuous normal mastication with hard pellets (HD); normal mastication followed by reduced mastication with equal periods of pellets followed by soft powder – HD/SD; or rehabilitated masticatory activity with equal periods of HD, followed by powder, followed by pellets – HD/SD/HD). Under each diet regimen, half of the individuals were raised in standard cages [impoverished environment (IE)] and the other half in enriched cages [enriched environment (EE)]. Animals behavior on the open field (OF) task were recorded by webcam and analyzed with Any Maze software (Stöelting). The locomotor and exploratory activities in OF task declined with age, and this was particularly evident in 18M HD EE mice. Although all groups kept their preference by the peripheral zone, the outcomes were significantly influenced by interactions between environment, age, and diet. Independent of diet regime, 6M young mice maintained in an EE where voluntary exercise apparatus is available, revealed significant less body weight than all other groups. Although body weight differences were minimized as age progressed, 18M EE

group revealed intragroup significant influence of diet regimens. We suggest that long life environmental enrichment reduces the tendency to avoid open/lit spaces (OF) and this is particularly influenced by masticatory activity. These measurements may be useful in discussions of anxiety-related tasks.

Keywords: reduced mastication, environmental changes, aging, locomotor and exploratory activities, anxiety-like behavior

INTRODUCTION

Mastication seems to contribute to maintain body weight within normal limits, and a soft diet has been associated with obesity in murine model (Desmarchelier et al., 2013). The latter outcome appears to be associated with significant changes in hypothalamic synaptic input organization and gliosis (Horvath et al., 2010). This type of diet-induced obesity progressively alters cognition and mouse performance in the elevated plus maze (EPM) task (André et al., 2014). In addition, it has been reported that inhibition of adequate masticatory function, due soft-diet feeding, occlusal disharmony or molar losses, affected mice cognitive behavior (Onozuka et al., 2000; Yamamoto and Hirayama, 2001; Tsutsui et al., 2007; Kubo et al., 2010; Ono et al., 2010; Frota De Almeida et al., 2012; Ekuni et al., 2013; Mendes et al., 2013; Sakatani et al., 2013; Nose-Ishibashi et al., 2014; Utsugi et al., 2014; Pang et al., 2015; Takeda et al., 2016; Aguirre Siancas, 2017). Furthermore, loss of molars early in life (Kawahata et al., 2014) or soft diet consumption (Fukushima-Nakayama et al., 2017) caused loss impaired hippocampal-dependent recognition memory and induced a lateralized preference of object location in recognition tasks (Kawahata et al., 2014). In a previous report, we mimicked an active and a sedentary-life styles in murine model and tested the effects of age, environment and diet regimes on spatial memory, using Morris water-maze task (Mendes et al., 2013). We found that an enriched environment (EE) and masticatory activity rehabilitation recover spatial memory decline in aged mice.

However, only a few reports have investigated the effects of a soft diet (Nose-Ishibashi et al., 2014) and aging on sedentary-like and active murine models and no studies have tested the combined effects of masticatory rehabilitation and environmental enrichment on innate repertoire to explore novel environments and to assess risk. In the present report, we tested the hypothesis that age, environment, and masticatory changes may interact and alter mice exploratory patterns of locomotor activity and preferences in an OF arena. Open arenas are widely used for measuring anxiety-like behavior in mice and rats. In these tasks, when animals explore an unfamiliar area, they remain close to the walls; this preference is taken as an indication of fear-induced anxiety (Lalonde and Strazielle, 2008; Ennaceur, 2014). Hiding behavior may contribute to avoiding attack and predation, and it may be included in the repertoire of animal survival instincts. The species-specific hiding response in mice appears to lie at the root of their natural preference for unlit and protected spaces. Thus, the OF tests are based on the natural tendencies in mice to avoid open/lit areas and to spontaneously explore unfamiliar areas (Komada et al., 2008). Thus, animals appear to innately avoid open and/or lit spaces in the central area of the OF (Ennaceur, 2014). Mouse preference for the safety of the peripheral zone of the OF may reflect this adaptive response; for a recent review (see Ennaceur, 2014).

Thus, we focused on these preferred regions to compare mouse behaviors and to detect potential differential effects of diet regimes, age and environmental changes on mouse locomotor and exploratory activities.

To that end we examined outcomes of mice exploratory and locomotor activities in an OF. Tested individuals were under influence of different masticatory regimens, environments and age. Mature (6-month-old; 6M), late mature (12-month-old; 12M), and aged (18-month-old; 18M) mice were maintained either in standard laboratory or enriched cages. Masticatory regimens included: continuous normal mastication with hard pellets (HD); normal mastication followed by reduced mastication, with equal periods of pellets followed by soft powder; and rehabilitated masticatory activity with equal periods of HD, followed by powder, followed by pellets.

MATERIALS AND METHODS

Female albino Swiss mice were maintained in animal housing, in accordance with the guidelines published by the National Institutes of Health (Guide for the Care and Use of Laboratory Animals). The experimental protocol was tested and approved prior to study initiation by the Ethics Committee on Experimental Animal Research (from the Institute of Biological Sciences, Federal University of Pará, Brazil, CEPAE-UFPA: BIO223-14).

Age, Diets, Environments, and Body Weight

Masticatory activity was manipulated with different diets. Each age group was subjected to either a diet of HD, which required substantial chewing, or alternation with powder soft food (SD), which required little chewing. Masticatory rehabilitation was employed by feeding different sequences of HD and SD to mice.

The mice were raised either in sedentary-like or active conditions (**Figure 1**). The impoverished environment (IE) comprised plastic cages (32 cm × 45 cm × 16.5 cm) limited to chopped rice straw on the floor (no equipment or toys) covered with metal grids. Each IE cage housed eight individuals per age group: young (6M), or middle-aged (12M), or aged (18M) mice. The EE consisted of two-level wire cages (100 cm × 50 cm × 100 cm) equipped with rod bridges, tunnels, ropes, toys, and running wheels. Toys were composed of different

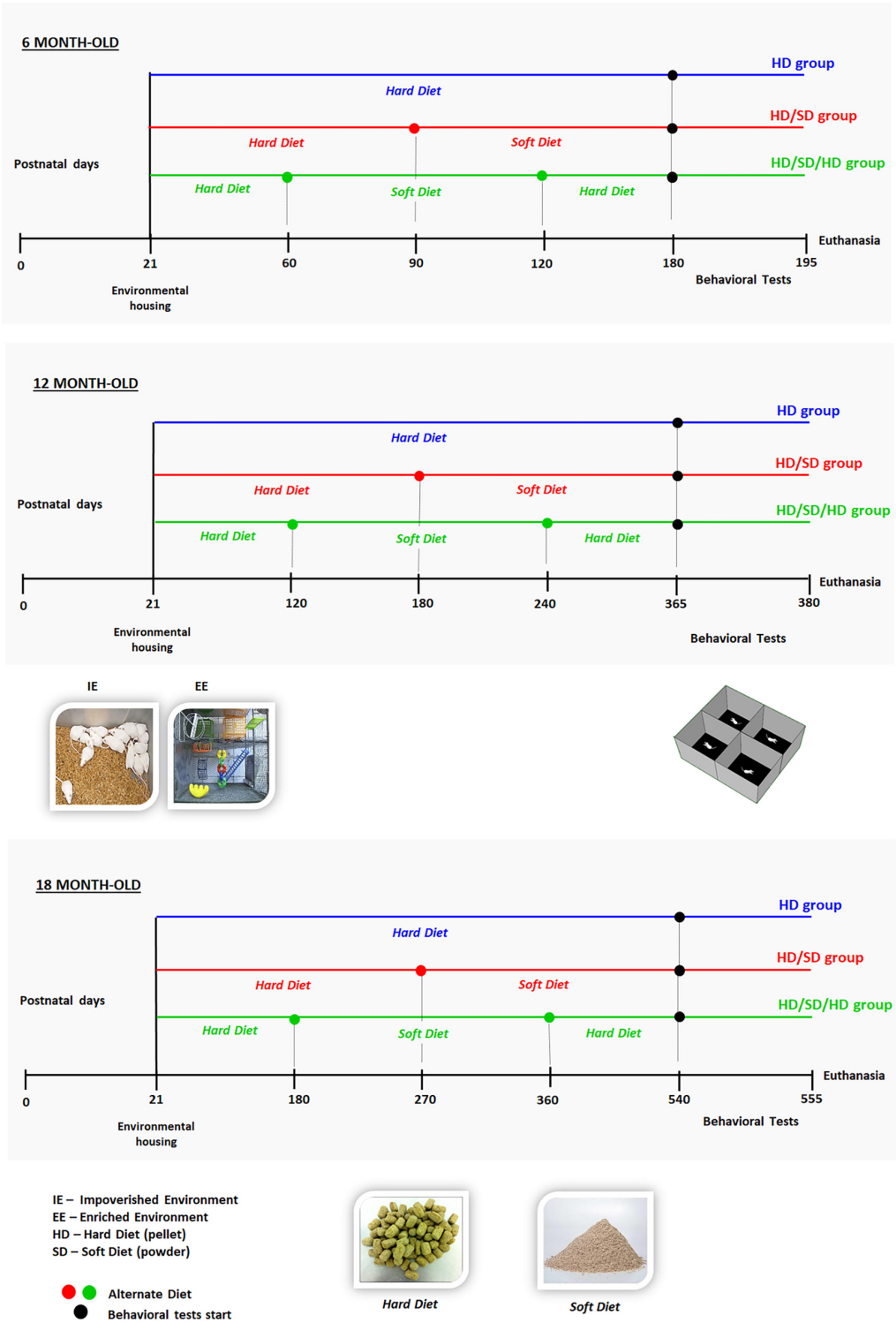

FIGURE 1 | Experimental setup and timeline. Time is indicated non-linearly, increasing from left to right, from birth (day 0) to euthanasia. Mice were housed in either an impoverished or enriched environment (IE or EE; images of environments are shown in the *middle panel, left*) from postnatal days 21 to 180 (*top*, 6 months old; 6M), 365 (*in the middle*, 12 months old; 12M), or 540 (*from bottom*, 18 months old; 18M). All animals were submitted to the open field (OF) tasks (test apparatus is shown in the *middle panel, right*); test was performed at the points indicated with filled circles on the corresponding timelines. Timelines are color-coded to indicate the following diet regimes: HD (blue): pellet diet; HD/SD (red): alternating pellet and powder diets, switched at 3, 6, or 9 months, respectively, for 6M, 12M, or 18M mice; and HD/SD/HD (green): alternating pellet, powder, and pellet diets, switched every 2, 4, or 6 months, respectively, for 6M, 12M, or 18M mice. *Bottom panel:* images show the hard pellets (HD) and soft powder (SD).

forms of plastic, wood, and metal of different colors, and they were changed periodically. Each EE cage housed 12 mice per age group: 6M or 12M, or 18M (the total number of animals per experimental group is described in **Table 1**). Water and food were delivered to the top and bottom levels, respectively. This arrangement obliged mice to move from one floor to the other, when they wanted to drink and eat. All animals had free access to food and water. All mice were raised in a controlled room temperature (23 ± 1°C) and a 12-h light–dark cycle. To detect potential influences of diet regimes and environments on body weight, all animals were weighed at the beginning of experiment and at the end of each time window (6M, 12M, and 18M).

Behavioral Tests

All mice were behaviorally assessed only once according to age (6, 12, or 18 months). All groups were tested in an OF before euthanasia. The apparatus and test protocol were slightly modified from previous report (Rodgers and Johnson, 1995).

The OF apparatus consisted of a gray polyvinyl chloride box (30 cm × 30 cm × 40 cm), with a floor divided into central and peripheral regions of equal areas (**Figure 4**). Each animal was placed at the center of the apparatus for 5 min. All experiments were carried out following the same protocol which included also the tests at the same time of day, conditions of luminosity (3 cd/m^2) and handling of the animals.

A video camera connected to a computer was placed one meter above the OF. Each training session was recorded for later analysis with Any-Maze software (Stöelting), and this allowed to record the precise position of each mouse throughout OF test. The following parameters were analyzed: distance traveled, minute-by-minute (m), time spent in the peripheral zone (seconds) and in the corners of the test apparatus. The times were also expressed as the percentage of the total time. After each session, the OF apparatus was cleaned with 70% ethanol.

TABLE 1 | Number of animals for each group.

Environment	Age	Diet	Number of animals
IE	6M	HD	8
		HD/SD	8
		HD/SD/HD	8
	12M	HD	8
		HD/SD	8
		HD/SD/HD	8
	18M	HD	8
		HD/SD	8
		HD/SD/HD	8
EE	6M	HD	12
		HD/SD	12
		HD/SD/HD	12
	12M	HD	12
		HD/SD	12
		HD/SD/HD	12
	18M	HD	12
		HD/SD	12
		HD/SD/HD	12

In the table are presented the environments [impoverished environment (IE); enriched environment (EE)], diets (HD, HD/SD and HD/SD/HD), and the age 6M, 12M, and 18M (6, 12, and 18 months old, respectively).

Statistical Analysis of Behavioral Changes

No blinding procedure was applied for behavioral tests since video records and software do not allow researcher influence. It is also important to mention that all experiments were performed according to the protocol described in previous session. Significant differences between groups with respect to the behavioral tasks were evaluated with three-way ANOVA and Tukey's *post hoc* test honestly significant difference. We also investigated the influences of age, diet regime, and environment on behavioral outcomes; differences between groups were considered significant with a 95% confidence level cutoff ($p < 0.05$). Analyses were performed with EzAnova or BioEstat 5.0 (Ayres et al., 2007) software. To apply three-way factorial ANOVA to the results of OF test, we used the percentage of time spent in peripheral area, as a function of the total time test. One-way ANOVA and the Tukey's *post hoc* test honestly significant difference were used to analyze significant differences in distance traveled along each minute of OF test. Differences between groups were considered significant at the 95% confidence level ($p < 0.05$).

RESULTS

Intergroup Analysis

We investigated the effects of masticatory activity changes, environment, and age on the pattern of exploratory activity in the OF test. Thus, three-way ANOVA analysis applied to time spent in peripheral zone revealed that it was significantly influenced by environment [$F_{(1,90)} = 4.55$; $p < 0.036$] and diet [$F_{(2,90)} = 8.9$; $p < 0.0003$], but not by age [$F_{(2,90)} = 3.07$; $p < 0.0512$]. The combination of environment and diet did not show a significant interaction [$F_{(2,90)} = 0.76$; $p < 0.470$].

However, all other variables acting together showed that OF test results were significantly influenced by interactions between the environment and age [$F_{(2,90)} = 11.3$; $p < 0.000042$]; age and diet [$F_{(4,90)} = 6.00$; $p < 0.00025$]; and environment, age, and diet [$F_{(4,90)} = 9.72$; $p < 0.000001$]. These variables (environment and age; age and diet; and environment, age, and diet) interacted either subtractive or additively to influence OF results.

Intragroup Analysis

In the OF task, the HD individuals of 6M mice group raised in IE, spent less time in the periphery as compared to mice of the same age under all other diet regimes maintained at the same environmental condition (**Figure 2**). Related to 6M mice raised in EE, the influence of diet regimes was limited, because the environmental stimulation seems to increase the preference of the control group (HD EE 6M) by the peripheral zone, minimizing the observed differences between diets among the IE animals. In **Figure 3**, this preference of the HD EE

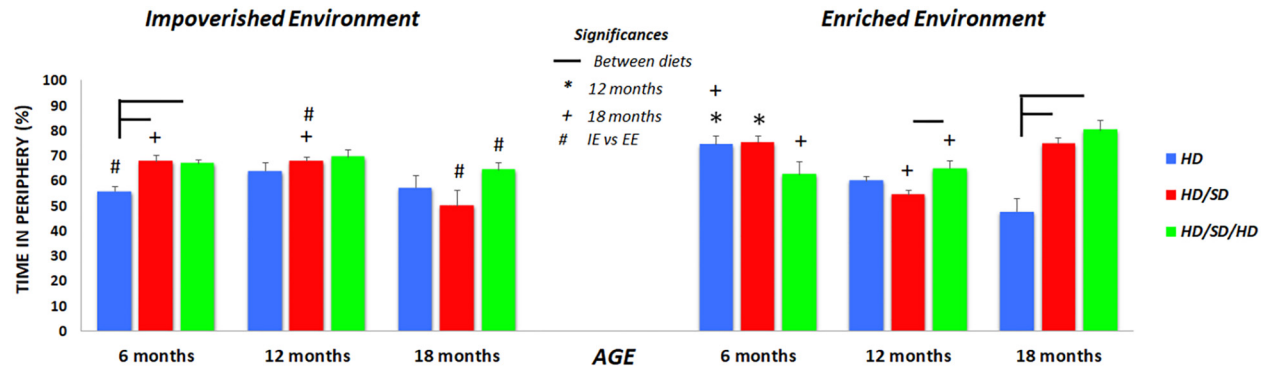

FIGURE 2 | The percentages of time spent in the periphery of the OF. (*) Significantly different from 12 months; (+) significantly different from 18 months; (#) significant difference between impoverished (IE) and enhanced environments (EE); line connectors indicate significant differences between diets. HD (blue) = hard diet; HD/SD (red) = hard diet followed by soft diet; HD/SD/HD (green) = hard diet followed by soft diet followed by hard diet.

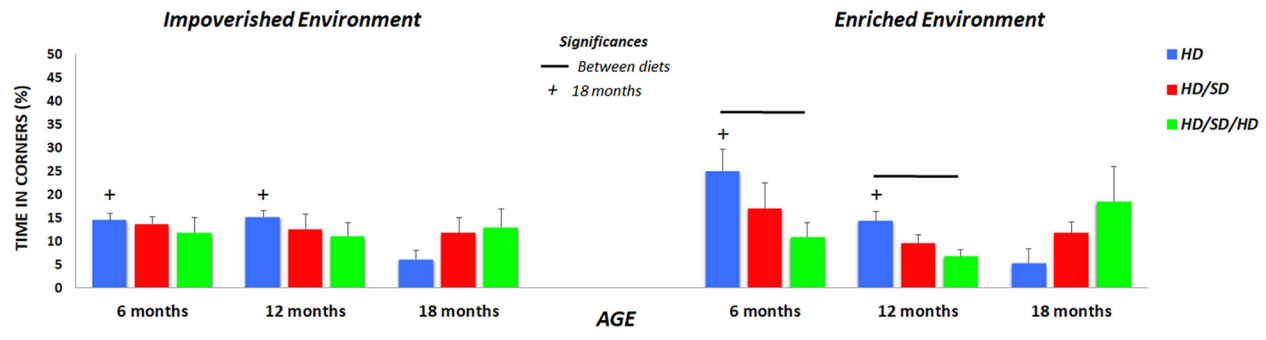

FIGURE 3 | Quantification of the time spent in the corners of the OF. Times are shown for mice raised in **(left)** impoverished or **(right)** enhanced environments. (+) Significantly different from the 18-month group; lines indicate significant differences between diets; HD (blue) = hard diet; HD/SD (red) = hard diet followed by soft diet; HD/SD/HD (green) = hard diet followed by soft diet followed by hard diet.

6M by the periphery and especially by the corners of the apparatus are illustrated.

Among 12M and 18M groups maintained in IE, no significant effect of diet changes is observed, but it is interesting to highlight that only the HD/SD IE group reduces their preference for the peripheral zone when the animals of 6M and 12M are compared with those of 18M (**Figure 2**). Another particular difference between diet regimens was observed in the old mice group from enriched environment (18M EE). Mice fed with hard diet (HD – control group), spent less time in periphery zone than mice from all other diets. Interestingly, as age progresses, mice tend to decrease their preference for the peripheral zone, and this behavior is more evident among animals kept in EE and with no change in masticatory activity.

In the case of animals raised in IE, this age effect seems to be present only in the HD/SD group as previously commented. In addition, groups that underwent alteration of masticatory activity also appeared to be more sensitive to environmental influences (IE vs. EE in HD/SD 12M and 18M; IE vs. EE in HD/SD/HD 18M).

Because independent of experimental variables, the experimental groups spent at least half of test time in OF peripheral zone, we investigated potential preferences for selected points of this zone such as its corners. We speculate that corners may be associated with less risk. Thus, **Figure 3** illustrates the percentage of test time spent at the corners of the apparatus. Note that reduction of time spent in corners was greater related to aging, and this is evident in HD group, independent of the environment where mice were raised.

More detailed comparisons (mean, standard error, *F*-values, and *p*-values) were performed for the different experimental groups (see **Supplementary Table 1**) to determine the effects of diet regime, environment, and age on OF test outcomes.

Figure 4 demonstrate experimental groups preferences for selected OF regions. In this figure, red color indicates higher preferences and blue one, a smaller preference. Note that mice rarely visited the central region of the OF test, independent of age, environment, or diet regimen. However, as discussed earlier, note that the control group 18M maintained in enriched environment (HD EE 18M) avoid the peripheral zone and corners of the OF apparatus while chewing alteration groups avoid the center of the arena (HD/SD and HD/SD/HD at EE 18M).

We also analyzed the total distance traveled in each minute of test, independent of spatial location occupied in the OF (**Figure 5**) and found significant reductions in the locomotor

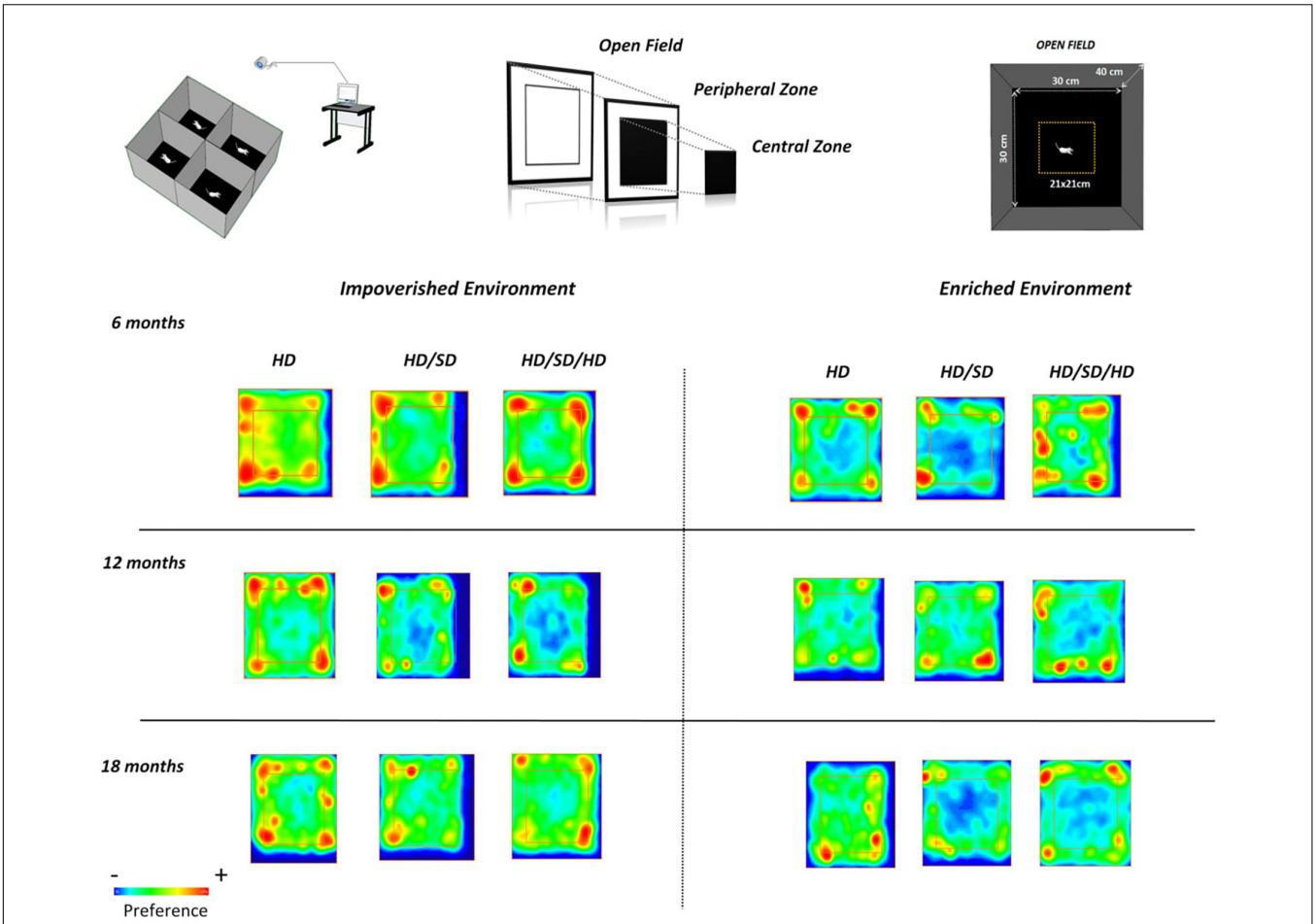

FIGURE 4 | Graphic representations of preferred areas in the OF test. (*Top row*, *left*) Experimental setup and video capture system for recording mouse behavior in the OF test; (*top middle and right*) the peripheral and central regions are equal in area. (*Lower panels*) Time spent in all areas by mice raised in impoverished (*left*) or enriched (*right*) environments, at different ages (*rows*). The square region more central (internal) delineates the central (external) and peripheral areas of the OF. Red, yellow, and blue indicate high, intermediate, and low preferences, respectively. Diet regimes are indicated at the top: HD = hard diet; HD/SD = hard diet followed by soft diet; HD/SD/HD = hard diet followed by soft diet followed by hard diet.

and exploratory activities over the 5 min. These minute-by-minute analysis revealed that traveled distance decrease as time progresses, and that the time-dependent reduction was accentuated by aging and environmental enrichment.

More detailed comparisons (mean, standard error, *F*-values, and *p*-values) were performed for the different experimental groups (**Supplementary Table 2**) to determine the effects of diet regime, environment, and age on OF outcomes.

Body Weight

At the beginning of the experiment no statistically significant difference was found related to weights of the animals in the different groups.

Intergroup Analysis

A three-way ANOVA of body weights recorded on the day before euthanasia of each group revealed that body weight was significant influenced by environment [$F_{(1,90)}$ = 32.4, $p < 0.000001$], age [$F_{(2,90)}$ = 3.45, $p < 0.036$], and diet [$F_{(2,90)}$ = 14.4, $p < 0.000004$]. Significant interactions between environment and diet [$F_{(2,90)}$ = 3.62, $p < 0.03$], and between environment and age [$F_{(2,90)}$ = 15.1, $p < 0.000002$] were observed. There was not significant interaction between age and diet [$F_{(4.90)}$ = 2.29, $p < 0.066$] or between environment, age, and diet [$F_{(4,90)}$ = 0.201, $p < 0.937$].

Intragroup Analysis

Within the 6M and 12M age group, created in IE, mice rehabilitated to normal masticatory activity (HD/SD/HD) showed significant higher body weights than that mice with reduced masticatory activity (HD/SD group) (**Figure 6**). This increasing condition was not observed in the groups created in EE at the same time windows. However, HD weighed significantly more than HD/SD in IE and EE at 6M, and more than HD/SD/HD in EE at the same age. The same occurred at 18 months of age.

Comparing the environments, the EE 6M was associated with a significant body weight reduction compared to

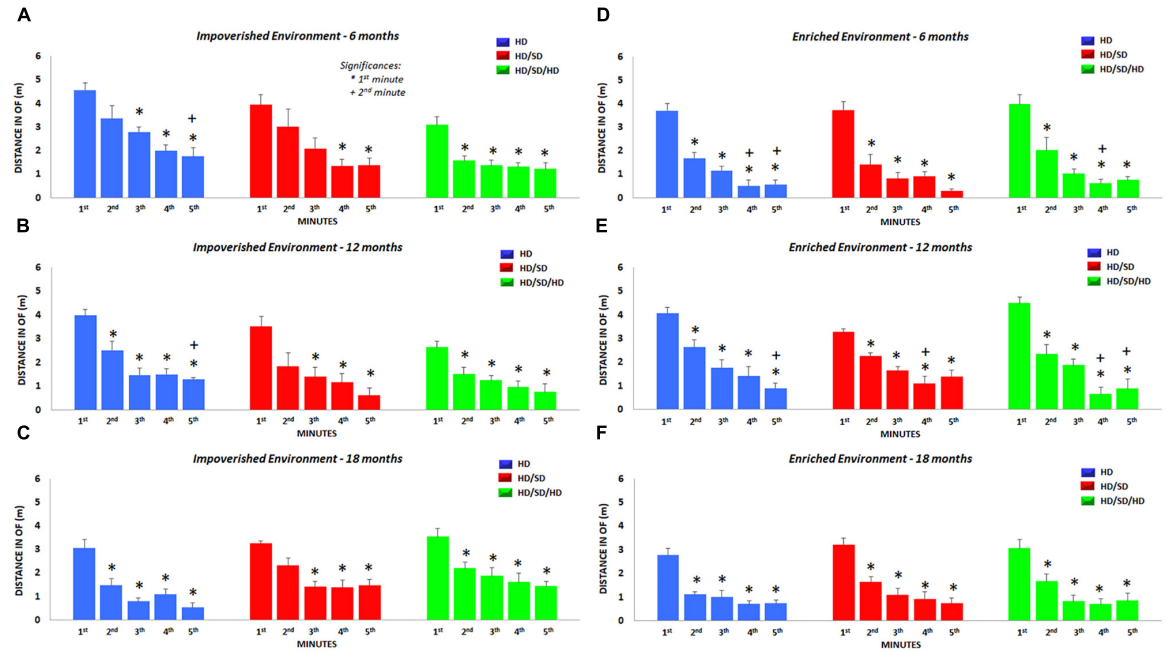

FIGURE 5 | Distance traveled in the OF test. Total distance per minute traveled during the OF test is shown for each diet regime (color-coded), age (*top row*: 6M, *middle row*: 12M, and *bottom row*: 18M), and environment (*left*: impoverished and *right*: enriched environment). (*) Significantly different from the 1st minute; (+) significantly different from the 2nd minute; HD (blue) = hard diet; HD/SD (red) = hard diet followed by soft diet; HD/SD/HD (green) = hard diet followed by soft diet followed by hard diet.

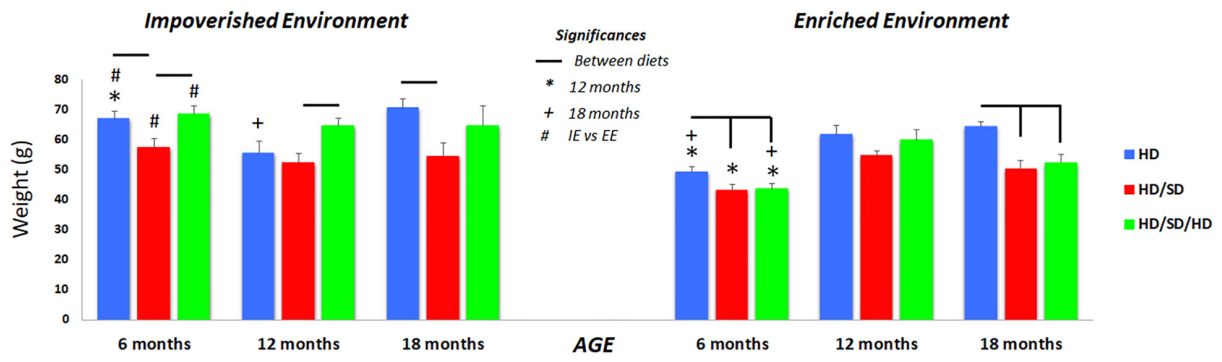

FIGURE 6 | Quantification of the weight body at the end of each age analyzed. (*) Significantly different from 12 months; (+) significantly different from 18 months; (#) significant difference between impoverished (IE) and enhanced environments (EE); line connectors indicate significant differences between diets. HD (blue) = hard diet; HD/SD (red) = hard diet followed by soft diet; HD/SD/HD (green) = hard diet followed by soft diet followed by hard diet.

IE mice, independent of diet regime, but no significant differences were detected between IE and EE mice at 12 or 18 months.

Related to different ages, the weight gain of animals in the 12M group, regardless of the diet regime, was significantly higher compared to 6M, as well as the weight gain at 18M, and this was especially noted in animals raised in EE.

More detailed comparisons (mean, standard error, *F*-values, and *p*-values) were performed for the different experimental groups to determine the effects of diet regime, environment, and age on weight outcomes and are depicted on **Supplementary Table 3**.

DISCUSSION

In a previous report, we demonstrated that a 24-h, complex EE had differential effects on performance in episodic-like and water maze memory tests, among young and aged female albino Swiss mice (Diniz et al., 2010). We also showed that imposing reduced masticatory activity, by offering a powder diet to mice previously fed a pellet (HD/SD), caused impaired spatial learning, but a diet that imposed masticatory rehabilitation (HD/SD/HD) reversed the impairments in aged mice (Mendes et al., 2013).

Part of these effects can be explained by studies that evidence that masticatory dysfunction, sustainably activates

the Hypothalamic-Hypophysis-Adrenal (H-H-A) axis increasing blood stream glucocorticoid levels. The continuous increase in circulating glucocorticoids in turn interrupts H-H-A axis negative feedback system, further increasing secretion of glucorticoids (Azuma et al., 2017). However, neurogenesis in dentate gyrus, which is critical to hippocampal-mediated modulation in H-H-A axis also appears to be impaired by masticatory dysfunction causing cognitive deficits which are dependent on morphofunctional integrity of the hippocampus (Snyder et al., 2011).

In this context, high levels of glucorticoids could still be modulating anxiety-like or fear behavior. The act of hiding to avoid attacks and predation ends up being an innate strategy to guarantee animal survival and this logic follows OF tests in which apparatus they have a natural tendency to avoid open areas (Komada et al., 2008).

The EPM, although widely used in these cases, seems to suffer undesirable influence from environmental manipulation that may increase or decrease locomotor activity generating false positive or negative test results (Dawson and Tricklebank, 1995). Exposure to a new environment immediately prior to EPM, for example, increases motor activity in maze increasing time spent in open arms (File and Wardill, 1975a,b; Pellow et al., 1985).

In our laboratory, mice are often subjected to a sequence of tasks, which include OF testing, object recognition, followed by EPM and forced swimming. In these cases, it is common to perform each task on separate occasions. However, in face of such evidence of pre-exposure to another environment influencing the EPM results we assume that careful consideration should be given when designing experiments using EPM.

Therefore, we tested the hypothesis that a combination of masticatory rehabilitation and EE would minimize the impaired exploratory and locomotor behavioral changes induced by a combination of IE and reduced masticatory activity. We demonstrated that all mice, independent of the masticatory condition, environment, and age, exhibited a similar temporal organization of their spatial horizontal exploratory activity in the OF task. Nevertheless, we found that the EE, aging, and normal masticatory activity (HD), interacting with each other, reduced the innate tendency to avoid open/lit spaces in the OF task. We also demonstrated that contrasting diet regimes, designed to reduce or rehabilitate masticatory activity, had differential effects at different time windows.

In the OF test sedentary young adults from IE with reduced masticatory activity or rehabilitation (HD/SD and HD/SD/HD IE 6M) showed a higher preference for the periphery of the OF than control animals from similar environment (HD IE 6M). This result may suggest that the preference for the peripheral zone observed in the groups with masticatory alterations may be associated with potential stress due to masticatory changes, either associated with masticatory reduction or rehabilitation. This result is in line with previous report, showing that the total distance of locomotion was significantly higher for animals fed with soft diet previously fed with hard diet, suggesting that the shift to powdered diet may affect the responsiveness of mice exposed to new environments (Nose-Ishibashi et al., 2014). Indeed, it has been proposed that early in life masticatory manipulation may increase vulnerability for mental disorders (Nose-Ishibashi et al., 2014).

Horizontal locomotor exploration comprises part of the innate repertoire used by animals to explore novel environments and to assess risk in the wild (Augustsson and Meyerson, 2004; Ennaceur, 2014). It has been suggested that this strategy arises from the drives to avoid and to explore a perceived threatening stimulus (Crusio, 2001). In the present study, we promptly recognized this stereotypical behavior, when mice explored the OF (Lister, 1990; Kalueff et al., 2006; Ennaceur, 2014).

With exposure to predator odors, a previous report (Sampedro-Piquero et al., 2016), demonstrated that EE could reduce anxiety in aged Wistar rats, when confronted with cat odor. That finding was indicated by a time reduction in freezing behavior. Similar EE effects were observed in aged Wistar rats on an elevated-zero maze task (Sampedro-Piquero et al., 2013, 2014).

Although a controversial issue due to methodological differences, species and strains used in the tests, it is important to highlight that EE may contribute either to reduce or increase anxiety-like behavior and depression (Greenwood et al., 2003; Burghardt et al., 2004; Van Hoomissen et al., 2004; Duman et al., 2008; Leasure and Jones, 2008).

In the present report, the HD EE 18M group compared with HD EE 6M individuals, exhibited a reduction in anxiety-like behavior, suggesting that open arena adaptive behavior in this group may be associate with a continuous non-aversive EE which includes normal chewing, voluntary exercise, and visuospatial stimulation as part of the necessary repertoire to adapt to an open arena with reduced stress level (Dupire et al., 2013). In agreement, our findings revealed that, as compared with individuals from IE, animals raised in EE, decreased traveled distance more rapidly along the test, and this was particularly significant to 18M mice.

An alternative view is that the environmental enrichment may increase stress level due to the weekly change in the location or substitution of old by new toys (Larsson et al., 2002). This chronic low level of stress may adapt old mice from EE to face novelty (Konkle et al., 2010).

Allied to this, as in the present work the tests were performed only in females, it is important to discuss some possible influences of estrous cycle. We have seen that estrus phases and respective ovarian hormones oscillations in rats may be mediating defensive behaviors (Blume et al., 2017), which are associated with fear and anxiety (Graham and Scott, 2018; Pentkowski et al., 2018), including neuronal changes in amygdala nuclei (such as the lateral and basal). However, despite evidence on these influences, it is considered that estrous cycle may be affected by animal housing conditions too. Andrade et al. (2002) reported that females from overpopulation cages, without males, appear to exhibit a phase designated as anestrous characterized by the absence of estrus cycles. In our case, the estrous cycle phases were not investigated in the animals and since we only worked with females and only females inhabited our laboratory, we expect

little influence of the cycle on the behavioral results detected. However, because we did not measure potential influences of hormones on behavior, future studies should take this as a limitation of the present study. Despite of this, it is consistent to say that the older mice was depleted of estrogenic protection. Thus, we reasoned that at least part of the behavioral changes may be related to the estropause.

When analyzed the differences in the body weight of the animals, as compared with controls HD or rehabilitated HD/SD/HD mice, the body weight from HD/SD mice, fed proportionally longer with soft diet revealed significant reduction of their body weight. This finding does not agree with previous data demonstrating long term soft diet-induced obesity (Nojima et al., 2006; Desmarchelier et al., 2013; André et al., 2014). Coherently, we found no correlation between preferred zone of the OF and body weight as previously described in rats with weight losses induced by gastrectomy as compared with rats fed with hypercaloric diets (Himel et al., 2018).

Notwithstanding, it is important to recognize that the environmental enrichment influenced the weight gain. Indeed, independently of the diet regime at 6M all EE groups weighed less than those from IE. In contrast to animals from IE, EE seemed to enhance body weight differences between 6M, 12M, and 18M mice reducing age related metabolic differences.

Therefore, we conclude that changes in masticatory activity, influence the pattern of exploration by zones in the OF test and environmental impoverishment seems to enhance the effects of aging, increasing the preference for the peripheral zone of OF by the animals that not have undergone chewing alteration. Finally, the environmental enrichment also influences mice weight gain, but this is not correlated with spatial pattern of explored zones in the OF.

AUTHOR CONTRIBUTIONS

FCCSM participated in the development and methodological design, collection and treatment of data, analysis and interpretation of data and writing. LTVBP participated in the collection and processing of data. CWPD participated in the development and methodological design, supervision, analysis and interpretation of data and writing. MCKS participated in the development and methodological design, supervision, analysis and interpretation of data and writing.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2019.00107/full#supplementary-material

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Maternal Protein Restriction in Two Successive Generations Impairs Mitochondrial Electron Coupling in the Progeny's Brainstem of *Wistar* Rats From Both Sexes

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Maternal protein deficiency during the critical development period of the progeny disturbs mitochondrial metabolism in the brainstem, which increases the risk of developing cardiovascular diseases in the first-generation (F1) offspring, but is unknown if this effect persists in the second-generation (F2) offspring. The study tested whether mitochondrial health and oxidative balance will be restored in F2 rats. Male and female rats were divided into six groups according to the diet fed to their mothers throughout gestation and lactation periods. These groups were: (1) normoprotein (NP) and (2) low-protein (LP) rats of the first filial generation (F1-NP and F1-LP, respectively) and (3) NP and (4) LP rats of the second filial generation (F2-NP and F2-LP, respectively). After weaning, all groups received commercial chow and a portion of each group was sacrificed on the 30th day of life for determination of mitochondrial and oxidative parameters. The remaining portion of the F1 group was mated at adulthood and fed an NP or LP diet during the periods of gestation and lactation, to produce progeny belonging to (5) F2R-NP and (6) F2R-LP group, respectively. Our results demonstrated that male F1-LP rats suffered mitochondrial impairment associated with an 89% higher production of reactive species (RS) and 137% higher oxidative stress biomarkers, but that the oxidative stress was blunted in female F1-LP animals despite the antioxidant impairment. In the second generation following F0 malnutrition, brainstem antioxidant defenses were restored in the F2-LP group of both sexes. However, F2R-LP offspring, exposed to LP in the diets of the two preceding generations displayed a RS overproduction with a concomitant decrease in mitochondrial bioenergetics. Our findings demonstrate that nutritional stress during the reproductive life of the mother can negatively affect mitochondrial metabolism and oxidative balance in the brainstem

of F1 progeny, but that restoration of a normal diet during the reproductive life of those individuals leads toward a mitochondrial recovery in their own (F2) progeny. Otherwise, if protein deprivation is continued from the F0 generation and into the F1 generation, the F2 progeny will exhibit no recovery, but instead will remain vulnerable to further oxidative damage.

Keywords: mitochondria, intergenerational, low-protein diet, brainstem, gender, rats

INTRODUCTION

The Developmental Origins of Health and Disease Hypothesis posits that environmental stimuli encountered during critical periods of development, including embryonic, fetal and neonatal life, can induce long-lasting changes in the morphology and physiology of the fully developed individual (Bateson et al., 2004; West-Eberhard, 2005). Among the mechanisms proposed for the developmental origin of adult disease are unbalanced levels of micro- or macronutrients that cause oxidative stress-related damage to critical biomolecular compounds (Luo et al., 2006; Martin-Gronert and Ozanne, 2012; Rashid et al., 2018) which, in turn, enter into signaling pathways underlying several chronic degenerative diseases, including cardiovascular diseases (CVD) (Mansego et al., 2011; Sheeran and Pepe, 2017).

According to the World Health Organization (WHO), nearly 17.5 million deaths per year around the world are associated with CVD, 9.4 million of which have hypertension as the main cause (WHO, 2014). Although, complex and multifactorial etiologies underlie the occurrence of chronic hypertension (Hering et al., 2017), it is suspected that approximately 50% of all cases of hypertension have a neurogenic origin, wherein sympathetic over-activation of central pathways of blood pressure regulation has been described (Guyenet, 2006; Esler et al., 2010). In addition, several studies have demonstrated that this neurogenic hypertension can be triggered by mitochondrial impairment and oxidative stress into the brainstem (Chan et al., 2006, 2009b; Hirooka, 2008; Lopez-Campistrous et al., 2008; Dampney, 2016).

Several reports showed that environmental insult during critical periods of development can induce oxidative imbalance in various components of the central nervous system (CNS) of the adult (Bonatto et al., 2005, 2006; Feoli et al., 2006; Ferreira et al., 2016c). Moreover, our laboratory has demonstrated that a low-protein maternal diet during the perinatal period of her offspring results in impaired mitochondrial function and antioxidant capacity throughout the lives of those progeny, and an increased risk of developing CVD in adulthood (Ferreira et al., 2015, 2016a, 2018; de Brito Alves et al., 2016; de Sousa et al., 2017). There is also evidence that the increased risk of CVD may not affect only the immediate offspring of an undernourished parent, but, it that might also be transmitted across successive generations (i.e., children, grandchildren, great-grandchildren) in several possible ways, including non-Mendelian inheritance via maternal mitochondria (Zambrano et al., 2005; Zambrano, 2009; Dunn and Bale, 2011; Bale, 2015; Aiken et al., 2016; Saben et al., 2016). This means of transmitting parental damage to succeeding generations is particularly relevant to nutritional deficits during development (Woods et al., 2018), as a number of experimental studies have demonstrated long-lasting changes in mitochondrial functions such as apoptosis, calcium control, redox homeostasis and energy supply (Bertram et al., 2008; Ponzio et al., 2012; Yin and Cadenas, 2015; Saben et al., 2016) following maternal malnutrition.

On the other hand, several studies have described the enhancement of cell-protective systems following repeated environmental insult, including the up-regulation of various antioxidant mechanisms that lend resilience to oxidative stress and the amelioration of oxidative damage in the progeny of malnourished mothers (Pickering et al., 2013; Banos-Gomez et al., 2017).

Among the factors contributing to the degree of oxidative damage, and conversely the upregulation of antioxidant defenses in individuals exposed to malnutrition during gestation/lactation, is the gender, which greatly influences the relative amount of estrogen present both during the period of mitochondrial damage in the mother, and later in life when damage first becomes evident in the progeny (Lagranha et al., 2010; Braz et al., 2017; Silva et al., 2018). Clearly then, a maternal low-protein diet would be expected to affect male and female offspring differently in the F1 generation, and perhaps into succeeding generations.

We have previously shown that maternal protein deficiency during a critical period of rat brain development results in mitochondrial dysfunction in the brainstem of her offspring throughout life (Ferreira et al., 2016a, 2018; de Sousa et al., 2017). In the current study, we investigate whether a maternal low-protein diet differentially affects mitochondrial bioenergetics and oxidative balance in the male and female brainstem not only in the F1 generation, but also in the generation that follows. In addition, we examine whether oxidative brainstem resilience through upregulation of protective anti-oxidant mechanisms occurs in the second filial (F2) generation when that generation is re-exposed to a maternal low-protein diet.

MATERIALS AND METHODS

Ethics Statement

This study was carried out in accordance with the recommendations of the National Institutes of Health guide for animal experimentation (NIH Publications N°. 80-23, revised 1978). The protocol was approved by the Ethical Committee of the Bioscience Center of the Federal University of Pernambuco (n° 23076.018417/2013-73).

Experimental Groups and Diet

F1 Offspring

Nulliparous Wistar rats at 90 days of age and weighing 250–270 g were mated in a ratio of two females to one male, and pregnancy was determined by the presence of spermatozoa in vaginal smears. The pregnant rats ($n = 16$) were divided into two groups according to the protein content of their respective diets, which were formulated in the Laboratory of Dietetic Techniques at the Federal University of Pernambuco, as previously described (Reeves et al., 1993; Ferreira et al., 2016a). These diets were: (1) 17% protein ($n = 8$; normo-protein, NP) or (2) 8% protein ($n = 8$; low-protein, LP). Both diets were isocaloric and had casein as the only source of protein, with other dietary components added based on recommendations of the American Institute of Nutrition (AIN). NP dams delivered 12–15 pups, while LP delivered 8–11. However, no difference in sex ratio between groups was observed. Twenty-four hours after birth, the litters were standardized to eight pups per litter in the first generation (F1). The litters were maintained on their respective NP and LP diets during a lactation period lasting 21 days, then received laboratory chow (Labina; Purina Agriband, Brazil) until they reached an age of 30 days of life, at which time a part of each group was sacrificed by decapitation for the experimental procedures. Two males and two females were sacrificed in each litter, and experimental analyses were performed with 1 rat from each litter (i.e., 1 male and 1 female for the mitochondria assays, as well as for the total homogenate) (see diagram in **Figure 1**).

F2 Offspring

Males ($n = 16$) and females ($n = 32$) in the F1 generation that were not sacrificed were maintained on standard laboratory chow until reaching 80–90 days of age, at which time they were mated to produce the F2 generation of offspring. Mating was always performed between rats from different litters, in a ratio of two females to one male. Once pregnancy was verified, the pregnant F1 rats were assigned to receive either (1) the diet that their mothers' had received, or (2) a diet of laboratory chow. Thus, the following groups were formed: (1) F2-NP ($n = 8$): pups descended from grandmothers that received a normoprotein diet and mothers that received laboratory chow during gestation and lactation; (2) F2-LP ($n = 8$): pups descended from grandmothers that received a low-protein diet and mothers that received laboratory chow during gestation and lactation; (3) F2R-NP ($n = 8$): pups descended from grandmothers and mothers that received normoprotein diet during gestation and lactation; and (4) F2R-LP ($n = 8$): pups descended from grandmothers and mothers that received low-protein diet during gestation and lactation. No difference in gender ratio or other litter characteristics was observed between the F1 and F2 animals. All groups were maintained on their respective diets until weaning (21 days of age), at which time they received laboratory chow (Labina; Purina Agriband) until, being sacrificed for experimental analyses at 30 days of age.

Preparation of Brainstem Mitochondria

After the brainstems were removed, they were immediately minced and homogenized in an ice-cold mitochondrial isolation buffer containing 225 mM mannitol, 75 mM sucrose, 4 mM HEPES, 2 mM taurine and 0.5 mM EGTA, pH 7.4, using a potter-Elvehjem pestle and glass tube connected to a homogenizer (IKA@ RW20 digital). Homogenates were centrifuged for 5 min at 1,180 × *g* maintained at 4°C and the resulting supernatants were centrifuged for an additional 10 min at 12,470 × *g* and 4°C. After the last centrifugation, the pellets containing isolated mitochondria were re-suspended in respiration buffer (RB) consisting of 120 mM KCl, 4 mM HEPES, 5 mM K_2HPO_4 and 0.2% BSA (w/v), pH 7.4. Mitochondria were kept on ice until assay (Lagranha et al., 2010; Ferreira et al., 2016a).

Measurement of Mitochondrial Respiration

Mitochondrial respiration is a cogent measure of the ability of the organelle to produce ATP in response to different energy demands. The assay is based on the mitochondrial capacity to consume O_2 in response to the electron coupling-related proton current (Chance and Williams, 1955; Brand and Nicholls, 2011). Here, we used a 600 SL chamber connected to a Clark-type oxygen electrode (Hansatech Instruments, Pentney King's Lynn, United Kingdom) at 28°C to monitor the O_2 consumption under different conditions (Ferreira et al., 2016a). Initially, mitochondria were incubated in RB (1 mg protein/mL) containing only Complex I substrates (10 mM glutamate and 0.4 mM malate) (basal state/state 2). This state restricts the mitochondria to a minimal consumption of O_2 due to the scarcity of ADP in the assay. Thereafter, ATP synthesis was stimulated by adding 0.5 mM ADP (state 3/ADP-stimulated), at which time, mitochondria use O_2 as the final electron acceptor while phosphorylating ADP to ATP via ATP synthase activation. State 3 was ended by the addition of 1.2 μM oligomycin (resting state) to decrease O_2 consumption through inhibition of ATP synthase activity. The last state evaluated was the uncoupled state, measured by adding 5 μM carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP), a protonophore that increases the velocity of O_2 consumption by uncoupling the mitochondria (Goldsby and Heytler, 1963). In addition, ADP-stimulated/resting states were used to assess the mitochondrial respiratory control ratio (RCR), a classic indicator of mitochondrial "health" (Nicholls and Bernson, 1977; Brand and Nicholls, 2011).

Measurement of Mitochondrial Reactive Species (RS) Production

RS production was assessed by the dihydrodichlorofluorescein diacetate (H_2DCF-DA) method (Ferreira et al., 2016a). Briefly, 0.1 mg of mitochondria were incubated in RB with complex I substrates, as described in Section "Measurement of Mitochondrial Respiration," followed by the addition of 5 μM (H_2DCF-DA), which in the presence of reactive species form a product that fluoresces at 485 nm excitation and 530 nm emission. The reaction was followed by gentle shaking for 8 min in a FLUOstar OMEGA spectrophotometer (BMG Labtech, United States) at 28°C.

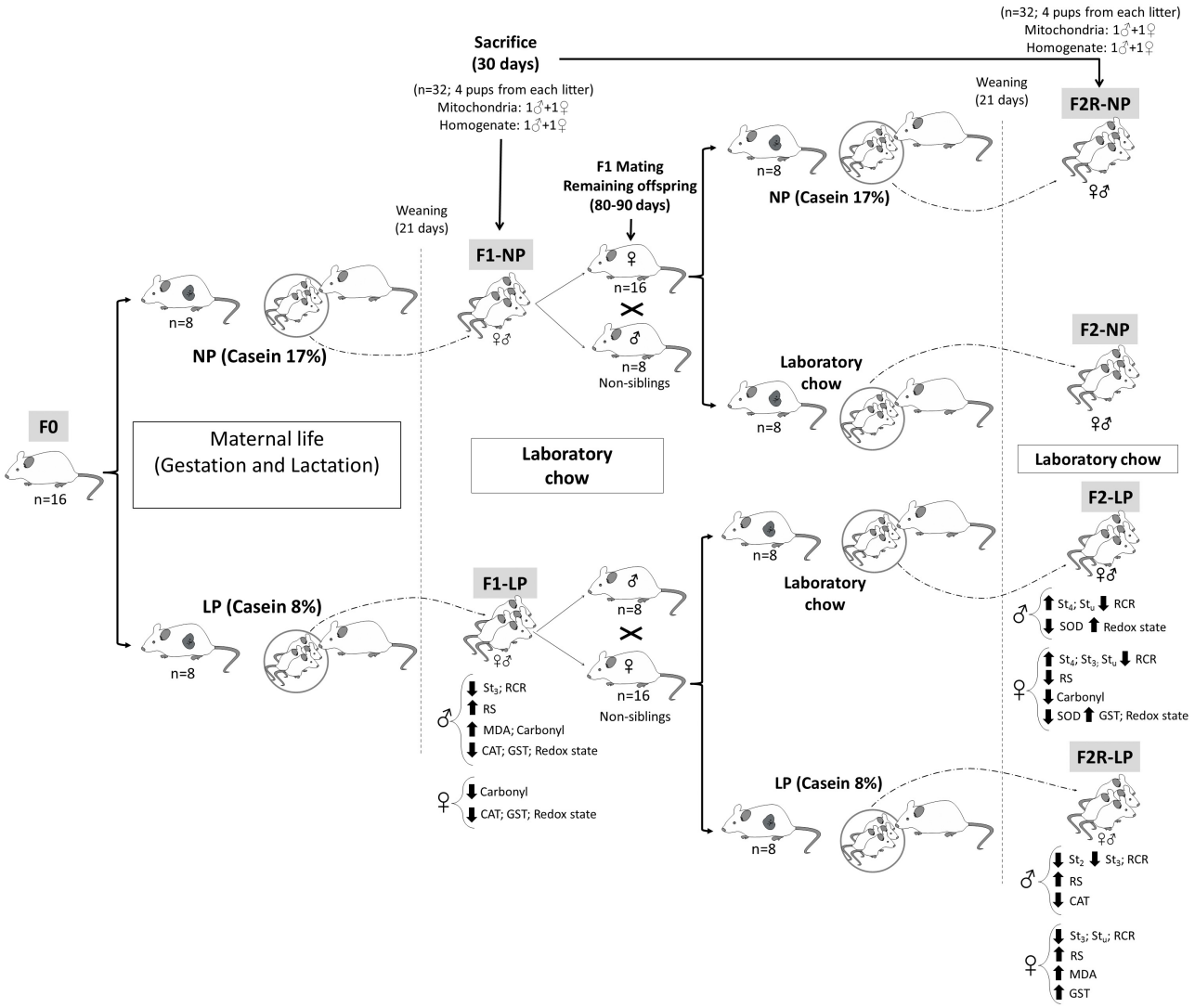

FIGURE 1 | Diagram of the experimental design. Normal protein diet (NP); Low-protein diet (LP); Female rats before mating (F0); First generation offspring of mothers that were fed either normal protein (F1-NP) or low-protein (F1-LP) diets; Offspring of F0 grandmothers that were fed normal protein (F2-NP) or low-protein (F2-LP) diets; Offspring of two successive female generations (grandmothers and mothers) that were fed normal protein (FR2-NP) or low protein (FR2-LP) diets; Basal consumption of O_2 by the mitochondria (St_2); Mitochondrial consumption of O_2 when stimulated with ADP (St_3); Mitochondrial consumption of O_2 following ATP synthase inhibition (St_4); O_2 consumption by an uncoupled mitochondria (St_u); Respiratory Control Ratio (RCR); Reactive species (RS); Biomarker of oxidative damage to lipids (MDA); The enzymatic activities of- Superoxide dismutase (SOD); Catalase (CAT); and Glutathione-S-transferase (GST).

The results were expressed as a percentage of H_2DCF-DA fluorescence yielded.

Sample Preparation for Oxidative Stress and Antioxidant Analyses in the Total Homogenate

Frozen brainstems were immersed in a cold buffer containing 50 mM TRIS and 1 mM EDTA, pH 7.4, with the addition of 1 mM sodium orthovanadate and 200 μg/mL phenylmethanesulfonylfluoride. Samples were then homogenized with an IKA@ RW20 digital homogenizer using a potter-Elvehjem pestle in a glass tube on ice. Homogenates were centrifuged at 1,180 × *g* for 10 min at 4°C. Protein concentration of the supernatants was determined by the Bradford method which measures absorption at 595 nm at RT (Bradford, 1976).

Evaluation of Lipid Peroxidation (MDA Levels)

Lipid peroxidation was analyzed using malondialdehyde (MDA) levels, as previously published (Buege and Aust, 1978). Three hundred μg protein was sequentially mixed to in 30% (w/v) trichloroacetic acid (TCA) and 10 mM TRIS buffer at 30°C, pH 7.4. This mixture was centrifuged at 2,500 × *g* for 10 min, and the supernatant was boiled for 15 min with 0.73% (w/v) thiobarbituric acid. The resulting pink pigment was then measured at 535 nm

absorption at RT and the extinction coefficient used to determine the MDA level.

Determination of Protein Oxidation (Carbonyl Content)

Protein oxidation was assessed using the procedures outlined by Reznick and Packer (1994). Briefly, the samples were placed on ice and 30% (w/v) TCA was added, and the mix was then centrifuged for 14 min at 1,180 × *g*. The pellet was re-suspended in 10 mM 2,4 dinitrophenylhydrazine and immediately incubated in a dark-room for 1 h with agitation every 15 min. Samples were washed and centrifuged three times in ethyl/acetate buffer, and the final pellet was re-suspended in 6 M guanidine hydrochloride, incubated for 30 min at 37°C and the absorbance read at 370 nm. Results were expressed as a percentage of the control group value.

Measurement of Total Superoxide Dismutase (SOD) Activity

Total SOD activity was evaluated following the protocol developed by Misra and Fridovich (1972). In brief, 300 μg of protein was added to 50 mM carbonate buffer with 0.1 mM EDTA, pH 10.2, and the reaction was started with the addition of 150 mM epinephrine. SOD activity was determined by measuring the inhibition epinephrine auto-oxidation at 30°C for 1.5 min as indicated by the decrease in absorbance at 480 nm. Results expressed as a percentage of the control group value.

Measurement of Catalase (CAT) Activity

The assay for CAT activity was performed as previously described by Aebi (1984). Briefly, 0.3 M hydrogen peroxide and 300 μg protein were added to a 50 mM phosphate buffer, pH 7.0 at 20°C and absorption decay in the mix was monitored for 3 min at 240 nm to indicate CAT activity. Results were expressed as a percentage of the control group value.

Measurement of Glutathione-S-Transferase (GST) Activity

GST activity was measured as described previously by Habig and Jakoby (1981). Three hundred μg of protein was incubated in a 0.1 M phosphate buffer, pH 6.5 containing 1 mM EDTA at 30°C. After the addition of 1 mM 1 chloro-2.4-dinitrobenzene and 1 mM reduced glutathione (GSH), the formation of 2.4-dinitrophenyl-S-glutathione was followed for 1 min at 340 nm. Results were expressed as a percentage of the control group value.

Measurement of Reduced Glutathione (GSH) and Oxidized Glutathione (GSSG)

To assess GSH levels, the samples were diluted (1:10) in a 0.1 M phosphate buffer containing 5 mM EDTA, pH 8.0, and an aliquot from the diluted sample was incubated with *o*-Phthaldialdehyde (OPT) at RT for 15 min. After incubation, the fluorescence intensity of the mix was measured at 420 nm emission and 350 nm excitation and then GSH in each sample was determined by comparison to a standard curve of known GSH concentrations. To determine GSSG levels, the samples were incubated with 0.04 M *N*-ethylmaleimide for 30 min at RT followed by addition of 0.1M NaOH, and after incubation, fluorescence measurements and calculations of GSSG levels were performed as described in the GSH assay above. The results for GSH and/or GSSG levels were expressed in units of μmol/mg protein. Redox state was determined by the ratio of GSH/GSSG as previously described (Hissin and Hilf, 1976).

Statistical Analyses

All values are expressed as mean ± SEM. Once the data were tested for normal distribution (Kolmogorov–Smirnov), either the unpaired Student's *t*-test or the Mann–Whitney test (depending on normality) was employed to assess the significance of differences between groups. Comparisons were considered statistically significant at $p \leq 0.05$. All statistical analyses were performed using GraphPad Prism 6.0® software (GraphPad Software, Inc.).

RESULTS

First Generation (F1)

It has been suggested that the females are more protected from oxidative damage than males. As a first step in this investigation, we addressed a possible gender influence on oxidative parameters in F1 rats born and nursed by normoprotein mothers. Our findings corroborate the existing literature on this subject by demonstrating that female F1 offspring from the normoprotein group have lower levels of oxidative biomarkers (i.e., oxidized lipid and protein), and enhanced antioxidant capacity (CAT activity and Redox status) in the brainstem compared with male offspring in the same cohort (**Supplementary Data**). After determining a protective effect of female gender on oxidative parameters in F1 animals, we addressed the effects of maternal low-protein diet in the brainstem of male and female in the subsequent (F2) generation.

In male offspring of the first generation, the basal state of the LP group showed higher O_2 consumption than the NP group (F1-NP: 6.95 ± 0.81 vs. F1-LP: 9.65 ± 0.89 nmol O_2/mL; $p = 0.0497$; $n = 6$–7) (**Figure 2A**). However, when stimulated with ADP, the LP animals were unable to increase their O_2 consumption as the NP animals did, culminating in a lower state 3 for the LP group (F1-NP: 42.40 ± 3.76 vs. F1-LP: 26.18 ± 3.62 nmol O_2/mL; $p = 0.013$; $n = 5$-6) (**Figure 2A**). Although no further differences were found across the coupling states, the reduced responsiveness to ADP in the LP offspring lowered the RCR (F1-NP: 6.81 ± 0.62 vs. F1-LP: 3.13 ± 0.68; $p = 0.004$; $n = 5$) (**Figure 2A**). Additionally, the F1-LP male offspring produced more reactive species than the F1-NP group (F1-NP: 100.0 ± 10.21 vs. F1-LP: 189.8 ± 22.48 percentage compared to the control; $p = 0.0066$; $n = 5$) (**Table 1**). As result, the male offspring exhibited increased oxidative damage to both lipids and proteins (Lipids - F1-NP: 100.0 ± 4.32 vs. F1-LP: 146.9 ± 7.61 percent MDA compared to the control; $p = 0.0007$; $n = 5$; proteins - F1-NP: 100.0 ± 9.72 vs. F1-LP: 190.6 ± 33.42 percent carbonyl compared to the control; $p = 0.0264$; $n = 6$) (**Table 1**). The augmented oxidative stress is consistent with the significant decreases in both enzymatic (**Figure 2B**) and non-enzymatic (**Figure 2C**) antioxidant systems ($n = 5$–6) observed

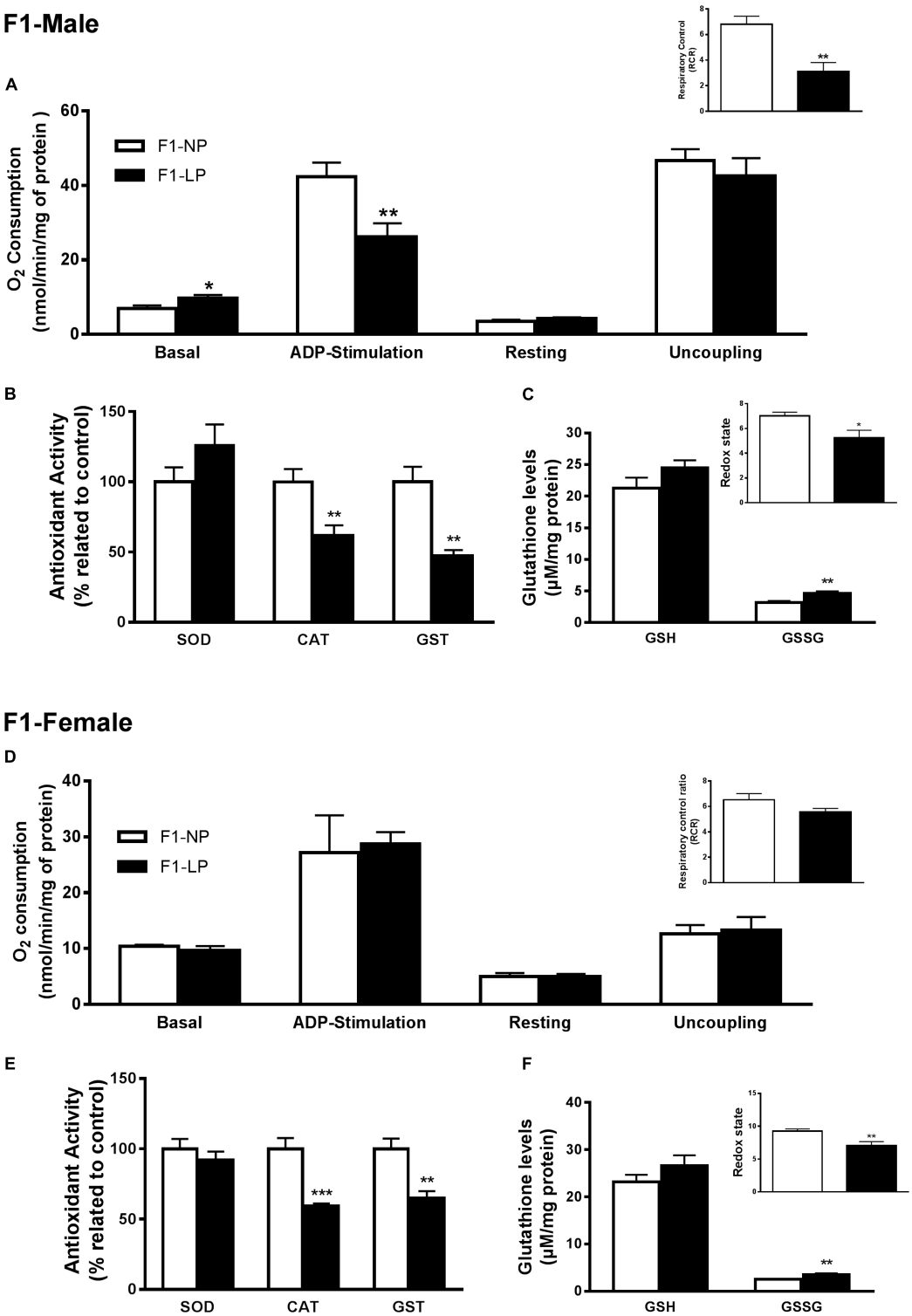

FIGURE 2 | Effects of maternal protein restriction in the brainstem metabolism of first generation (F1) progeny. Male offspring of mothers that were fed either normal protein or low protein diets (F1-NP vs. F1-LP, respectively): mitochondrial O_2 consumption and respiratory control ratio **(A)**; enzymatic antioxidant capacity **(B)**; non-enzymatic antioxidant system and redox state **(C)**. Data are presented as mean ± SEM. $^*p \leq 0.05$. $^{**}p \leq 0.01$; $^{***}p \leq 0.001$ by unpaired Student's *t*-test (n = 4–8). Female offspring of normal protein vs. low protein mothers (F1-NP vs. F1-LP, respectively): mitochondrial O_2 consumption and respiratory control ratio **(D)**; enzymatic antioxidant capacity **(E)**; non-enzymatic antioxidant system and redox state **(F)**. Data are presented as mean ± SEM. $^*p \leq 0.05$. $^{**}p \leq 0.01$; $^{***}p \leq 0.001$ using an unpaired Student's *t*-test (n = 4–8).

TABLE 1 | Production of reactive species (RS) and oxidative biomarkers in the brainstem of male and female progeny of F0 mothers that were protein restricted during gestation and the lactation.

		Male		Female	
		NP	**LP**	**NP**	**LP**
F1	RS	100.0 ± 10.21	189.8 ± 22.48**	100.0 ± 7.86	75.64 ± 13.63
	MDA	100.0 ± 4.32	146.86 ± 7.61***	100.0 ± 9.40	113.26 ± 8.51
	Carbonyl	100.0 ± 9.72	190.60 ± 33.42*	100.0 ± 5.09	76.05 ± 1.99**
F2	RS	100.0 ± 8.17	93.62 ± 8.32	100.0 ± 5.26	77.49 ± 7.26*
	MDA	100.0 ± 6.56	86.85 ± 15.94	100.0 ± 4.62	90.04 ± 3.16
	Carbonyl	100.0 ± 2.42	88.47 ± 13.25	100.0 ± 9.06	72.10 ± 3.26*
F2R	RS	100.0 ± 14.05	285.5 ± 22.93**	100.0 ± 6.64	239.9 ± 37.42**
	MDA	100.0 ± 12.5	106.63 ± 15.47	100.0 ± 13.83	173.78 ± 16.21**
	Carbonyl	100.0 ± 4.20	98.87 ± 5.72	100.0 ± 9.15	73.38 ± 9.54

*F1, first generation progeny; F2, second generation progeny; F2R, second generation progeny re-exposed to nutritional insult during development by protein restriction of their F1 mothers; RS, reactive species; MDA, lipid peroxidation. Data are expressed as a percentage of the control group ± SEM; n = 4–6. *$p \leq 0.05$, **$p \leq 0.01$, and ***$p \leq 0.001$.*

in male F1 offspring with an LP background compared to those with an NP background.

In marked contrast to the males, female offspring of the first generation showed no differences in either mitochondrial coupling states (**Figure 2F**) or the production of RS (**Figure 2D**) between NP and LP individuals. Interestingly, however, the F1-LP female offspring showed increased protection from oxidative damage to protein (F1-NP: 100.0 ± 5.09 vs. F1-LP: 76.06 ± 1.99 percentage carbonyl compared to the control; $p = 0.0024$; $n = 5$) (**Table 1**) even though their antioxidant capacity was reduced ($n = 5$–6) (**Figures 2E,F**).

Second Generation

Our findings for the F2 groups showed that impairments in mitochondrial bioenergetics induced by a maternal low-protein diet were extended to the second generation while the non-enzymatic capacity increased, possibly as a compensatory mechanism for the oxidative damage. In males of the F2 generation, no differences were found in either the basal or ADP-stimulated states, although both the resting and uncoupled states were increased in F2-LP offspring compared to NP offspring (F2-NP: 4.74 ± 0.15 vs. F2-LP: 25.8 ± 3.30 nmol O_2/mL; $p < 0.0001$) and (F2-NP: 19.29 ± 0.84 vs. F2-LP: 38.26 ± 2.78 nmol O_2/mL; $p = 0.0002$), respectively ($n = 5$–7) (**Figure 3A**), lowering the RCR in the LP animals (F2-NP: 11.26 ± 0.64 vs. F2-LP: 9.13 ± 0.57; $p = 0.0294$; $n = 7$) (**Figure 3A**). In regard to oxidative balance, our data demonstrate similar RS production as well as oxidative biomarkers in the F2-NP and F2-LP groups ($n = 5$) (**Table 1**). On the other hand, the F2-LP group showed a lower capacity to deal with superoxide ($n = 6$) (**Figure 3B**) that might be offset by the increase in the redox status ($n = 4$) (**Figure 3C**).

Female LP offspring, despite showing a higher phosphorylation capacity in the ADP-stimulated state (F2-NP: 50.80 ± 1.42 vs. F2-LP: 64.55 ± 6.9 nmol O_2/mL; $p = 0.0005$), and higher basal and uncoupling states ($n = 4$–6) had a decreased respiratory control ratio, which represents an overall decline in mitochondrial capacity (F2-NP: 7.77 ± 0.72 vs. F2-LP: 3.75 ± 0.33; $p = 0.0001$; $n = 5$) (**Figure 3D**). Regarding oxidative balance, females of the F2 generation down-regulated their RS production ($n = 5$–6) (**Table 1**), thereby protecting proteins from oxidative damage ($n = 5$) (**Table 1**) by dealing with the general electrophilic compounds ($n = 4$–5) (**Figures 3E,F**).

Second Generation Re-exposed to Low Protein or Normoprotein

Several studies suggest that chronic population-wide conditions in (such as malnutrition) affecting individuals of reproductive age can result in increased susceptibility to those conditions in their F1 offspring and in subsequent generations. To investigate this hypothesis in our model, we evaluated oxidative parameters in the F2 progeny of F1 rats that were exposed to the effects of a low-protein diet during their early development, and then were themselves exposed to the same low protein diet during their own gestation and nursing periods. In male offspring 're-exposed' to a low-protein diet, mitochondria increased their consumption of O_2 in state 2 (F2R-NP: 5.18 ± 0.51 vs. F2R-LP: 8.011 ± 0.65 nmol O_2/mL; $p = 0.007$; $n = 4$-5) and decreased the both ADP-stimulated state (F2R-NP: 40.64 ± 2.52 vs. F2R-LP: 31.16 ± 3.0 nmol O_2/mL; $p = 0.042$; $n = 4$-5) and the RCR (F2R-NP: 8.01 ± 0.53 vs. F2R-LP: 4.27 ± 0.25; $n = 5$) compared to control (**Figure 4A**). Following re-exposure to LP, RS production increased markedly in LP males (F2R-NP: 100.0 ± 14.05 vs. F2R-LP: 285.5 ± 22.93 percentage compared to the control; $p < 0.0001$; $n = 5$) (**Table 1**). However, no differences were found in biomarkers of either lipid or protein oxidation ($n = 4$–6) (**Table 1**) or in antioxidant parameters ($n = 4$–6) (**Figures 4B,C**).

In contrast to males of the F2 generation, when female offspring were re-exposed to an LP diet, their mitochondria decreased the O_2 consumption in most of the respiratory states ($n = 6$) (ADP-stimulated, $p < 0.0001$; resting, $p = 0.0003$ and uncoupling, $p = 0.001$), culminating in the decrease of respiratory control ratio and RCR ($p < 0.0001$; $n = 6$) (**Figure 4D**). As in the males, however, low-protein re-exposure of F2 females promoted RS overproduction (F2R-NP: 100.0 ± 6.64 vs. F2R-LP: 239.9 ± 37.42 percentage compared to the control; $p = 0.0043$; $n = 4$–5) (**Table 1**) which in the females was accompanied by an increase in lipid damage (F2R-NP: 100.0 ± 13.84 vs.

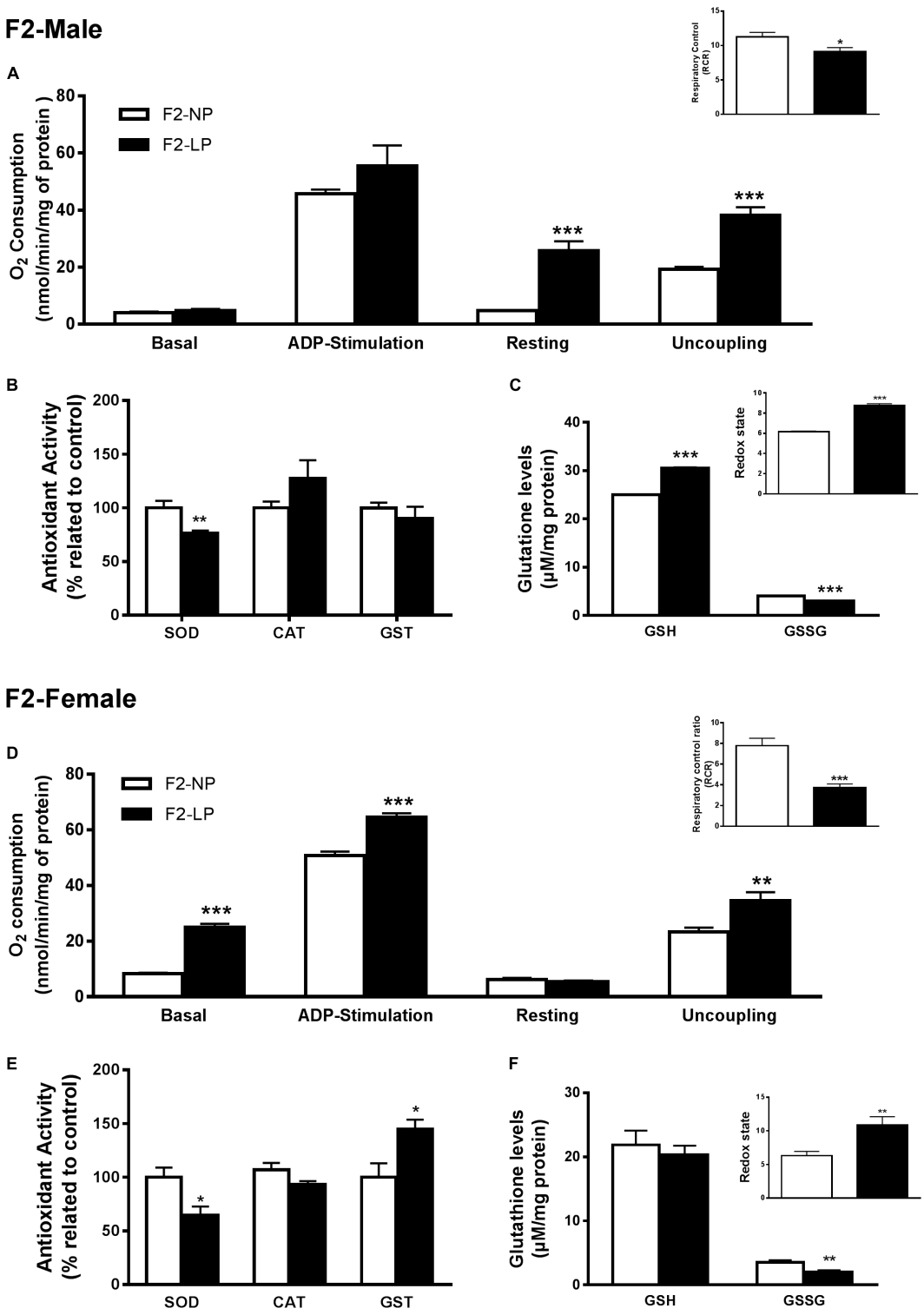

FIGURE 3 | Effects of maternal protein restriction in the brainstem metabolism of the second generation (F2) progeny. Male offspring of F0 grandmothers that were fed normal protein vs. low protein diets (F2-NP vs. F2-LP, respectively): mitochondrial O_2 consumption and respiratory control ratio **(A)**; enzymatic antioxidant capacity **(B)**; non-enzymatic antioxidant system and redox state **(C)**. Data are presented as mean ± SEM. $*p \leq 0.05$. $**p \leq 0.01$; $***p \leq 0.001$ using an unpaired Student's *t*-test ($n = 4–8$). Female offspring of F0 grandmothers that were fed normal protein vs. low protein diets (F2-NP vs. F2-LP, respectively): mitochondrial O_2 consumption and respiratory control ratio **(D)**; enzymatic antioxidant capacity **(E)**; non-enzymatic antioxidant system and redox state **(F)**. Data are presented as mean ± SEM. $*p \leq 0.05$. $**p \leq 0.01$; $***p \leq 0.00$l using an unpaired Student's *t*-test ($n = 4–8$).

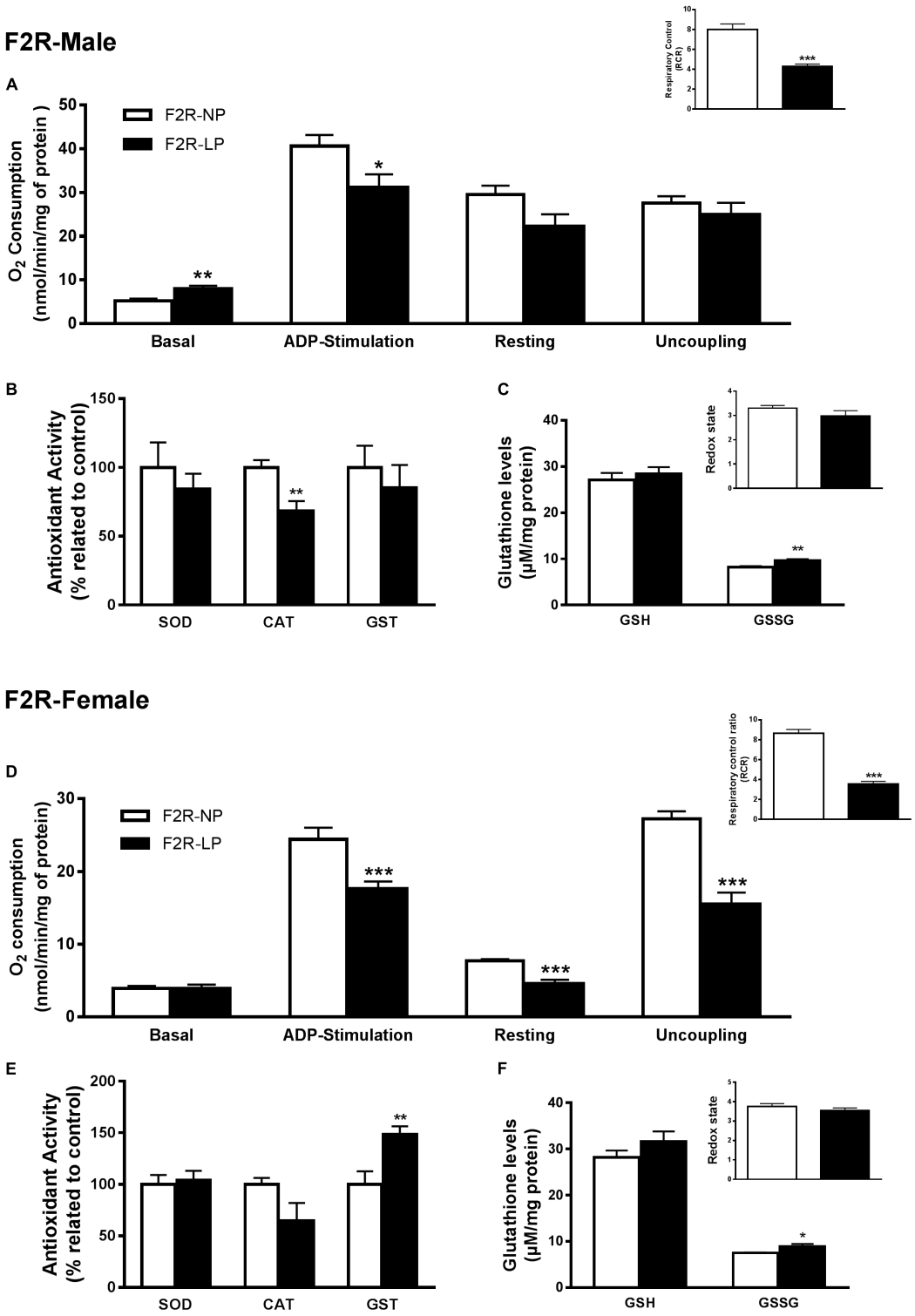

FIGURE 4 | Effects of maternal F0 protein restriction in the brainstem metabolism of second generation (F2) progeny, reinforced by re-imposition of low protein during their gestation and early development. Male offspring of successive female generations (grandmothers and mothers) that were fed normal protein vs. low protein diets (FR2-NP vs. FR2-LP, respectively): mitochondrial O_2 consumption and respiratory control ratio **(A)**; enzymatic antioxidant capacity **(B)**; Non-enzymatic antioxidant system and redox state **(C)**. Data are presented as mean ± SEM. $*p \leq 0.05$. $**p \leq 0.01$; $***p \leq 0.001$ using an unpaired Student's *t*-test (n = 4–8). Female offspring of successive female generations (grandmothers and mothers) that were fed normal protein vs. low protein diets (FR2-NP vs. FR2-LP, respectively): mitochondrial O_2 consumption and respiratory control ratio **(D)**; enzymatic antioxidant capacity **(E)**; non-enzymatic antioxidant system and redox state **(F)**. Data presented as mean ± SEM. $*p \leq 0.05$. $**p \leq 0.01$; $***p \leq 0.001$using an unpaired Student's *t*-test (n = 4–8).

F2R-LP: 173.8 ± 16.21 MDA percentage compared to the control; $p = 0.0085$; $n = 5$) (**Table 1**), despite the up-regulation of GST activity ($p = 0.0122$; $n = 4$-5) (**Figure 4E**), and unchanged non-enzymatic defense (**Figure 4F**).

Information regarding the animal body composition, and additional statistic results can be accessed in the **Supplementary Data**.

DISCUSSION

In the present study, we have employed a maternal low-protein diet model to assess the effect of the protein deprivation on the mitochondrial bioenergetics and oxidative balance in the brainstems of progeny born to the protein-deprived mothers, and in the brainstems of rats in the succeeding generation. Furthermore, we evaluated whether the F2 offspring born and nursed by protein-deprived mothers can compensate for oxidative damage when they themselves are 're-exposed' to the same low-protein nutritional insult. Our findings demonstrate that maternal protein restriction disrupts mitochondrial coupling states in all experimental groups except females of the F1 generation. We hypothesize that despite the impairments to mitochondrial function, F2 animals were able to avoid oxidative damage by altering their redox status in response to renewed protein restriction.

Compelling evidence has suggested a relationship between mitochondrial dysfunction and oxidative stress in the brain, and the neuropathogenesis of hypertension (Peterson et al., 2006; Hirooka, 2008; Lopez-Campistrous et al., 2008; Chan and Chan, 2013). Studies from our laboratory have demonstrated that maternal protein restriction during gestation and lactation disrupts mitochondrial bioenergetics, increases RS production, and impairs redox homeostasis in the young and adult brainstem, increasing the risk in those progeny of developing autonomic activity-related hypertension in later life (de Brito Alves et al., 2014; Barros et al., 2015; Ferreira et al., 2015, 2016b; de Brito Alves et al., 2016; de Sousa et al., 2017).

According to Guyenet (2006), impairments in either afferent or efferent autonomic pathways are the underlying cause of neurogenic hypertension, a blood pressure defect characterized by a flaw in autonomic control rather than a dominant renal or vascular irregularity. As previously demonstrated, at 30 days of life, F1-LP male offspring exhibit higher respiratory frequency and ventilation-related chemosensitivity than NP individuals, however, no change in blood pressure was observed (de Brito Alves et al., 2014). Maximo Cardoso et al. (2006) demonstrated that an increase in reactive oxygen species in the fourth ventricle induces pressor responses by unbalancing autonomic control. In spontaneously hypertensive rats, Chan et al. (2006) and Nishihara et al. (2012) demonstrated that the reduction in antioxidant capacity in the rostral ventrolateral medulla (RVLM), a brainstem nucleus involved in the efferent arm of pressure control, contributes to neurogenic hypertension. Further studies from Chan's laboratory suggested that an impairment in the mitochondrial electron transport chain (ETC), in addition to mitochondrial uncoupling-induced oxidative stress, could contribute to the development of neurogenic hypertension (Chan et al., 2009a,b). Here, we demonstrate that young male F1-LP rats overproduce RS while exhibiting decreased antioxidant capacity. In addition, we observe decreased mitochondrial phosphorylative capacity (low RCR and state 3) that would deprive the brainstem of energy and thus suggest an autonomic imbalance in male offspring.

In isolated mitochondria, energy metabolism relies on three components - substrate oxidation, ATP turnover, and proton leak - which together, control the outward and inward transport of protons across the mitochondrial inner membrane (Hafner et al., 1990). Substrate oxidation involves reactions that prompt the mitochondrial proton motive force (pmf) (substrate uptake, metabolism and electron transport). ATP turnover refers to the reactions that employ the pmf to phosphorylate ADP to ATP and to export it, while proton leak employs the pmf without yielding ATP (Hafner et al., 1990; Brand and Nicholls, 2011). In the brain, Olorunsogo (1989) described how protein restriction affects substrate oxidation by decreasing dehydrogenase activities (e.g., isocitrate and succinate), which in turn could diminish supplies of critical co-factors to the mitochondrial ETC. The authors also stated that the mitochondria of LP animals had decreased their capacity for coupling electron transport, proton pumping, and ATP synthesis, possibly due to the decrease in the cytochrome C oxidase activity in rats with an LP background. Ferreira et al. (2016a) demonstrated that, at 22 days of life, lower phosphorylative capacity in the male F1-LP brainstem is associated with a disruption in mitochondrial membrane potential due to the deceleration of the Krebs cycle and the lower mitochondrial co-factor supply. Such impairments could persist to at least 30 days of life (the age of sacrifice for F1 animals in the present study). However, further studies must be done to address how the maternal LP diet affects mitochondrial components involved in the pmf maintenance in her offspring.

In the present study, we've shown that F1-LP mitochondria in the basal state consume more O_2 than the normal rats, indicating the occurrence of mitochondrial uncoupling (Parker, 1965). Moreover, we've shown that the different responsiveness to ADP stimulation is not related to the ATP synthase, since the consumption of O_2 across the resting and uncoupling states are unaltered. In the adult LP offspring, however, both states following state 3 consumed more O_2 than in the NP offspring, especially with complex II, suggesting the presence of defects in ATP synthase (Ferreira et al., 2018). In the present study, since, mitochondria appear to respirate equally in the last two states evaluated, we believe as do Olorunsogo (1989), that mitochondria in LP individuals may have lost their capacity for coupling to electron transport, the proton pump and the ATP synthesis. Studies with hypertensive rats have demonstrated similar patterns. Chan et al. (2009b) describe a flaw in the electron coupling across the mitochondrial complexes in the RVLM, while Lopez-Campistrous et al. (2008) point to a dysfunction in brainstem complex I (anti Fe–S protein expression and complex activity).

Regarding mitochondrial bioenergetics in the female offspring, it is recognized that genomic and phenotypic sex differences assure the female a degree of cardioprotection that

is unavailable to the males (Lagranha et al., 2010). Rodriguez-Rodriguez et al. (2015) argued that the more favorable global plasma antioxidant status established in malnourished females during perinatal life can contribute to their protection against hypertension in adulthood. In the present study we show that, despite the decrease in antioxidant capacity occurring in the young female F1-LP group, both the mitochondrial bioenergetics and the production of RS were unchanged in face of the nutritional insult.

Estrogens regulate oxidative phosphorylation and mitochondrial oxidative stress in the CNS (Gaignard et al., 2018). Furthermore, according to Rettberg et al. (2014), estrogen modulates glucose transport, the glycolytic pathway, the activity of pyruvate dehydrogenase, which is essential in linking glycolysis and the Krebs cycle and other dehydrogenases including succinate and alpha ketoglutarate dehydrogenase, as well as ETC complexes I, III, and IV. Therefore, it seems likely that in females with an LP background, estrogen-modulated bioenergetics (substrate oxidation module) in the brain could protect against detrimental outcomes found in male LP animals, even with the observed reduction in antioxidant capacity.

In regard to the F2 offspring, although several studies have demonstrated an effect of nutritional insult during maternal life in their health, of their F1 offspring, and in the succeeding F2 generation (Reyes-Castro et al., 2015; Saben et al., 2016; Ambeskovic et al., 2017), few reports have evaluated the multigenerational impact of a maternal low-protein diet on parameters related to CVD risk. Harrison and Langley-Evans (2009) deprived pregnant rats of protein (18 vs. 9%) and found that low-protein offspring had higher arterial blood pressure in the following two generations. They also reported that despite an increased blood pressure in the offspring of both sexes, in protein deficient animals, the F1 females had lower arterial blood pressure than the males, corroborating a cardioprotective effect of estrogen in the immediate descendants of protein-deprived females. On the contrary, in the F2 generation, no influence of gender was observed, with blood pressure increased in all LP offspring up to 6 weeks of age. Torrens et al. (2008), using much the same protocol as Harrison and Langley-Evans (2009) also reported that LP offspring in the second generation (at 100 days of age) have higher blood pressure than NP offspring, a result that they associated with endothelial dysfunction.

In the present study, our results demonstrated that F2-LP rats of both sexes responded to a normal laboratory diet in their adulthood by recovering from the nutritional insult suffered by their F1 antecedents. Despite the lower respiratory control in F2 animals of both genders, it is noteworthy that LP mitochondria exhibited increased O_2 consumption in the ADP-stimulation state, thereby equalizing O_2 consumption between the NP- and LP- F2 male groups, while allowing higher O_2 consumption in the females. Moreover, the high O_2 consumption observed in the uncoupling state suggests an effort by the brainstem mitochondria to deal with oxidative stress (Mailloux and Harper, 2011). It has been shown that uncoupling proteins regulate RS production by modulating the mitochondrial membrane potential (Azzu and Brand, 2010), which might, in turn, result in the increased resistance of CNS neurons to metabolic and oxidative stress through an adaptive shift in energy metabolism (Liu et al., 2006). Furthermore, we show in this investigation that antioxidant capacity is up-regulated in F2-LP progeny, wherein both CAT and GST activity were restored (i.e., F1 vs. F2 pattern), which together with the increase in the redox status, kept oxidative damage in the F2-LP-animals under control. Banos-Gomez et al. (2017) demonstrated that offspring from undernourished grandmothers increased their CAT activity, thereby preventing oxidative damage to the brain. This would suggest an adaptation in the activity of at least one enzymatic antioxidant mechanism that reduces oxidative damage in the second filial generation.

Evidence from the present study suggests that in at least two filial generations following a maternal low-protein insult, brainstem mitochondria may be recovering, or attempting to recover from the detrimental effects programmed by their antecedents by increasing their resilience to peroxides. In regard to possible mechanisms underlying this recovery, Saben et al. (2016) demonstrated that a high-fat/high-sugar diet increases oxidative stress, disrupts mitochondrial function, and alters organellar shape in response to mitochondrial dynamics in the skeletal muscle of mice persisting across three generations, suggesting a possible explanation for the persistence of changes in oxidative parameters that we see in the present report. Moreover, the mitochondrial impairments observed by Saben et al. (2016) were progressively attenuated with each succeeding generation, reinforcing the idea of a gradual recovery/resilience following a one-time occurrence of oxidative damage.

Despite the apparent multigenerational recovery of brainstem mitochondria from an 'acute' instance of maternal protein impairment in the F0 generation, however, it appears that F2 offspring in our study remain predisposed to mitochondrial malfunction and oxidative impairments if they themselves experience a low-protein environment during gestation and nursing. This was demonstrated when the re-exposure of second generation animals to the low protein diet (i.e., F2R-LP) increased the production of RS and reduced the O_2 consumption in the phosphorylated state, regardless of gender. This observation would suggest that during the period of brain development in the F2 generation, a new nutritional insult such as low protein can modify the reparative mitochondrial reprogramming of the brainstem that would normally occur in the second generation post-insult, and result in increased RS and oxidative damage. Furthermore, the damage to F2 generation mitochondria does not appear to be prevented or ameliorated by estrogen, in stark contrast to LP animals of the F1 generation, where a relative resistance to oxidative damage was detected in the female offspring. The higher levels of lipoperoxidation and the lower O_2 consumption in the mitochondrial uncoupled state in F2-LP females would suggest that the ability of the estrogen to protect mitochondrial function is partially or fully impaired if low-protein is encountered not only in the grandmother's generation, but in the mother's generation as well. According to Rodell et al. (2013), mitochondrial function/morphology may fluctuate between maladaptive episodes and adaptive specialization, wherein mitochondrial selection over the course of several generations improves the overall metabolism in an

'attempt' to correct the insult that was suffered. The specific mechanisms involved in these effects are unclear, and will need to be investigated in future studies, but those mechanisms could involve epigenetic imprints that can be transferred to the offspring via both nuclear and mitochondrial transcription.

In the present study, since we used male and female from the same nutritional background (i.e., NP or LP) to produce the F2 generation, it remains possible that, the paternal germline could also influence the findings described herein. In this regard (Reyes-Castro et al., 2015) have demonstrated that LP male rats give rise to offspring with different anxiety-related behaviors the appeared to depend on the sex of the progeny. On the other hand, recent, studies have described the possibility of the paternal inheritance of mitochondrial DNA (mtDNA) (Breton and Stewart, 2015; Luo et al., 2018), raising additional possibilities for the inheritance of nutrition-related mitochondrial defects, and challenging the dogma of exclusively matrilineal mtDNA inheritance.

CONCLUSION

Our results indicate that a maternal low-protein diet during pregnancy and lactation affects mitochondrial metabolism in the brainstems of F1 progeny of both sexes. However, the response in the male was the overall elevation of oxidative parameters, whereas in the female, both RS and protein oxidation were reduced compared to the control, suggesting an estrogen-dependent protection from nutrition-induced damage in the F1 generation. Moreover, we show that despite the recovery of oxidative balance in the F2 generation of LP rats with normal nutrition in the succeeding generation, the LP-F2 progeny that were 're-exposed' to poor nutrition via their mothers showed marked elevations in RS and in lipid peroxidation. This would suggest that F2 animals with an LP background via their grandmothers remain especially vulnerable to oxidative damage due to later episodes of malnutrition. Results such as these suggest a means by which mitochondrial damage due to malnutrition can span several generations, and leads us to hypothesize a mechanism involving disruption of normal mitochondrial bioenergetics due to impaired ATP synthesis linked to electron coupling-related ADP phosphorylation. Further study will be required, however, to fully investigate and integrate the biochemical parameters that contribute to the transgenerational inheritance of nutrition-dependent mitochondrial damage that eventually leads to CVD.

AUTHOR CONTRIBUTIONS

DS conducted the majority of the experiments and analyzed the data. GB and DF conducted several experiments together with DS. TdAS and SS helped with some experiments and take care of the animals. DG and MF helped in the discussion of the experimental design, and with the discussion of the results. DF, BA-d-C, and CL helped with the construction of the experimental design, analyzed the data, discussed the results and manuscript construction.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2019.00203/full#supplementary-material

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Effect of GABA-Fortified Oolong Tea on Reducing Stress in a University Student Cohort

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GABA-containing tea has gained popularity as an accessible intervention to reduce the impact of chronic stress-induced autonomic imbalance and increased risk for cardiovascular disease despite a lack of evidence concerning the γ-aminobutyric acid (GABA) content in a cup of the tea and its effects on physiological and psychological stress as measures of cognitive function. We aimed to measure the effects of GABA-fortified tea consumption on heart rate variability (HRV) and stress in 30 participants using a pre-post cohort study design. Ten minute lead II ECG recordings were analyzed with Kubios software. Frequency domain parameters including total power, high and low frequency power, along with heart rate, were determined. A control group that consumed a non-fortified tea was included in the research. Statistical analysis was by two-way ANOVA for two-group comparison with time as an interaction and a significance level of $p < 0.05$. Oolong tea consumption led to a significant decrease in the immediate stress score and a significant improvement in HRV. We conclude that autonomic imbalance and HRV in people with acute stress is significantly reduced following a cup of GABA fortified oolong tea and highlights the complex interaction between autonomic nervous system function and mood.

Keywords: oolong tea, GABA, heart rate variability, stress, autonomic nervous system

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INTRODUCTION

Stress is defined as a disruption of the body's homeostasis. Activation of the stress response is crucial for survival by enabling an organism to cope with and adapt to internal and external factors. Stressors may be psychological or physiological, or both. The body's ability to maintain homeostasis in the face of continually changing environmental circumstances and stressors is controlled in part by the autonomic nervous system, and the balance between parasympathetic and sympathetic innervation of viscera, vasculature, the heart, skeletal muscle, and the control of energy metabolism (1). Stress results in sympathetic nervous system (SNS) activation and parasympathetic nervous system (PNS) withdrawal. Input by the sympathetic and parasympathetic branches of the ANS to the heart lead to a continuous change in heart rate that can be expressed as alterations in heart rate variability (HRV). Chronic or acute stress leads to ANS imbalance and decreased HRV (2, 3). Importantly, HRV measures fluctuations in heart rate, which are used as an indicator

of autonomic involvement in the control of the heart (4–6). HRV is defined as the statistical variability of the time interval between consecutive heartbeats, that is, between successive *R* waves of an electrocardiograph (ECG) the RR interval (7). Many physiological factors are involved in controlling heart rate, including cardiac sino-atrial node innervation by SNS efferent fibers and the PNS vagus nerve, which decrease and increase the RR interval length, respectively. Each of these factors inherently produces a characteristic and distinct frequency of fluctuation in the RR interval (8, 9), with the overall RR interval series representing their summation. In this way HRV represents a sensitive indicator of adaptability to stress (10), and therefore provides a useful measure of stress response. Cognitive therapy and pharmacological intervention are the mainstream treatment options for anxiety and stress but are often not accessible to all experiencing these conditions (11).

HRV can be analyzed using frequency domain analysis, which analyses the components of the total power in sinus rhythm. Total power indicates the total variability within the heart rate tachogram and can be subdivided into high frequency and low frequency components. High frequency power indicates parasympathetic function, however low frequency power reflects a combination of sympathetic and parasympathetic activity (12).

Tea (*Camellia sinensis*) is reported to be the most widely consumed beverage worldwide (13–15) and has been shown to reduce physiological stress and anxiety, and induce relaxation (14, 16–19). Green tea consumption was shown to improve relaxation in women who enjoyed high social support at work (20), and 6 weeks of black tea consumption led to reduced cortisol levels, and increased subjective relaxation following an acute stress task in healthy non-smoking men (18). Mice exposed to green tea extract perinatally showed reduced anxiety and fear responses on the elevated plus maze (21). Further, decaffeinated tea reduced blood pressure and lowered arousal in mice (17). GABA-fortified green tea was also shown to reduce behavioral indicators of depression and stress in mice in a forced swim test (19). Tea contains numerous constituents including polyphenols such as epigallocatechin gallate (EGCG), amino acids including theanine and GABA, and purine alkaloids like caffeine (13, 14, 22) that may facilitate the effects of tea on stress and mood.

A popular intervention in complementary medicine practices to improve chronic stress involves dietary supplementation with γ-aminobutyric acid (GABA) (23). The effect of GABA on stress reduction is due to both peripheral, acting on the autonomic nervous system ganglia (24), and central processes (25). One such commercially available product is GABA-enriched oolong tea. HRV, as a measure of the stress response, provides an ideal tool with which to investigate the anti-stress or antianxiety effect of GABA fortified tea. HRV is regulated via a feedback loop by higher order nervous system processes including cortical, subcortical and brainstem areas (26). Due to this central and peripheral interaction, chronic psychological stress is able to induce changes in HRV (3). GABA content in tea or other consumables is considered an option to reduce this risk.

This study aimed to investigate the effect of GABA-enriched tea on HRV, and on subjective stress.

METHODS

All experimental procedures were conducted in a quiet room with air-conditioning set at 22° Celcius and ambient light controlled via a dimmer switch, between 10 am and 12 noon to minimize the effects of external stimuli and diurnal HRV fluctuations (27). Upon arrival, participants were randomly allocated into either a GABA-fortified oolong tea group or control group. Both groups habituated to the environment for 10 min, during which time they completed the pre-tea acute stress score as a measure of immediate stress state and Cohen's Perceived Stress Scale (PSS) (28) as a measure of chronic stress status. A 10-min baseline supine ECG was then recorded, as detailed in Experimental measures. The GABA-fortified oolong tea was then consumed over a maximal 5-min period by the GABA-fortified group. After 30 min, a second supine 10 min ECG was recorded, after which the post-tea immediate stress score was completed. The same protocol was followed for the control group who consumed a non-GABA-fortified tea.

Participants

Thirty healthy volunteers (11 males, 19 females) were recruited from the student cohort at the University of Sydney by advertisements posted around the university. Inclusion criteria were: age 18-30 years, non-smoker, no history of CVD or diabetes, not taking any medications and not pregnant. Group number was determined by a power analysis with Type 2 error set at 0.8, a median effect size and $p < 0.05$. This gave a suggested participant number of 27 (29). Thirty participants were included in the study and the female to male ratio nearly equal (not significant). Participants were instructed to refrain from consuming alcohol or caffeine or engaging in strenuous physical activity, for 3 h prior to the study. This study was carried out in accordance with the recommendations of the NHMRC Human Research Ethics Guidelines and the University of Sydney Human Research Ethics Committee with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the University of Sydney Human Research Ethics Committee (approval number: 12715).

Tea Preparation

Oolong tea was sourced commercially from Taiwan and prepared fresh for each participant by adding a standard cup volume (200 mL) of tap water at 90° Celcius to 5 g of tea leaves. The tea was allowed to steep for 10 min before being strained into a cup, left to cool for 10 min and served. This protocol was designed to the manufacturer's recommendations to replicate consumer practices. High performance liquid chromatography showed the GABA content of the GABA-enriched tea to be 2.01 mg/200 mL, while that of the regular oolong tea was 0.25 mg/200 mL.

Experimental Measures

A pre-test post-test study design was used for this research. A self-assessment, single item of immediate stress level (hereon referred to as the immediate stress score) was conducted before and after tea consumption by asking the participant to rate

"how stressed you feel right now, at this exact moment in time" on a scale of 1 ("relaxed") to 10 ("highly stressed") after participants were accustomed to the recording room and recording apparatus at a previous visit. This validated single item stress assessment was adapted from Elo et al. (30). Chronic stress levels were assessed using the PSS (28), a 14-point questionnaire validated to quantify the degree to which the participant perceived their life over the past month as stressful. Participants were divided into high or low chronic stress groups based on whether their PSS score was above or below the sample median of 25.5. HRV was the other main outcome measure determined from a 10 min ECG recording using a standard three lead configuration representing Einthoven's triangle (31). Lead I was selected as the monitoring lead unless a well-defined R wave could not be detected, whereby Lead II was used instead. Following removal of ectopic beats, time and frequency domain parameters were determined including the average heart rate, total power, high frequency and low frequency power of the beat-to-beat interval changes over the 20 min recording period.

Upon arrival, participants were provided 10 min to habituate to the environment, during which they completed the Perceived Stress Score and pre-tea acute stress score. A 10 min baseline supine ECG was then conducted. Participants were then provided 5 min to consume their allocated tea, which they were blinded to. After 30 min, a second supine 10 min ECG was conducted, after which the post-tea acute stress score was completed. During all ECGs, participants were instructed to lie with their arms by their sides with eyes open and to refrain from moving or speaking or intentionally altering their respiration. Importantly, reviewing the overall range of RR intervals from participants indicated no outliers, which would have suggested other variables such as respiration contributed to HRV measures.

Data were recorded using a PowerLab 2/20 (ADInstruments; Sydney, Australia) at a sampling rate of 1 kHz and analyzed using Chart version 7 with HRV Module (ADInstruments). A 3 Hz high-pass filter removed wandering baselines while a 45 Hz low-pass filter removed power supply interference. In accordance with recommendations of the Task Force (1996), each ECG was manually inspected for missing and incorrectly classified beats, which were corrected. Remaining ectopic beats were replaced by linear interpolation (32), a method with minimal impact on the underlying HRV structure (14), and the edited RR interval series exported to Kubios HRV version 2.0 (Biosignal Analysis and Medical Imaging Group, Department of Physics, University of Kuopio) for subsequent analyses (33). As the RR interval series intrinsically consists of samples unequally spaced in time, it was interpolated and resampled at 4 Hz to produce the equally sampled data points required by the Fast Fourier Transform (Welch window, width 256 s, 50% overlap) to determine total power (TP), low frequency power (LF), high frequency power (HF), the LF:HF ratio of the power spectral density associated with the RR interval tachogram. Frequency domain analysis was chosen as it has been shown to best represent parasympathetic and sympathovagal balance of the autonomic nervous system modulation of the heart. **Table 1** summarizes the various parameters arising from frequency domain analyses of HRV data to provide their physiological meaning with respect to stress.

TABLE 1 | Physiological meaning of HRV parameters and their relation to stress.

HRV parameter	Physiological meaning	Normal values (34)
Total power (TP)	Overall variability (ANS functionality and adaptability). Reduced under stress (35)	3466 ± 1018 ms^2
Low frequency power (LF)	Baroreflex (SNS and PNS activity depending on orthostatic state). Variable effects under stress depending on posture (26)	1170 ± 416 ms^2
High frequency power (HF)	PNS activity. Reduced under stress (26)	975 ± 203 ms^2
Ratio of low frequency to high frequency power LF/HF	SNS activity and/or sympathovagal balance (controversial). Increased under stress (36)	1.5 – 2.0

Statistics

Complete data for all participants were analyzed using SPSS version 22.0.0 (IBM; Chicago, IL, U.S.A.) and are reported as mean ± standard deviation. Normality of the data was determined by the Kolmogorov-Smirnov test. The immediate stress score, TP, LF, HF, and LF:HF were not normally distributed and thus log transformed prior to analysis. To determine the effect of tea consumption on HRV and immediate psychological stress, data were analyzed using a mixed two-way between-subject (type of tea) × within-subject (time: pre, post) ANOVA design. The repeated measure dependent variable was either the immediate stress score or the HRV parameters before and after tea consumption (herein referred to as the factor of time). Threshold for significance was set at $p = 0.05$

RESULTS

Table 1 shows the baseline characteristics for the 30 participants divided by tea consumed (collapsed across stress level) or by stress level (collapsed across tea consumed).

A significant main effect of time was found for the decrease in immediate stress score (0.547 ± 0.279 to 0.387 ± 0.256; $F = 34.422$, $p < 0.001$) and increase in TP (3.364 ± 0.276 to 3.519 ± 0.331; $F = 15.221$, $p < 0.001$), LF (2.726 ± 0.338 to 2.834 ± 0.374; $F = 5.564$, $p < 0.05$) and HF (2.852 ± 0.385 to 3.022 ± 0.397; $F = 9.343$, $p < 0.005$). **Figure 1** shows this effect of time for each participant grouped according to tea consumed.

The immediate stress score was significantly higher ($t = -4.000$, $p < 0.05$) and LF:HF significantly lower ($t = 2.234$, $p < 0.05$) in the high compared to the low stress group. No other comparisons were significant after confounder variables including age were considered. The mean immediate stress score, average RR interval, TP, LF, HF, and LF:HF before

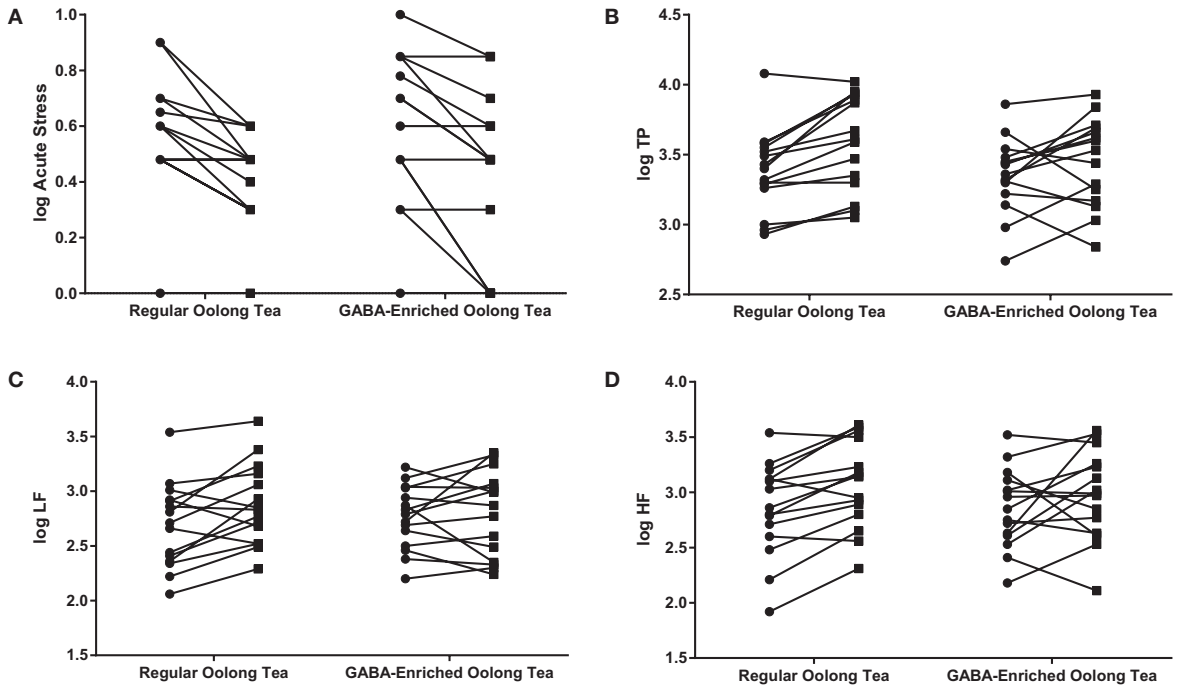

FIGURE 1 | Changes in **(A)** the acute stress score, **(B)** TP, **(C)** LF, and **(D)** HF in the GABA-enriched and regular oolong tea groups. Data are expressed as log values. Each line represents a separate participant. ●— indicates pre-tea value; —■ indicates post-tea value.

and after tea consumption, with subjects divided by tea and stress level, are shown in **Table 2**.

Only the average RR interval showed a significant time x tea x stress level interaction ($F = 4.977$, $p < 0.05$). The regular oolong tea increased the average RR interval by 83.0 and 39.2 ms, and the GABA-enriched oolong tea by 49.2 and 85.6 ms, in the low and high stress groups, respectively (**Table 3**).

DISCUSSION

This study aimed to measure the effects of GABA-fortified vs. regular oolong tea consumption on HRV and self-reported stress in 30 young participants using a pre-post cohort study design. This is not the first study examining GABA-fortified supplementation and its effect on the stress response. However, it is the largest study to report an effect of GABA-fortified oolong tea and effect on stress as measured by HRV and self-reported stress levels.

A significant increased main effect of oolong tea for HRV and acute stress score (decreased) was found. The significant time x tea x chronic stress level interaction observed for the average RR intervals suggests that in both stress groups, both teas raised the average RR intervals. An increase in RR interval reflects more stable autonomic function through an increase in vagal activity (elevated PNS activity), reflective of a reduction in stress response (8, 35). However, the greater influence of GABA-enriched tea on overall HRV (measured by a change in the RR interval) compared with regular tea for highly chronically stressed respondents suggests an additional benefit of GABA-fortification in tea for this group of individuals. The greater influence of the regular oolong tea in the low, chronic stress group further indicates that the different teas do affect autonomic cardiac control to a different extent (when chronic stress level is also considered), providing a rationale for further studies. Moreover, this finding suggests that other active constituents in oolong tea in addition to GABA have stress-reducing effects.

"The ability for GABA to reduce stress levels depends in part on its capacity to cross the blood brain barrier and to affect neurons in areas such as the amygdala, as well as in the paraventricular nucleus of the hypothalamus and monoamine brainstem nuclei where GABA inhibits sympathetic outflow (15). The finding of a GABA-transporter in the brain suggests that GABA can cross the blood brain barrier and therefore central action of GABA supplementation is likely (37). Nonetheless there are no data on GABA blood-brain-barrier permeability in humans, and whether oral GABA consumption increases its concentration in the brain. However, there is some evidence that GABA oral supplementation reaches the brain in concentrations able to exert biological effects in humans. For example, 100 mg GABA in water increased the alpha:beta EEG wave ratio compared with water alone, suggesting improved relaxation (38) and chronic GABA tea consumption increased sleep efficiency and reduced latency to sleep onset (39). Further understanding of GABA bioavailability following oral consumption is required. GABA may also reduce stress levels by acting on GABA receptors located on sympathetic pre- and post-ganglionic neurons where they inhibit noradrenaline activity (18–21).

TABLE 2 | Sample characteristics at baseline as divided by tea consumed or chronic stress level.

Parameter	Tea consumed		Chronic stress level	
	Control ($n = 15$)	GABA-enriched oolong ($n = 15$)	Low ($n = 15$)	High ($n = 15$)
Gender (% male)	33	40	47	27
Age (years)	21.00 ± 1.414	21.73 ± 1.710	21.27 ± 1.534	21.47 ± 1.685
PSS score	23.47 ± 8.551	28.87 ± 9.094	19.20 ± 5.401	33.13 ± 6.186*
Acute stress score	0.537 ± 0.259	0.557 ± 0.306	0.381 ± 0.270	0.712 ± 0.171*
Average RR interval (ms)	856.8 ± 120.0	864.1 ± 203.3	829.9 ± 112.5	891.0 ± 202.8
TP	3.379 ± 0.293	3.349 ± 0.268	3.377 ± 0.325	3.350 ± 0.228
LF	2.689 ± 0.387	2.762 ± 0.289	2.811 ± 0.380	2.641 ± 0.276
HF	2.849 ± 0.423	2.854 ± 0.357	2.816 ± 0.471	2.887 ± 0.287
LF/HF	−0.159 ± 0.314	−0.092 ± 0.322	−0.006 ± 0.325	−0.246 ± 0.260*
UNTRANSFORMED VALUES				
Acute stress score	3.97 ± 0.522	4.40 ± 0.675	2.83 ± 0.401	5.53 ± 0.559
TP (ms^2)	3026 ± 689	2636 ± 420	3099 ± 698	2563 ± 399
LF (ms^2)	729 ± 214	699 ± 111	909 ± 215	520 ± 80
HF (ms^2)	1017 ± 222	973 ± 217	1023 ± 234	967 ± 204
LF/HF	0.880 ± 0.189	0.996 ± 0.160	1.218 ± 0.207	0.658 ± 0.091

**Significantly different ($p < 0.05$) than the corresponding value in the low chronic stress group.*

In addition GABA has been shown to activate extrasynaptic $GABA_A$ receptors to generate a long lasting inhibitory state (40). As GABA is extensively distributed within the enteric nervous system, it has been suggested that a bidirectional activity or modulation via the PNS through the vagus nerve could also affect stress responses (41), though this requires further investigation.

Of interest in the current research is that the concentration of GABA in the GABA-fortified tea sample (2.01 mg/200 mL which equates to 40.2 mg/100 g as determined by HPLC) was less than previously reported in similar research but still led to a positive response. The significant increased HRV, measured as total power, indicates elevated overall variability, associated with relaxation, while the rise in the high frequency component of the HRV power spectrum suggests improved parasympathetic tone (12, 26, 35, 36). This stress-reducing effect is reflected in the significant decreased immediate stress score. Thus, the experimental protocol of this study, which involved consumption of GABA-fortified and regular oolong tea, induced HRV changes (suggestive of correction of stress-induced autonomic nervous system imbalance) and reduction of psychological stress.

Delaney and Brodie showed that an acute stressor (mental arithmetic) led to a significant decrease in total power and the high frequency component with a concurrent increase in the LF:HF ratio (42). This reflects parasympathetic withdrawal and greater sympathetic regulation typical of the acute stress response (36, 42–44). Comparatively, chronic stress as measured by the PSS (45), a visual-analog scale (46) or trait anxiety (47–49) was associated with reduced total power and parasympathetic tone. Combined, these findings suggest that under prolonged psychogenic stress, the sympathetic hyperactivity of the acute stress state is attenuated, while parasympathetic withdrawal persists. Thus, it follows that tea consumption under the setting of the present study, increased HRV features that indicate parasympathetic tone, while those proposed to indicate primarily sympathovagal balance (such as LF:HF) remained unaffected.

The HRV effects observed in this study differ from those previously reported for GABA-fortified foods. Okita and colleagues assessed the effects of a single dose of the vegetable kale (containing 31.8 mg GABA), formulated into a tablet compared to a control tablet with no GABA, on chronic stress-induced changes in HRV (50). GABA attenuated the increase in LF and LF:HF reported in the control group without affecting HF. GABA reduced the LF without a detectable effect on peripheral nervous system activity. In support of this finding, oral ingestion of 50–100 mg GABA in water reduced salivary chromogranin A, a protein co-released with noradrenaline in the SNS, indicating a reduction of SNS activity (51). In contrast, both tea options in the present study exerted their effects by parasympathetic augmentation with comparatively smaller sympathetic effects.

In humans the minimum effective oral dose of GABA leading to HRV changes was suggested at 20–30 mg (52). Interestingly in our study the concentration of GABA in the tea was much lower, yet a reduction in perceived immediate stress decreased and HRV parameters improved. This reduction may however have also been due to the experimental condition or other bioactive constituents in the tea (53). Two main bioactive components of tea that may have contributed to the observed stress-reduction are (–)-epigallocatechin gallate (EGCG) and L-theanine. Both are able to cross the blood-brain barrier (54, 55) and are therefore able to influence the central stress processes, which regulate HRV. Both have

TABLE 3 | Psychological stress and heart rate variability parameters before and after ingestion of either GABA-enriched or control tea, separated by low, or high chronic stress level.

Parameter		Regular tea		GABA-enriched oolong tea	
		Low stress ($n = 8$)	High stress ($n = 7$)	Low stress ($n = 7$)	High stress ($n = 8$)
Immediate stress score	Pre	0.396 ± 0.253	0.698 ± 0.159	0.365 ± 0.308	0.724 ± 0.192
	Post	0.307 ± 0.218	0.476 ± 0.107	0.197 ± 0.261	0.553 ± 0.272
Average RR (ms)	Pre	849.8 ± 130.7	864.8 ± 116.4	807.2 ± 92.1	914.0 ± 263.3
	Post	932.8 ± 134.3	904.1 ± 83.2	856.4 ± 112.7	999.5 ± 242.6
TP (ms^2)	Pre	3.397 ± 0.365	3.358 ± 0.210	3.355 ± 0.301	3.343 ± 0.258
	Post	3.537 ± 0.342	3.649 ± 0.355	3.396 ± 0.248	3.494 ± 0.378
LF (ms^2)	Pre	2.839 ± 0.405	2.518 ± 0.305	2.778 ± 0.379	2.748 ± 0.209
	Post	2.937 ± 0.387	2.795 ± 0.361	2.752 ± 0.376	2.837 ± 0.420
HF (ms^2)	Pre	2.809 ± 0.535	2.894 ± 0.281	2.825 ± 0.428	2.880 ± 0.311
	Post	2.995 ± 0.464	3.154 ± 0.317	2.819 ± 0.458	3.113 ± 0.320
LF:HF	Pre	0.031 ± 0.215	−0.376 ± 0.271	−0.047 ± 0.453	−0.132 ± 0.201
	Post	−0.059 ± 0.31	−0.359 ± 0.197	−0.067 ± 0.544	−0.276 ± 0.121
UNTRANSFORMED VALUES					
Immediate stress score	Pre	2.81 ± 1.252	5.29 ± 1.976	2.86 ± 1.952	5.75 ± 2.435
	Post	2.25 ± 1.035	3.07 ± 0.732	1.86 ± 1.125	4.13 ± 2.100
TP (ms^2)	Pre	3497 ± 3584	2489 ± 995	2645 ± 1269	2629 ± 1978
	Post	4424 ± 3206	5636 ± 3413	2832 ± 1432	4098 ± 2752
LF (ms^2)	Pre	1014 ± 1056	403 ± 266	788 ± 538	622 ± 322
	Post	1266 ± 1357	844 ± 760	756 ± 576	988 ± 816
HF (ms^2)	Pre	1092 ± 1088	932 ± 577	945 ± 720	998 ± 980
	Post	1543 ± 1407	1805 ± 1368	1037 ± 1109	1619 ± 1113
LF:HF	Pre	1.227 ± 0.854	0.484 ± 0.248	1.208 ± 0.802	0.810 ± 0.371
	Post	1.103 ± 0.589	0.476 ± 0.201	1.284 ± 0.883	0.548 ± 0.148

Light gray shading indicates a significant ($p < 0.05$) time x tea x stress level interaction.

also shown anxiolytic effects in humans and animals (36, 40–45). On the other hand, teas are known to contain caffeine. Caffeine in tea has also been shown to weakly inhibit $GABA_A$ receptors (56). Weak inhibition may have a mildly stimulating effect. It has also been suggested that the stimulant effects of tea may be due, in part, to the caffeine (57). Our results suggest, however, that the observed stress-reducing effects of tea are not contributed to by any potentially stimulating effects of caffeine.

As teas contain a number of active constituents, including catechins, theanine and caffeine, the concentrations of these may differ between GABA-fortified and non-fortified teas owing to the additional processing required to increase GABA content in the GABA-fortified tea (19). It cannot be ignored that differences observed in the effects of the two teas may be due to differences in concentrations in other active constituents in the teas. Further studies to evaluate the concentrations of these active constituents in the teas are warranted, and blood samples for measuring their serum concentrations in participants would also be valuable.

In future studies it will be useful to ascertain the regularity of consumption of teas and other stimulant beverages by participants as a further mediating factor in our findings. Blood pressure would further provide a useful measure of stress, although it is difficult to ascertain blood pressure acutely without influencing the stress state. Glycaemia is another important consideration which may impact results and would be a valuable measure in future studies via a simple finger prick test. While studies of acute GABA or GABA-fortified food and beverage consumption have not documented side effects, the safety and tolerability of GABA consumption and overconsumption should also be considered in future investigations.

In conclusion, our results indicate that even a very small amount of GABA-fortified oolong tea leads to a significant effect on acute stress levels reflected by the improved HRV measures.

AUTHOR CONTRIBUTIONS

SM, GJ, and VV conceived the research questions. VV carried out the experiments. SM, TH, HJ, and GJ supervised the project. VV, TH, SM, and HJ undertook the statistical analysis. All authors discussed and contributed to the interpretation of the results of the research and writing of the paper. All authors contributed equally to this work. All authors designed the experiments and research protocol.

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REVIEW
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Diet-Derived Fatty Acids, Brain Inflammation, and Mental Health

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Western societies experienced drastic changes in eating habits during the past century. The modern nutritional profile, typically rich in saturated fats and refined sugars, is recognized as a major contributing factor, along with reduced physical activity, to the current epidemics of metabolic disorders, notably obesity and diabetes. Alongside these conditions, recent years have witnessed a gradual and significant increase in prevalence of brain diseases, particularly mood disorders. While substantial clinical/epidemiological evidence supports a correlation between metabolic and neuropsychiatric disorders, the mechanisms of pathogenesis in the latter are often multifactorial and causal links have been hard to establish. Neuroinflammation stands out as a hallmark feature of brain disorders that may be linked to peripheral metabolic dyshomeostasis caused by an unhealthy diet. Dietary fatty acids are of particular interest, as they may play a dual role, both as a component of high-calorie obesogenic diets and as signaling molecules involved in inflammatory responses. Here, we review current literature connecting diet-related nutritional imbalance and neuropsychiatric disorders, focusing on the role of dietary fatty acids as signaling molecules directly relevant to inflammatory processes and to neuronal function.

Keywords: fatty acids, high-fat diet, Western diet, mood disorders, neuroinflammation

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INTRODUCTION

Western society experienced a marked nutritional transition during the past century. Multiple factors, stemming primarily from the industrial revolution and mass urbanization, have driven the nutritional profile of the population toward increased consumption of processed and animal-derived foods, saturated fats and refined sugars, while reducing the intake of vegetables, fruits, fibers, and fish (Popkin and Gordon-Larsen, 2004; Popkin et al., 2012). Moreover, these altered eating habits have been associated with larger portion sizes and reduced energy expenditure, making modern lifestyle highly obesogenic (Bray and Popkin, 1998; Hill and Peters, 1998; Kopelman, 2000; Berthoud, 2012). During the same time span, the prevalence of mood disorders has increased significantly in Western countries, even when accounting for evolving diagnostic criteria and other confounding factors (Hagnell, 1989; Hidaka, 2012). As mechanisms linking diet to mental health become better understood, accumulating evidence suggests that the modern/Western diet may be one of the drivers of this increase (Jacka et al., 2010; O'Neil et al., 2014).

To address this link, the nascent field of "nutritional psychiatry" (Logan and Jacka, 2014) focuses on clinical studies examining the impact of both isolated nutrients and overall quality of diets on the incidence and progression of prevalent psychiatric conditions, most often anxiety and

mood disorders. In parallel, the field strives to overcome challenges associated with the combined complexities of human diet and behavior, both of which are difficult to control and evaluate consistently (Jacka, 2017).

Although it is not disputed that the modern lifestyle and nutritional behavior promote a surplus of energy and its storage in the form of expanding adipose tissue (Spiegelman and Flier, 2001), the exact relevance of macronutrient composition–the balance of protein, carbohydrate and fat—to body weight regulation remains under discussion. While some authors argue for a disproportionate contribution of a single type of nutrient to the development of obesity and metabolic disorders, others sustain that such pathologies are not significantly dependent on diet composition, resting instead squarely on a positive energy balance.

Bray and Popkin (2014), for instance, have suggested that increased intake of carbohydrates—mainly in the form of glucose/fructose-sweetened beverages, a primary source of added sugars in modern society—is a key driver of the modern pandemic of obesity and metabolic conditions (Bray et al., 2004; Bray and Popkin, 2014). Indeed, numerous meta-analyses show a positive correlation between sugar consumption and increased risk of insulin resistance, non-alcoholic fatty liver disease (NAFLD), obesity and type 2 diabetes (Ludwig et al., 2001; Bray et al., 2004; Malik et al., 2006, 2010; Montonen et al., 2007; Ouyang et al., 2008; Bray and Popkin, 2014; Mosca et al., 2017). Although such correlations have not been consistently demonstrated when total energy intake is controlled, authors argue that added calories obtained from sugar-sweetened beverages tend to not be compensated elsewhere, as they do not effectively suppress intake of other calories. Mechanistically, increased fructose uptake and metabolism in the liver stimulates *de novo* lipogenesis (DNL), which results in increased intrahepatic lipid content, leading to increased production and secretion of very low-density lipoprotein and triglycerides. In the long term, these alterations may result in increased fat storage in visceral adipose tissue and ectopic lipid deposition in tissues such as muscle, further contributing to insulin resistance (Lê et al., 2009; Stanhope et al., 2009; Stanhope, 2016; Mock et al., 2017).

On the other hand, as highlighted by Khan and Sievenpiper (2016), more recent trends of reduced sugar intake by United States adults have not been accompanied by a reduction in obesity and metabolic disorders. In addition, as mentioned above, most controlled trials using isocaloric diets have not shown a specific contribution of any type of nutrient to obesity, suggesting total energy content is the most relevant variable (Kahn and Sievenpiper, 2014; Khan and Sievenpiper, 2016).

Regarding the role of fat intake, Hu et al. (2018) recently published a compelling report comparing the long-term effects of 29 types of diet, with varying proportions of fat, carbohydrates and protein, on five different mouse strains. Surprisingly, they found fat content in a diet to be the only factor involved in increased energy intake and adiposity. This observation was explained by a hedonic drive linked to fat, but they did not observe a similar effect with sugar (Hu et al., 2018). The fact that a certain diet composition may drive higher energy intake independent of the diet's own energy density adds an additional layer of complexity to the field, particularly in the design and execution of clinical trials.

Hu et al. (2018) present an interesting discussion on the translatability of this finding, and the feasibility of performing an equivalent study in humans. However, be it due to its higher energy content compared to other nutrients, to its reward value that drives increased caloric intake, or to specific but not yet fully understood signaling and metabolic dysregulation, the impact of increased fat consumption on the development of diet-related diseases has been well documented over the years (for an excellent recent review, see Ludwig et al., 2018). Although controversy remains on the relevance of total fat intake toward body fat accumulation, with several studies indicating no causal relationship (Curb and Marcus, 1991; Willett, 1998, 2002; Vergnaud et al., 2013), excessive energy intake from dietary fat is established as an important factor to increased adiposity (Horton et al., 1995; Bray and Popkin, 1998).

The fantastic remodeling capacity of adipose tissue allows for adipocyte hypertrophy and hyperplasia in response to nutrient availability and energy surplus. However, under pathological conditions, the need for adaptation exceeds the capacity of the tissue. Hypoxia and adipocyte cell death result in macrophage recruitment and polarization, increasing inflammatory markers, cytokine and chemokine secretion, and dysregulation in free fatty acid (FFA) fluxes (Sun et al., 2011).

Increased circulating FFAs and proinflammatory factors are also central to insulin resistance and deregulation of glucose homeostasis, the core aspects of type 2 diabetes mellitus (T2DM) (Greenberg and Obin, 2006). Obesity and T2DM currently affect a large portion of the world population and are considered a global epidemics, with obesity as the leading risk factor for T2DM (Barnes, 2011).

Notably, the fatty acid composition of diets has been shown to impact their obesogenic profile and overall toxicity. Particularly, enrichment in saturated fatty acids (SFAs) results in a diet that induces greater accumulation of body fat and lower satiety than diets enriched in polyunsaturated fatty acids (PUFAs) (Lawton et al., 2000; Piers et al., 2003; Moussavi et al., 2008; Phillips et al., 2012). Moreover, excessive SFA consumption was shown to increase SFAs in the circulation, increase expression of genes involved in inflammatory processes in adipose tissue, reduce insulin sensitivity and increase intrahepatic triglyceride content in humans (Vessby et al., 2001; van Dijk et al., 2009; Rosqvist et al., 2014).

Long-term longitudinal studies have linked a low intake of PUFAs and a high intake of cholesterol and SFAs to increased risk of impaired cognitive function and development of dementia, including Alzheimer's disease (Kalmijn et al., 1997; Morris et al., 2003a,b, 2004; Barnard et al., 2014; Reichelt et al., 2017). In this context, it has been suggested that metabolic imbalance caused by high-fat diets and a sedentary lifestyle constitutes an important AD risk factor, particularly due to its association with higher levels of plasma FFAs, chronic low grade inflammation, insulin resistance and T2DM (De Felice, 2013). Further, whereas moderate intake of PUFAs at midlife appears to decrease the risk of dementia in aging (Laitinen et al., 2006), saturated and trans-unsaturated fat consumption have been found to be positively

associated with increased risk of AD (Morris et al., 2003b). Adherence to a Mediterranean diet and frequent consumption of fruits and vegetables, fish, and ω-3 (n-3 PUFA) rich oils has been proposed as a factor capable of preventing AD and dementia (Scarmeas et al., 2006; Barberger-Gateau et al., 2007, 2011). Moreover, cognitive performance in elderly people (65–90 years old), free from significant cognitive impairment, was better in subjects having high intakes of vegetables, fruits, and vitamins and lower intakes of monounsaturated fatty acids, SFAs, and cholesterol (Ortega et al., 1997).

A close relationship exists between metabolic syndrome, T2DM and brain dysfunction, encompassing both mood and cognitive disorders (Ott et al., 1996; De Felice, 2013; Santos et al., 2016; Rebolledo-Solleiro et al., 2017). The mechanisms underlying this connection appear largely based on neuroinflammation and dysregulated brain insulin signaling, both of which can result from nutritional imbalance (reviewed in Luchsinger, 2012; De Felice and Ferreira, 2014; Holt et al., 2014; Sevilla-González et al., 2017).

In the following sections, we focus on data available on the connection between dietary fatty acids and their potential role in mental health, particularly in depressive disorders. We explore how, due to their potential as modulators of neuroinflammation and insulin signaling, fatty acids may be key to the interplay between diet and mental health. We also discuss some of the more recent work exploring how the dopaminergic system, increasingly implicated in the pathophysiology of depression, may be affected by dietary choices.

HUMAN POPULATIONAL STUDIES

While numerous observational studies have been carried out, randomized controlled trials (RCTs) on the relationship between diet and mood disorders are comparatively rare. A meta-analysis conducted in 2014 (Lai et al., 2014) examined links between dietary patterns and depression, and found 20 observational studies meeting inclusion criteria, but only one RCT. These authors concluded that 'healthy' diets, including a high intake of fruit, vegetables, fish and whole grains, were inversely correlated with depression. Around the same time, another meta-analysis carried out by Psaltopoulou et al. (2013) reached similar conclusions while examining nine observational studies that had depression as the main outcome and eight studies evaluating cognitive function. They found a significant association between adherence to the Mediterranean diet – a diet pattern similar to what Lai et al. (2014) classified as healthy – and lower rates of both depression and cognitive impairment.

Since humans do not typically consume any single type of food in isolation, studies with isolated nutrients, such as fatty acids, are not only difficult to perform, but also trade potential relevance for increased power. Thus, to approach questions dealing with the roles of specific nutrients, authors often rely on supplementation or on observational studies to seek correlations between study outcomes and specific biomarkers reflecting a nutrient's level of intake or metabolism. Interestingly, a meta-analysis of 13 randomized placebo-controlled trials enrolling a total of 1,233 patients with major depressive disorder (MDD) demonstrated a beneficial effect of omega-3 PUFA supplementation on depressive symptoms (Mocking et al., 2016), with a larger effect at higher doses and in patients being simultaneously treated with antidepressants, suggesting a potential adjuvant role of omega-3 fatty acids in MDD treatment.

Results from several other meta-analyses and epidemiological studies suggest that reduced levels of PUFAs could be involved in the pathogenesis of cognitive and mood disorders, and may be therapeutic targets in those diseases. A meta-analysis of 14 studies found that subjects with depressive symptoms or social anxiety disorders had lower circulating levels of the n-3 PUFAs, eicosapentaenoic acid, 20:5n-3 (EPA) and docosahexaenoic acid, 22:6n-3 (DHA), and/or higher levels of the n-6 PUFA, arachidonic acid, 20:4n-6 (ARA), than control subjects (Lin et al., 2010). In addition, post-mortem analysis of orbitofrontal and prefrontal cortex of patients with major depression showed lower DHA levels compared to controls (McNamara et al., 2007, 2013). Moreover, a recent 7-year follow-up study of 69 young individuals with an ultra-high risk phenotype for psychosis demonstrated that lower levels of EPA and/or DHA, and higher n-6/n-3 PUFA ratio in the phosphatidylethanolamine fraction of erythrocyte membranes, specifically predicted mood disorders (in this cohort, 24 patients received a diagnosis of MDD and 2 of bipolar disorder during the follow-up period; Berger et al., 2017). Altogether, these and other studies implicate PUFAs in the pathogenesis of mood and cognitive disorders, providing a basis for nutritional psychiatry approaches in these highly prevalent and incapacitating diseases.

HIGH-FAT DIET IN RODENT MODELS

Many of the current inferences on the impact of HFD on human health have been based on or influenced by studies in animal models, mostly rodents. As in humans, fat-enriched diets induce rapid weight gain and metabolic alterations in animal models. Although the term 'high-fat diet' is widely used to describe studies in which fat corresponds to the highest proportion of energy intake, that percentage may range from 20 to 60% of total energy intake and diet composition may include animal-derived fats or plant oils. Moreover, the composition of the control diet is often not standardized, with non-purified chow being used as a control and some studies omitting to mention the composition of the control diet altogether (Buettner et al., 2007; Hariri and Thibault, 2010). Further, the age at which exposure to HFD is initiated is also variable among studies. Lack of standardization in studies under the HFD umbrella leads to great variability in observed outcomes and difficulty in establishing comparisons between studies.

Despite the different protocols, some of the effects of excess fat in the diet appear to be central and robust in mice. In a landmark report by Xu et al. (2003), for instance, obesity induced in C57BL/6J mice by long-term exposure to a HFD (containing up to 60% calories from fat) produced increases in adipocyte number and size, body weight, fasting blood glucose levels, and induced hyperinsulinemia (Xu et al., 2003). Those metabolic

changes were later shown to occur even after a short period of fat-enriched diet consumption (Lee et al., 2011).

Hotamisligil et al. (1993) demonstrated a central role of TNF-α in diabetes and obesity-induced insulin resistance, using db/db, ob/ob, tub/tub, and fa/fa mice, genetic models of metabolic disorders (recently reviewed in Kleinert et al., 2018). In those mice, Hotamisligil et al. (1993) showed that increased expression of TNF-α in adipocytes as well as high levels of this cytokine in the circulation resulted in insulin resistance. In line with those findings, Xu et al. (2003) went on to show that excessive macrophage recruitment and upregulated expression of ADAM8, MIP-1α, MCP-1, MAC-1, F4/80, and CD68 in white adipose tissue contribute to the establishment of chronic inflammation and increased production and release of pro-inflammatory cytokines, notably TNF-α and IL-6, into the circulation. These results were later corroborated by others, and positioned obesity-induced inflammation into a broader picture (Hotamisligil et al., 1993; Wellen and Hotamisligil, 2003; Xu et al., 2003; Lumeng et al., 2007; Eder et al., 2009).

The HFD mouse model has been instrumental in dissecting the molecular mechanisms involved in FFA-induced T2DM. It was first shown in humans that excessive FFAs in the circulation inhibit insulin signaling and glucose metabolism in several tissues, such as adipocytes, liver, and muscle. Excessive FFAs were shown to reduce muscle glucose transport and metabolism via decreased GLUT4 translocation to the plasma membrane (Roden et al., 1996) and to inhibit insulin signaling by increasing IRS-1 serine phosphorylation and reducing insulin-stimulated PI3-kinase activity (Goodyear et al., 1995; Dresner et al., 1999). The important role of inflammatory responses in this process, which culminate in the activation of stress kinases such as JNK and IKKβ, which in turn target IRS, was described in HFD mouse models (Yuan et al., 2001; Hirosumi et al., 2002; Arkan et al., 2005).

Increased saturated FFAs, observed in obesity and high fat intake models, have an intrinsic pro-inflammatory potential that impacts important cell functions. Fatty acids may activate Toll-like receptor 4 (TLR4) signaling in adipocytes and macrophages and induce inflammatory signaling (Shi et al., 2006), and mice lacking TLR4 were shown to be protected against high-fat diet-induced obesity and insulin resistance (Poggi et al., 2007; Davis et al., 2008). TLRs are a family of type I transmembrane receptors that recognize a variety of microbial danger-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs) and orchestrate an intracellular signaling response, playing an important role in infectious and inflammatory disorders. Amongst at least 13 members of TLRs described in mammals, TLR2 and TLR4 are best characterized in terms of their involvement in the immune response (Fessler et al., 2009). TLR4 plays a critical role in the innate immune system by activating MyD88-dependent and MyD88-independent proinflammatory signaling pathways, as well as the NFκB response (Lu et al., 2008).

Mood disorders cannot be fully reproduced in rodent models. In addition to their incompletely understood etiology, they involve symptoms that may not exist outside of the human experience, such as inappropriate guilt and suicidality (Krishnan and Nestler, 2011). However, rodent models may exhibit depressive-like symptoms, such as behavioral correlates of hopelessness or anhedonia. Recent data on rats and mice fed HFD suggest a positive association between HFD and such depressed phenotypes (Yang et al., 2016; Arcego et al., 2018; Hassan et al., 2018), which may be causally linked to diet-induced inflammatory processes, as discussed below.

SATURATED FATTY ACIDS AND NEUROINFLAMMATION: POSSIBLE LINKS TO MOOD DISORDERS

Microglial cells respond rapidly to pathological changes in the brain, altering their morphology and phagocytic behavior, and increasing cytotoxic responses by secreting NO, proteases and cytokines, such as TNF-α and IL-1β (Kreutzberg, 1996). SFAs, such as palmitic acid, have been shown to induce activation of TLR4 receptors in hypothalamic microglia and to stimulate cytokine release (Valdearcos et al., 2014), indicating a potential mechanism by which HFD leads to brain inflammation. Notably, the hippocampus—a key brain region involved not only in learning and memory but also in depression and the effect of antidepressants—is vulnerable to altered levels of IL-1β, IL-6, and TNF-α, as these cytokines have important roles in synaptic plasticity and may inhibit neurogenesis (Sheline, 2011; Calabrese et al., 2014).

Microglia and astrocytes are essential to normal synaptic function. Synaptic pruning by microglia is essential to synaptic maturation and neurotransmission (Paolicelli et al., 2011), while astrocytes hold important metabolic and plasticity functions (Beattie et al., 2002; Singh and Abraham, 2017). Importantly, HFD-induced depressive-like behavior in rodents, as well as cognitive impairment, has been associated with brain inflammation. For instance, Dutheil et al. (2016) showed that, in addition to the classical metabolic alterations, rats fed an HFD (60% of calories as fat) for 16 weeks show anhedonic behavior, which presents alongside insulin signaling impairment and increased levels of cytokines such as IL-6, IL-1β, and TNF-α in the hippocampus. In turn, mice exposed to long term HFD were shown to have spatial memory deficits in the Morris water maze, with increased serum and hippocampal levels of TNF-α and presence of activated microglia in the hippocampus, as well as reduced dendritic branching and complexity (Heyward et al., 2012; Jeon et al., 2012).

In line with the structural similarity between SFAs and the lipid portion of bacterial lipopolysaccharide (LPS), several lines of evidence suggest that SFAs act as ligands of TLRs. *In vitro* experiments have shown that SFAs activate TLR2 to induce an inflammatory response (Erridge and Samani, 2009; Huang et al., 2012), and numerous reports have linked SFAs to TLR4-mediated signaling pathways in immune cells (Park et al., 2009; Rogero and Calder, 2018). Using both the BV-2 microglial cell line and primary microglial cultures, Wang et al. (2012) demonstrated that palmitic acid and stearic acid induce a reactive microglial phenotype and increase levels of inflammatory markers in a TLR4-dependent manner. The SFAs, lauric, palmitic, and stearic

acids, but not unsaturated fatty acids or PUFAs, were shown to induce NF-κB activation and expression of COX-2 and other inflammatory markers in macrophages, effects inhibited in dominant-negative TLR4 cells (Lee et al., 2001). Further, the liver secretory protein fetuin-A (FetA) has been suggested as an adaptor protein between FFAs and TLR4 activation, connecting FFAs to TLR-mediated inflammation (Pal et al., 2012). Importantly, however, the role of TLRs as SFA receptors is still a matter of debate. The most recent challenge to this notion was a compelling report by Lancaster et al. (2018) suggesting that SFAs are not direct ligands of TLR4 in macrophages, but instead contribute to pro-inflammatory signaling by altering lipid metabolism in these cells. They reconcile these results with past literature findings by showing that, despite not being a direct target, TLR4-dependent priming is a requirement for SFA-induced inflammatory signaling.

Inflammation has emerged as an important factor in mood disorders. Patients presenting mood disorders show elevated plasma levels of cytokines such as TNF-α, IL-6, and IL-1β, as well as increased expression of inflammatory markers in blood cells (reviewed by Mechawar and Savitz, 2016). Increased consumption of high fat diet is related to depressive-like behavior and emotional disorders in mice (Wang et al., 2017; Arcego et al., 2018; Vagena et al., 2018; Xu et al., 2018), and neuroinflammation could be an important modulator of these behavioral alterations. Palmitic acid abolished the migration and phagocytic activity of microglia in response to interferon-γ, thus affecting the protective response of these cells after an inflammatory challenge *in vitro* (Yanguas-Casás et al., 2018). Post-mortem analysis of brain tissue from MDD patients indicated a 6.5% increase in palmitic acid and a 6.2% decrease in oleic acid in the amygdala, as compared to controls (Hamazaki et al., 2012), further suggesting that altered levels of specific fatty acids may be implicated in brain dysfunction.

One potential mechanistic connection between neuroinflammation and mood disorders is the positive effect of pro-inflammatory cytokines on microglial expression of indolamine-2,3-dioxygenase (IDO), the enzyme that converts tryptophan to kynurenine (Wichers and Maes, 2004; Dantzer et al., 2008). Lower availability of tryptophan in the brain due to upregulation of this alternative pathway could slow down its conversion to 5-hydroxytryptophan, the rate-limiting step in serotonin synthesis, carried out by tryptophan hydroxylase. Notably, while far from the only factor involved, serotonin depletion has been shown to induce depressive-like symptoms in animal models and impact mood in humans under certain conditions (Ruhé et al., 2007; O'Connor et al., 2009). Furthermore, increased kynurenine metabolism may result in excessive production of 3-hydroxykynurenine, a generator of reactive oxygen species (ROS), and quinolinic acid, an NMDA receptor agonist, both of which could have their own implications to depression (Müller and Schwarz, 2007).

Another possible mechanism linking neuroinflammation to mood involves precisely the vulnerability of monoaminergic pathways to oxidative stress. Tetrahydrobiopterin (BH_4) is an essential cofactor, required for certain enzymatic reactions such as those carried out by tryptophan hydroxylase, phenylalanine hydroxylase (which coverts phenylalanine to tyrosine) and tyrosine hydroxylase (which converts tyrosine to L-DOPA, the rate limiting step in dopamine synthesis). BH_4 may be readily inactivated by ROS, a likely event in strong proinflammatory contexts, thus affecting dopamine and serotonin levels (reviewed by Swardfager et al., 2016). Notably, in addition to the role of serotonin mentioned above, recent reports have shown that dopamine neurotransmission, particularly in the ventral tegmental area-nucleus accumbens circuit, is essential for the expression of depressed phenotypes and social behavior, and thus its depletion could contribute to mood disorders (Tye et al., 2012; Gunaydin et al., 2014; Matthews et al., 2016).

POLYUNSATURATED FATTY ACIDS, NEUROINFLAMMATION AND LINKS TO MOOD DISORDERS

The nutritional transition observed worldwide in the past few decades has introduced high amounts of SFAs and omega-6 (n-6) PUFAs in the human diet through increased intake of dairy products, vegetable oils and red meat. This change in dietary profile was further accompanied by a reduction in consumption of fruits, vegetables, legumes, grains and fish, important sources of omega-3 (n-3) PUFAs. These changes resulted in an increase in omega-6/omega-3 ratio from about 1:1 to 10:1, reaching up to 20–25:1 or higher, and an alarming omega-3 deficiency in the global population, mainly in Western countries (Simopoulos, 2011).

Omega-3 and omega-6 PUFAs are categorized in these two groups according to the position of the double bond closest to the methyl terminus of the hydrocarbon chain, and, together, comprise the very-long chain family of polyunsaturated fatty acids (VLC-PUFAs). The main VLC-PUFAs in humans are the omega-3 PUFAs, EPA and DHA, and the omega-6 PUFA, ARA, which are components of membrane phospholipids and important signaling molecules (Zárate et al., 2017). In humans, VLC-PUFAs are endogenously synthesized in small amounts from dietary intake of the essential fatty acids, linoleic acid (LA) and alpha-linolenic acid (ALA). These are precursors of ARA, EPA, and DHA synthesis through the action of elongase and desaturase enzymes, which successively elongate and include double bonds into the carbon chain. Thus, adequate balance of these nutrients in the diet is necessary for healthy development, survival and aging (Calder, 2018).

The brain is a lipid-rich organ, and approximately 35% of those lipids are PUFAs (Yehuda et al., 1999). DHA and ARA are major PUFA components in brain cells. They are predominantly found esterified as glycerophospholipids at the plasma membrane (approximately 10,000 nmol per gram of brain tissue) but are also found at much lower amounts in non-esterified form (about 1 nmol per gram of brain tissue). They act as structural components and signaling molecules in neurons, glial cells, and endothelial cells (Bazinet and Layé, 2014). Studies in humans and, mainly, in animal models have revealed that PUFAs enter the brain via lipoproteins or albumin transport in esterified form, as lysophosphatidylcholine, or in non-esterified form,

by passive diffusion through a flip-flop mechanism or through protein transporters, such as fatty acid binding proteins (FABPs), fatty acid transport protein (FATP), fatty acid translocases (FAT/CD36) and major facilitator superfamily domain-containing protein 2 (Mfsd2a) (Lauritzen et al., 2001; Umhau et al., 2009; Domenichiello et al., 2014; Nguyen et al., 2014; Chen et al., 2015; Liu et al., 2015; Pan et al., 2015, 2016; Hachem et al., 2016). PUFAs play important roles in brain function, including synaptic plasticity, neurotransmission, metabolism, neurogenesis, neuroinflammation and neuroprotection (Bazinet and Layé, 2014). Not surprisingly, therefore, reduced or unbalanced dietary supply and brain levels of PUFAs (notably, DHA) are associated with brain disorders, including cognitive and mood disorders (see below).

In addition to modulation of serotonin (5-HT1 and 5-HT4), beta-adrenergic and dopamine (D1 and D2) receptor signaling through increased adenylate cyclase and protein kinase A (PKA) activities (Liu et al., 2015), PUFAs play an important role in neuroinflammation, an important etiologic factor of mood disorders (Chang et al., 2015; Yirmiya et al., 2015; Chen et al., 2018). Omega-6 and omega-3 PUFAs have opposite effects on inflammatory modulation. ARA is an important precursor of eicosanoids, bioactive molecules that regulate the inflammatory process in immune cells. In response to inflammatory stimuli, membrane phospholipids are cleaved by phospholipase A2 (PLA-2) and release ARA, a substrate of cyclooxygenase (COX), lipoxygenase (LOX) and cytochrome P450. This stimulates synthesis of prostaglandins (PGs), thromboxanes (TXs), and leukotrienes (LTs), key pro-inflammatory mediators (Innes and Calder, 2018). Post-mortem analysis of brains from patients with bipolar disorders indicated a dysregulation of ARA release and downstream metabolism in frontal cortex (Kim et al., 2009), and mood stabilizers such as lithium, valproate and carbamazepine have been found to modify the ARA cascade in the brain (Kim et al., 2009). These findings suggest that increased levels of ARA from the diet could lead to exacerbation and dysregulation of the inflammatory response in brain cells, thus contributing to mechanisms associated with mood disorders.

In vitro studies showed that omega-3 PUFAs modulate microglial functions. For instance, EPA treatment inhibited microglial production of proinflammatory cytokines (IL-1β, IL-6, and TNF-α) (Liuzzi et al., 2007) *in vitro*, and supplementation with omega-3 PUFAs inhibited microglial activation and shifted microglial profile from the so-called classical pro-inflammatory M1 to the neuroprotective M2 phenotype in a model of brain injury in rats (Chen et al., 2018). When incorporated into microglial membranes, DHA, which has been described as a potent immunomodulator in brain cells (Antonietta Ajmone-Cat et al., 2012), blocked the recognition of LPS by cell surface receptors and inhibited nuclear factor kappa B (NF-κB) activation and synthesis of IL-1β and TNF-α (De Smedt-Peyrusse et al., 2008). In addition, DHA prevented LPS-induced neuroinflammation and restored synaptic structure and functions in hippocampal CA1 pyramidal neurons (Chang et al., 2015). In Fat-1 mice, which convert n-6 to n-3 PUFAs in the brain, feeding with a DHA-enriched diet prevented LPS-induced increases in pro-inflammatory cytokines, microglial activation, depressive-like behavior and reduction in BDNF levels (Gu et al., 2018).

Omega-3 PUFAs, mainly EPA, are competitive substrates for enzymes involved in the biosynthesis of inflammatory mediators derived from ARA. Increased PUFA consumption results in a membrane phospholipid composition with increased levels of these fatty acids, and in a reduction of ARA-derived inflammatory mediators (reviewed in Calder, 2015). Moreover, DHA and EPA are precursors of important lipid mediators with anti-inflammatory and pro-resolutive actions, such as resolvins and protectins. Resolvin D1 (RvD1) and resolvin E1 (RvE1), for example, decrease LPS-induced microglial expression of proinflammatory cytokines, namely TNF-α, IL-6, and IL-1β (Rey et al., 2016).

FATTY ACIDS, MICROBIOTA CHANGES, AND MOOD DISORDERS

Recently, the gut–microbiota–brain axis has been implicated in neuroinflammation and the development of neuropsychiatric disorders. A comparative analysis between children from a rural African village in Burkina Faso (fed a rural diet) and European children (fed a modern Western diet) indicated significant differences in gut microbiota between the two groups (De Filippo et al., 2010), and suggested an important role of the nutritional transition in altering the human gut microbiome and in the development of inflammatory diseases.

The gut microbiome rapidly responds to dietary composition. Using mouse models, David et al. showed that short-term exposure to diets enriched in animal or plant products changed microbiota composition and microbial gene expression (David et al., 2014). Feeding a HFD caused shifts in the gut bacterial ecosystem in mice (Daniel et al., 2014). More recently, mice fed a HFD for 8 weeks were shown to present a depressive-like phenotype accompanied by a relative reduction in the population of Bacteroidetes and increase in the population of Firmicutes and Cyanobacteria in their caecal microbiome (Hassan et al., 2018). Interestingly, MDD patients showed different abundances of Firmicutes, Actinobacteria and Bacteroidetes when compared to healthy controls. In the same study, transplantation of fecal microbiota from MDD patients into mice resulted in depressive-like behaviors compared with colonization with microbiota derived from healthy control individuals (Zheng et al., 2016). Similarly, transplantation of fecal microbiota from depressed patients to microbiota-depleted rats induced anhedonia and anxiety-like behaviors (Kelly et al., 2016).

The detailed mechanisms underlying how changes in microbiota may lead to mood disorders remain unclear, but neuroinflammation appears as a potential mechanism. Microglia from germ-free mice showed decreased expression of genes associated with inflammation and defense responses, and an immature profile when compared with microglia from control mice (Matcovitch-Natan et al., 2016; Fung et al., 2017). Moreover, microbiota complexity has a central role in microglia function, regulating the neuroinflammatory response in health and disease (Erny et al., 2015).

ADIPONECTIN

Adiponectin, a hormone released by adipocytes and found abundantly in plasma and at lower concentrations in the CSF (Ebinuma et al., 2007), has been linked to mood disorders, and may connect dietary changes to behavior, particularly with respect to long-term effects. Circulating levels of adiponectin and the response elicited by activation of its receptors, AdipoR1 and AdipoR2 (found in several organs, including the brain), have been shown to be modulated by inflammatory and metabolic conditions, such as obesity and diabetes (Hotta et al., 2000; Yang et al., 2002). Although consistent human data are lacking, adiponectin has anti-depressant (Liu et al., 2012) and anti-inflammatory (Ouchi and Walsh, 2007) properties in mice. It has also been shown to be a candidate mediator of the positive effects of exercise and environmental enrichment on neurogenesis, mood, and cognition (Chabry et al., 2015).

THE INSULIN-DOPAMINE LINK

Kleinridders et al. (2015) showed that reduced insulin signaling in the brain, as a result of insulin resistance, led to increased levels of monoamine oxidases and increased dopamine clearance. They further showed that this change in dopamine metabolism led to age-related anxiety and depressive-like behavior in mice, results consistent with the above mentioned increasingly important role of dopamine signaling in mood disorders.

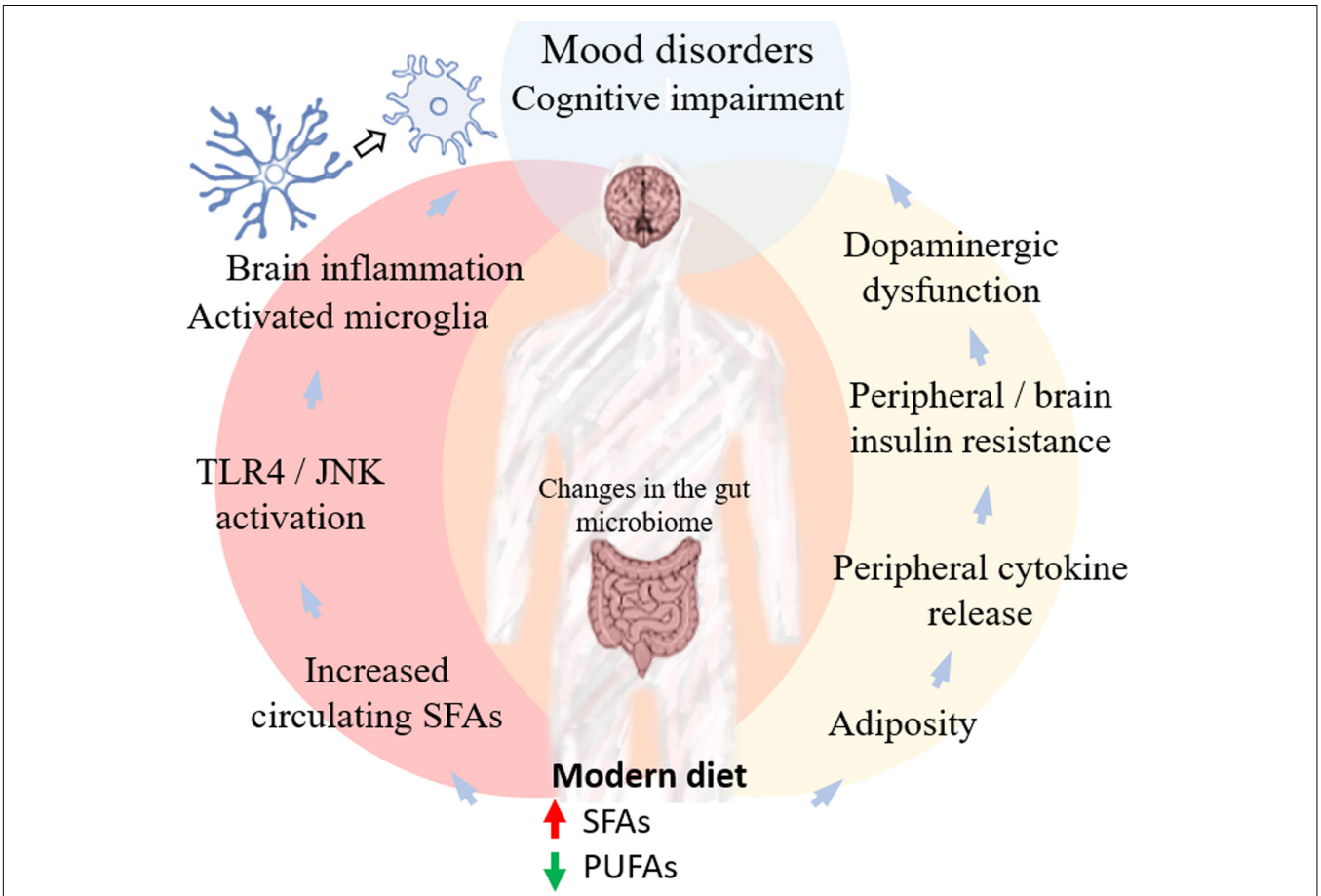

FIGURE 1 | Pathways linking diet and mental health. The nutritional transition observed in modern society, mainly in Western countries, has resulted in increased consumption of SFAs and reduced intake of PUFAs. Excessive energy intake from fat-enriched diets increases fatty acid storage and surpasses the remodeling capacity of adipose tissue, resulting in macrophage recruitment and increasing circulating levels of proinflammatory cytokines. Increased cytokine levels lead to the activation of stress kinases, such as JNK and IKKβ, resulting in increased IRS-1 serine phosphorylation and reduced insulin-stimulated PI3-kinase activity, causing central and peripheral insulin resistance. Brain inflammation and insulin resistance have been implicated in cognitive deficits (reviewed in De Felice et al., 2014; Ferreira et al., 2014). A recent report further suggested that reduced insulin signaling in the brain affects dopamine metabolism and release, contributing to mood disorders (Cai et al., 2018). Whether this dopaminergic dysfunction is also related to cognitive impairment is a possibility yet unexplored. Excessive energy intake from dietary fat further results in dysregulation of free fatty acid (FFA) fluxes, reducing PUFAs and increasing SFAs in the circulation. Although the exact mechanism is still controversial (Lancaster et al., 2018), several reports have shown that SFAs activate TLR4-dependent signaling pathways that increase inflammatory responses in microglia and induce brain inflammation, another potential mechanism involved in the development of both mood disorders and cognitive impairment. At the same time, dietary choices are known to impact the gut microbiota, which may regulate neuroinflammatory responses. Together, these interconnecting mechanisms suggest relevant links between lipid imbalance associated with fat-enriched diets and the onset and progression of neuropsychiatric and cognitive disorders.

Complementing their previous results (Kleinridders et al., 2015), the same group later used conditional insulin receptor knockout mice to show that insulin signaling in astrocytes has a role in regulating dopaminergic transmission, via the release of the gliotransmitter ATP (Cai et al., 2018). Their results suggest that activation of insulin receptors in astrocytes activates Munc18c to promote ATP exocitosis, which acts on P2X receptors on dopaminergic neurons to modulate dopamine release and normal mood behavior. These results also led to the conclusion that dopamine signaling may be altered and contribute to mood disorders in an insulin resistance scenario (Cai et al., 2018).

Moreover, Fordahl and Jones (2017) demonstrated in mice that prolonged consumption of an HFD impairs insulin signaling in the nucleus accumbens and reduces dopamine reuptake in dopaminergic terminals. Notably, restoring insulin signaling could revert this deficit, suggesting that loss of insulin sensitivity may be the cause of altered dopaminergic in the region (Fordahl and Jones, 2017).

PERSPECTIVES AND CONCLUDING REMARKS

Given the high rate of failure of antidepressant therapies, with at least 30% of patients being unresponsive to multiple rounds of pharmacological treatment (Sinyor et al., 2010), and the lack of effective, disease modifying treatments for dementia, the prospect of dietary interventions for mood and cognitive disorders is appealing. Notably, the targets involved in potential dietary approaches to mental health may in fact overlap with targets for pharmacotherapy in current clinical trials, including neuroinflammation (e.g., TLR and cytokine receptors) and brain insulin signaling (**Figure 1**). To reach this goal, an important step will be to understand and dissect the distinct but interdependent roles of fatty acids as nutrients and signaling molecules in the brain, and their impact on brain function and dysfunction. Finally, since no food is consumed in isolation by humans, this should happen as part of a larger effort to explore the already proposed potential of other nutrients, particularly carbohydrates, as competing players in both inflammation and insulin signaling (DiNicolantonio et al., 2018).

AUTHOR CONTRIBUTIONS

All authors discussed and contributed ideas to the manuscript. HM and LS shared most of the writing work. SF advised the process and edited the manuscript.

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frontiers in Neuroscience

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Evidence of Aquaporin 4 Regulation by Thyroid Hormone During Mouse Brain Development and in Cultured Human Glioblastoma Multiforme Cells

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Accumulating evidence indicates that thyroid function and the thyroid hormones L-thyroxine (T4) and L-triiodothyronine (T3) are important factors contributing to the improvement of various pathologies of the central nervous system, including stroke, and various types of cancer, including glioblastoma multiforme (GBM). Low levels of T3 are correlated with the poorest outcome of post-stroke brain function, as well as an increased migration and proliferation of GBM tumor cells. Thyroid hormones are known to stimulate maturation and brain development. Aquaporin 4 (AQP4) is a key factor mediating the cell swelling and edema that occurs during ischemic stroke, and plays a potential role in the migration and proliferation of GBM tumor cells. In this study, as a possible therapeutic target for GBM, we investigated the potential role of T3 in the expression of AQP4 during different stages of mouse brain development. Pregnant mice at gestational day 18, or young animals at postnatal days 27 and 57, received injection of T3 (1 μg/g) or NaOH (0.02 N vehicle). The brains of mice sacrificed on postnatal days 0, 30, and 60 were perfused with 4% paraformaldehyde and sections were prepared for immunohistochemistry of AQP4. AQP4 immunofluorescence was measured in the mouse brains and human GBM cell lines. We found that distribution of AQP4 was localized in astrocytes of the periventricular, subpial, and cerebral parenchyma. Newborn mice treated with T3 showed a significant decrease in AQP4 immunoreactivity followed by an increased expression at P30 and a subsequent stabilization of aquaporin levels in adulthood. All GBM cell lines examined exhibited significantly lower AQP4 expression than cultured astrocytes. T3 treatment significantly downregulated AQP4 in GBM-95 cells but did not influence the rate of GBM cell migration measured 24 h after treatment initiation. Collectively, our results showed that AQP4 expression is developmentally regulated by T3 in astrocytes of the cerebral cortex of newborn and young mice, and is discretely downregulated in GBM cells. These findings indicate that higher concentrations of T3 thyroid hormone would be more suitable for reducing AQP4 in GBM tumorigenic cells, thereby resulting in better outcomes regarding the reduction of brain tumor cell migration and proliferation.

Keywords: aquaporin 4, thyroid hormone, brain development, GBM, brain tumor

INTRODUCTION

Thyroid hormones play important roles during the development and maturation of the nervous system, being involved in the processes of myelination, cell growth, cell migration, in addition to their well-known metabolic effects (Oppenheimer et al., 1991; Trentin and Moura-Neto, 1995; Mullur et al., 2014).

Several studies have shown that the main water channel protein in the brain, aquaporin 4 (AQP4) also participates in important brain processes, including cell migration (Saadoun et al., 2005; Papadopoulos et al., 2008) and regulation of the flow of metabolites and ions (Ho et al., 2009) and that its expression can be regulated by the changes in metabolism (Deng et al., 2014), extracellular fluid volume, and tumorigenesis (Saadoun et al., 2002; Noell et al., 2012).

The aquaporins (AQPs) are a family of integral membrane carrier proteins that mediate bidirectional water transport across the membrane cells in response to an osmotic gradient. Currently, 14 members (AQP 0–13) of the AQP protein family have been identified and characterized in humans and rodents. The AQPs are structurally organized into tetramers within the cell membrane and each monomer acts as a pore for conducting water. Certain isoforms of AQPs may also mediate the transport of small solutes, such as glycerol, in addition to the transport of gasses (CO_2, NH_3, NO, and O_2) and ions (K^+ and Cl^-) (Papadopoulos and Verkman, 2013).

Thyroid hormones are essential for brain development and metabolic homeostasis. Their deprivation during pregnancy, even if modest, causes abnormal cortical development and changes of synaptic function affecting fetal development (Goodman and Gilbert, 2007). A correlation between low levels of thyroid hormones and a predisposition to the emergence of diseases affecting the central nervous system (CNS) has been demonstrated. Thus, patients with low serum levels of the hormone L-triiodothyronine (T3) exhibit a greater predisposition to strokes (Jiang et al., 2017), and there also appears to be a correlation between the levels of thyroid hormones and certain types of astrocytomas (Ding et al., 2013; Xiong et al., 2018), such as glioblastoma multiforme (GBM).

Gliomas are tumors originated in glial cells, which are classified as astrocytomas, oligodendrogliomas, ependymomas, and glioblastomas. The GBM is a diffuse high-grade astrocytoma with high invasiveness and migration capacity, which makes it very difficult to treat and thus, reducing patients' life expectancy to about 12 to 14 months after the diagnosis (Lacroix et al., 2001).

The expression of AQP4 is extremely correlated with the degree of severity of the astrocytoma (Warth et al., 2005; Zhao et al., 2012), tissue edema formation in the peritumoral region (Saadoun et al., 2002), increased cell migration (Saadoun et al., 2005; Papadopoulos et al., 2008) and disorganization of the characteristic arrangement of AQP4 in orthogonal arrays of particles (OAPS) in perivascular astrocytes endfeet (Noell et al., 2012).

These changes together, would contribute to the increase in brain swelling, rupture of the blood-brain barrier and disorganization of extracellular matrix (ECM) proteins, found in GBM tumors (Dubois et al., 2014).

Glioblastoma has also been reported to be a thyroid hormone-dependent tumor, in which these hormones would act promoting growth, migration, and development of tumor cells (Nauman et al., 2004; Davis et al., 2006; Sudha et al., 2017). Conversely, other studies have reported that, acting in non-genomic pathways thyroid hormone could reduce malignant cell proliferation and therefore, be a potential therapeutic agent in GBM (Martínez-Iglesias et al., 2009, 2016).

In astrocytes, T3 regulates protein expression in the ECM during brain development and the secretion of growth factors, which, in the cerebellum, act in an autocrine manner, inducing astrocyte proliferation, ECM reorganization, and cerebellar neuroblast proliferation, and are assumed to affect other pathways in the CNS via astrocytic cells (Trentin et al., 1995, 2001; Trentin, 2006).

Studies on the molecular mechanisms of thyroid hormone action and in the development and course of cancer have indicated a route of gene action, in which the pro-hormone thyroxine (T4) acts via integrin αvβ3, MAPK signaling, and ERK mitogen (1/2-protein kinase and extracellular signal-regulated kinase 1/2), mediated by phosphorylation of the thyroid β1 receptor (TRβ1), thereby inducing angiogenesis and tumor proliferation (Bergh et al., 2005). Although the effects of thyroid hormones on AQP expression, particularly in the CNS, are still largely unknown, current studies have shown T3 modulation of the expression of the liver mitochondrial isoform of aquaporin AQP8 in hypothyroid rats, indicating that T3 negatively regulates the AQP8 gene (Calamita et al., 2007).

In the present study, we aimed to evaluate whether the main water channel in astrocytes of the CNS (AQP4) could be regulated by T3 in normal brains at several stages of development (mice at P0 to P60) and also in brain glioma tumors.

The initial evaluation was carried out in cell culture using lines of human glioblastoma cells (GBM-11, GBM-95, and GBM-02), generously donated by Dr. Vivaldo Moura-Neto, as well as U-87 and SCC-4 [squamous cell carcinoma (SCC-4) of the tongue] cell lines. The effects on healthy cells were evaluated through the human HaCat keratinocyte cell line, and in a secondary culture of astrocytes derived from E16 mouse embryos. Finally, the effect of T3 on the expression of AQP4 and tumor cell mobility and invasiveness were analyzed in cell culture using a "scratch assay."

MATERIALS AND METHODS

Animals

Swiss mice at approximately the 18th day of pregnancy, provided by the Central Vivarium of the Federal University of Alagoas (BIOCEN-UFAL), were maintained in an air-conditioned room at 22°C and under a light-dark cycle of 12 h (lights on: 0700–1900). Pregnant mice at gestational day 18, or young animals on postnatal days 27 and 57, received injection of T3 (1 μg/g) or NaOH (0.02 N vehicle) during 3 consecutive days, intraperitoneally. The animals were anesthetized at postnatal days P0, P30, and P60 (n = 3–4 for each group) and then perfused with 0.9% NaCl followed by 4% paraformaldehyde,

after which their brains were dissected and post-fixed in 4% paraformaldehyde at 4°C.

This study was carried out in accordance with the recommendations of the Brazilian guide for the care and use of laboratory animals and local ethics committee. The protocol was approved by Animal Ethics Committee from Federal University of Alagoas (approval number 25/2013).

Immunohistochemistry

After post-fixation in 4% paraformaldehyde for 4 h, the brains were immersed in 30% sucrose solution at 4°C until subsequent preparation for microscopic analysis. Coronal section (40 μm) were cut using a cryostat (−20°C) and arranged on gelatinized slides. For immunohistochemical analysis, glial fibrillary acidic protein (GFAP) labeling was used to identify the location of AQP4, specifically in astrocytes. Briefly, sections were washed with phosphate-buffered saline (PBS) for 5 min (three times), immersed in 0.5% Triton X-100 in PBS solution (30 min), rinsed with PBS for 5 min (three times), and then blocked with 1% bovine serum albumin (BSA) for 90–120 min. Thereafter the sections were incubated overnight at 4°C with primary antibodies diluted in 1% BSA (anti-AQP4 1:200, Merck # AB3594; anti-GFAP 1:200, Dako #Z0334). The following day, after washing three times with PBS for 5 min, the sections were incubated with secondary antibodies (Alexa Fluor 448, Invitrogen #A11008 and Alexa Fluor 568 Invitrogen #A11004, 1:1000) diluted in 5% normal goat serum (1 h at room temperature), rinsed with PBS for 5 min (three times) and arranged on slides with PBS + glycerol solution (1:1). To evaluate the distribution of AQP4 in the brains of mice treated and non-treated with T3, the sections were observed under a fluorescence optical microscope (Nikon^{TM}). Cells showing immunoreactivity for AQP4 were quantified using the ImageJ imaging program.

Cell Culture

Human glioblastoma cells (GBM-95, GBM-02, and GBM-11) were kindly provided by Dr. Vivaldo Moura-Neto, and U87, HaCat, and SCC-4 cell lines were obtained from the American Type Culture Collection. These cells were cultured in Dulbecco's modified Eagle's medium (DMEM) F12 containing 10% fetal bovine serum (FBS), 10,000 U/mL penicillin, and 10,000 μg/mL streptomycin. Cultures were incubated at 37°C in a humidified atmosphere at 5% CO_2/95% atmospheric air.

E16 Astrocyte Secondary Culture

Pregnant Swiss mice, anesthetized with 100 mg/kg ketamine and 10 mg/kg xylazine, were submitted to cesarean surgery on the 16th embryonic day (E16). The uterus was placed in a Petri dish containing PBS and the embryos were removed. The brains were dissected and their cortices were placed in serum-free DMEM F12 culture medium for punching and cell dissociation. After centrifugation at 1500 rpm and 4°C, the supernatant was discarded and the pellet resuspended in serum medium for cell counting in a Neubauer chamber. The cells were plated in 25 mL bottles and the medium changed on alternate days to prevent neuronal growth. After 7 days, the cells were removed and plated again for treatment.

Cell Treatment

After reaching confluency, the cells were treated with 50 nM T3 in serum-free medium or only with serum-free medium for 24 h. Control cells were treated with serum-containing medium. After 24 h, the conditioned medium of the T3-treated cells was withdrawn and maintained at −20°C.

Immunocytochemistry

After washing with PBS, the cells were fixed in 4% paraformaldehyde, permeabilized with 0.3% Triton X-100, and blocked for non-specific binding with 5% BSA. Cells were incubated overnight in a refrigerator at 4°C in 0.3% blocking solution containing a polyclonal anti-AQP4 antibody (1:100) as described above. The following day, after washing with PBS, the cells were incubated with Alexa Fluor 546, Invitrogen # Z25304 anti-rabbit secondary antibody and/or Alexa 488-conjugated phalloidin, Invitrogen #A12379 (both 1:500). The nuclei were labeled with 1 mg/mL DAPI (4′,6-diamidino-2-phenylindole) and the coverslips glued with fluoromount. Photographs of cell were obtained at ×60 magnification.

Scratch Assay

Cells were plated in six-well plates and maintained under the same culture conditions until they reached confluence, at which time they were crossed with two cross-shaped scratches, performed using a 10-μL pipette tip, and photographed under a ×20 objective lens. After treatment with 50 nM T3 for 24 h, the cells were photographed once again.

Fluorescence Analysis

For quantification of fluorescence intensity, sections of mice brains were viewed at 10× using a fluorescence optical microscope (Nikon^{TM}). Images captured by a camera were saved in .jpeg format and quantified using the ImageJ program. An average of 6–10 sections by animal was analyzed, corresponding to AP: −2.1; and −2.5 mm from Bregma. The images were opened and a 'threshold' was settled. After putting a dark background, fluorescent stained areas for AQP4 were selected, and measured. The fluorescence intensities of the total cerebral cortex were averaged for each section and by group. AQP4 fluorescence intensity in cell lines HaCat, GBM 95, SCC-4, U-87 and secondary culture of astrocytes was quantified based on area of selected cell vs. mean fluorescence of background readings.

Statistical Analysis

Fluorescence quantification was performed based on the integrated densities of selected areas, using the ImageJ imaging program, and significance analysis of the differences between the conditions was performed with a one-way ANOVA. A Bonferroni post-test was used to compare the integrated densities between different treatments of the same cell type. Analysis of the scratch assay results was performed by comparing the means of the measures of the risk area at time zero and after 24 h of treatment. Statistical analyses were performed with GraphPad Prism 6.0 (GraphPad Software, Inc., San Diego, CA, United States).

RESULTS

Immunohistochemistry

Immunofluorescence analysis was performed on serial coronal sections (n = 3 or 4 animals, 6 to 10 sections). The results, shown in **Figure 1**, indicate that, relative to the control group, the treatment of mice with 1 μg/g T3 (E18–E20) reduced the distribution of AQP4 in the cerebral cortex on postnatal day P0 (Student's t-test: $P < 0.02$). However, T3-treated young mice showed a significant increase in AQP4 at postnatal day 30 (Student's t-test: $P < 0.0001$), which remained stable until the 60th postnatal day (P60). In the control animals, there was a significantly increased variation in AQP4 over the three postnatal developmental stages (P0, P30, and P60), indicating that T3 treatment contributes to the increased distribution of AQP4 in the cerebral cortex (one-way ANOVA: $P < 0.0001$).

Figure 1 shows the fluorescence microscopic images depicting the distribution of AQP4. A diffuse fluorescence can be observed in the entire cerebral cortex, with a more pronounced distribution of AQP4 being observed on the surface of astrocytic end feet surrounding the cerebral capillaries and the cortical surface near the pia mater, as has previously been described in the literature. Treatment with T3 resulted in a biphasic expression of AQP4 in the cerebral cortex of mice, with a decrease in expression being observed at the beginning of postnatal life (P0: $P < 0.01$), followed by an increase in expression at 30 days of life, relative to the control group.

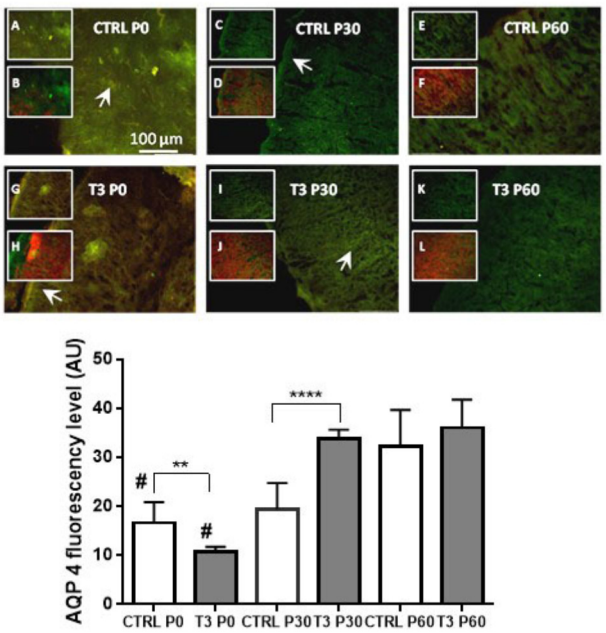

FIGURE 1 | (Upper) Aquaporin 4 (AQP4) distribution in the cerebral cortex of mice at postnatal days 0, 30, and 60 in control (CTRL) and T3-injected animals. Insets depict AQP4 immunoreactivity (green: A, C, E, G, I, and K) or merged GFAP (red) colocalization (B, D, F, H, J, and L). Immunoreactivity of AQP4 showed polarization to perivascular astrocytic end feet surrounding the cerebral microvessels (arrowheads in CTRL P0 and T3 P30) or in subpial astrocytes as depicted in CTRL P30 and T3 P0 slices. T3 injection caused an increase in AQP4 immunoreactivity with developmental progression. **(Lower)** AQP4 fluorescence levels in coronal sections of total brains. Mice treated with T3 showed higher levels of AQP4 from 30 to 60 days of age. N = 3 or 4 for each group; fluorescence analysis from 6 to 10 coronal sections of 40-μm thickness. Student's t-test; $^{**}P < 0.01$; $^{****}P < 0.0001$; $\#P < 0.0001$ one-way ANOVA followed by Tukey's test (CTRL P0 vs. CTRL P60; T3 P0 vs. T3 P30 and T3 P60).

In vitro Studies

Figures 2, **3** depict phase contrast microscopic images of GBM-11 and GBM-95 (**Figure 2**), HaCat and SCC-4 (**Figure 3**) cells grown under three different conditions: DMEM-F12 culture medium supplemented with 10% FBS, the same medium without serum supplementation (FBS-free), and T3 supplemented FBS-free medium (FBS-free + T3). Analyses of the images indicated that treatment of cells with T3 in FBS-free medium caused morphological changes in all the studied cell lines, leading to an increase in cytoplasmic volume and cellular processes. The images of GBM-95 and GBM-11 cells indicate that treatment with T3 in the FBS-free medium caused protoplasmic changes in the cells, producing a more elongated morphology (C, D, E, and F) relative to cells maintained in FBS medium (A and B). Moreover, T3 treatment also led to an increased number of cells with increased cytoplasmic volume, compared with cells maintained in FBS-free medium. HaCat cells treated with T3 and those maintained in FBS-free also exhibited a more elongated protoplasmic morphology (C, D, E, and F), relative to cells maintained in FBS (A and B), whereas no difference was observed between the T3-treated group and the cells maintained in FBS-free medium. In the SCC-4 cell line, T3-treated cells and those maintained in FBS-free medium were found to be more disorganized (C and D), less delimited, and more heterogeneous in shape than cells maintained in FBS medium (A and B).

Immunofluorescence – Cell Culture

Figure 4 presents immunofluorescence results obtained from an analysis of the integrated densities of images using the Prism program. **Figures 5A,B** show the results obtained using immunofluorescent markers for AQP4, red (left) and the merged images of AQP4, phalloidin, and DAPI (right). As shown in **Figure 5A** (D, E, and F), treatment with T3 negatively regulated AQP4 expression in the GBM-95 glioblastoma culture only when compared with AQP4 expression in cells maintained in FBS. Observation of a secondary culture of E16 astrocytes showed that the two groups maintained in FBS-free medium exhibited a significant difference in the expression of AQP4 relative to the group maintained with FBS only, although no statistically significant difference was observed between the FBS-free groups, suggesting that not only the T3 treatment but also the FBS-free conditions may have negatively regulated AQP4 expression, as shown in **Figure 5A** (A, B, and C). Analysis of SCC-4 cells indicated that the T3 treatment negatively regulates AQP4 expression compared with the cells maintained in FBS-free medium; however, this difference was not statistically significant according to **Figures 4**, **5B** (M, N, and O). **Figures 4**, **5** indicate that there were no significant differences in AQP4 expression in U-87 and HaCat cells related to T3 treatment or in response

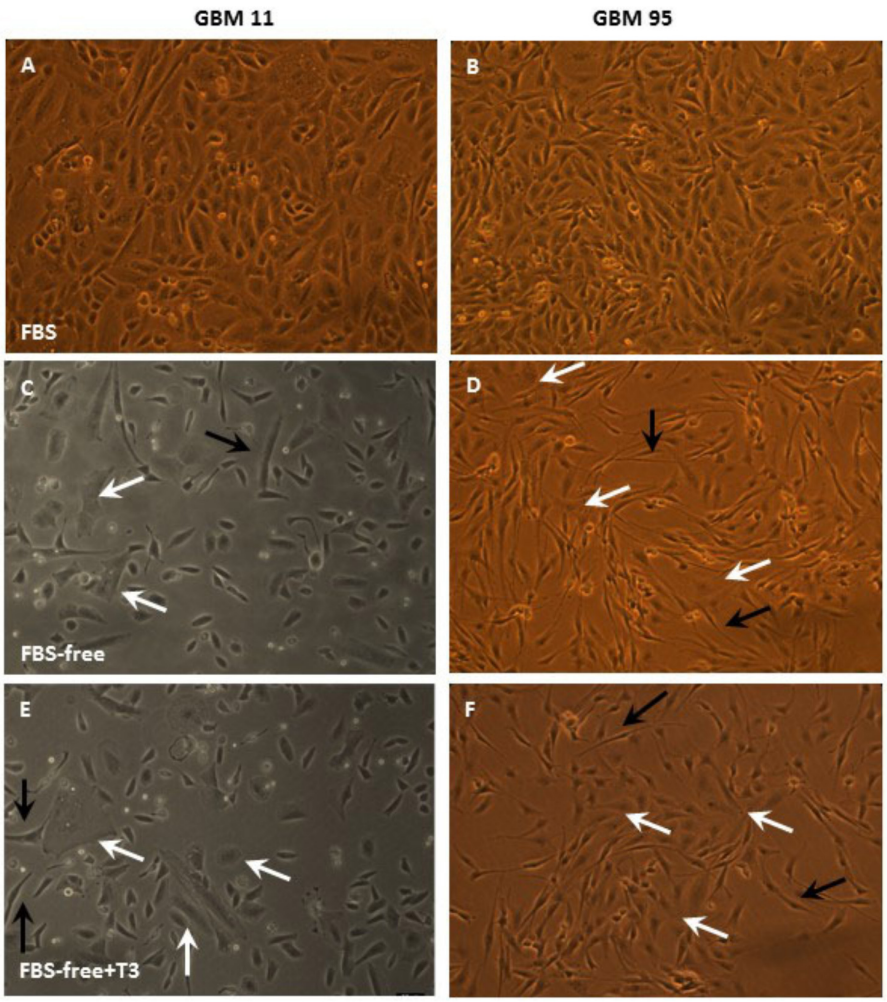

FIGURE 2 | Phase-contrast microscopic images of glioblastoma multiforme GBM-11 and GBM-95 cells cultured in medium containing 10% fetal bovine serum (FBS), medium without serum (FBS-free), and treated with T3 in medium without serum (FBS-free+T3). GBM tumor cells treated with T3 showed large cytoplasmic volume (**E,F**, white arrows) and processes (**E,F**, black arrows) when compared with those maintained in FBS-free medium **(C,D)**. GBM-11 and GBM-95 cells maintained in serum-containing medium **(A,B)**.

to the different culture media. However, as shown in **Figure 4**, all the cell lines examined showed significantly lower AQP4 fluorescence staining compared with the secondary cultures of astrocytes. Thus, our initial studies indicated that, compared with the treatment control (FBS-free medium), T3 treatment had no significant influence on the expression of AQP4 in any of the cell types examined.

Scratch Assay

Figure 6 shows the migration of cells of the GBM-95 line and the secondary culture of E16 astrocytes under our three treatment conditions: FBS, FBS-free, and FBS-free + T3. In this study, we used the FBS-free medium to reduce cell proliferation. For each treatment, we subjected the crossed out area to statistical analysis. Time T0 represents the beginning of treatment and T24 represents cell migration after 24 h.

We found that after 24 h, there was no statistically significant difference between cells grown in FBS-free +T3 and control (FBS-free) media. The total scratched area of GBM95 in FBS revels rather cell proliferation, than only cell migration ($P < 0.01$). In control astrocytes (AST E16) treated with both FBS and FBS-free medium, unexpectedly cell migration difference was not observed, although it was noted a tendency to decreased in cell migration.

DISCUSSION

In this study, we sought to determine whether the administration of T3 has a regulatory effect on the expression of AQP4 in astrocytes during the normal development of the CNS and in cells derived from glioblastomas, the major type of brain tumor. The results indicated that the expression of AQP4 in the

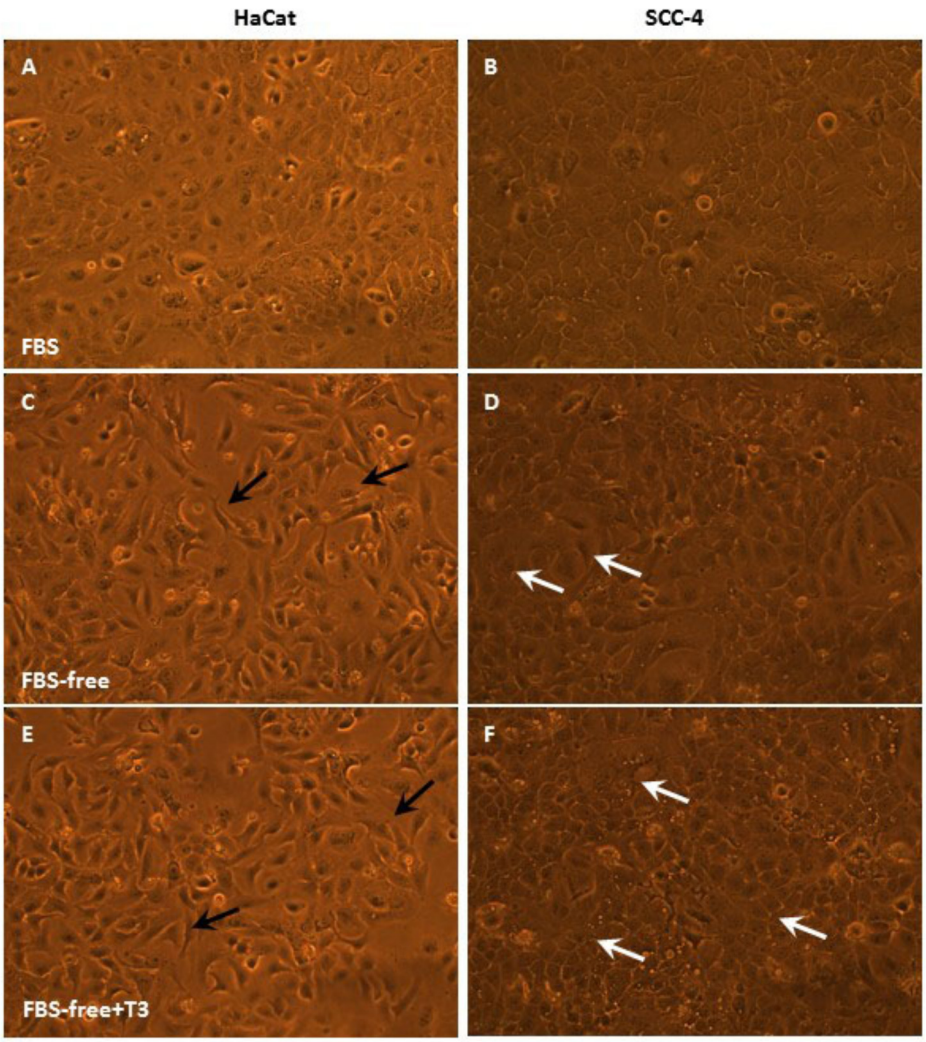

FIGURE 3 | Phase-contrast microscopic images of HaCat and SCC-4 cells in medium containing 10% FBS, medium without serum (FBS-free), and treated with T3 in medium without serum (FBS-free+T3). In the SCC-4 line, cells grown in T3-treated and FBS-free medium showed more disorganized cell–cell contacts **(C,D)** compared with those of cells maintained in serum-containing medium **(A,B)**. White arrows indicate large cytoplasmic volume **(D,F)** and black arrows cytoplasmic processes **(C,E)**.

mouse brain varies according to the developmental phase of the nervous system and that the thyroid hormone T3 modulates this expression during the different phases. In addition, experiments performed on a culture of normal astrocytes and glioma cells showed a potential effect of T3 on the expression of AQP4.

The lower expression of AQP4 at the onset of postnatal life observed in the control animals of our study could be related to a reduction in water volume in the brain that occurs during development. In mice, the decreased brain water content is more marked from postnatal day 14 to 21, corresponding to 84 and 79% of the adult brain water content, respectively. In this regard, it has previously been demonstrated that the decrease in postnatal brain water content is delayed in AQP4-null mice (Li et al., 2013). Interestingly, a higher expression of AQP4 during the period between postnatal days 7 and 14 has been found to precede a marked reduction of brain water volume in wild-type mice, indicating that higher expression AQP4 is pivotal for reduced CSF during the early postnatal weeks (Wen et al., 1999; Hsu et al., 2011; Li et al., 2013).

Fallier-Becker et al. (2014) demonstrated that during the development of the CNS in the P1–P3 stage, the distribution of AQP4 immunoreactivity became increasingly restricted to the subpial and perivascular end feet. The results of the present study are consistent with those of the aforementioned studies, in that we observed a lower level of AQP4 in the P0 control group relative to that in the adult animals, and that these difference were more marked in animals treated with T3, which exhibit a significant reduction of AQP4 at P0, and an increased brain expression of this protein at P30 and P60.

During pregnancy, there is limited T3 transport through the placenta, and T4 produced in the placenta is the main source of T3 for the developing fetal brain. In the brain, T4 is converted

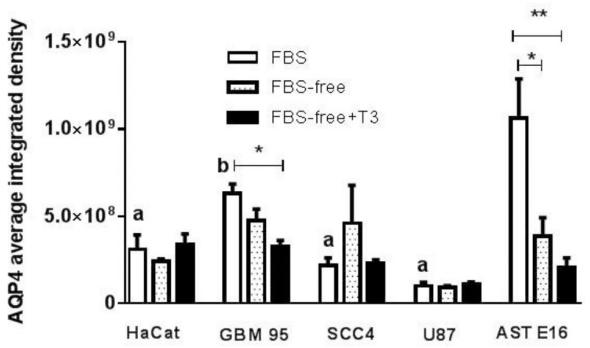

FIGURE 4 | Fluorescence analysis for AQP4 in HaCat, GBM-95, SCC-4, and U-87 cell lines and a secondary culture of astrocytes (AST E16). The integrated density is the sum of the values of the pixels in the area considered fluorescent. T3 treatment significantly downregulated AQP4 in GBM-95 cells maintained in FBS. Among all the cell lines examined, astrocytes exhibited the highest expression of AQP4. T3 treatment in FBS-free medium reduced AQP4 expression in astrocytes. HaCat, SCC-4, and U87 cell lines showed no change in AQP4 expression. Cells were cultured in medium containing 10% FBS, medium without serum (FBS-free), and treated with T3 in medium without serum (FBS-free+T3). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; and (a) $P < 0.001$; (b) $P < 0.05$ are related to differences between the tumor cells in FBS medium vs. secondary culture of Ast E16 in FBS.

to T3 by the enzyme deiodinase 2 (D2) present in astrocytes. Experiments carried out by Bárez-López and Guadaño-Ferraz (2017) and Bárez-López et al. (2018) using mice whose mothers had received thyroid hormone T4 in their drinking water during the E12–E18 embryonic phase revealed an increase in T3 and a reduction in T4 levels in maternal blood, whereas pups in the perinatal phase (P1) showed low levels of T4 and no change in T3 levels in the blood and brain. In the present study, pregnant females received subcutaneous injections of T3 for three consecutive days (E18–E20), and although thyroid hormone levels were not measured, it can be assumed that a similar alteration in the levels of these hormones occurred in the P0 animals, since during fetal life the transport of T3 through the placenta is limited. Thus, we conclude that the contribution of T4 in the animals of the P0 group would be decreased, which may have led to an increase in the activity of the D2 enzyme in the fetal brain to compensate for the fall of T4, thereby ensuring local T3 formation in the fetal brain.

It has recently been demonstrated in mice that T4, mostly of maternal origin, is transported via the cerebrospinal fluid reaching the fetal brain where it is converted into T3 through the astrocytic D2 enzyme present in the blood-brain barrier (meninges and choroid plexus) and lateral ventricles (Bárez-López et al., 2018). From the cerebrospinal fluid, the T3 thus produced can disperse throughout the brain and access the cells to exert its action. D2 enzyme activity decreases around P3 and was not detected in the meninges, choroid plexus, or lateral ventricles of adult rodents. Assuming that T3 injection in the mothers caused a decrease in T4 in fetal blood of the animals examined in the present study, there would be an increase in the activity of D2 enzyme in astrocytes to compensate for the decrease in T4 to ensure the maintenance of T3 levels in the brain. Concomitantly, a decrease in the action of the enzyme D3 in neurons would ensure the reduction in the metabolism of T3 to rT3 and T2, thereby ensuring the supply of T3 to the brain. Accordingly, T3 would act on astrocytes, leading to a reduction in AQP4 expression, as observed in the P0 animals examined in this study.

It is known that thyroid hormones play a role in the reduction of AQP4 expression through non-genomic pathways by protein kinase C (PKC) activation (Sadana et al., 2015). The PKC pathway when activated leads to the phosphorylation of AQP4 (residue Ser 180), causing a reduction of this water channel (Zelenina et al., 2002). Reduced AQP4 expression found in the brains of P0 T3-treated animals in our study could have been mediated by the conversion of circulating T4 to T3 in the astrocytes, promoting a reduction in AQP4 via PKC activation. Subcutaneous injections of T3 in young and adult animals in our study could be supposed to promote an increase of T3 supply to the brain, via the MCT8 transporter present in the blood–brain barrier increasing neuronal D3 activity to regulate cerebral T3 levels (Roberts et al., 2008). Decreased activity of T3 in the astrocytes could reduce AQP4 phosphorylation, thereby increasing the expression of this protein in the brain of young mice. Supporting this, studies have shown that adult Dio3KO mice exhibit an increase in T3 concentration, leading to a state of central hypothyroidism and decreased expression of T3-regulated genes (Hernandez et al., 2010, 2012), which is consistent with the data obtained for adult P60 animals in the present study, in which we found no significant difference in the expression of AQP4 between the control and T3-treated animals.

We assume that the effects of T3 administration on the expression of AQP4 observed in the present study are not attributable to the possible toxic effects of T3 on the brain or thyroid, given that the experimental protocols used to induce hyperthyroidism in animals involve administration of this hormone for approximately 14 consecutive days and at a dosage of 250 μg/kg (Drover and Agellon, 2004), which is considerably higher than the used in the present study.

In the present study, we have also examined the effects of T3 on the expression of AQP4 in cultured human glioblastoma cells. We accordingly found that all the cell lines examined showed significantly lower AQP4 expression compared with that observed in the secondary culture astrocytes, thereby confirming the previously established principal site of AQP4 in this cell type. Nevertheless, a significant effect on AQP4 expression was not observed under all conditions upon treatment with T3, relative to the treatment control, although we did observe a tendency for a reduction in AQP4 expression in GBM-95 cells and normal astrocytes.

Evaluation of the effects of T3 on cell migration after 24 h of treatment showed that in both normal astrocytes and GBM95 cells there was no difference in the migratory process. The reduction found in GBM95 FBS-free and GBM95 FBS-free + T3 medium was probably attributed to cell proliferation decrease, more than cell migration, as a result of the absence of FBS in the two treated groups (**Figure 6**). Our studies examining astrocyte and glioblastoma cell cultures indicated a tendency for T3 to negatively regulate the expression of AQP4 under the conditions

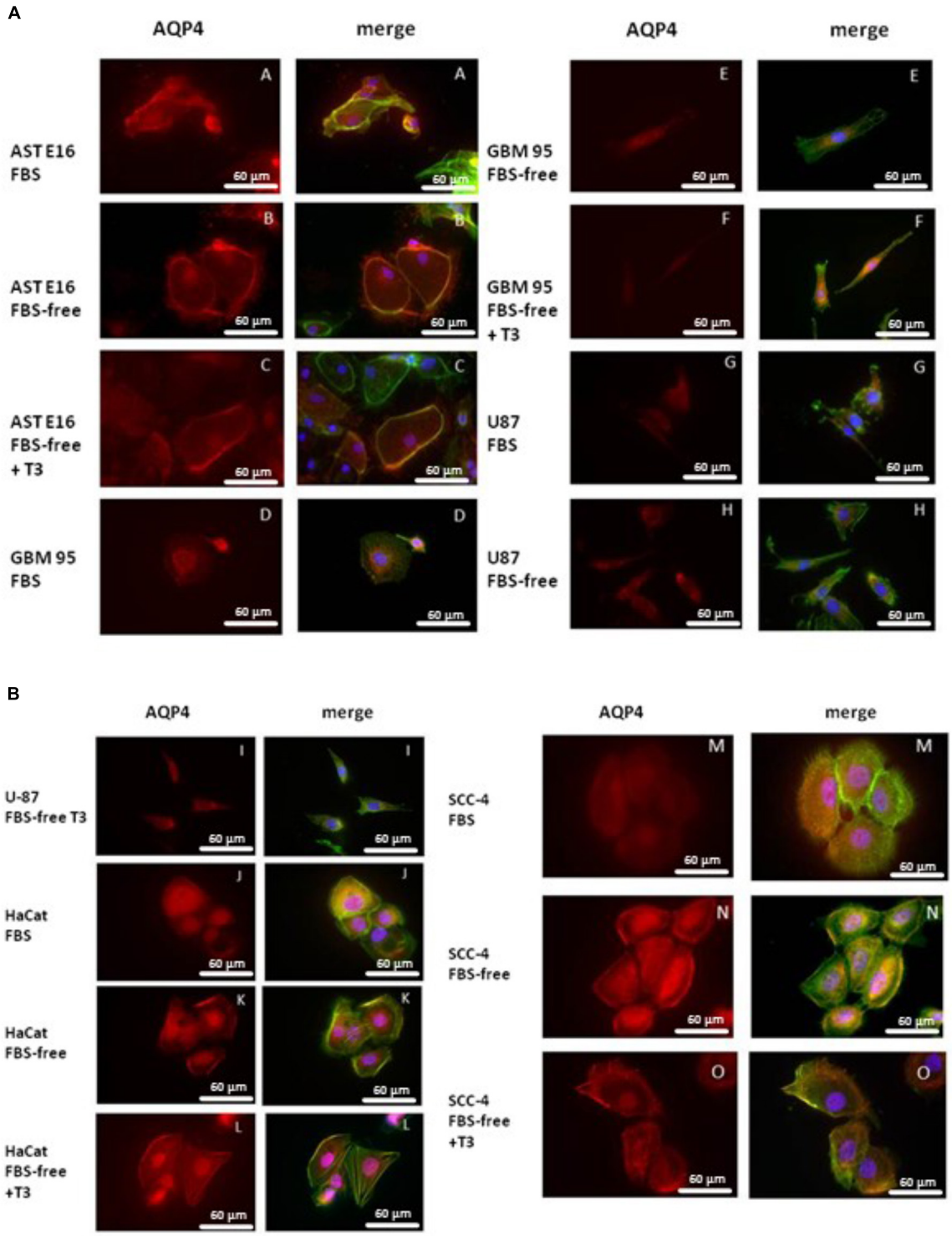

FIGURE 5 | (A) Immunofluorescence staining for AQP4 (red) and phalloidin (green) or DAPI (blue). T3 treatment (50 mM) negatively regulated AQP4 expression only in GBM-95 cells when compared to AQP4 expression in cells maintained in FBS, as shown in **Figure 6**. Cells were cultured in medium containing 10% FBS, medium without serum (FBS-free), and treated with T3 in medium without serum (FBS-free+T3). **(B)** Immunofluorescence staining for AQP4 (red) and phalloidin (green) or DAPI (blue). HaCat, SCC-4, and U87 cell lines treated with T3 (50 mM) showed no change in AQP4 expression.

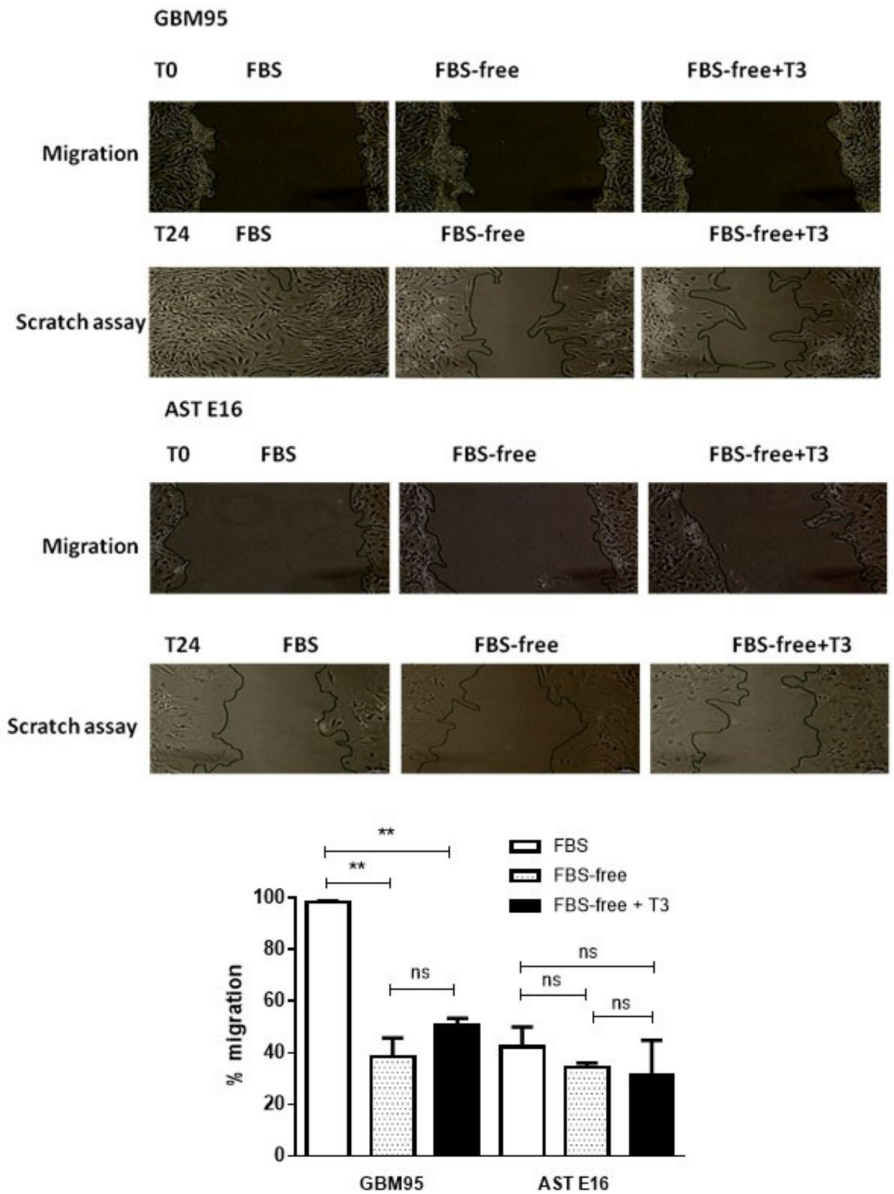

FIGURE 6 | (Upper) Analysis of cell migration in GBM-95 cells and cultured E16 astrocyte cells. The migration and invasion of the GBM cell line was not significantly altered by treatment with T3, whereas the migration of cultured astrocytes was significantly decreased by 24.47% in response to treatment with T3. **(Lower)** Cell migration assay of GBM-95 cells and cultured E16 astrocytes treated with T3. Assay measurements were performed at two different time points: T0, represents the beginning of the treatment and T24 represents 24 h after the start of treatment. Cells were cultured in medium containing 10% FBS, medium without serum (FBS-free), and treated with T3 in medium without serum (FBS-free+T3). The images were analyzed by measuring the reduction in scratch area (***$P < 0.001$: one-way ANOVA).

described above (**Figure 4**), which did not influence the cellular migration process.

Preliminary data on cell cultures indicated a discrete down-regulation of AQP4 expression in GBM-95, SCC-4, and astrocyte secondary culture cells subjected to the serum-free medium and T3 treatments. Previous studies on the effect of thyroid hormones on the development, migration, and growth of gliomas, in particular GBM, have yielded contradictory data, indicating either a protective action of these hormones on tumor development or a proliferative action in the development of gliomas (Sudha et al., 2017). In this regard, some epidemiological studies have provided evidence of the role of thyroid hormones in promoting tumor cell proliferation, arguing that several different types of tumors exhibit increased growth in patients with a history of hyperthyroidism (Moeller and Führer, 2013). For example, it has been found that patients with a history of hyperthyroidism are more likely to develop ovarian cancer, whereas patients with pancreatic cancer have a two-fold

higher risk of developing this type of tumor when they have hyperthyroidism. Notably, these epidemiological studies report an increased probability of enhanced proliferation of cancer cells under conditions of a pathological increase in the release of thyroid hormones (hyperthyroidism).

Studies by Davis et al. (2006) have highlighted the role of thyroid hormones in the growth of gliomas. These hormones have been shown to act as growth factors on gliomas, acting non-genomically via the αVβ3 receptor, to promote cell growth. There is evidence that T3 binding to the S1 site of the αVβ3 receptor activates PI3K (lipid kinase) and increases cellular invasiveness and metastasis, whereas the binding of T4 to the S2 site of this ERK1/2 active receptor alters FGF2 expression and promotes angiogenesis, with both these pathways favoring tumor growth. In contrast, more recent studies have indicated the more prominent action of T4 in tumorigenesis, relative to T3. Using a deaminated T4 derivative, tetraiodothyroacetic acid, linked to nanoparticles as an anticancer agent in the treatment of human GBM U87MG xenografts cells in immunodeficient mice, Sudha et al. (2017) demonstrated a reduction in tumor cell density (of up to 80%) and tumor necrosis caused by the T4 derivative possibly acting by inhibitory effect of extracellular domains of integrin αvβ3 T4 receptor. Other studies appear to highlight the greater activity of T4 in the processes of tumorigenesis, when compared to the direct effects of T3.

CONCLUSION

In conclusion, in this study we demonstrated that T3 induces morphological alterations in normal and GBM-95, GBM-11, HaCat, and SCC-4 cells. Moreover, T3 treatment resulted in a positive regulation of AQP4 expression in the brain of mice at three different stages of brain development: immediately after birth and at 30 and 60 days of age. Astrocyte and glioblastoma cell culture studies revealed the tendency of T3 to negatively regulate the expression of AQP4 under the experimental conditions described herein, without significantly disrupting cellular migration. Further studies should be carried out to confirm the present results regarding the effects of T3 on the decreased expression of AQP4 and its relationship with tumor cell migration, and thereby unravel the possible role of the biologically active form of this hormone in altering the migration pattern of these cells.

DATA AVAILABILITY

The datasets for this manuscript are not publicly available because the data are the results of research projects that are being submitted for the first time for publication. Requests to access the datasets should be directed to ximenes.adri@gmail.com.

AUTHOR CONTRIBUTIONS

AX-d-S and VM-N conception and design of the experiments. LC, JC-N, CM, NF, and EC collected the data. AX-d-S, LC, JC-N, and CM analyzed and interpreted the data. AX-d-S, JC-N, and CM drafted the article and critically revised for important intellectual content. All authors approved the final version of the manuscript.

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REVIEW
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Efficacy and Mechanism of Panax Ginseng in Experimental Stroke

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Stroke is one of the leading causes of death and long-term disability worldwide. However, effective therapeutic approaches are still limited. The disruption of blood supply triggers complicated temporal and spatial events involving hemodynamic, biochemical, and neurophysiologic changes, eventually leading to pathological disturbance and diverse clinical symptoms. Ginseng (*Panax ginseng*), a popular herb distributed in East Asia, has been extensively used as medicinal and nutritional supplements for a variety of disorders worldwide. In recent years, ginseng has displayed attractive beneficial effects in distinct neurological disorders including stroke, involving multiple protective mechanisms. In this article, we reviewed the literature on ginseng studies in the experimental stroke field, particularly focusing on the *in vivo* evidence on the preventive or therapeutic efficacy and mechanisms of ginseng and ginsenosides in various stroke models of mice and rats. We also summarized the efficacy and underlying mechanisms of ginseng and ginsenosides on short- and long-term stroke outcomes.

Keywords: ginsenosides, global cerebral ischemia, intracerebral hemorrhage, middle cerebral artery occlusion, permanent MCAO, subarachnoid hemorrhage, transient MCAO

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INTRODUCTION

Ginseng (*Panax ginseng C. A. Meyer*) has been extensively used as medicinal and nutritional supplements for a variety of disorders worldwide (Rastogi et al., 2014; Colzani et al., 2016). Asian ginseng has a history of herbal use over thousands of years, first described in the ancient Chinese pharmacopeia, Shen Nong Ben Cao Jing (300 BC—200 AD, also *Divine Farmer's Classic of Materia Medica*) (Unschuld, 1985; Yang and Wu, 2016). It is one of the most highly regarded herbs in the Orient used to promote health, general body vigor, and to prolong life span. The Greek word "*Panax*" originates from the word "panacea," which means "cure all diseases," and true to its name, ginseng has been proven to have a wide variety of medicinal uses, including benefits in cardiovascular disorders (Karmazyn et al., 2011; Sun et al., 2016; Kim, 2018), aging-related disorders (Bjorklund et al., 2018), and others (Sotaniemi et al., 1995; An et al., 2011; Shergis et al., 2014; Zhang et al., 2017; Arring et al., 2018). In recent years, preclinical and clinical studies revealed that ginseng displayed attractive beneficial effects in multiple neurological disorders like stroke, hypertension, cancer, and maintenance of hemostasis in the immune system, involving multiple protective mechanisms (Lee et al., 2009; Im and Nah, 2013; Rastogi et al., 2014; Gonzalez-Burgos et al., 2015; Ong et al., 2015; Oh and Kim, 2016; Wang et al., 2016b; Kim et al., 2018).

Stroke is a leading cause of death and long-term disability worldwide (Feigin et al., 2017; Benjamin et al., 2018); however, effective therapies are limited (Feigin et al., 2016). The disruption of blood supply triggers complicated temporal and spatial events involving hemodynamic,

biochemical, and neurophysiologic changes, eventually leading to pathological disturbance and diverse clinical symptoms (Lo et al., 2003; Iadecola and Anrather, 2011; Annunziato et al., 2013; Bernhardt et al., 2018). The severity and dynamic progression of brain injury depend on the degree of cerebral blood flow (CBF) interruption, lesion volume and site, duration of stroke, and the coexisting complications (Shen and Duong, 2008; Sun et al., 2014b; Fu et al., 2015; Ward, 2017). Accumulated evidence shows that oxidative stress and inflammation play key roles in the pathophysiology of stroke (Iadecola and Anrather, 2011; Li et al., 2011a; Carbone et al., 2015; Fu et al., 2015). Although the ginseng remedy has been widely applied to improve cardiac health and circulation, their studies in the stroke field are still limited (Gan and Karmazyn, 2018; Kim, 2018). Over the last decade, much promising advancements were made in the therapeutic effects of ginseng or ginsenosides on experimental stroke brain injury.

In this article, we reviewed the literature on ginseng and ginsenosides studies in the experimental stroke field, particularly focusing on the *in vivo* evidence in diverse stroke models of mice and rats. We summarized the efficacy of ginseng and ginsenosides on short- and long-term stroke outcomes, as well as the underlying molecular and cellular mechanisms. This review provides current understanding of the pharmacological benefits of ginseng that contribute to stroke prevention and recovery.

PANAX GINSENG AND ITS ACTIVE CONSTITUENTS

Two common products of ginseng are red ginseng, prepared by a process of steaming or heating, and dried white ginseng, prepared by air-drying after harvest (Wang et al., 2016a; He et al., 2018). Due to the presence of different active components, they have distinct pharmacodynamics profiles (Karmazyn et al., 2011). The major active components responsible for the pharmacological activities of ginseng are a group of unique triterpene glycosides or saponins called ginsenosides. The first attempt to isolate the active constituents of ginseng began many years ago, and the isolation of ginsenosides was started in 1963 (Shibata et al., 1963). To date, more than 150 ginsenosides have been isolated from ginseng, 40 of which have been found in *Panax ginseng* (Christensen, 2009).

Ginsenosides are divided into two different structural classes: (1) The 20(S)-protopanaxadiol (PPD) type that includes Ra1, Ra2, Ra3, Rb1, Rb2, Rb3, Rc, Rd, Rg3, Rh2, F2, and compound K; (2) The 20(S)-protopanaxatriol (PPT) type that includes Re, Rf, Rg1, Rg2, Rh1, and F1 (Baek et al., 2012). They share a four-ring hydrophobic steroid-like structure with sugar moieties, but differ in the carbohydrate moieties at C3, C6, and C20. **Figure 1** shows the chemical structures of some of the most commonly studied ginsenosides. Quantitative and statistical analyses of the plasma indicate that PPD ginsenosides exhibit higher concentration and longer half-life than PPT ginsenosides (Zhang et al., 2014b). The peak concentrations of ginsenosides Rb1, Rb2/b3, Rc, Rd, Rg1, and Re are 55.32, 30.22, 21.42, 8.81, 7.15, 2.83 mg/l, while their mean values of half-lives are 18.41, 27.70, 21.86, 61.58, 15.26, and 2.46 h, respectively.

Intact ginsenosides are absorbed only through the intestines with a very low absorption rate at 1–3.7%. Most ginsenosides are metabolized in the stomach (acid hydrolysis) and/or intestine (bacterial hydrolysis) and transformed to other ginsenosides (Oh and Kim, 2016). For instance, ginsenoside Rb1 is processed by gastric acid/intestinal microorganisms into smaller molecules, such as Rd, F2, and compound K, and further into PPD. Similarly, ginsenoside Rg1 is converted into Rh1 and F1, and further into PPT, which is better absorbed in the gastrointestinal tract and therefore more bioactive than parent compounds. Collective evidences suggest that the metabolism and transformation of intact ginsenosides is a crucial process, influencing the bioavailability and potential health benefits of ginseng (Chen et al., 2008a).

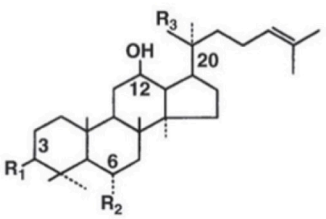
STROKE MODELS OF MICE AND RATS

Stroke can be classified into two types: ischemic stroke and hemorrhagic stroke. In ischemic stroke patients, the middle cerebral artery (MCA) is the artery most often blocked. Accordingly, focal cerebral ischemia models (permanent or transient) that aim at MCA territory have been most widely used (Dorr et al., 2007; Mehta et al., 2007). In contrast, global cerebral ischemia occurs when cerebral blood flow (CBF) is disrupted throughout whole brain. Hemorrhagic stroke is a devastating stroke subtype with a high mortality rate within 1 month; it mainly includes intracerebral and subarachnoid hemorrhage (Maclellan et al., 2010; Ma et al., 2011; Leclerc et al., 2018).

(i) Permanent focal cerebral ischemia (pdMCAO and pMCAO): The MCA can be occluded at distal or relatively proximal site; consequently they are termed as pdMCAO or pMCAO. Comparably, the pdMCAO model generates a reproducible ischemic lesion that is mainly restricted in cortex region and leads to definable sensorimotor deficits. Because it closely mimics human ischemic stroke, it serves as one of the most useful stroke models, allowing to assess long-term recovery with high survival rate (Doyle and Buckwalter, 2014). pMCAO can be produced by the intraluminal suture MCAO.

(ii) Transient focal cerebral ischemia (tMCAO): The rodent MCAO with intraluminal suture is the most widely used animal stroke model, displaying reproducible MCA territory infarctions and allowing reperfusion by retracting the suture. Usually, MCAO generates ischemic infarct damage in the striatum, overlying frontal, temporal, parietal, and portions of cortex. Around 60 to 120 min of ischemia following MCAO is required to generate reproducible infarct volumes.

(iii) Global cerebral ischemia (GCI): Due to cardiac arrest, GCI results in delayed neuronal death in the hippocampal CA1 region and subsequent cognitive decline (Traystman, 2003; Tu et al., 2015; Ostrowski et al., 2016). The four-vessel occlusion (4VO) model provides a method of reversible



	Ginsenoside	R1	R2	R3
PPD	Rb1	-O-Glu-Glu	-H	-O-Glu-Glu
	Rg3	-O-Glu-Glu	-H	-OH
	Rd	-O-Glu-Glu	-H	-O-Glu
PPT	Rg1	-OH	-O-Glu	-O-Glu
	Re	-OH	-O-Glu-Rha	-O-Glu

FIGURE 1 | Chemical structures of most commonly studied ginsenosides. Glu, glucose; Rha, rhamnose.

forebrain cerebral ischemia-reperfusion, whereas the two-vessel (2VO) model was developed to characterize the incomplete ischemia (Traystman, 2003).

(iv) Cerebral hypoxia-ischemia (HI): HI is a transient unilateral cerebral ischemia model, which produces reproducible brain lesion in the ipsilateral hemisphere (Liu et al., 2019). Following the occlusion of one side of the common carotid artery and after a short recovery, the animal will be exposed to systemic hypoxia for no more than 1h.

(v) Intracerebral hemorrhage (ICH): ICH is a most devastating type of stroke without effective therapies. Two available models are used to mimic spontaneous intracerebral bleeding, either by the stereotactic injection of autologous blood or collagenase (Maclellan et al., 2010; Ma et al., 2016; Ahmad et al., 2017). Till now, no ginseng study has been performed in the ICH model.

(vi) Subarachnoid hemorrhage (SAH): SAH claims one of the highest rates of mortalities and morbidities. None of therapeutic options has effectively to reduce mortality rate in a clinical setting. Rodent models have been predominantly made by approaches involving intravascular perforation of a vessel in the circle of Willis or direct injection of blood into the cisterna magna or prechiasmatic cistern (Leclerc et al., 2018).

Therapeutic Effects of Panax Ginseng on Stroke Outcomes: The *in vivo* Evidence

Multiple administration strategies of ginseng have been employed in the experimental stroke studies, including mice or rats with different genetic backgrounds, pre-treatment or post-treatment, administration routes, dosage range and duration, and various histological and neurobehavioral stroke outcomes. Infarct volume is designed to evaluate the temporal evolution of stroke damage that can be easily measured with different techniques. Neurobehavioral assessment is an essential measure of stroke outcome since functional recovery is universally used as a primary endpoint in clinical trials. Both histological and neurobehavioral measurements are considered as pivotal components for examining efficacy of potential therapeutics in the translational stroke research field. Here, we outlined the short-term (usually referring to the acute stage of recovery following stroke, about 1–3d) and long-term (usually referring to 3d to weeks or months following stroke) effects of ginseng and ginsenosides on stroke outcomes. **Table 1** summarized the details of these studies.

Red Ginseng

The standard extracts of red ginseng (such as Korean red ginseng, KRG) are manufacture by the traditional preparation method (by a steaming or heating process) and contain most of the primary effective components, coordinately controlling the pharmacological efficacy in the body (Lee et al., 2015; Wang et al., 2016a). Many are converted from the major ginsenosides Rb1, Rb2, Rc, Rd, Rg1, and Re (Lee et al., 2015). The therapeutic efficacy of KRG on ischemic brain damage, at the dosage of 100–360 mg/kg/d for 7–14d, has been revealed in permanent and transient cerebral ischemia models by several groups. In the pdMCAO mouse model, KRG pretreatment prevented the acute enlargement of ischemic brain lesion (36.37 $\pm$ 7.45% on d3) and the definable sensorimotor deficits indicated by optimized cylinder and corner tests, and such functional benefits extended over 28d (Liu et al., 2018b). KRG pretreatment also reduced the infarct volume at 24 h and improved the coordinated motor deficits, indicated by the rotarod test, at 3 and 7d after MCAO (1 h) (Cheon et al., 2013). Recently, it was reported that pretreatment with Ginseng elicited robust neuroprotection against the deterioration of acute cerebral hypoxia-ischemia damage in an Nrf2-dependent manner, evidenced by the reductions of neurological deficits and brain infarction and edema at 6 h, 1 and 3d after HI (Liu et al., 2019). Such beneficial outcomes could be associated with the enhanced expression levels of Nrf2 target antioxidant proteins and anti-inflammation mediators. Meanwhile, KRG post-treated rats showed significant improvement in the neurological deficits for 7d indicated by the modified neurological deficits score (NDS) and corner test, as well as the infarct volume at 7d, following ischemia-reperfusion injury after MCAO (2 h) (Lee et al., 2011; Ban et al., 2012). In addition, ethanolic P. ginseng extracts post-treatment was reported to reduce rat hippocampal CA1 neuronal death 7d after global cerebral ischemic injury (Kim et al., 2009), further supporting the beneficial role of KRG in ischemic stroke.

Ginsenoside Rb1

Ginsenoside Rb1 (Rb1) is a representative component of *Panax genus*, including *Panax ginseng* (Asian ginseng), *Panax quinquefolius* (American ginseng), and *Panax notoginseng*

TABLE 1 | The effects of various ginseng extracts on stroke outcomes in rodent stroke models.

Species	Genetic background	Type of ginseng extracts	Dosage/Administration route	Time of termination	Brain lesion /Edema	Neurobehavioral deficits	Authors/year
CEREBRAL ISCHEMIA							
Permanent Distal Middle Cerebral Artery Occlusion (pdMCAO)							
Mouse	C57BL/6	Red ginseng (KRG)	• Pre;100 mg/kg/d; gavage • For 7d	28d	• Infarct volume (1d, 3d ↓) • Brain edema (1, 3d) ↓	(pre, 1, 3, 7, 14, 21, 28d for all tests) • Open field • Cylinder (3, 7, 28d) ↓ • Corner (3, 7, 14, 28d) ↓	Liu et al., 2018b
Rat	SHR-SP (spontaneous hypertensive rats-stroke prone)	Ginsenoside Rb1	• Post; 60 ul (6 or 60 μg/60 ul/d); i.v.; immediately after MCAO; infusion for 4 wks • 60 μl (6, 60, 3,000, or 12,000 μg/60 μl/d); i.v.; at 2 h after MCAO; infusion for 1d	2, 4 wks	• Infarct size ↓ • (28d or delayed treatment 1d; 6 or 60 μg/60 ul/d)	• Water maze (2, 4 wks) ↓	Zhang et al., 2006
Rat	SH-SP	Ginsenoside Rb1	• (0.006 to 6.0) μg/d; i.c.v.; started 2 h before MCAO; infusion for 4 wks; and (0.06, 0.6, 6.0) μg/d; i.c.v.; started immediately after MCAO • Infusion for 4 weeks	2, 4 wks	• Infarct size ↓ 32d; Rb1 (0.6 μg/d)	• Water maze (2, 4 wks); Rb1 (0.6 μg/d) ↓ • Inclined Screen (muscle strength, 2, 4 wks) • Rotarod (2, 4 wks)	Zhang et al., 1998
Permanent Middle Cerebral Artery Occlusion (pMCAO)							
Rat	SD	Ginsenosides Rb1 and Rg1	pMCAO • Pre; Rb1 (10, 20 and 40 mg/kg); Rg1 (40 mg/kg); i.v.; 30 min before MCAO MCAO (2 h) • Pre/Post; Rb1 (10, 20, and 40 mg/kg); Rg1 (40 mg/kg); i.v.; 30 min before MCAO or immediately after MCAO	24 h	• Infarct size (24 h) • pMCAO: Rb1 (40 mg/kg) ↓; • tMCAO (2 h): Rb1 (10, 20, and 40 mg/kg) ↓ • Brain edema (24 h) • pMCAO: Rb1 (40 mg/kg) ↓ • tMCAO (2 h): Rb1 (10, 20, and 40 mg/kg) ↓Rg1 (40 mg/kg) ↓	pMCAO: • Neurologic deficits score (24 h) Rb1 (40 mg/kg) ↓ • MCAO (2 h) • Neurologic deficits score (24 h) Rb1 (10, 20 and 40 mg/kg) ↓	Zhang and Liu, 1996
Rat	SD	Ginsenoside Rd	• Pre; dose-response study: 1–50 mg/kg; i.p.; 30 min before MCAO • Post; therapeutic window study: 50 mg/kg; i.p.; at 0, 2, 4, or 8 h after the reperfusion for transient ischemia or after the onset of artery occlusion for permanent ischemia	24 h for pMCAO; 42d for tMCAO (2 h)	dose-response study • Infarct volume (1,3,7d; 10 and 50 mg/kg) ↓ • Brain edema (24 h; 10 and 50 mg/kg) ↓ therapeutic window study • Infarct volume (24 h; pMCAO; 2 h or 4 h treatment, not 8 h) ↓ • Infarct volume (1,3,7d; tMCAO; 2 or 4 h treatment, not 8 h) ↓	dose-response study (pre, 1, 3, 7, 14, 21, 28, 42d) • Modified neurological severity score (10,50 mg/kg) ↓ • Modified sticky-tape test (10,50 mg/kg) ↑ • Corner (50 mg/kg) ↓ therapeutic window study (pre, 1, 3, 7, 14, 21, 28, 42d) • Modified neurological severity score (2 or 4 h treatment) ↓ • Modified sticky-tape test (2 or 4 h treatment) ↑ • Corner (2 or 4 h treatment) ↓	Ye et al., 2011a

(Continued)

TABLE 1 | Continued

Species	Genetic background	Type of ginseng extracts	Dosage/Administration route	Time of termination	Brain lesion /Edema	Neurobehavioral deficits	Authors/year
Rat	Wistar	Ginseng total saponins (GTS)	• Pre/Post; 25 mg/kg/d; i.p.; start 3d before MCAO; twice daily • For 1,3,7,14d	1, 3, 7, 14d	• ND	• Neurological deficits score (14d) ↓	Zheng et al., 2011
Transient Middle Cerebral Ischemia (MCAO, 0.5 h)							
Mouse	C57BL/6	Compound K	• Pre; 30 mg/kg; i.p.;	3d	• Infarct volume (3d; total, cortex, but not striatum) ↓	• ND	Park et al., 2012
Transient Middle Cerebral Ischemia (MCAO, 1 h)							
Mouse	C57BL/6	Red ginseng (KRG)	• Pre; 360 mg/kg; i.p.; before MCAO • For 14d	4 and 24 h, 1, 3, 7, 14d	• Infarct volume (24 h) ↓	• Rotarod (3, 7d) ↑	Cheon et al., 2013
Mouse	C57BL/6J	Ginsenoside Rb1	• Pre; 0.5, 1, 5 or 10 mg/kg, respectively; oral gavage; every 3d • From 1 year till 2 years old	1, 2, 3, 7d	• Infarction volume (48 h; 1, 5, 10 mg/kg) or (1, 3, 7d; 5 mg/kg) ↓ • Brain edema (48 h; 1, 5, 10 mg/kg) or (1, 3, 7d; 5 mg/kg) ↓	• Neurological Bederson score (48 h; 1, 5, 10 mg/kg) or (1, 3, 7d; 5 mg/kg) ↓	Dong et al., 2017
Mouse	ICR	Ginsenoside Rb1	• Post; 5, 20 or 40 mg/kg; i.p.; after 3 h reperfusion	24, 48 h	• Infarct volume (48h; 20 and 40 mg/kg) ↓ • Brain edema (48 h; 20 and 40 mg/kg) ↓	• Neurological behavior deficits (5 point test; 48 h; 20 and 40 mg/kg) ↓	Chen et al., 2015
Mouse	C57BL/6	Ginsenoside Rd	• Pre (dose-response study); 0.1, 1, 10, 50, and 200 mg/kg; i.p.; 30 min before MCAO onset; once • Post (therapeutic window study); 50 mg/kg; i.p.; 2, 4, 6, or 10 h after the onset of MCAO • Pre; 50 mg/kg; i.p.; 30 min before MCAO; once • Post; 50 mg/kg/d; i.p.; immediately after reperfusion till 7d	1, 14d	• dose-response study: • Infarct Volume (1d; 10 and 50 mg/kg) ↓ • therapeutic window study: Infarct Volume (1d; treatment at 2 or 4 h after reperfusion) ↓ • Pre: Infarct volume (14d; 50 mg/kg) ↓ • Post: no difference from pre	dose-response study: • NDS on a scale of 0-12 (1d; 10 and 50 mg/kg) ↓; NDS on a scale of 3-18 (1d; 10 and 50 mg/kg) ↑ therapeutic window study: • NDS on a scale of 0-12 (1d; treatment at 2 h) ↓; NDS on a scale of 3–18 (1d; treatment at 2 or 4 h) ↑ • Pre: NDS on a scale of 0–12 (14d) ↓ NDS on a scale of 3–18 (14d) ↑ • Post: no difference from pre	Ye et al., 2011b
Mouse	BALB/c	Ginsenoside Rg1	• Post; 20 or 40 mg/kg; i.p.; 0.5 h after ischemia and 12 h after reperfusion	24 h	• Infarct volume (24 h; 40 mg/kg) ↓ • Brain edema (24 h; 40 mg/kg) ↓	• Neurological deficits score (24 h; 40 mg/kg) ↓	Sun et al., 2014a
Rat	SD	Ginsenoside Rg2	• Post; 2.5, 5, and 10 mg/kg; i.v.; 15 min before and 24 h after reperfusion • Twice	48 h	• Infarct volume (2.5 mg/kg) ↓	• Neurological deficits score (2.5, 5 and 10 mg/kg) ↓ • Y-maze ↓	Zhang et al., 2008a

(Continued)

TABLE 1 | Continued

Species	Genetic background	Type of ginseng extracts	Dosage/Administration route	Time of termination	Brain lesion /Edema	Neurobehavioral deficits	Authors/year
Transient Middle Cerebral Ischemia (MCAO, 1.5 h)							
Rat	SD	Ginsenoside Rb1	• Post; 1.25 or 12.5 mg/kg; intranasal; right after MCAO • Once	24 h	• Infarct volume (24 h) ↓ • Nissl-positive neurons (24 h) ↑	• ND	Lu et al., 2011
Rat	SD	Ginsenoside Rd	• Post; 1, 2.5, and 5 mg/kg/d, i.p. • From 1d to 3d after MCAO	7d	• Infarct volume (7d; 5 and 2.5 mg/kg) ↓	• Neurological deficits score (24 h and 7d; 5 mg/kg) ↓	Liu et al., 2015
Rat	SD	Ginsenoside Rg1	• Post; 30, 60 mg/kg; i.p.; 0 and 6 h after reperfusion	24, 72 h	• Brain edema (24 h; 60 mg/kg) ↓	• Longa's Neurological deficits score (24 h, 72 h; 60 mg/kg) ↓	Li et al., 2017b
Rat	SD	Ginseng extracts	• Post; 200 mg/kg; orally after reperfusion; once daily • For a week	1, 3, 7, 10, and 15d	• Infarct volume (15d) ↓	• Rotarod (3d) ↓	Park et al., 2010
Transient Middle Cerebral Ischemia (MCAO, 2 h)							
Mouse	C57BL/6J	Ginsenoside Rg1	• Pre; 20,40 mg/kg/d; gavage • For 7d	24 h	• Infarct volume (40 mg/kg; 24 h) ↓	• Neurological deficits score (40 mg/kg; 24 h) ↓	Wang et al., 2018a
Rat	SD	Red ginseng (KRG)	• Post; 100 mg/kg/d, orally; after the onset of reperfusion; once daily • For 7d	1, 3, 7d	• ND	• Modified neurological severity score (3, 7d) ↓ • Corner (3, 7d) ↓	Ban et al., 2012
Rat	SD	Red ginseng (KRG)	• Post; 100 mg/kg/d; orally after reperfusion	1, 3, 7d	• Infarct volume (7d) ↓	• Modified neurologic severity score (1, 3, 7d) ↓ • Corner (1, 3, 7d) ↓	Lee et al., 2011
Rat	SD	Black ginseng (produced from red ginseng)	• Post; 100 or 400 mg/kg; p.o.; after MCAO; daily for 2 wks	2 wks	• Cresyl violet stained neurons in hippocampus ↓	• Morris water maze (2nd wk)	Park et al., 2011
Rat	SD	Fermented red ginseng	• Post; 100 mg/kg; orally; promptly prior to reperfusion • Once	22 h	• Infarct volume (22 h)	• ND	Bae et al., 2004
Rat	SD	Ginsenoside Rb1	• Pre; 12.5 mg/kg/d; Intranasal administration • For 7d	6, 12, 24,72 h	• Infarct Volume (24 h) ↓	• Modified neurological severity score (72 h) ↓	Zhu et al., 2012
Rat	SD	Ginsenoside Rb1	• 40 mg/kg/d; i.p.; start 3d before MCAO; once daily • Till the animals were sacrificed	6 h, 1, 3, and 7d	• Infarct volume (6 h, 1, 3, and 7d) ↓	• Neurological deficits score (6 h, 1, 3 and 7d) ↓	Li et al., 2018b
Rat	SD	Ginsenoside Rb1	• Post; 20, 40, and 80 mg/kg/d; i.p.; start immediately after ischemia • Once	24 h	• Infarct volume (40 and 80 mg/kg) ↓	• Neurologic deficits (24 h; 40 and 80 mg/kg) ↓	Liu et al., 2013
Rat	Wistar	Ginsenoside Rb1	• Post; 50, 100 and 200 mg/kg/d; i.v.; after ischemia	24 h	• Infarct volume (24 h; 50, 100, and 200 mg/kg) ↓	• Neurological deficits score (24 h; 100 and 200 mg/kg) ↓	Liu et al., 2018a

(Continued)

TABLE 1 | Continued

Species	Genetic background	Type of ginseng extracts	Dosage/Administration route	Time of termination	Brain lesion /Edema	Neurobehavioral deficits	Authors/year
Rat	Wistar	Ginsenoside Rb1	• Post; 40 mg/kg; i.p.; Immediately after the onset of reperfusion • Once	3 h, 12 h, 1, 2, 3, 5, and 10d	• ND	• Modified Neurological severity score (3, 5d) ↓	Gao et al., 2010
Rat	SD	Ginsenoside Rd	• Pre; 0.1, 1, 10, 50, 200 mg/kg, i.p. 30 min before MCAO • Once	1, 3, 7, 14, 21, 28 and 42d	• Infarct size (1d; 10 and 50 mg/kg) ↓	• Modified neurological severity score (14, 21, 28 and 42d; 10 and 50 mg/kg) ↓	Ye et al., 2011c
Rat	SD	Ginsenoside Rd	• Pre; 50 mg/kg; i.p.; 30 min before MCAO • Once	1,14d	• Infarct volume (1, 14d) ↓	• Belayev's neurological score (1, 14d) ↓ • Garcia's neurological score (1, 14d) ↑	Ye et al., 2011d
Rat	SD	Ginsenoside Rd	• Post; 50 mg/kg; i.p.; at 0, 2, 4, 8 h after reperfusion • pMCAO 24 h • tMCAO 2 h, reperfusion 42d	1, 3, 7d	• Infarct volume (1, 3, and 7d) • Brain edema(1d)	pre, d1, 3, 7, 14, 21, 28, and 42d • Modified neurological severity score • Modified sticky-tape • Corner	Ye et al., 2011a
Rat	SD	Ginsenoside Rd	• Pre/Post; 30 mg/kg; i.p.; 1 h before MCAO + 10 mg/kg/d after MCAO	1, 7, 14, 26–32d	• Infarct volume (24 h) ↓	• Novel object recognition (26–32d) ↑ • Morris water maze (26–32d) ↓	Zhang et al., 2014a
Rat	SD	Ginsenoside Rd	• Pre/Post; 30 mg/kg 1 h before MCAO; + 10 mg/kg/d after MCAO • For 7d	2 h, 8 h, 24 h, 2–7d	• ND	• Zea-Longa neurological deficits score (3–7d) ↓	Yang et al., 2016
Rat	SD	Ginsenoside Rd	• Pre/Post; 50 mg/kg; i.p.; 30 min before MCAO or immediately after MCAO • Single dose	2, 12, 24 h	• Infarct volume (24 h) ↓	• Neurological deficits (Bederson's scoring system; 24 h) ↓	Xie et al., 2016
Rat	SD	20(R)-Ginsenoside Rg3	• Pre; 5, 10, and 20 mg/kg; i.p.; twice daily before MCAO	24 h	• Infarct volume ↓ (24 h; 10 and 20 mg/kg)	• Neurological deficits score (24 h; 10 and 20 mg/kg) ↓	He et al., 2012
Rat	SD	Ginsenoside Re	• Pre; 5 or 10 or 20 mg/kg/d; p.o. • For 7d	2, 24 h	• ND	• Neurological deficits (5 point; 24 h)	Chen et al., 2008b
Rat	SD	Ginsenoside Rg1	• Pre; 20, 40, 60 mg/kg; i.v.; 1 h before MCAO	24 h	• ND	• Neurological deficits score (24 h) ↓	Yang et al., 2015b
Rat	SD	Ginsenoside Rg1	• Post; 30, 60 mg/kg/d; i.v.; after 2 h reperfusion; twice daily • For 3d	1, 3d	• Infarct volume (3d) ↓	• Neurological deficits score (60 mg/kg on 1d, 30 and 60 mg/kg on 3d) ↓	Lin et al., 2015
Rat	SD	Ginsenoside Rg1	• 20 mg/kg; i.p.; 1 h before MCAO and repeated each 12 h • Till each experiment was completed	2, 24 h	• Cortical damage size (4 h, 1, 2, and 5d) ↓; • Nissl stained neurons in cortex (24 h) ↑	• Neurological deficits score (24 h) ↓	Zhang et al., 2008b
Rat	SD	Ginsenoside Rg1	• 20 mg/kg; i.p.; started 3d before MCAO; twice daily • Till the animals were killed;	6 h, 1, 3, 7, and 14d	• ND	• Neurological deficits score (6 h, 1, 3, 7, and 14d) ↓	Zhou et al., 2014

(Continued)

TABLE 1 | Continued

Species	Genetic background	Type of ginseng extracts	Dosage/Administration route	Time of termination	Brain lesion /Edema	Neurobehavioral deficits	Authors/year
Rat	SD	Ginsenoside Rg1	• 20 mg/kg; i.p.; started 3d before MCAO; twice daily • Till the animals were sacrificed	6 h, 1, 3, 7, and 14d	• Infarct volume (3d) ↓	• Neurological deficits score (Zea-Longa; 1, 3, 7, and 14d) ↓	Xie et al., 2015
Rat	SD	Ginsenoside Rh2	• Post; 100 mg/kg; orally.; immediately prior to reperfusion	22 h	• Infarct volume (22 h) ↓	• ND	Park et al., 2004
Global Cerebral Ischemia (GCI)							
Rat (2VO)	Wistar	Ginsenoside Rb1	• Pre; 20 or 40 mg/kg; i.v.; 15 min before ischemia	24, 72 h	• CA1 neuronal death ↓	• ND	Luo et al., 2014
Rat (4VO)	Wistar	Panax Ginseng extracts	• Post; 100, 200, 500, 1,000 mg/kg; i.p.; two injections at 0 and 90 min after occlusion	7d	• CA1 Neuronal death ↓	• ND	Kim et al., 2009
Rat (4VO)	SD	Ginsenosides Rb + Ro	• Pre; 100 mg/kg; i.v.; 30 min before 4-vessel occlusion	1 h	• Brain edema (1 h) ↓	• ND	Chu and Chen, 1990
Hypoxia-Ischemia (HI)							
Mouse	C57BL/6	Red ginseng (KRG)	• Pre;100 mg/kg/d; gavage • For 7d	6 h, 1 and 7d	• Neuronal intensity (6 h) ↓ • Infarct volume (6 h, 1d, 7d) ↓ • Brain edema (6 h, 1d, 7d) ↓	• (pre, 6 h, 1, 3, and 7d) • Neurological deficits score (6 h, 1, 3, and 7d) ↓ • Open field (3, 7d ↓) • Cylinder (3, 7, 28d) ↓ • Corner (7d) ↓	Liu et al., 2019
INTRACEREBRAL HEMORRHAGE (ICH)							
NA							
SUBARACHNOID HEMORRHAGE (SAH)							
Rat	SD	Ginsenoside Rb1	• Post; 20 mg/kg; via vena caudalis; 30 min after the first SAH • Followed by additional 7d	6 and 24 h after the 1st SAH; 6, 24, 48, 72, 96, and 120 h after the 2nd SAH	• Brain edema (24 h after the second SAH) ↓ • Arterial vasospasm (120 h after the second SAH)	• Spontaneous activity score (96 h after the second SAH) ↓	Li et al., 2011b
Rat	NA	Ginsenoside Rb1	• 5 or 20 mg/kg	NA	• Cerebral vasospasm; • Brain edema	• Neurological deficits	Li et al., 2010

MCAO, middle cerebral artery; pdMCAO, permanent distal middle cerebral artery occlusion; pMCAO, permanent (proximal end of) middle cerebral artery occlusion that is generated by the intraluminal suture MCAO; Pre, pretreatment; Post, posttreatment; i.p., intraperitoneal; i.v., intravenous; i.c.v., intracerebroventricular; The changes in brain lesion/edema and neurobehavioral deficits (↑ or ↓, exacerbated or attenuated stroke outcomes at indicated time point; no label at indicated time point, no significant difference); h, hour; d, day; wk, week; NA, no answer; ND, non-discussed.

(Ahmed et al., 2016), which has exhibited potent efficacy on cardiovascular disorders like myocardial Ischemia-reperfusion Injury (Zheng et al., 2017). In pdMCAO rats with stroke-prone spontaneous hypertension, Rb1 pretreatment by intravenous infusion ameliorated ischemia-induced place navigation disability at 2 and 4 weeks evidenced by the water maze test, reduced muscle strength deficit in the inclined screen test, impaired coordinated four-leg movements function in the rotarod test, and decreased the volume of the cortical infarct lesion at 28 and 32d after ischemia (Zhang et al., 1998, 2006). In pMCAO rats, Rb1 pretreatment reduced acute ischemic brain damage in infarct volume and overall neurological deficits 24 h after ischemia (Zhang and Liu, 1996). In MCAO (1–2 h) rats, Rb1 pre- or post-treatment significantly reduced acute brain lesion, evidenced by infarct volume at 24 h (Lu et al., 2011; Zhu et al., 2012) or 48 h (Chen et al., 2015; Dong et al., 2017), brain edema (Dong et al., 2017) at 48 h, and neurobehavioral deficits indicted by the overall neurological deficits score at 48 h (Chen et al., 2015; Dong et al., 2017), 72 h (Gao et al., 2010; Zhu et al., 2012), and 5d (Gao et al., 2010) after reperfusion onset. In GCI (2VO) rats, Rb1 pretreatment protected against hippocampal CA1 neuronal death at the acute stage of ischemia (Luo et al., 2014). Besides its favorable role in ischemic stroke, Rb1 also exhibited extensive neuroprotection in subarachnoid hemorrhage brain damage. Rb1 treatment dramatically reduced brain edema, cerebral vasospasm, and neurological deficits including spontaneous activity (Li et al., 2010, 2011b), indicating the extensive benefits to stroke outcomes.

Ginsenoside Rg1

Ginsenosides Rb1 and Rg1 (Rg1) are the most abundant ginsenosides in ginseng roots, exhibiting pharmacological properties in multiple neurological conditions (Gao et al., 2017b; Song et al., 2017; Mohanan et al., 2018). Multiple studies have revealed the preventive and therapeutic efficacy of Rg1 on acute ischemia-reperfusion brain damage and long-term recovery in MCAO (1–2 h) of mice and rats. Rb1 pre- or post-treatment reduced the infarct volume at 24 h (Sun et al., 2014a; Li et al., 2017b; Wang et al., 2018a) and 3d (Lin et al., 2015) and brain edema at 24 h, and attenuated overall neurological deficits at 6 h (Zhou et al., 2014), 24 h (Zhang et al., 2008b; Sun et al., 2014a; Zhou et al., 2014; Lin et al., 2015; Xie et al., 2015; Yang et al., 2015b; Li et al., 2017b; Wang et al., 2018a) and 3d (Zhou et al., 2014; Xie et al., 2015) following MCAO (1–2 h). The neurobehavioral protection was also observed at late stage of stroke, evidenced by the reduced neurological deficits at 7 and 14d after MCAO (1–2 h) (Zhou et al., 2014; Xie et al., 2015).

Ginsenoside Rd

Similar as the ginseng extracts above, ginsenoside Rd (Rd) is another important ingredient of ginsenosides and widely investigated in the stroke field (Ye et al., 2013; Nabavi et al., 2015). In pdMCAO model mice, either pre-treatment or post-treatment of Rd prevented acute ischemic brain injury and promoted the long-term histological and neurobehavioral recovery, evidenced by the reduction of infarct volume at 1, 3, and 7d and neurological deficits score, sticky-tape test, and corner test over 42d after ischemia (Ye et al., 2011a). This benefit was also observed in ischemia-reperfusion rodent models. In MCAO (1.5 h) model rats, Rd post-treatment exhibited sustained neuroprotection against ischemic brain damage, indicated by the reduced neurological deficits at 1 and 7d and infarct volume at 7d after the onset of reperfusion (Liu et al., 2015). In MCAO (2 h), several studies showed that Rd treatment alleviated ischemia-reperfusion induced infarct volume at 24 h (Ye et al., 2011a,c,d; Zhang et al., 2014a; Xie et al., 2016), 3d, 7d (Ye et al., 2011a), and 14d (Ye et al., 2011d), and reduced overall neurological deficits at 1–42d (Ye et al., 2011a,c,d; Zhang et al., 2014a; Xie et al., 2016; Yang et al., 2016).

Ginsenoside Rg3

Ginsenoside Rg3 (Rg3) is abundantly present in red ginseng preparation, which is highly known for its anticancer effects (Sun et al., 2017; Mohanan et al., 2018). A report showed that Rg3 pretreatment reduced ischemia-reperfusion injury, indicated by reduced infarct volume and overall neurological deficits score at 24 h after MCAO (2 h) (He et al., 2012).

Ginsenoside Re

Ginsenoside Re (Re) is a major ginsenoside and important ingredient in ginseng leaf, berry, and root, exhibiting multiple pharmacological activities via different mechanisms (Peng et al., 2012). Re protected rats against acute brain lesion, indicated by the reduction of infarct volume at 24 h after MCAO (2 h) (Chen et al., 2008b).

Ginsenoside Rh2

Ginsenoside Rh2 (Rh2), an important ginsenoside (Smith et al., 2014), was reported to reduce the acute ischemia-reperfusion damage indicated by reduced infarct volume at 22 h after MCAO (2 h) (Park et al., 2004).

Compound K

Compound K is one of the major metabolites of ginseng, exhibiting a variety of pharmacological activities, including anti-inflammatory, antitumor, and other effects (Shin et al., 2015; Yang et al., 2015a). Compound K pretreatment significantly reduced the infarct volume (hemisphere, cortex, but not striatum) of ischemic brain after MCAO (0.5 h) (Park et al., 2012).

Black Ginseng

Black ginseng is a more recent type of processed ginseng with a unique components profile, implying potent *in vitro* and *in vivo* pharmacological activities (Liu et al., 2010; Jin et al., 2015). A study showed that 2 weeks' black ginseng post-treatment improved the impairment of learning and memory in rats, indicated by the Morris water maze 2 weeks after MCAO (2 h) (Park et al., 2011).

Ginseng Total Saponins

Ginsenosides (ginseng total saponins, GTS) may be mainly responsible for the pharmacological effects of ginseng. GTS treated rats have better neurological scores compared with those in control group at 14d after pMCAO (Zheng et al., 2011).

Ginsenosides Rb and Ro Mixture

It was reported that pretreatment with ginsenosides Rb and Ro mixture (which was hard to purify due to similar polarity), markedly reduced ischemic brain edema in rats at 1 h following GCI (4VO) (Chu and Chen, 1990).

Fermented Red Ginseng

Fermented red ginseng was reported to be prepared from red ginseng extract, and the primary components were compound K > ginsenoside Rg3 > or = ginsenoside Rh2 (Bae et al., 2004). It was shown to protect against ischemic brain injury, indicated by the significant reduction of infarct volume after 22 h of reperfusion.

NEUROPROTECTIVE MECHANISMS OF PANAX GINSENG IN STROKE

The discovery of the beneficial effects of ginseng or ginsenosides on ischemic and hemorrhagic stroke has spurred interest in their mechanisms of action. Multiple potential neuroprotective mechanisms were evaluated during the studies (**Table 2**).

Anti-oxidative Stress

Redox homeostasis in the cell is maintained by the counterbalance between reactive oxygen and nitrogen species (ROS/RNS) generation and the antioxidant defense system (Lin and Beal, 2006; Ma, 2013). Oxidative stress is a result of imbalance between the ROS/RNS and the antioxidant defense system. ROS/RNS are constantly produced by oxygen metabolism in accordance with the rate of oxidant formation and elimination, most of which comes from mitochondria (Balaban et al., 2005). Under normal conditions, only 1–2% of molecular oxygen is converted into superoxide radicals (Orrenius et al., 2007; Drummond et al., 2011) and then removed by the potent and extensive antioxidant system. However, under stress conditions like stroke attack, the overproduction of ROS and the reduced antioxidant capacity result in oxidative damage to DNA, RNA, lipids, and other cell components, eventually leading to cell death. The central nervous system (CNS) is typically vulnerable to oxidative stress as it consumes a higher amount of oxygen and has a lower level of endogenous antioxidant defense capacity than other organs (Sims and Muyderman, 2010; Chen et al., 2011; Sinha and Dabla, 2015). The rapid increase in the production of ROS/RNS immediately following stroke overwhelms the antioxidant defense system, damaging cellular macromolecules which leads to apoptosis, autophagy, and necrosis (Rodrigo et al., 2013). Moreover, the restoration of blood flow further increases the tissue oxygenation level and initiates a second burst of ROS/RNS overproduction, triggering reperfusion injuries (Sims and Muyderman, 2010; Rodrigo et al., 2013). Given that oxidative stress occurs early and acts causally in stroke pathogenesis (Chen et al., 2011), therapies targeting basic oxidative processes, such as free-radical generation or specific antioxidants that interact with stroke-related proteins, hold great promise (Becerra-Calixto and Cardona-Gomez, 2017; Bhatti et al., 2017).

Accumulated evidence demonstrated the beneficial efficacy of ginseng against various CNS diseases, mainly owing to its anti-oxidative and anti-inflammation properties (Gonzalez-Burgos et al., 2015; Ahmed et al., 2016; Lee et al., 2017). Several studies supported that, associated with their benefits on stroke outcomes, ginseng, or ginsenosides have the antioxidant potential against stroke damage by scavenging overproduced ROS/RNS via modulating endogenous antioxidant defense system. KRG attenuated the oxidative damages indicated by the reduced levels of 8-hydroxyguanosine (8-OHG) (Cheon et al., 2013), a biomarker of oxidative DNA damage, and lipid peroxidation (Ban et al., 2012) and the increased antioxidant related protein levels in superoxide dismutase 2 (SOD2), glutathione peroxidase 1 (Gpx1), heme oxygenase 1 (HO1), and NAD(P)H quinone dehydrogenase 1 (NQO1) (Liu et al., 2018b, 2019) compared to controls. Mitochondrial SOD2 is one critical component of the antioxidant system, accounting for the removal of superoxide ions in the mitochondria (Flynn and Melov, 2013). GPx is another key antioxidant enzyme that catalyzes the reduction of lipid peroxides and hydroperoxide to non-toxic species. Superoxide in the mitochondrial matrix is metabolized to hydrogen peroxides by SOD2 and decomposed to water by GPx (Ghosh et al., 2011). HO1, an inducible enzyme, has emerged as a major protective mechanism against oxidative stress (Zeynalov et al., 2009). In addition, these findings above are also supported by the results in GCI model (Luo et al., 2014). Rb1 was shown to have neuroprotective effects on brain damage by anti-oxidant activity, indicated by the levels of glutathione (GSH), MDA, nitric oxide (NO), nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) expression and NADPH oxidase activity (Dong et al., 2017). Rg1 increased the activity or content of antioxidant enzymes SOD and catalase (CAT) (Li et al., 2017b), as well as HO1 (Yang et al., 2015b), contributing to the histological and functional benefits after stroke. Rd treated animals exhibited a reduced level in free radical generation revealed by microdialysis, oxidative DNA damage (8-OHG), oxidative proteins carbonyl and advanced glycosylation end products (AGEs), lipid peroxidation [malondialdehyde (MDA) and 4-hydroxynonenal formations (4-HNE)] following MCAO (Ye et al., 2011c). Rd administration also reduced mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) damages, which contributed to an improvement in survival rate and neurological function (Yang et al., 2016). It was observed that Re significantly ameliorated lipid peroxidation by raising the activities of SOD and GSH-Px, and reduced the content of MDA in rat brains protecting against cerebral ischemia-reperfusion injury (Zhou et al., 2006), which was supported by another Re study in MCAO (Chen et al., 2008b).

In recent years, fundamental progress in the oxidative stress research field was the discovery of transcriptional factor Nrf2/antioxidant response element (ARE) pathway, which is the master regulator of redox hemostasis by tightly controlling multiple ARE-driven antioxidant proteins likeNQO1 and HO1 (Cuadrado et al., 2018; Raghunath et al., 2018; Yamamoto et al., 2018). In response to stress conditions or Nrf2

TABLE 2 | The putative neuprotective mechanisms of Panax ginseng in experimental stroke.

Stroke model	Species	Genetic background	Type of ginseng extracts	Main mechanisms (*in vivo*)	Authors/year
pdMCAO	Mouse	C57BL/6	Red ginseng (KRG)	• Nrf2 pathway • Oxidative stress • Reactive astrogliosis and microglia activation • Glutamine synthetase (GS), Aquaporin-4 (AQP4)	Liu et al., 2018b
MCAO (1 h)	Mouse	C57BL/6	Red ginseng (KRG)	• Oxidative stress (8-hydroxyguanosine) • Apoptosis signal-regulating kinase 1 (ASK1)	Cheon et al., 2013
MCAO (2 h)	Rat	SD	Red ginseng (KRG)	• Levels of lipid peroxidation	Ban et al., 2012
MCAO (2 h)	Rat	SD	Red ginseng (KRG)	• Inflammatory cytokines [tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), and IL-6, IL-10]	Lee et al., 2011
Hypoxia-ischemia	Mouse	C57BL/6	Red ginseng (KRG)	• Nrf2 pathway • Oxidative stress • Neuroinflammation • Reactive astrogliosis and microglia activation • Glutamine synthetase (GS), Aquaporin-4 (AQP4)	Liu et al., 2019
pdMCAO	Rat	SHR-SP	Ginsenoside Rb1	• Upregulation of Bcl-x(L) expression • (activation of mitochondrial cell death signaling)	Zhang et al., 2006
MCAO (1 h)	Mouse	C57BL/6J	Ginsenoside Rb1	• oxidative stress • Extracellular signal-regulated Kinase (ERK) signaling activation	Dong et al., 2017
MCAO (1 h)	Mouse	ICR	Ginsenoside Rb1	• BBB (evans blue, ZO1, and occludin proteins) • Inflammation (iNOS, IL-1β, IL-10) • Oxidative stress	Chen et al., 2015
MCAO (1.5 h)	Rat	SD	Ginsenoside Rb1	• Autophagy [LC3 (I, II) and Beclin1 proteins]	Lu et al., 2011
MCAO (2 h)	Rat	SD	Ginsenoside Rb1	• Inflammation (IL-6, gene, and protein levels) • Nuclear factor-κB (NF-κB) pathway • (expression of total and phosphorylated NF-κB/p65, inhibitor protein of κB (IκB)-α, and IκB-kinase complex (IKK)-α)	Zhu et al., 2012
MCAO (2 h)	Rat	Wistar	Ginsenoside Rb1	• Modulations of apoptotic-related genes and glial-derived neurotrophic factor (GDNF) expression	Yuan et al., 2007
MCAO (2 h)	Rat	NA	Ginsenoside Rb1	• Neural cell apoptosis • Expressions of Bcl-2 and Bax	Yang et al., 2008
MCAO (2 h)	Rat	Wistar	Ginsenoside Rb1	• Brain-derived neurotrophic factor (BDNF) • Caspase-3 protein	Gao et al., 2010
MCAO (2 h)	Rat	Wistar	Ginsenoside Rb1	• BBB permeability • Aquaporin-4 (AQP4)	Li et al., 2018b
MCAO (2 h)	Rat	Wistar	Ginsenoside Rb1	• Inflammation [tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), iNOS, and NO] • High mobility group box 1 (HMGB1)	Liu et al., 2018a
MCAO (2 h)	Rat	SD	Ginsenoside Rb1	• IL-1 beta	Liu et al., 2013
GCI (2VO)	Rat	Wistar	Ginsenoside Rb1	• Augophagy (LC3II and Beclin1) • Phosphatidylinositol 3-kinase (PI3K)/Akt pathway	Luo et al., 2014
GCI (4VO)	Rat	NA	Ginsengoside Rb1	• Improve cerebral glucose utilization	Choi et al., 1996
SAH	Rat	NA	Ginsenoside Rb1	• Apoptosis (P53, Bax, and Caspase-3 proteins)	Li et al., 2010
pdMCAO	Rat	SH-SP	Ginsenoside Rb1	• ND	Zhang et al., 1998
SAH	Rat	SD	Ginsenoside Rb1	• ND	Li et al., 2011b
pMCAO	Rat	SD	Ginsenosides Rb1 and Rg1	• ND	Zhang and Liu, 1996
MCAO (1.5 h)	Rat	SD	Ginsenoside Rg1	• Oxidative stress [myeloperoxidase (MPO), superoxide dismutase (SOD), catalase (CAT)] activities • Inflammation (IL-6, TNFα) • Peroxisome proliferator-activated receptor γ (PPARγ), NF-κB	Li et al., 2017b
MCAO (2 h)	Mouse	C57BL/6J	Ginsenoside Rg1	• Inflammation (IL-1β, IL-6, and TNFα) • Excitatory amino acids such as the contents of Glu and Asp (by High-performance liquid chromatography)	Wang et al., 2018a

(Continued)

TABLE 2 | Continued

Stroke model	Species	Genetic background	Type of ginseng extracts	Main mechanisms (*in vivo*)	Authors/year
MCAO (2 h)	Rat	SD	Ginsenoside Rg1	• Apoptosis (TUNEL) • Extracellular signal-regulated kinase 1/2 (ERK1/2), phosphorylated extracellular signal-regulated kinase 1/2 (p-ERK1/2), c-Jun N-terminal kinases (JNK), and phosphorylated c-Jun N-terminal kinase (p-JNK)	Wang et al., 2013a
MCAO (2 h)	Rat	SD	Ginsenoside Rg1	• PPARγ/Heme oxygenase1 (HO1) signaling (suppress both apoptosis and inflammation) • PPARγ, bcl-2, cleaved caspase-3, cleaved caspase-9, IL-1β, TNF-α, High mobility group box 1 (HMGB1), and Receptor for advanced glycation end products (RAGE)	Yang et al., 2015b
MCAO (2 h)	Rat	SD	Ginsenoside Rg1	• Metabolic regulation	Lin et al., 2015
MCAO (2 h)	Rat	SD	Ginsenoside Rg1	• Ca^{2+} influx through NMDA receptors and L-type voltage-dependent Ca^{2+} channels	Zhang et al., 2008b
MCAO (2 h)	Rat	SD	Ginsenoside Rg1	• BBB integrity • Aquaporin 4	Zhou et al., 2014
MCAO (2 h)	Rat	SD	Ginsenoside Rg1	• BBB integrity • Regulation of protease-activated receptor-1 expression	Xie et al., 2015
MCAO (2 h)	Rat	SD	Ginsenoside Rg1	• BBB integrity • matrix metalloproteinases (MMPs)	Wang et al., 2013b
MCAO (1 h)	Mouse	BALB/c	Ginsenoside Rg1	• ND	Sun et al., 2014a
MCAO (1 h)	Mouse	C57BL/6	Ginsenoside Rd	• Mitochondrial dysfunction • Antioxidant activities	Ye et al., 2011b
MCAO (1.5 h)	Rat	SD	Ginsenoside Rd	• Neurogenesis	Liu et al., 2015
MCAO (2 h)	Rat	SD	Ginsenoside Rd	• Early oxidative damage and sequential inflammatory response (Free radical generation (microdialysis), oxidative DNA damage (8-hydroxy-deoxyguanosine), oxidative protein (protein carbonyl and advanced glycosylation end products), lipid peroxidation (the malondialdehyde and 4-hydroxynonenal formations)	Ye et al., 2011c
MCAO (2 h)	Rat	SD	Ginsenoside Rd	• Mitochondrial enzyme activities, mitochondrial membrane potential (MMP), production of ROS, energy metabolites, and apoptosis	Ye et al., 2011d
MCAO (2 h)	Rat	SD	Ginsenoside Rd	• AIF mitochondrio-nuclear translocation and NF-κB nuclear accumulation by inhibiting poly (ADP-ribose) polymerase-1	Hu et al., 2013
MCAO (2 h)	Rat	SD	Ginsenoside Rd	• Microglial activation • Pro-inflammatory Cytokines (IL-1β, IL-6, IL-18, TNFα, and IFN-γ) • Alpha (IκBα) phosphorylation and NF-κB nuclear translocation	Zhang et al., 2016
MCAO (2 h)	Rat	SD	Ginsenoside Rd	• Tau protein phosphorylation • Pl3K/AKT/GSK-3β pathway	Zhang et al., 2014a
MCAO (2 h)	Rat	SD	Ginsenoside Rd	• Mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) damages	Yang et al., 2016
MCAO (2 h)	Rat	SD	Ginsenoside Rd	• The phosphorylation of the NMDAR 2B subunit (NR2B subunit)	Xie et al., 2016
pMCAO	Rat	SD	Ginsenoside Rd	• ND	Ye et al., 2011a
MCAO (1.5 h)	Rat	SD	Ginsenoside Rd	• ND	Zhang et al., 2012
MCAO (2 h)	Rat	Wistar	Ginsenoside Re	• Oxidative stress [lipid peroxidation: malondiadehyde (MDA) formation], superoxide dismutase (SOD) and glutathion peroxidase (GSH-Px)]	Zhou et al., 2006
MCAO (2 h)	Rat	SD	Ginsenoside Re	• Oxidative stress (MDA)	Chen et al., 2008b
MCAO (2 h)	Rat	SD	Ginsenoside Rh2	• ND	Park et al., 2004
MCAO (2 h)	Rat	SD	20(R)-Ginsenoside Rg3	• Apoptosis (TUNEL) • Calpain I and caspase-3	He et al., 2012

(Continued)

TABLE 2 | Continued

Stroke model	Species	Genetic background	Type of ginseng extracts	Main mechanisms (*in vivo*)	Authors/year
MCAO (0.5 h)	Mouse	C57BL/6	Compound K	• Inflammation • Microglial activation	Park et al., 2012
MCAO (1.5 h)	Rat	SD	Black ginseng (produced from red ginseng)	• ND	Park et al., 2011
pMCAO	Rat	Wistar	Ginseng total saponins (GTS)	• Neurogenesis	Zheng et al., 2011
GCI (4VO)	Rat	Wistar	Panax Ginseng extracts	• Oxidative (lipid peroxidation: MDA, SOD and GPx)	Kim et al., 2009
GCI (4VO)	Rat	SD	Ginsenosides Rb + R0	• Oxidative stress [Anti-lipid peroxidation: creatine phosphokinase (CK) and SOD]	Chu and Chen, 1990

MCAO, middle cerebral artery; pdMCAO, permanent distal middle cerebral artery occlusion; pMCAO, permanent (proximal end of) middle cerebral artery occlusion that is generated by the intraluminal suture MCAO; GCI, global cerebral ischemia; SAH, subarachnoid Hemorrhage; BBB, blood brain barrier; NA, no answer; ND, non-discussed.

inducers, Nrf2 protein is liberated from Kelch-like ECH-associating protein 1 (Keap1)-mediated repression, translocates into the nucleus, binds to the ARE sequence in the promoter region of Nrf2 target proteins, thereby activating a wide range of cytoprotective genes (Hayes and Dinkova-Kostova, 2014). Very recently, pretreatment with KRG, as an Nrf2 inducer, significantly increased the expression levels of Nrf2 target cytoprotective and antioxidant proteins after pdMCAO, which was abolished in ischemic-Nrf2$^{-/-}$ mice, supporting the Nrf2-dependent neuroprotection of KRG in ischemic stroke (Liu et al., 2018b). This is supported by other *in vivo* (Yang et al., 2015b; Gao et al., 2017a; Li et al., 2017a) and *in vitro* (Hwang and Jeong, 2010) reports. In addition, pretreatment of ginsenoside Rb1 was reported to have anti-oxidant neuroprotective effects through promoting ERK1/2 pathways in cerebral ischemia-induced injuries in aged mice (Dong et al., 2017).

Astrocytes are recognized to exert essential and complex functions for maintaining normal neural activity in the healthy CNS and respond to various forms of CNS injury or disease. Reactive astrogliosis, regulated in a context specific manner, alters astrocytic functions and thereby exerts beneficial effects on neural functions. Given the important role of astrocytes in oxidative stress and inflammation process (Hamby and Sofroniew, 2010; Sofroniew, 2014; Ong et al., 2015), reactive astrogliosis was considered to contribute to the neuroprotection of ginseng in stroke. Indeed, in permanent cerebral ischemia model mice, ginseng pretreatment robustly attenuated the acute reactive astrogliosis progression but not the microglia activation in the ischemic cortex region in an Nrf2-dependent manner. The spatial and temporal pattern correlated well with the acute ischemic damage expansion during the acute stage of ischemia (Liu et al., 2018b). In addition, ginseng pretreatment was found to attenuate the deterioration of glutamine synthetase, the key enzyme for glutamate metabolism, and aquaporin 4 (AQP4), the unique water channel that is predominantly distributed in astrocytes. One of the major causes of morbidity and mortality after stroke is brain edema; the influence of ginseng on cellular water penetrability at least partly involves its favorable effects on stroke damage. In agreement with this observation, it was reported that the neuroprotection of Rg1 against ischemic-reperfusion brain injury might be associated with the reduced expression AQP4 level (Zhou et al., 2014).

Anti-inflammation

Inflammation is another major player that is involved in stroke pathogenesis, which contributes to all the stages of the stroke pathophysiology (Iadecola and Anrather, 2011; Fu et al., 2015; Esenwa and Elkind, 2016; de Oliveira Manoel and Macdonald, 2018; Drieu et al., 2018). The inflammatory responses are typically mediated by pro-inflammatory prostaglandins, cytokines and chemokines. These components attract immune cells, interact with the adaptive immune system, and evoke the systemic release of acute phase reactants (Esenwa and Elkind, 2016). These pro-inflammation proteins include IL-1β, IL-6, tumor necrosis factor α (TNFα), interferon γ (IFNγ), complement proteins, C-reactive protein (CRP), etc., which are implicated in the pathogenesis and progression of atherosclerosis and intravascular thrombosis (Sofroniew, 2015; Drieu et al., 2018). Anti-inflammatory mediators include IL-4, IL-10, TGFβ, etc. (Mandolesi et al., 2015; Sofroniew, 2015). Microglial activation plays an important role in inflammation, and activated microglia have both pro- and anti-inflammatory properties (Hoogland et al., 2015).

Anti-inflammation might be another intriguing neuroprotective effect of ginseng. Suppression of inflammation contributed to the neuroprotection of Rb1 on cerebral ischemic injury and the integrity of blood-brain barrier (BBB), indicated by the downregulated expression of pro-inflammatory factors nitric oxide synthase, IL-1β, IL-6, and upregulated expression of anti-inflammatory markers arginase 1 and IL-10 in the ischemic brain (Zhu et al., 2012; Chen et al., 2015). Rg1 was reported to suppress inflammation and preserve the brain tissue from stroke insults (Wang et al., 2018a), and the underlying mechanism was related to the activation of PPARγ/HO-1 (Yang et al., 2015b) and PPARγ-regulated pathways (Li et al., 2017b). The beneficial effect in inhibition of inflammation was also observed in MCAO rats treated with KRG (Ban et al., 2012) or Rd (Zhang et al., 2016). In addition, Rb1 and Rd have been shown to repress microglial

activation and decrease the pro-inflammatory cytokines IL-6 in a transientMCAO rat model, which resulted in a decrease in infarct volume and neurological deficits score (Ye et al., 2011c; Zhu et al., 2012).

Nuclear factor-κB (NF-κB) is a critical transcription factor involved in the regulation of inflammation through the target genes such as cycloygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), and IL-6 (Harari and Liao, 2010). A study showed that Rb1 can suppress NF-κB and its DNA binding activity thus suppressing neuronal death as well as decreasing IL-6 levels in the brain with cerebral ischemia (Zhu et al., 2012). Rg1 was shown to exert its neuroprotective action through antioxidative and anti-inflammatory effects mediated by the activation of PPARγ signaling, and the beneficial effect was abolished by a selective PPARγ antagonist GW9662 (Li et al., 2017b). The administration of Rd after stroke inhibited ischemia-induced microglial activation, decreased the expression levels of various proinflammatory cytokines, and suppressed nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor, alpha (IκBα) phosphorylation and NF-κB nuclear translocation (Zhang et al., 2016). The anti-inflammatory effect of Rd was also supported by another report. It was shown that Rd significantly eliminated inflammatory injury as indicated by the suppression of microglial activation and reduced pro-inflammatory factors levels (Ye et al., 2011c). Mitogen-activated protein kinases (MAPKs) mediate another group of signal transduction pathways activated by stress and inflammation that enhance the formation of pro-inflammatory proteins in stroke. The p38 MAPK can downregulate HO1 expression, which has potent anti-inflammatory, antioxidant and anti-apoptotic properties (Naidu et al., 2009; Jang et al., 2012; Wang et al., 2015). Compound K showed a neuroprotective effect on experimental stroke in mice through inhibiting phosphorylation of MAPKs and enhancing HO1 expression, thus decreasing production of pro-inflammatory proteins in activated microglia (Park et al., 2012).

Anti-apoptosis

Another important role of ginseng on ischemic stroke is the inhibition of apoptosis or cell death. There is a dynamic balance between anti-apoptotic proteins (such as Bcl-2) and pro-apoptotic proteins [such as Bcl-2-associated X protein (Bax)], playing a major role in regulating apoptosis. Rb1 was shown to increase Bcl-2 protein and decrease BAX protein in MCAO model rats (Yuan et al., 2007). Similarly, Rg2 was shown to increase Bcl-2 protein and decrease Bax protein in rats after MCAO (Zhang et al., 2008b). Bcl-2 is mainly located in the mitochondrial outer membrane, and cytochrome c, a small heme protein, is mainly located in the mitochondrial inner membrane, signifying the important role of mitochondria in apoptosis process. Rd was reported to attenuate mitochondrial release of AIF, caspase 3 and cytochrome c in MCAO rats, leading to the benefit of Rd on ischemic brain lesion (Ye et al., 2011d). Rb1 can also decrease the activity of caspase 3 in the ischemic brain of rats, thus inhibiting cell death after MCAO (Gao et al., 2010). KRG extract decreased the number of apoptosis signal-regulating kinase 1 (ASK1)-positive cells and the expression level of ASK1 protein in the ischemic region at 4 and 24 h after MCAO, resulting a better performance in ischemic rats (Cheon et al., 2013).

Anti-autophagy

Autophagy is a self-eating cellular catabolic pathway, degrading and recycling damaged organelles and misfolded proteins for cellular homeostasis (Wang et al., 2018b). Due to its important homeostatic role in regulating cell survival, emerging evidence showed that autophagy is implicated in the destructive process in stroke (Wu et al., 2016; Li et al., 2018a). LC3, a crucial protein for autophagy, is mainly located in the cytoplasm and concentrated in autophagosomes during autophagy. Beclin1 also plays a key role in the regulation of autophagosome formation. In MCAO model rats, Rb1 attenuated autophagy via a decrease in the associated proteins LC3 and Beclin 1 in transient MCAO rat models (Lu et al., 2011). In GCI (2VO) model rats, Rb1 administration inhibited autophagy in hippocampal CA1 neurons, evidenced by the expression level of autophagy hallmark proteins LC3 (I and II) and Beclin1 in CA1 neurons by confocal microscopy and Western blot (Luo et al., 2014).

Other Beneficial Mechanisms

Stroke is a heterogeneous and multi-factorial cerebrovascular disease; multiple cell death pathways are evoked in response to acute brain injury (Kellner and Connolly, 2010; Fisher, 2011; Tasker and Duncan, 2015). Such injury induces various endogenous protective mechanisms, including neurogenesis, angiogenesis, and vascular remodeling responses (Marti and Risau, 1999; Greenberg, 2014; Seto et al., 2016; Koh and Park, 2017). To enhance the endogenous neurogenesis driven by ischemia and promote the survival of newborn neurons are considered as the promising therapeutic interventions for stroke (Lu et al., 2017). It was shown that GST and Rb1 increased the numbers of neuronal precursors and promoted the proliferation of endogenous neural stem cells, thus promoting the behavior recovery post-ischemia (Gao et al., 2010; Zheng et al., 2011). Re was shown to improve the fluidity of the mitochondrial membrane that was important for energy generation (Zhou et al., 2006).

Angiogenesis refers to the process of new blood vessel formation from the existing vasculature (Adair and Montani, 2010). Although the vascular system in the adult brain is extremely stable under normal conditions, pathological angiogenesis is induced in response to brain ischemia. The angiogenesis induction, mainly in the ischemic area, enhances the supply of oxygen and nutrients. Therefore, post-stroke angiogenesis facilitates the process of vascular remodeling and is considered a harmonized target for neurological recovery (Mennel, 2000; Beck and Plate, 2009; Dejana, 2010; Xiong et al., 2010; Ergul et al., 2012). The angiogenic factors are induced within hours following stroke, and new capillaries are developed within days (Greenberg, 2014). Ginsengosides have indicated salutary effects on angiogenesis in stroke through inducing various angiogenesis regulators. Ginsenoside Rg1 was shown to facilitate angiogenesis after hypoxia/ischemia brain injury, and the pharmacological effects of Rg1 may be attributed to the

regulation of the vascular endothelial growth factor (VEGF) and cleaved caspase 3 expression levels (Tang et al., 2017). Ginsenoside Rg1 was also reported to improve angiogenesis in the diabetic ischemic hind limb, and the potential mechanism might be related to the eNOS activation and upregulation of the VEGF expression (Yang et al., 2012).

TRANSLATIONAL POTENTIAL OF GINSENG AND GINSENOSIDES IN STROKE THERAPEUTICS

Since 1996 till now, one strategy for improving functional recovery after ischemic stroke is to restore blood flow to salvage ischemic tissue by introducing intravenous recombinant tissue plasminogen activator (rtPA) in acute ischemic stroke, while the other protocol if removal of the blood clot by thrombectomy (Prabhakaran et al., 2015; Romano and Sacco, 2015). Despite that only <40% patients who are treated with rtPA alone regain functional independence (Saver et al., 2015), more than 95% of patients receive only supportive care without rtPA treatment due to the narrow therapeutic window (up to 4.5 h) and limited indications (Hacke et al., 2008; Fonarow et al., 2011; Sandercock et al., 2012; Emberson et al., 2014). The other strategy is neuroprotection targeting various components of the cascade during ischemic insult, which is supported by preclinical data for many agents (Fisher, 2011; Dirnagl and Endres, 2014; Fisher and Saver, 2015). Unfortunately, all prior drug development of neuroprotective agents has been unsuccessful, no neuroprotective drug demonstrated unequivocal efficacy in clinical trials (Fisher, 2006; Hossmann, 2006; Della-Morte et al., 2013).

Many single-target stroke intervention strategies have failed to provide efficacy in clinical trials. The field is in tremendous need of new targets that exert pleiotropic effects on cellular viability through multiple mechanisms. Interestingly, ginseng could be beneficial for the prevention or treatment of stroke through regulating multipronged mechanisms that can provide the brain/cells with resistance against acute and chronic debilitating neurodegenerative conditions. Living organisms are continuously threatened by the damage caused by free radicals produced during normal oxygen metabolism and mitochondrial function or generated by exogenous damage. For centuries, ginseng has been reported as a preventive medicine capable of boosting the nervous system, but the effects on stroke and the underlying cellular mechanisms are still unclear. Increasing *in vivo* pre-clinical stroke studies of either pretreatment or posttreatment will provide a better understanding of the unique properties of ginseng and its derivatives in the preventive and therapeutic treatment of stroke.

CONCLUDING REMARKS

The promising preventive and therapeutic efficacy of ginseng or ginsenosides on experimental stroke damage has been illuminated during the last decade. The putative neuroprotective mechanisms of ginseng or ginsenosides include anti-oxidant, anti-inflammation, anti-apoptosis, anti-autophagy, neurogenesis, and others. These effects have the potential to influence short- and long-term complex neurobehaviors such as the overall deficits, motor, sensorimotor, and cognition. It is known that stroke injury results in severe motor, sensory, emotional, and cognitive deficits (Ferro et al., 2016), and long-term functional recovery is considered as the ultimate goal of stroke intervention. Accordingly, more effective and long-term histological and neurological assessments are expected for future preclinical stroke studies. In addition, the responses to various forms of stroke insults involve complicated interactions among brain cells with numerous functions and lineages, including intrinsic neural cells, intrinsic non-neural cells, and extrinsic cells that come from the circulation. The contributions of different non-neuronal cell types to the progress after acute brain injury are of robust interests for future studies as the impetus toward understanding and ameliorating stroke insults.

REVIEW CRITERIA

We searched the PubMed and Embase databases by Jan 31, 2019 for the following terms individually or in combination: "ginseng," "ginsenoside," "stroke," "ischemi*," "ischaemi*," "hemorrhage," "hemorrhagic," "subarachnoid," "mouse," "rat," and their abbreviations. Study selection for inclusion and exclusion was performed based on predefined criteria. Selection of articles: (1) The studies were published in English; (2) The study clearly described the stroke model and administration route of ginseng or ginsenosides; (3) Ginseng or ginsenosides were administrated without the combination of other compounds. (4) The study was an original full paper that presented the data. Totally 402 articles in PubMed and 454 articles in Embase were identified. After screening analysis in title, abstract and full text and duplication analysis, 54 articles met inclusion criteria. Three independent investigators reviewed articles and extracted data for study design elements, such as animals, animal models, administration strategies, stroke outcomes, and mechanisms. We specifically focus on the *in vivo* evidence for the effects of ginseng and ginsenosides on various stroke damages and mechanism.

AUTHOR CONTRIBUTIONS

LL and SD conceived the study, designed the databases analysis, and wrote the manuscript. LL, GA, and TF searched databases, collected data, performed analyses, and prepared the tables and figure. All authors discussed and approved the final manuscript.

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frontiers in Neuroscience

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Effect of Conjugated Linoleic Acid on Memory and Reflex Maturation in Rats Treated During Early Life

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In the critical period of neurodevelopment (gestation and lactation), maternal consumption of essential fatty acids (FAs) can alter the offspring cognitive function permanently causing damage. Lipids can regulate neurotrophin and compose brain tissue. However, the effects of maternal consumption of a mixture of conjugated linoleic acid (CLA) on an offspring nervous system are not completely clear. We aimed to investigate the impacts of different CLA concentrations mixed into the maternal diet during early life on neonatal reflex maturation and cognitive functions of the offspring. Three groups were formed: control (CG): receiving a standard diet; CLA1: receiving a diet containing 1% of CLA, and CLA3: receiving a diet containing 3% of CLA, offered during gestation and lactation. After birth, the reflex responses of the offspring were observed from the 1st to the 21st day. After weaning, the animals' anxiety and memory were assessed using open field (OF) and novel object recognition tests. Fatty acids in the breast milk and the offspring's brain were also quantified. The data were analyzed using one-way ANOVA and the Kruskal–Wallis test. CLA1 presented accelerated palmar grasp disappearance versus CLA3 and negative-geotaxis versus CG; and the CLA3 presented increases for most reflexes (cliff-avoidance, vibrissa-placing, negative-geotaxis, and auditory-startle response), and decrease in reflexes palmar grasp and free-fall righting versus CG ($p < 0.05$). CLA3 group explored less of the OF in the second exposure. CLA1 and CLA3 presented an increased exploration ratio for new objects, which indicates memory improvement. The milk tested from CLA3 demonstrated an increase in polyunsaturated fatty acids (PUFAs), and a decrease in monounsaturated fatty acids. The amount of CLA in milk was greater in CLA1 and CLA3 and in the brain offspring both presented moderated amounts of CLA. Maternal treatment with the CLA mixture induced anticipated reflex maturation and improved memory in the offspring. Even

though CLA was detected in the brains in only trace amounts, offspring's brain PUFA and SFA levels were increased. Further studies aimed to delineate the effect of maternal CLA supplementation on offspring's brain lipid metabolism and long-term neurologic outcome are needed to confirm these findings.

Keywords: conjugated linoleic acid, neurodevelopment, reflex maturation, memory, physical parameters, fatty acids, maternal nutrition

INTRODUCTION

The central nervous system first appears in the human embryo at around the 3rd or 4th week after fertilization; development continues until roughly to 2 years of age. In rats, development occurs from the second week of pregnancy until the end of lactation. This phase is known as "the critical period of development" and any injury can cause permanent damage (Morgane et al., 1993; Hsieh and Brenna, 2009).

In this critical period of development, there is an increased need for polyunsaturated fatty acids (PUFAs) in brain; chiefly arachidonic acid (AA, 20:4n-6) and docosahexaenoic acid (DHA, 22:6n-3), which together comprise about 20% of the brain tissue (Valenzuela and Nieto, 2003). DHA provides better blood flow and optimizes the development and functions of the neuronal membrane (Valenzuela and Nieto, 2003; Balogun et al., 2014). Regular PUFA intake is important for neurotrophin regulation. Neurotrophins perform essential functions during the development of the fetal nervous system (Balogun et al., 2014). During lactation, breast milk replaces the placental function by carrying nutrients from the nursing mother to the neonate. The lipid fraction present in breast milk, in addition to its energy supply function, is also responsible for myelin sheath structuring.

Found in breast milk, linoleic (18:2n-6) and linolenic (18:3n-3) are PUFAs, and also essential fatty acids (FAs). Adequate intake of FAs is necessary for proper neurological and cognitive development in infants, and deficiencies during the brain development phase are associated with behavioral abnormalities (Hayat et al., 1999; Herrera, 2002; Gustavsson et al., 2010). Conjugated linoleic acids (CLAs) are a family of linoleic acid isomers presenting conjugated double bonds. CLAs are naturally produced by ruminant animals, found in their milk fats and muscle tissue, and in food products derived from them (Pariza et al., 2001; Banni, 2002). CLA isomers are commercially prepared by partial hydrogenation of linoleic acid (Banni, 2002). CLAs have been widely investigated due to their many beneficial health effects (Halade et al., 2010; Park et al., 2010; Furlan et al., 2013; Jelińska et al., 2014). It was found that CLAs cross the blood–brain barrier (Jelińska et al., 2014), inhibit angiogenesis in the mammalian brain (Sikorski et al., 2008), and *in vitro* were found to protect cortical cells against neurotoxic elements (Hunt et al., 2010). A maternal diet containing goat milk fat (as a source of CLAs), has also been found to affect cortical electrical activity (Soares et al., 2012) and anxiety in rats (Soares et al., 2013).

In the previous studies, goat milk was used as a source of CLA, but also of other lipids such as AA and DHA. Lipids of the n-3 series, present in goat's milk, are already known for their beneficial effects on the nervous system when supplemented during pregnancy and lactation. Research has found improvement in the memory of pups whose mothers were supplemented during pregnancy with this lipid and with valproic acid. This medication is used indiscriminately in pregnancy and can cause adverse effects, such as fetal malformation and cognitive defects (Chalon et al., 1998). Improvement in brain development has also observed in mice receiving series-3 fatty acids (DHA, EPA, and AA) during lactation (Gaoa et al., 2016). However, studies analyzing the effects of CLA alone in these phases of life are still scarce.

It is well-known that dietary lipids, when offered during the initial phases of life, may alter reflexes, maturation (Santillan et al., 2010; Aquino et al., 2015), and behavior in animals (Soares et al., 2013). The hypothesis of this study is that maternal feed supplementation containing a mixture of CLA isomers during pregnancy and lactation positively influences reflex maturation (short term), and improves memory (long term) in rat offspring. This study aims to investigate the impact of supplementing the maternal diet with differing concentrations of a commercial CLA mixture on reflex ontogeny and memory in offspring.

MATERIALS AND METHODS

Animals and Diets

Female Wistar rats (n = 12, four female per each group), acquired from the Federal University of Paraíba (UFPB), aged 90 days and weighing 230 ± 30 g were used to obtain pups (n = 36, only males were used). One female was maintained for each male during the mating period.

After confirmation of pregnancy, the mothers were housed in individual polypropylene maternity cages under standard conditions: temperature 22 ± 1°C, with a light-dark cycle (12 h; first light at 6:00 h), humidity of approximately 65%, and food and water *ad libitum*. During the first week of gestation, the rats received a commercial diet (Presence Purina®, São Paulo, Brazil), and an experimental diet was then offered starting from the second week of gestation and throughout lactation. During pregnancy and lactation, maternal feed intake and body weight were measured weekly. Three groups were formed: the control group (CG) receiving a standard diet without CLA (n = 11); the CLA1 group receiving an experimental diet containing 1% CLA (n = 13); and the CLA3 group receiving an experimental diet containing 3% CLA (n = 12) (**Table 1**); all diets were in accordance with the recommendations of the American Institute of Nutrition (AIN-93G) (Reeves et al., 1993). The CLA mix used was Clarinol® powder (Stepan Lipid Nutrition, Maywood, NJ, United States); the composition is shown in **Table 2**.

TABLE 1 | Composition of control and experimental diets.

Ingredient (g/kg)	Diets		
	Control	CLA1	CLA3
Corn starch	530	520	500
Casein	199.5	199.5	199.5
Sucrose	100	100	100
Soybean oil	70	70	70
CLA mix isomers	–	10	30
Fiber	50	50	50
Mineral mix	35	35	35
Vitamin mix	10	10	10
L-Cystine	3.0	3.0	3.0
Choline bitartrate	2.5	2.5	2.5
Gross energy (Joules)	16.568,6	16.777,8	17.196,2

TABLE 2 | Fatty acid composition of the commercial CLA mix.

Fatty acid	Mean	Standard deviation
14:0	0.1	0.011
16:0	4.3	0.038
18:0	1.4	0.021
18:1c9	10.7	0.135
18:1c11	0.6	0.024
18:2n-6	1.0	0.027
20:0	0.2	0.014
CLA-c9t11	39.2	0.097
CLA-t10c12	38.3	0.071
CLA c/t and t/c isomers	2.5	0.128
CLA-trans,trans	1.5	0.042
22:0	0.2	0.010

The litters were standardized with six pups and some parameters were evaluated: Litter size, Number of males, Number of females, Birth weights. After weaning, at 21 days of age, the animals were separated in polypropylene cages, two animals per cage, where they received water and commercial feed *ad libitum*, containing 1.589,9 J of energy, 23 g of proteins (24.21%), 63 g of carbohydrates (66.31%), and 4 g of lipids (9.47%).

The research followed an experimental protocol in accordance with the ethical recommendations of the National Institutes of Health (Bethesda, MD, United States), and was approved by the ethics research committee of the Federal University of Paraíba No. 0407/13 (**Figure 1**).

Physical Maturation

The pups were weighed throughout lactation at 28, 49, and 70 days of life.

Reflex Ontogeny in Newborn Pups

From the 1st day through the 21st postnatal day, the reflex responses were observed each day at 12:00 pm. Response consolidation was considered positive when a reflex reaction was repeated for three consecutive days (Smart and Dobbing, 1971), have established an experimental model for reflex maturation in rats, as presented in **Table 3**.

Open Field Habituation Test

The open field habituation test is used to evaluate the animal to long-term habituation capacity on the open field device, consisting of a circular metallic arena (painted white) delimited by white walls with an open ceiling. The floor of the arena is divided into 17 fields (with lines painted black), 3 concentric circles (15, 34, and 55 cm in diameter, respectively) which are subdivided into a total of 16 segments and a central circle.

In rodents, habituation is analyzed by locomotor activity on the open field and is considered an indicator of non-associative learning (Leussis and Bolivar, 2006; Rachetti et al., 2013).

At 42 days of age, the animals were exposed to the open field in two phases, the second exposure occurred seven (7) days after the first. Four parameters were evaluated in the first and second expositions, each during 10 min (Rachetti et al., 2013; Gamberini et al., 2015; Speight et al., 2017).

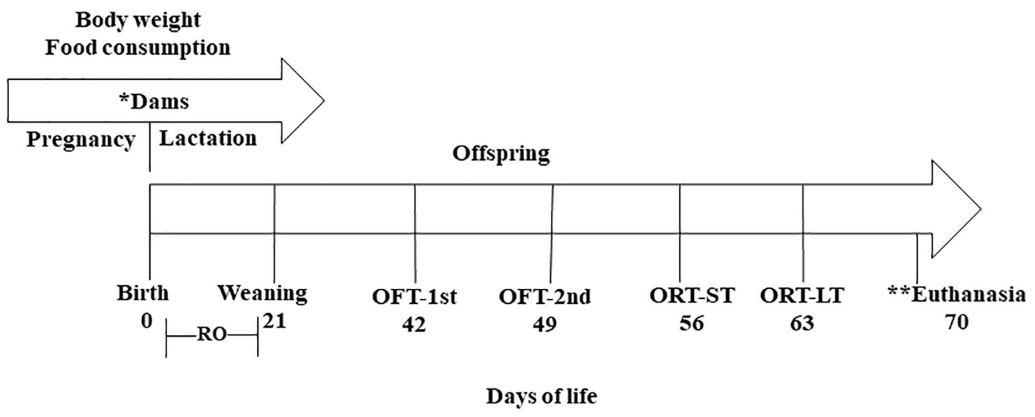

FIGURE 1 | Experimental design. (*) Treatment period where the mothers received: control diet (CG) (n = 11); diet containing 1% (n = 13); and diet containing 3% CLA (n = 12). (**) Brain collection for fatty acid analysis. RO, reflex ontogeny; OFT-1st, open field test 1st exposure; OFT-2nd, open field test 2nd exposure; ORT-ST, object recognition test, short-term; ORT-LT, object recognition test, long-term.

TABLE 3 | Description of the reflex test.

Reflex	Stimulus	Response
Palmar grasp	Light percussion on the palm of the right foreleg	Quick bending of ankles
Righting	The rat is placed in supine position on a surface	Return to the prone position with all paws in 10s
Cliff-avoidance	The rat is placed on a flat and high surface (table), with legs toward the extremity	Moves to one side and walks in the opposite direction to the edge
Vibrissa-placing	The is was suspended by the tail and its vibrissae lightly touch the edge of a flat surface	Both front legs are placed on the table, performing march movements
Negative-geotaxis	The rat is placed at the center of an inclined ramp with head facing downwards	Body spin at an angle of 180°, positioning head upwards
Auditory-startle response	Intense and sudden sound stimulus	Retraction of anterior and posterior legs, with rapid and involuntary body immobilization
Free-fall righting	Held by the four legs, at a height of 30 cm, it is released in free fall on a synthetic foam bed	Position recovery during freefall on the surface supported on four paws

- Duration of locomotion – Time spent by the animal moving in the open field.
- Number of crossings in open field – The ambulation was evaluated by the total of segments covered. It was counted when the animal inserted the four legs inside the segments.
- Number of entries into inner zone – Number of entries into inner zone – Quantified when the animal placed the four legs inside each inner zone of the open field.
- Duration in inner zone – Time spent by the animal in the inner zone of the open field.

All of the sessions were recorded with a video camera attached to the laboratory ceiling and the videos were analyzed afterward. The videos containing the data were analyzed randomly and by a single evaluator.

Object Recognition Test

To evaluate declarative memory, when the animals reached 56 days of life, object recognition testing was applied on the previously used open field arena. Here, the animals underwent two open field exposures (the second exposure at 7 days after the first). The first test is associated with short-term memory, the second test relates to long-term memory (Rachetti et al., 2013).

The testing assesses the amount of time an animal spends in sniffing or touching an object with its nose and/or front legs. First, habituation is performed in the absence of any objects; animals may freely explore the arena for 3 min. Next, in the training session, the animals are placed in the arena when containing two different objects (A1 and A2) allowing free exploration for 10 min, for the animals to recognize and identify object A1 (a familiar object). The test session is held at 180 min after the training session to evaluate short-term memory, in which the animals are placed in the arena now containing two objects, A1 (the familiar object) and A3 (a new object), and they are allowed to freely explore for 5 min. After 7 days, another test is performed to assess long-term memory, in which the animals are placed in the arena to freely explore object A1 (the familiar object) together with object A4 (a new object). The videos were subsequently analyzed by a single evaluator on a random basis, that is, the evaluator was not aware of which group was being evaluated.

Before and after each test, the device and the objects were cleaned with 10% alcohol, and when exchanging animals, both the device and objects were cleaned with 10% alcohol and paper towels.

With completion of the test, the results obtained were analyzed using both the total time spent exploring the objects, and the novel object/(total familiar + novel object) ratio (Gustavsson et al., 2010).

Profile of Brain and Milk Fatty Acids

The milk was collected at the end of lactation (21th day after weaning). Oxytocin (0,5 ml) was administered to facilitate lactation. For collection, the animals were anesthetized with ketamine hydrochloride and xilasin (1 ml/kg body weight). The milk was removed by hand (squeezing the rat's breast) and placed in encoded Eppendorf tubes, 3 per group, where 2 CG Eppendorf tubes and 1 Eppendorf tube for CLA3 were lost during the journey, leaving 1 sample of CG, 3 samples of CLA1 and 2 of CLA3. After collection, the rats were sacrificed by cervical detachment.

At the end of the experiment, at 70 days of age and after a 6-h fast, the offspring were anesthetized and sacrificed. After euthanasia, the brain was removed using a scalpel and pliers, and then lyophilized.

Milk and brain samples were sent to the Faculty of Veterinary Medicine at the University of Lisbon where the FA analyses were conducted. Fatty acid methyl esters (FAMEs) from the freeze-dried milk fat samples were prepared by direct trans-esterification using potassium hydroxide (2M) in methanol, in accordance with (Rego et al., 2009) and FAMEs and dimethyl acetal (DMA) from the brain samples were prepared by reaction with HCl 1.25 M in methanol for 20 h at 50°C. Fatty acid methyl esters and DMA were analyzed by gas chromatography with flame ionization detection using a Shimadzu GC 2010-Plus (Shimadzu, Kyoto, Japan) equipped with a SP-2560 (100 m × 0.25 mm, 0.20 μm film thickness, Supelco, Bellefonte, PA, United States) capillary column. The chromatographic conditions were as follows: injector and detector temperatures were set at 250 and 280°C, respectively; helium was used as the carrier gas at 1 mL/min constant flow; the initial oven temperature of 50°C was held for 1 min, increased at 50°C/min to 150°C and held for 20 min; then increased at 1°C/min to 190°C; and finally increased at 2°C/min to 220°C and held for 40 min. Identification of FAME and DMA were achieved using electron impact mass spectrometry using a Shimadzu GC–MS QP2010 Plus (Shimadzu) and published chromatograms (Alves et al., 2013). The chromatographic column and the GC conditions were similar to the GC-FID analyses. Additional mass spectrometer conditions were as follows: ion source temperature, 200°C; interface temperature, 240°C; and emission voltage, 70 eV. The

fatty acids and DMA inside the incubation tubes were expressed as milligrams per flask, and determined using the internal standard, assuming a direct proportionality between GC-FID peak area and FA weight.

Statistical Analysis

Intergroup differences for reflex maturation were analyzed using one-way Kruskal–Wallis testing, followed by Dunn testing on Sigma Stat software (San Jose, CA, United States). Milk sample FA profile data were presented as means and as standard deviations when more than one sample per treatment was available. Brain tissue FA profile data were analyzed using PROC MIXED, SAS 9.4 (SAS Inst., Cary, NC, United States) using a model that considered the treatment as a single fixed effect, and allowed for variance heterogeneity between treatments. When significant ($p < 0.05$) treatment effects were detected the least square means were compared using the Tukey procedure.

RESULTS

Maternal Feed Intake and Pup Weights

There were no significant differences in maternal food intake and body weight among the different groups.

Body weight analyses showed that the CLA3 group of pups presented higher body weights than the other two groups at 1, 14, and 21 days of age. However on day 7, the CLA3 group body weight was significantly higher only as compared to the CLA1 group, and not to the CG ($p < 0.05$). When assessing body weight after lactation, when animals were 28 days old, CLA3 had a higher body weight when compared to CLA1 and CG. At 49 days of age, the animals of the CLA1 and CLA3 groups presented higher body weights versus CG ($p < 0.05$). At the end of the experiment no significant statistical differences were observed between the groups (**Figure 2**).

Birth Data

Table 4 shows the parameters evaluated after birth. The CLA1 and CLA3 groups presented larger litters as compared to CG ($p < 0.05$). For the other parameters, there were no significant statistical differences.

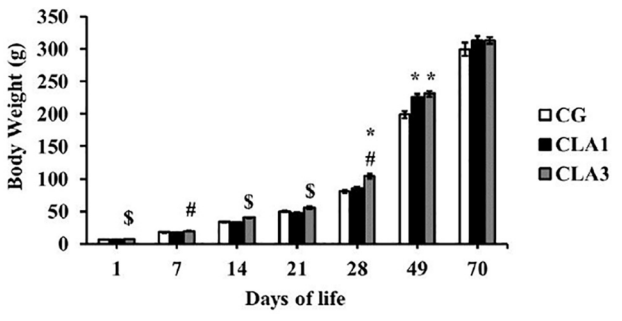

FIGURE 2 | Body weight of rats during lactation to the beginning of adulthood, treated with diet containing 1% CLA (CLA1) or 3% CLA (CLA3). Values are expressed as means and standard error (one way ANOVA, Holm–Sidak); * versus CG # versus CLA1 group; $ versus all group.

Reflex Ontogeny in Newborn Pups

From evaluation of reflex maturation as measured in this study (**Table 5**), we observed that palmar grasp disappearance in the CLA1 and CLA3 groups was delayed as compared to the CG; and the CLA3 group was delayed as compared to the CLA1 group ($p < 0.05$). When maturation of cliff avoidance, vibrissa placing, negative geotaxis, and auditory startle were investigated, the CLA3 group presented acceleration as compared to the control group (CG) ($p < 0.05$). The CLA3 animals also presented increased righting and vibrissa placing in relation to the CLA1 group ($p < 0.05$). CLA1 showed acceleration of palmar grasp versus CLA3 and negative-geotaxis versus CG ($p < 0.05$). In summary, the CLA3 presented acceleration of four reflexes and CLA1 three reflexes of the seven evaluated.

Open Field Habituation

In **Figure 3A**, the CLA1 and CLA3 groups spent less time ambulating in the second exposure to the open field when compared to the first exposure ($p < 0.05$). In **Figure 3B**, it is possible to observe a smaller number of crossings in the fields of the open field in CLA3 in the second exposure ($p < 0.05$). The other groups did not present a statistically significant difference ($p > 0.05$). When the time spent in the internal zone was evaluated, CG spent less time in the internal zone when compared to the first and second exposures. CLA1 and CLA3 spent a longer

TABLE 4 | Evaluation of parameters after birth.

Birth data	Control	CLA1	CLA3
Litter size*	8.0 ± 3.6	13.0 ± 0.9*	13.0 ± 0.5*
Number of males	4.0 ± 1.0	7.0 ± 2.6	7.0 ± 0.5
Number of females	4.0 ± 2.7	6.0 ± 1.9	6.0 ± 1.1
Birth weights (g)	6.0 ± 0.6	6.0 ± 0.2	7.0 ± 0.9

Three litters per each group.

TABLE 5 | Reflex maturation of rats which mothers were treated during gestation and lactation with standard diet containing soybean oil and other two groups with experimental diets, one containing 1% CLA (CLA1) and the other with 3% CLA (CLA3).

Reflex maturation	Diets		
	Control (*n* = 11)	CLA1 (*n* = 16)	CLA3 (*n* = 12)
Palmar grasp[a]	3 (3–6)	3.5 (2–10)*	5 (2–10)*#
Righting[b]	3 (1–6)	3 (2–6)	2 (1–4)#
Cliff-avoidance[b]	10 (6–13)	7.5 (3–13)	6 (5–7)*
Vibrissa-placing[b]	11 (8–12)	10 (4–13)	6 (3–10)*#
Negative-geotaxis	17 (8–18)	13 (11–17)*	13 (8–16)*
Auditory-startle response[b]	14 (12–17)	13 (12–15)	12 (12–13)*
Free-fall righting[b]	11 (8–15)	12 (9–14)	13 (10–16)*

*Values expressed as median (minimum–maximum); (one-way ANOVA, Kruskal–Wallis); * versus Control group; # versus CLA1; [a]desappearance; [b]appearance.*

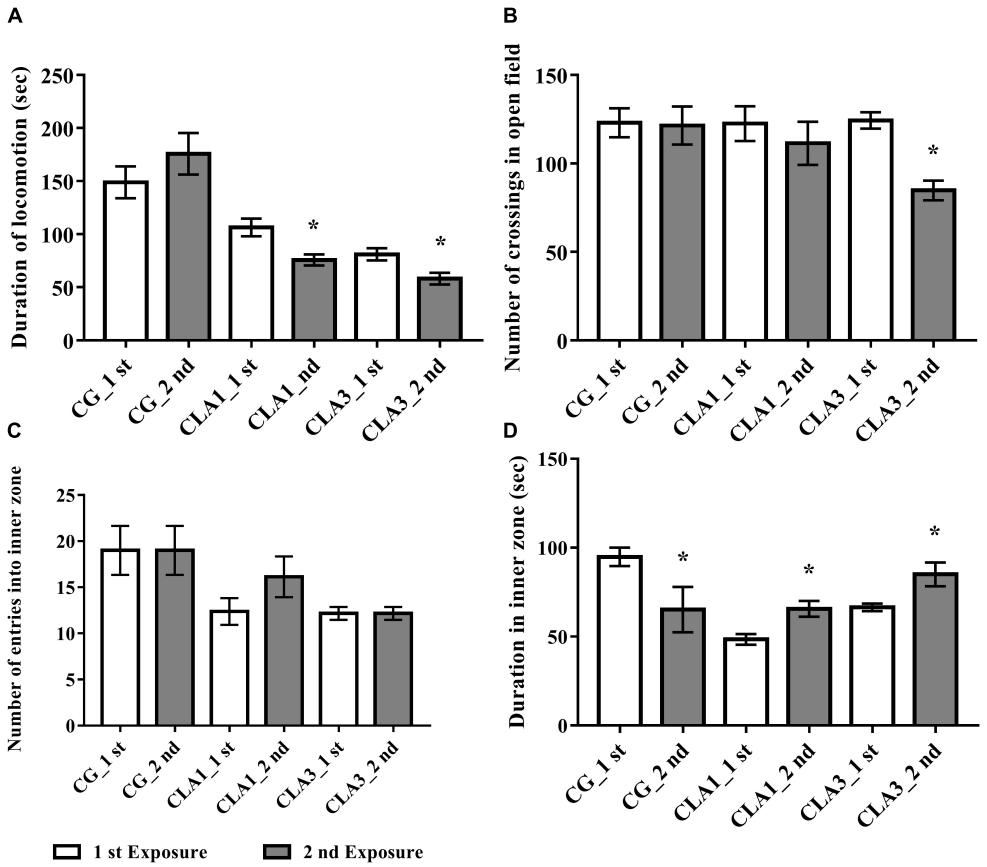

FIGURE 3 | Habituation test with newborn rats treated with standard diet (CG), with 1% CLA (CLA1), or 3% CLA (CLA3) during pregnancy and lactation (maternal diet). Values are expressed as means and standard deviation (one way ANOVA, Holm–Sidak); 1st exposure to 42 days of life and 2nd exposure to 49 days of life; CG (n = 11), CLA1 (n = 13), CLA3 (n = 12); * versus first exposure in the same group. **(A)** Duration of locomotion: time spent by the animal moving in the open field. **(B)** Number of crossings in open field: the ambulation was evaluated by the total of segments covered. It was counted when the animal inserted the four legs inside the segments. **(C)** Number of entries into inner zone: quantified when the animal placed the four legs inside each inner zone of the open field. **(D)** Duration in inner zone – time spent by the animal in the inner zone of the open field.

time in the internal zone when comparing the two exposures ($p < 0.05$) (**Figure 3D**). The number of entry in the internal zone did not differ between the groups (**Figure 3C**).

Object Recognition

For short-term memory testing, there were no significant differences between the groups (**Figure 4A**). In the long-term memory test, all groups (CG, CLA1, and CLA3) explored unfamiliar objects more than familiar objects (**Figure 4B**) ($p = 0.0373$), the CLA1 group presented an increased long-term exploration ratio as compared to the CG and the CLA3 group (**Figure 5B**). In the short-term CLA1 and CLA3 presented higher exploration ratio compared with CG ($p < 0.05$) (**Figure 5A**).

Fatty Acid Profiles in Milk

The means and standard deviations of FAs obtained from the available milk samples are presented in **Table 6**. The FA milk profiles from the animals (three differing diets) was in general similar, although a decrease in *cis*-monounsaturated fatty acid (MUFA), and an increase of total PUFA with CLA3 treatment as compared to the other treatments is suggested. The increase in total PUFA was due to the proportional increase of CLA in the milk presented by maternal (lactating) rats fed CLA supplemented diets. In fact, the control milk samples contained only trace amounts of the 18:2c9,t11 isomer, and presented no 18:2t10c12, whereas the CLA1 and CLA3 group milk samples respectively contained about 1 and 3% of total FA for each CLA isomer.

Fatty Acids and Dimethyl Acetal in the Brain

The fatty acid and DMA profiles in the brain tissue of the pups are presented in **Table 7**. The treatments significantly affected most of the FA and DMA profiles, although they did not follow the expected response patterns. In fact, most of the FA and DMA profiles differed between the controls and the CLA1 pups, whereas the CLA3 pups were either similar to the controls (14:0, 23:0, 16:1c7, 18:1c9, 18:1c11, 20:1c11, 24:1c15, 18:2n-6, 20:2n-6, 20:3n-6, *cis*-MUFA, n-6 PUFA, total PUFA, DMA-17:0, DMA-18:1c9, DMA-18:1c11, and total DMA), or between the controls

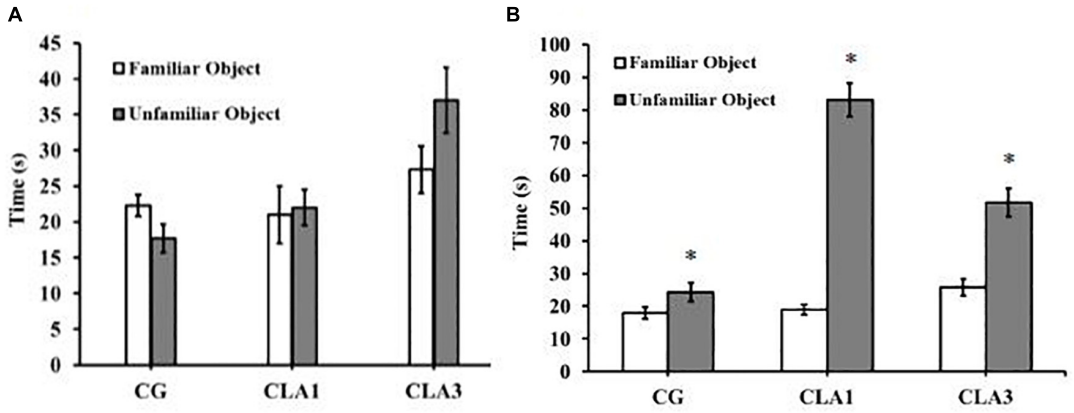

FIGURE 4 | Object recognition test in rats treated with 1% (CLA1) or 3% CLA (CLA3) during pregnancy and lactation (maternal diet). CG: Control group without CLA in the diet. Values are expressed as means and standard deviation (one way ANOVA, Holm–Sidak). **(A)** Short-term test using familiar object (A1) and unfamiliar object (A3). **(B)** Long-term test using familiar object (A1) and unfamiliar object (A4); CG (n = 11), CLA1 (n = 13), CLA3 (n = 12); * versus familiar object in the same group.

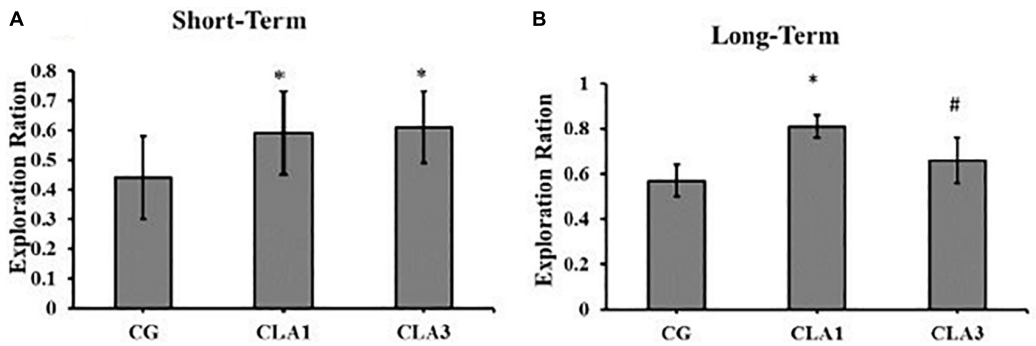

FIGURE 5 | Exploration Ratio for object recognition test in rats treated during pregnancy and lactation (maternal diet) with 1% (CLA1) or 3% CLA (CLA3). CG: Control group without CLA in the maternal diet. Values are expressed as means and standard deviation (one way ANOVA, Holm–Sidak). **(A)** Short-term test using familiar object (A1) and unfamiliar object (A3). **(B)** Long-term test using familiar object (A1) and unfamiliar object (A4); CG (n = 11), CLA1 (n = 13), CLA3 (n = 12); * versus CG; # versus CLA1 group.

and the CLA1 pups (16:0, 20:0, 24:0, 16:1c9, 20:1, 22:1c13, 24:1, 20:4n-6, 22:4n-6, 22:5n-6, 22:6n-3, SFA, n-3 PUFA, and DMA-18:0). Thus, the brains of the CLA1 pups presented higher ($p < 0.05$) proportions of SFA and PUFA, but lower ($p < 0.05$) proportions of MUFA and total DMA than the controls. The CLA isomers were not detected in the brains of the CG pups, and were present only as trace amounts in the CLA1 and CLA3 pups.

DISCUSSION

This study evaluated the developmental effects of maternal supplementation with differing CLA concentrations (1 and 3%) administered during gestation and lactation in their offspring. The data presented the significant effects of CLA on physical growth (with increases in body weight), and on neurodevelopment (acceleration in reflex maturation and memory improvement) in the newborn rats.

Neurodevelopment occurs in the perinatal period, and lipids are increasingly recognized as playing an important role in neuronal function in the brain (Morgane et al., 1993; Salvati et al., 2000). During this phase, essential fatty acids of the n-3 and n-6 families together constitute a lipid substrate that is required for adequate formation of nerve cell membranes. The lipids are also involved in cell signaling and regulate synaptic throughput (Muller et al., 2015). The effects of n-3 in the maternal diet on brain development of the offspring are already known (Rathod et al., 2014; Mucci et al., 2015), However, the effect of other fatty acids like CLA have not yet been investigated. In this study, a maternal diet that included CLA, induced improvements in memory, and accelerated neurodevelopment in the offspring.

Maternal diets containing different n-6/n-3 ratios in rats have been associated with reflex ontogeny and physical growth modifications in the offspring (Santillan et al., 2010; Aquino et al., 2015). Such results demonstrate early life vulnerability of developing nervous systems to an inadequate balance of essential fatty acids. Treatment of Wistar rats during lactation with goat milk (fat containing CLA) has been shown to result in cliff avoidance delays, but there were anticipations in free-fall righting (Soares et al., 2013). Another study (Santillan et al., 2010) found

TABLE 6 | Fatty acid composition (means ± standard deviation expressed as g/kg of total fatty acids) of breast milk of dams fed control, CLA1 and CLA2 diets.

Fatty acids	Control	CLA1	CLA3
n[1]	1	3	2
8:0	29.5	37.9 ± 11.1	52.5 ± 3.07
10:0	92.7	104 ± 42.9	148 ± 15.9
12:0	61.9	56.0 ± 27.3	80.1 ± 14.2
14:0	54.3	41.3 ± 15.7	49.9 ± 10.4
15:0	1.05	1.24 ± 0.18	1.12 ± 0.29
16:0	219	201 ± 24.0	159 ± 3.3
17:0	0.98	1.16 ± 0.20	1.03 ± 0.19
18:0	31.0	36.4 ± 4.44	31.5 ± 1.77
20:0	0.72	0.80 ± 0.07	0.95 ± 0.04
22:0	0.20	0.38 ± 0.04	0.28 ± 0.02
23:0	0.23	0.29 ± 0.05	0.31 ± 0.10
24:0	0.36	0.40 ± 0.04	0.51 ± 0.06
SFA[2]	492	481 ± 68.6	524 ± 44.6
14:1*c*9	1.00	0.74 ± 0.29	0.34 ± 0.12
16:1*c*7	3.25	3.05 ± 0.90	1.99 ± 0.39
16:1*c*9	36.4	26.6 ± 9.27	12.0 ± 1.50
17:1*c*9	1.16	1.09 ± 0.29	0.68 ± 0.14
18:1*t*6/*t*7/*t*8	0.17	0.29 ± 0.02	0.25 ± 0.02
18:1*t*9	0.22	0.27 ± 0.08	0.23 ± 0.01
18:1*t*10	0.17	0.35 ± 0.09	0.56 ± 0.16
18:1*t*11	0.53	0.68 ± 0.01	1.01 ± 0.44
18:1*t*12	0.24	0.41 ± 0.06	0.34 ± 0.01
18:1*c*9	230	222 ± 48.1	159 ± 19.5
18:1*c*11	17.6	18.1 ± 4.40	11.7 ± 1.89
18:1*c*13	0.46	0.52 ± 0.03	0.42 ± 0.18
24:1*c*15	0.10	0.23 ± 0.10	0.11 ± 0.004
cis-MUFA[3]	290	273 ± 61.3	186 ± 20.6
trans-MUFA[4]	1.34	2.27 ± 019	3.05 ± 0.61
18:2n-6	187	193 ± 15.3	187 ± 18.1
18:3n-6	1.83	1.41 ± 0.76	0.94 ± 0.08
20:2n-6	2.67	3.43 ± 0.47	3.14 ± 0.30
20:3n-6	1.59	1.63 ± 0.58	1.06 ± 0.05
20:4n-6	5.35	6.52 ± 1.70	6.26 ± 0.64
n-6 PUFA[5]	199	206 ± 18.1	198 ± 19.0
18:3n-3	13.0	14.7 ± 2.47	16.4 ± 0.78
20:3n-3	0.43	0.47 ± 0.06	0.50 ± 0.03
20:5n-3	0.38	0.42 ± 0.08	0.49 ± 0.01
22:5n-3	0.63	0.70 ± 0.09	0.66 ± 0.09
22:6n-3	0.78	1.06 ± 0.43	1.24 ± 0.01
n-3 PUFA[6]	15.2	17.3 ± 2.77	19.3 ± 0.69
18:2*c*9*t*11	0.42	10.6 ± 1.84	35.0 ± 1.86
18:2*t*10*c*12	0.00	7.8 ± 1.85	29.6 ± 1.03
18:2c9c11	0.19	0.53 ± 0.09	0.91 ± 0.13
Other CLA	0.50	1.17 ± 0.06	2.22 ± 0.44
Total CLA	1.11	20.1 ± 3.73	67.8 ± 3.47
Total PUFA	216	244 ± 18.2	286 ± 23.4

[1]Number of samples; [2]Sum of saturated fatty acids; [3]Sum of cis monounsaturated fatty acids; [4]Sum of trans monounsaturated fatty acids; [5]Sum of n-6 polyunsaturated fatty acids; [6]Sum of n-3 polyunsaturated fatty acids.

a delay for negative geotaxis in animals consuming soybean oil, yet an acceleration for cliff avoidance in animals treated with sunflower oil. The cerebellum presents peak development during lactation, and reflex maturity is directly related to the continuous differentiation and maturation of cerebellar neurons (Allam and Albo-Eleneen, 2012), which involve the visual and postural systems (Boyle, 2001).

In the present study, 1% CLA induced acceleration for palmar gasp and negative geotaxis, however when the dose was tripled, cliff-avoidance, vibrissa-placing, negative-geotaxis, auditory-startle response, were accelerated. The group treated with CLA3% obtained a better response in four parameters of the seven evaluated for reflex. On the other hand, the CLA1 group presented two accelerated reflexes. When analyzing the two groups it can be stated that the effects were moderate. It is unclear what mechanisms the body uses to transform CLA into PUFAs, what is known is that no large doses of omega-3 lipids are required for a better response in the nervous system. In addition, high doses of this lipid may cause depletion of omega-6 lipids and the consequent imbalance between n-3 and n-6 (Zanarini and Frankenburg, 2003; Salvati et al., 2006). These findings suggest that CLA positively affects neurodevelopment, anticipating reflex maturation in the offspring.

Studies show that CLA can pass through the blood–brain barrier (Fa et al., 2005; Soares et al., 2012), reaching the brain, where it performs beneficial functions. However in our study, CLA was found in the brain only in trace amounts. In the milk, in the CLA1 and CLA3 groups, apparently the quantities of CLA were higher, but this cannot be stated with accuracy since statistical analysis was not performed, since some of the samples were lost during transit. This may be considered a limitation of our study.

Thus, we believe that in the present study the effects of this fatty acid occurred indirectly. Study analyzed aspects of maternal metabolism on milk composition show that the transfer of fatty acids to the mother's milk may vary depending on their quality as consumed in the maternal diet (Demmelmair and Koletzko, 2018). The authors observed that alpha-linolenic acid offered in the diet contributes more than that of the normal maternal reserve to its final quantity as found in the milk. However, linoleic acid in breast milk is derived more from the liver than from the diet itself (Demmelmair and Koletzko, 2018). The literature reports on metabolism and omega 3 and 6 in breast milk (Demmelmair and Koletzko, 2018). However, little is known about the influence of maternal consumption of CLA on the composition of breast milk.

In rats fed a diet rich in *trans*-FAs, offered during early life and after weaning, spatial memory was modified (Souza et al., 2012), and a maternal diet containing high levels of lard and saturated fats was found to induce damages to the memory and learning ability of the offspring (Yu et al., 2010). However, both studies cited, evaluated memory using the Morris water maze test. In our study, new object recognition test was used to assess working memory. Both groups treated with CLA (CLA1 and CLA3) demonstrated increased time of exploration of unfamiliar (novel) objects, indicating a better working memory. This findings differ from the results reported by Yu et al. (2010), who showed that offsprings of mice treated with saturated fats performed worse in Morris water maze test. We should point

TABLE 7 | Fatty acids (FAs) and dimethyl acetals (DMAs) composition (least square means ± standard error, expressed as g/kg of total FA + DMA) cerebral tissue of offspring from dams fed control, CLA1 and CLA3 diets.

	Control	CLA1	CLA3	*P*-value
n[1]	6	4	4	
FA				
14:0	1.13 ± 0.07	3.54 ± 0.21*	1.77 ± 0.43#	< 0.001
15:0	0.38 ± 0.03	0.63 ± 0.06*	0.52 ± 0.02*	0.003
16:0	179 ± 0.8	216 ± 4.0*	197 ± 10.8	< 0.001
17:0	1.52 ± 0.04	1.40 ± 0.08	1.65 ± 0.17	0.289
18:0	207 ± 0.9	212 ± 1.92*	211 ± 1.5	0.025
20:0	5.86 ± 0.08	4.25 ± 0.35*	5.02 ± 0.54	0.003
22:0	5.87 ± 0.12	4.93 ± 0.41	5.08 ± 0.52	0.075
23:0	1.60 ± 0.07	0.45 ± 0.09*	1.23 ± 0.33#	< 0.001
24:0	12.3 ± 0.16	8.39 ± 0.87*	9.75 ± 1.41	0.002
SFA[2]	414 ± 0.6	452 ± 3.7*	433 ± 9.7	< 0.001
16:1*c*7	1.21 ± 0.06	3.12 ± 0.11*	1.82 ± 0.44	< 0.001
16:1c9	3.12 ± 0.18	4.06 ± 0.01*	3.55 ± 0.28	0.001
17:1c9	0.36 ± 0.04	0.27 ± 0.05	0.44 ± 0.08	0.209
18:1*trans*	2.18 ± 0.16	2.75 ± 0.16	2.23 ± 0.29	0.066
18:1c9	154 ± 0.6	122 ± 3.1*	141 ± 7.9#	< 0.001
18:1c11	29.7 ± 0.17	24.9 ± 0.61*	28.7 ± 0.93#	< 0.001
20:1c11[3]	12.9 ± 0.21	5.46 ± 0.49*	9.79 ± 1.87#	< 0.001
20:1[4]	4.03 ± 0.05	1.77 ± 0.19*	3.08 ± 0.58	< 0.001
22:1c13	1.85 ± 0.05	0.95 ± 0.08*	1.46 ± 0.28	0.012
24:1c15	18.1 ± 0.46	7.41 ± 0.84*	14.3 ± 2.96#	< 0.001
24:1[4]	1.92 ± 0.05	0.94 ± 0.09*	1.58 ± 0.32	< 0.001
cis-MUFA[5]	230 ± 0.8	173 ± 5.4*	208 ± 14.1#	< 0.001
18:2n-6	6.12 ± 0.06	12.1 ± 0.32*	7.00 ± 1.25#	< 0.001
20:2n-6	1.32 ± 0.07	2.58 ± 0.09*	1.50 ± 0.20#	< 0.001
20:3n-6	3.51 ± 0.08	5.97 ± 0.34*	3.65 ± 0.26#	< 0.001
20:4n-6	98 ± 0.65	115 ± 2.12*	104 ± 4.47	< 0.001
22:4n-6	29.1 ± 0.27	30.9 ± 0.52	29.7 ± 0.55	0.029
22:5n-6	7.05 ± 0.21	9.13 ± 0.27*	7.68 ± 0.87	< 0.001
n-6 PUFA[6]	145 ± 1.0	176 ± 2.7*	154 ± 7.4#	< 0.001
22:5n-3	2.33 ± 0.04	3.19 ± 0.18*	2.14 ± 0.05*#	< 0.001
22:6n-3	116 ± 0.9	123± 1.9*	120 ± 3.2	0.017
n-3 PUFA[7]	118 ± 0.9	127 ± 1.8	122 ± 3.1	0.030
20:2[4]	1.09 ± 0.05	0.79 ± 0.06*	0.86 ± 0.03*	0.005
20:3n-9	0.82 ± 0.06	0.69 ± 0.02	0.66 ± 0.03	0.101
18:2c9*t*11	*nd*	0.08 ± 0.06	0.24 ± 0.08	0.154
18:2*t*10c12	*nd*	0.05 ± 0.04	0.15 ± 0.05	0.171
Total CLA	*nd*	0.13 ± 0.09	0.39 ± 0.13	0.159
Total PUFA	266 ± 0.9	304 ± 3.9*	277 ± 10.3#	< 0.001
DMA				
16:0	20.6 ± 0.35	23.8 ± 0.27*	22.1 ± 0.27*#	< 0.001
17:0	0.85 ± 0.05	0.45 ± 0.06*	0.84 ± 0.16#	< 0.001
18:0	42.3 ± 0.36	33.4 ± 0.62*	37.7 ± 2.69	< 0.001
18:1c9	15.4 ± 0.15	8.1 ± 0.57*	12.4 ± 1.95#	< 0.001
18:1c11	11.5 ± 0.18	5.01 ± 0.29*	9.14 ± 1.72#	< 0.001
Total DMA	90.6 ± 0.66	70.8 ± 1.44*	82.3 ± 6.20#	< 0.001

*[1]number of samples; [2]Sum of saturated fatty acids; [3]Coelutes with minor amounts of 18:3n-3; [4]Double bond position not determined; [5]Sum of cis-monounsaturated fatty acids; [6]Sum of n-6 polyunsaturated fatty acids; [7]Sum of n-3 polyunsaturated fatty acids; * versus CG # versus CLA1 group.*

out that we did different memory test and CLA isomers are unsaturated fat.

In early stage of life in the rat, neurodevelopment happens very fast, as demonstrated when was measured the reflex maturation in the present study. The reflex maturation occurred from birth to 18 days of life. In addition, maternal supplementation with CLA altered the deposition of fatty acids in the brain as demonstrated in **Table 7**. Unfortunately, we evaluated the composition of fatty acids in this tissue only at the end of the experiment (70 days of life), which made it impossible to evaluate the transition of fatty acids in this brain tissue and to relate to the transitional period that led to the consolidation of the memory by the animals. However, the exploration index by CLA groups were always higher than CG (short and long time). Thus, these data confirm how neurodevelopment occurs fast in the nervous system, even during lactation with the evaluation of reflex maturation and in the postnatal period with evaluated of memory by the object recognition test.

The experimental diets significantly affected most of the fatty acids and DMA, although they did not follow the expected response patterns. The CLA1 group presented higher amounts of PUFAs as compared to the CG, and the CLA3 group's PUFA levels were intermediates between those of the CLA1 and CG groups. Among the polyunsaturated fats found in the brain for the CLA1 group we detected AA and DHA. In the brain, DHA (22:6n-3) and AA (20:4n-6) have been correlated with better spatial memory performance (Fernandes et al., 2011; Harauma et al., 2017).

As was seen in the results, CLA is present in breast milk, and it may be affirmed that the pups received CLA by breastfeeding. However, in the brain, the lipid was found only in small amounts. Yet maternal consumption of CLA induced memory improvement in their offspring. Although CLA was not deposited in large amounts in the brain, its consumption significantly increased n-3 and n-6 fatty acids in the rat brain. Among these fatty acids is AA, an n-6 fatty acid that plays an important role in brain functions, including neuronal signal transmission and long-term potentiation. In addition, AA preserves hippocampal neuron membrane plasticity, protects the brain against oxidative stress, improves memory, and helps in the synthesis of new proteins in brain tissues (Hadley et al., 2016).

AA can be synthesized from substrates such as CLA and LA (Banni, 2002). These fatty acids likely share the same enzymes (desaturases and elongases). Thus, increased CLA ingestion may impede metabolism of linoleic acid, and consequently pro-inflammatory metabolites (Białek et al., 2015).

The principally known metabolites of CLA belong to conjugated diene (CD) structured compounds, such as conjugated octadecetrienoic acid (CD18: 3), conjugated eicosatrienoic acid (CD 20: 3), and conjugated eicosatetraenoic acid (CD 20: 4) which are synthesized in the desaturase and elongase pathways (Banni, 2002). However, CLA metabolism may interfere with the formation of eicosanoids, and CLA hydroxylation and its metabolites conjugated in LOX pathways, or cytochrome P450 may form eicosanoid-like molecules that

compete with regular eicosanoids (Banni, 2002), thus potentially exerting anti-inflammatory action in many, including brain tissue (Shen et al., 2018). Further studies would be needed to elucidate the mechanism by which CLA can induce increased DHA.

Polyunsaturated fatty acids are essential for brain development and memory because they modulate synaptic plasticity, and thus improve learning ability. In human infants (our study investigates this same stage of life), accumulation of these fatty acids occurs during gestation and lactation, through the placenta and breast milk (Banni et al., 1996).

Another memory index used was the open field habituation test, and lower locomotor activity in a repeated exposure indicates good recognition (Rachetti et al., 2013). In the present study CLA1 and CLA3 presented less locomotion and spent longer time in the internal area in the second exposure into the open field. This behavior indicates that the animals moved less because they remembered the place and lost interest in exploration. The time spent in the central area confirms that this decrease in locomotion in the second moment did not occur due to the behavior like anxiogenic of the animals.

A diet enriched in n-3 PUFA has induced an increase in exploratory activity for young rats, which was not observed in the mature or older rats tested (Das, 2003). However, a maternal CLA enriched diet reduced ambulatory activity, as demonstrated in the present study. Thus, it is demonstrated for the first time that CLA is able to affect these learning parameters. With greater CLA levels in the diet, levels of CLA isomers (18:2 *cis*-9, *trans*-11 and 18:2 *trans*-10, *cis*-12) increase in the milk. Yet in the brain, these CLA isomers were present only in trace amounts; and although proportionally higher in the CLA3 group as compared to the CLA1 group, the difference was not significant.

Maternal dietary lipids may also affect body weight in offspring. In this study, the body weight of pups whose mothers received 3% CLA was higher throughout and after lactation. A similar result was observed when during gestation and lactation (Soares et al., 2012) rats received a diet containing goat milk (which is a source of CLA). A different result was observed when lactating rats received 1.35% CLA (Hayashi et al., 2007). Maternal treatment during pregnancy and lactation with 1.47% CLA resulted in pups presenting decreased body weights (Ringseis et al., 2004). The authors attribute this effect to lipid reduction in the breast milk; and yet such a reduction was not induced in the present study. The data demonstrate that maternal dietary lipids may differently affect the physical parameters of their offspring. Evaluating the body weight and feed intake of the progenitors, there was no statistical difference between the groups. The mothers who had the analyzed milk are representatives of their groups when evaluated body weight and feed intake.

The animals from the groups experimental presented anticipations of certain reflexes, as well as improvements in memory. These findings are important because they demonstrate the benefits that CLA consumption can bring to the developing brain. The present research will serve to guarantee a safety indication of the consumption of CLA by pregnant and lactating women by doctors and nutritionists. In addition, the supplement used in this research is a trademark and can be freely purchased by individuals and verify the effects of its consumption is very important. However, it is interesting to conduct research with humans to guarantee the similarity of the results found in the present research with women and infants.

CONCLUSION

Based on our results, it may be concluded that maternal supplementation with CLA influenced development of the offspring central nervous system, accelerating reflex maturation (Cliff-avoidance, Vibrissa-placing, Negative-geotaxis, Auditory-startle response) and delay in only two reflexes (Palmar grasp and Free-fall righting) in the group which received 3% CLA. In the group which received 1% CLA was observed acceleration in reflexes (Palmar grasp and Negative-geotaxis). Memory improvement was also observed in CLA treated groups where there was greater exploration of the new object in the object recognition test. It is suggested that further studies be performed to prove the effects of CLA on the central nervous system.

ETHICS STATEMENT

The research followed an experimental protocol in accordance with the ethical recommendations of the National Institutes of Health (Bethesda, MD, United States), and was approved by the ethics research committee of the Federal University of Paraíba No. 0407/13.

AUTHOR CONTRIBUTIONS

JS, CB, MM, MO, and RQ designed the theme of the study. MB and ML performed the designed experimental methods. RB, SA, and MS for conducting the analysis of fatty acids. JS, MM, MB, and MQ analyzed the data. JS and MQ interpreted the results and wrote the article. This research was carried out by all authors.

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Dopamine-Induced Ascorbate Release From Retinal Neurons Involves Glutamate Release, Activation of AMPA/Kainate Receptors and Downstream Signaling Pathways

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Ascorbate, the reduced form of Vitamin C, is one of the most abundant and important low-molecular weight antioxidants in living tissues. Most animals synthesize vitamin C, but some primates, including humans, have lost this capacity due to disruption in L-gulono-gamma-lactone oxidase gene. Because of this incapacity, those animals must obtain Vitamin C from the diet. Ascorbate is highly concentrated in the central nervous system (CNS), including the retina, and plays essential roles in neuronal physiology. Ascorbate transport into cells is controlled by Sodium Vitamin C Co-Transporters (SVCTs). There are four SVCT isoforms and SVCT2 is the major isoform controlling ascorbate transport in the CNS. Regarding ascorbate release from retinal neurons, Glutamate, by activating its ionotropic receptors leads to ascorbate release via the reversion of SVCT2. Moreover, dopamine, via activation of D_1 receptor/cyclic AMP/EPAC2 pathway, also induces ascorbate release via SVCT2 reversion. Because the dopaminergic and glutamatergic systems are interconnected in the CNS, we hypothesized that dopamine could regulate ascorbate release indirectly, via the glutamatergic system. Here we reveal that dopamine increases the release of D-Aspartate from retinal neurons in a way independent on calcium ions and dependent on excitatory amino acid transporters. In addition, dopamine-dependent SVCT2 reversion leading to ascorbate release occurs by activation of AMPA/Kainate receptors and downstream ERK/AKT pathways. Overall, our data reveal a dopamine-to-glutamate signaling that regulates the bioavailability of ascorbate in neuronal cells.

Keywords: SVCT2, vitamin C, D-aspartate, excitatory amino acid transporters, D_1R, EPAC, AKT, ERK

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INTRODUCTION

In spite of not being an amine, ascorbate is also called Vitamin C as an extension of the term "vital amine" introduced by Casimir Funk in early twenties to indicate the nutritional factor necessary to prevent scurvy. At that time, the chemical identity of Vitamin C was not known. Later on, Albert Szent-Giörgyi identified a 6-carbon sugar obtained from acid fruits and adrenal glands, the "hexuronic acid" that was later termed ascorbic acid because of its anti-scurvy properties (Szent-Györgyi, 1963). Vitamin C is highly concentrated in the central nervous system (CNS), including the retina, where it is best known for its antioxidant properties. Besides, Vitamin C plays important roles in neural physiology, for instance, participating in the formation of the myelin shaft (Carey and Todd, 1987; Eldridge, 1987), regulating the release of acetylcholine (Kuo and Yoshida, 1980; Feuerstein et al., 1993), modulating NMDA receptor function (Majewska et al., 1990; Domith et al., 2018), modulating GABAergic neurotransmission (Calero et al., 2011) and acting as a co-factor in a plethora of enzymatic reactions such as the conversion of dopamine into norepinephrine (Rebec and Pierce, 1994) and the synthesis of neuropeptides (Glombotski et al., 1986). Regarding its oxidative states, Vitamin C can be found in two forms in living tissues, the oxidized form, dehydroascorbate (DHA), and the reduced form, ascorbate. DHA is taken up by glucose transporters (GLUT) 1, 2, 3 and 4 (Rumsey et al., 1997, 2000; Mardones et al., 2011) whereas ascorbate is taken up by the Sodium Vitamin C co-Transporter – SVCT (Slc23) (Daruwala et al., 1999; Tsukaguchi et al., 1999; Wang et al., 2000; Takanaga et al., 2004). There are two different SVCT isoforms, SVCT1 and 2 (Slc23a1 and Slc23a2). SVCT1 is found in epithelial tissues involved in ascorbate (re)absorption, while SVCT2 is highly expressed in the CNS. SVCT2 is a glycoprotein with 12 transmembrane domains that transports ascorbate in a sodium-dependent manner (Tsukaguchi et al., 1999), with potential N-glycosylation sites in the extracellular loop between transmembrane segments three and four (Tsukaguchi et al., 1999). This transporter can be regulated by multiple signaling pathways including PKA (Wu et al., 2007), PKC (Daruwala et al., 1999; Liang et al., 2002) and NO-cGMP-PKG-NF-kB (Portugal et al., 2012).

Although SVCTs are the major transporter systems regulating ascorbate uptake into cells, several mechanisms can be employed to mediate the release of ascorbate from cells (Corti et al., 2010). In one of such mechanisms, ascorbate can be released via the reversion of its high-affinity transporter SVCT2 (Portugal et al., 2009; da Encarnação et al., 2018). In neuronal cells, the neurotransmitters glutamate, acting on AMPA/kainate ionotropic receptors (Portugal et al., 2009), and dopamine (DA), acting through the D_1R/cAMP/EPAC2 pathway (da Encarnação et al., 2018), can trigger SVCT2 reversion leading to ascorbate release. Because both glutamate and DA (two major neurotransmitter systems in the CNS) promote SVCT2-induced ascorbate release from neurons, we asked if these two neurotransmitter systems were, somehow, linked to mediate the release of ascorbate. Here we revealed that DA triggers D-aspartate release (a measure of glutamate release) through a calcium-independent and excitatory amino acid (EAA) transporter-dependent mechanism. Glutamate then activates AMPA/kainate receptors and downstream ERK and AKT pathways leading to SVCT2-dependent ascorbate release from neurons. Our data describe a DA-to-glutamate signaling regulating the bioavailability of ascorbate in the CNS.

MATERIALS AND METHODS

Animals

Fertilized White Leghorn chicken eggs were obtained from a local hatchery and incubated at 38°C in a humidified atmosphere until the 8th day *in ovo*. All experiments were performed in compliance with ARRIVE guidelines and under institutional approval of COBEA (Ethical principles of animal experimentation) and the Committee on Ethics in Animal Research (CEPA) of the Universidade Federal Fluminense (number 00146/09).

Reagents

Dopamine; SKF-38393 [1-phenyl-2,3,4,5-tetrahydro-(1H)-3-benzazepine-7,8-diol hydrochloride]; 8-pCPT-2′-O-Me-cAMP [8-(4-chlorophenylthio)-2′-O-methyladenosine 3′,5′-cyclic monophosphate monosodium hydrate]; HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid); DNQX (6,7-dinitroquinoxaline-2,3-dione); dimethyl sulfoxide; MK-801 (5S,10R)-(+)-5-Methyl-10,11-dihydro-5H-dibenzo[a, d]cyclohepten-5,10-imine hydrogen maleate); BAPTA-AM [1,2-bis-(o-Aminophenoxy)-ethane-N,N,-N′,N′-tetraacetic acid tetraacetoxy-Methyl ester]; PD 98,059 [2-(2-Amino-3-methoxyphenyl)-4H-1-benzopyran-4-one]; UO126 [1,4-Diamino-2,3-dicyano-1,4-bis (o-aminophenylmercapto) butadiene monoethanolate] and bovine serum albumin (BSA) were from Sigma Aldrich. Trypsin; minimum essential medium (MEM); glutamine; Penicillin; streptomycin and fetal bovine serum (FBS) were from Thermo Fisher Scientific. Acrylamide; ammonium persulphate (APS); N,N′-methylene-bisacrylamide; sodium dodecyl sulfate (SDS); tetramethyl-ethylenediamine (TEMED); ECL kit; polyvinylidene fluoride (PVDF) membranes; anti-mouse and anti-rabbit HRP-conjugated secondary antibodies were from GE Healthcare. DL-threo-β-benzyloxyaspartic acid (TBOA) was purchased from Tocris. 2-amino-5-phosphonopentanoic acid (APV) and LY294002 (2-(4-Morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one hydrochloride) were purchased from Biomol. [^{14}C] Ascorbic Acid (13 mCi/mmol) and [^{3}H] D-aspartic acid (12.2 Ci/mmol) were from PerkinElmer. Primary antibodies against phospho-AKT (Ser473), AKT, phospho-ERK (Thr202/Tyr204) and ERK were from Cell Signaling.

Primary Cultures of Retinal Cells

Monolayer cultures of chick retinal cells were prepared as previously described (de Mello, 1978). Briefly, retinas from 8-day-old chick embryos (E8) were dissected from other ocular tissues, including the retinal pigment epithelium, and digested with 0.2% trypsin in calcium and magnesium-free Hank's balanced salt solution (HBSS), for 15 min at 37°C. Cells were then physically dissociated in MEM supplemented with

3% FBS, penicillin (100 U/mL), streptomycin (100 mg/mL), and glutamine (2 mM). After that, cultures were seeded (2×10^4cells/mm^2) in plastic dishes and maintained at 37°C in a humidified incubator with 95% air and 5% CO_2. Culture medium was completely exchanged for fresh medium after 1 day in culture (C1) and experiments were performed at C3–C4. The proportion of neurons (80%) to Müller glia (20%) has been characterized elsewhere (Portugal et al., 2012).

[^{14}C] Vitamin C Release

Vitamin C release experiments were done exactly as previously described (Portugal et al., 2009). Firstly, the medium was removed and cultures were rinsed twice with HBSS (140 mM NaCl; 5 mM KCl; 20 mM HEPES; 4 mM glucose; 1 mM $MgCl_2$, and 2 mM $CaCl_2$, pH 7.4). After that, cultures were incubated with [^{14}C] ascorbate (0.3 μCi/mL) for 40 min at 37°C and then rinsed twice with HBSS and further incubated with HBSS during four periods of 3 min to completely remove extracellular radioactivity. Cells were then incubated for 10 min with HBSS in order to estimate the [^{14}C] ascorbate basal release and incubated for another period of 10 min with HBSS containing different antagonists. A third period of 10 min of incubation was performed with HBSS containing DA, SKF-38393, or Me-cAMP in the absence or in the presence of different antagonists. After all incubation periods, cells were then lysed with 5% trichloroacetic acid. All three supernatants sequentially collected from the same well, in addition to the cell lysates, were reserved to measure the radioactivity by liquid scintillation spectroscopy. Results were normalized to percent of control after calculation of the percent fractional release, that is, the percent of radioactivity released compared to intracellular radioactivity at each period of time. All Ascorbate release assays were conducted at 37°C.

[^{3}H] D-Aspartate Release

Firstly, the medium was removed, the cultures rinsed twice with HBSS and then pre-incubated with [^{3}H] D-Aspartate (1 μCi/mL) for 90 min. After this uptake period, the cultures were rinsed twice with HBSS and further washed for four periods of 3 min to completely remove the extracellular radioactivity. Cells were then incubated for 10 min with HBSS in order to measure the basal release. After this time, cultures were incubated for another period of 10 min with HBSS containing different drugs or antagonists. A third period of 10 min of incubation was performed with HBSS containing DA in the absence or presence of different antagonists. After this time, the cells were lysed with water and all three supernatants sequentially collected from the same well, in addition to the cell lysates, were reserved to measure the radioactivity by liquid scintillation spectroscopy. Results were normalized to percent of control after calculation of the percent fractional release, that is, the percent of radioactivity released compared to intracellular radioactivity at each period of time. All D-Aspartate release assays were conducted at 37°C.

Western Blotting

Cultures were washed twice with HBSS and starved for 40 min in HBSS to reduce basal phosphorylation of AKT and ERK. Afterward, cultures were incubated with DA, SKF-38393, or 8-pCPT-2′-O-Me-cAMP for different periods, washed twice with HBSS, lysed, and the total protein amount was estimated by Bradford's method. Samples containing 60 μg protein were submitted to 9% SDS-PAGE and proteins transferred to PVDF membranes, which were then incubated overnight with specific antibodies against phosphorylated forms of ERK (1:1,000) or AKT (1:1,000). Subsequently, membranes were washed in TBS-T buffer, incubated with anti-rabbit HRP-conjugated secondary antibody (1:2,000) and developed using an ECL kit. After stripping with 0.2M glycine, pH 2.2, for 30 min, membranes were re-probed with anti-ERK and anti-AKT (1:1,000). Subsequently, membranes were washed in TBS-T and incubated with anti-rabbit (1:2,000) peroxidase-conjugated secondary antibody. Western blot quantifications were performed using the ImageJ software.

Statistical Analysis

Data in all histograms display the mean ± SEM. Statistical analyses were performed using Student's *t*-test or one-way ANOVA followed by the Bonferroni or Dunnet post-test using the GraphPad Prism Software.

RESULTS

We previously demonstrated that glutamate, by interacting with its ionotropic receptors, stimulates the release of ascorbate from cultured retinal cells (Portugal et al., 2009). The glutamate-induced ascorbate release is independent of calcium and mediated by the reversion of SVCT2 (Portugal et al., 2009). Moreover, by interacting with D_1R and activating downstream EPAC2 pathway, DA induces ascorbate release also via SVCT2 reversion (da Encarnação et al., 2018). Because both the glutamatergic and the dopaminergic systems induce ascorbate release from neurons via the reversion of SVCT2, and the glutamatergic and dopaminergic systems are tightly associated in the retina, we studied whether the DA- and the glutamate-induced ascorbate release were, somehow, interconnected.

Activation of D_1R/EPAC2 Pathway by DA Drives Excitatory Amino Acid Release via Excitatory Amino Acid Transporter

Because the dopaminergic and the glutamatergic systems are interconnected in the retina and both systems induce ascorbate release by SVCT2 reversion, we asked if ascorbate release, elicited by DA might be mediated via EAA, including glutamate. To study EAA release from retinal cells we performed release assays using the non-metabolizable EAA [^{3}H] D-Aspartate, which shares some transport mechanisms with endogenous glutamate (Palmer and Reiter, 1994; Muzzolini et al., 1997). We stimulated primary cultured cells with DA or the D_1R agonist SKF-38393 and observed that both treatments increased [^{3}H] D-Aspartate release compared with the basal condition (**Figure 1A**). Because the DA-dependent modulation of D_1R induces ascorbate release via the activation of the guanine exchange factor EPAC2 instead of PKA (da Encarnação et al., 2018), we evaluated the effect of EPAC2

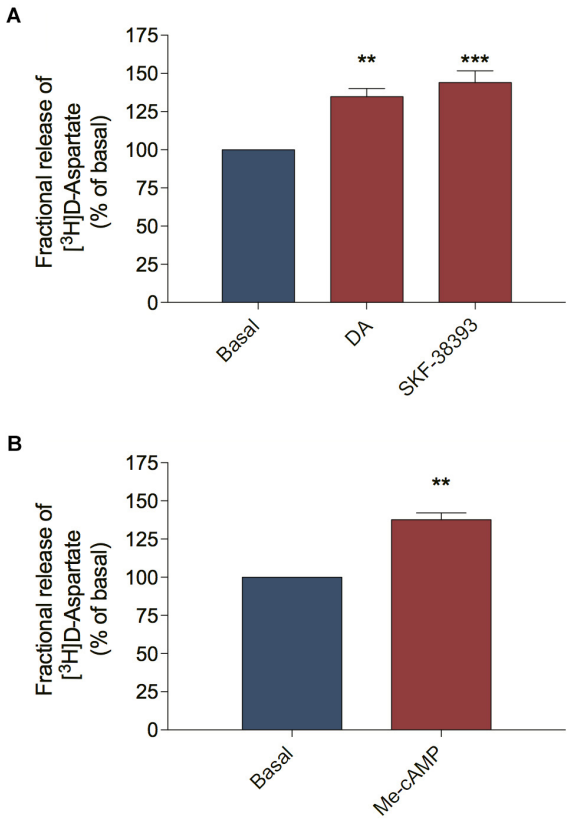

FIGURE 1 | D_1 receptor activation and EPAC stimulation trigger D-aspartate release from retinal cultures. Retinal cultures were incubated for 90 min with [^{3}H] D-aspartate (1 μCi/mL), washed and processed for release experiments as described in section "Materials and Methods." Cultures were incubated with DA (50 μM; **A**) or the D_1 receptor agonist SKF-38393 (10 μM; **A**) or the selective EPAC activator Me-cAMP (100 μM; **B**) for 10 min. The results represent the mean ± SEM of three independent experiments. Statistical analyses were performed using one-way ANOVA followed by the Dunnet post-test **(A)** or the unpaired *t*-test **(B)**. $^{**}p < 0.01$ (relative to basal); $^{***}p < 0.001$ (relative to basal). DA, Dopamine; Me-cAMP, 8-pCPT-2′-O-Me-cAMP.

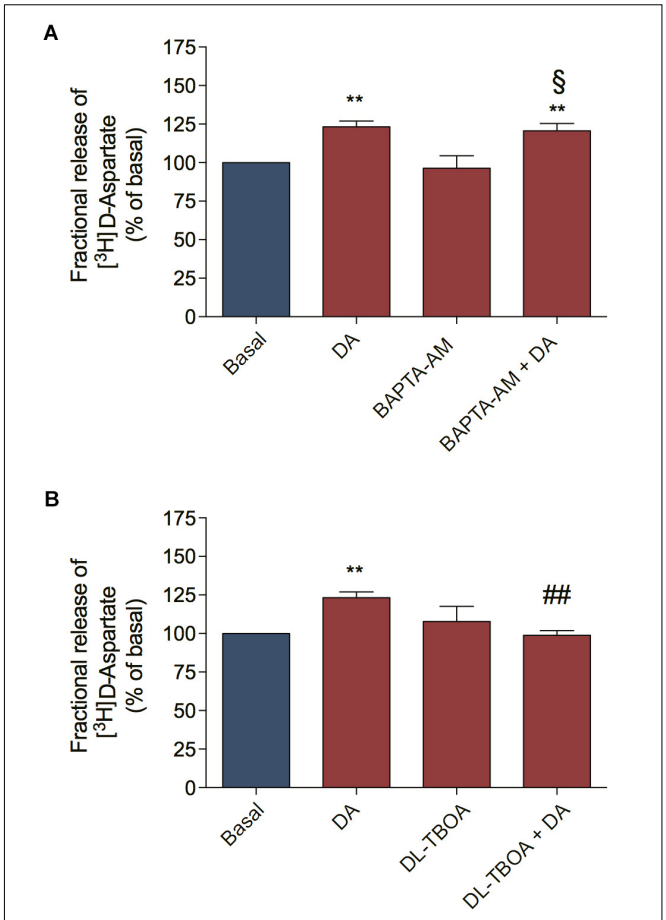

FIGURE 2 | Dopamine-induced D-aspartate release is mediated by EAATs. Retinal cultures were incubated for 90 min with [^{3}H] D-aspartate (1 μCi/mL), washed and processed for release experiments as described in section "Materials and Methods." Cultures were pre-incubated with the intracellular Ca^{2+} chelator, BAPTA-AM (50 μM; **A**) or the selective non-transportable inhibitor of EAATs DL-TBOA (100 μM; **B**) for 10 min. Then, cultures were incubated with DA (50 μM; **A,B**) for an additional period of 10 min. The results represent the mean ± SEM of four independent experiments. Statistical analyses were performed using one-way ANOVA followed by the Bonferroni post-test. $^{**}p < 0.01$ (relative to basal); $^{\#\#}p < 0.01$ (relative to DA). § Not statistically different compared with DA. DA, Dopamine.

activation in the release of [^{3}H] D-Aspartate. Indeed, 8-pCPT-2′-O-Me-cAMP (Me-cAMP), a selective EPAC stimulator, also induced [^{3}H] D-Aspartate release from retinal cells (**Figure 1B**), suggesting that the DA/D_1R/EPAC2 signaling pathway can induce EAA release from retinal cultured cells.

We also asked by which mechanism DA could mediate [^{3}H] D-Aspartate release from retinal cells. [^{3}H] D-Aspartate release in the retina can occur via calcium-dependent and calcium-independent mechanisms (Santos et al., 1996; de Freitas et al., 2016). In order to distinguish between them, we pre-incubated retinal cell cultures with BAPTA-AM to abolish cytosolic calcium mobilization and observed that BAPTA-AM treatment did not block the DA-induced [^{3}H] D-Aspartate release (**Figure 2A**), concluding that DA induces [^{3}H] D-Aspartate release through a mechanism independent of cytosolic calcium mobilization. Because reversion of EAATs is the main calcium-independent mechanism regulating the release of glutamate in the retina (de Freitas et al., 2016), we blocked EAATs-dependent transport with DL-TBOA and observed that incubation of retinal cells with DL-TBOA abrogated the DA-induced [^{3}H] D-Aspartate release (**Figure 2B**). Altogether, these data suggest that DA, by activating a D_1R/EPAC2 signaling pathway, induces EAA release from cultured retinal cells through an EAAT-dependent mechanism.

EAA Released in Response to Dopamine Activates Ionotropic Glutamate Receptors Eliciting Ascorbate Release

As we demonstrated above, DA is capable of inducing [^{3}H] D-Aspartate release by the activation of D_1R/EPAC2 signaling pathway. To demonstrate that the DA-induced EAA release and activation of glutamate ionotropic receptors is important for

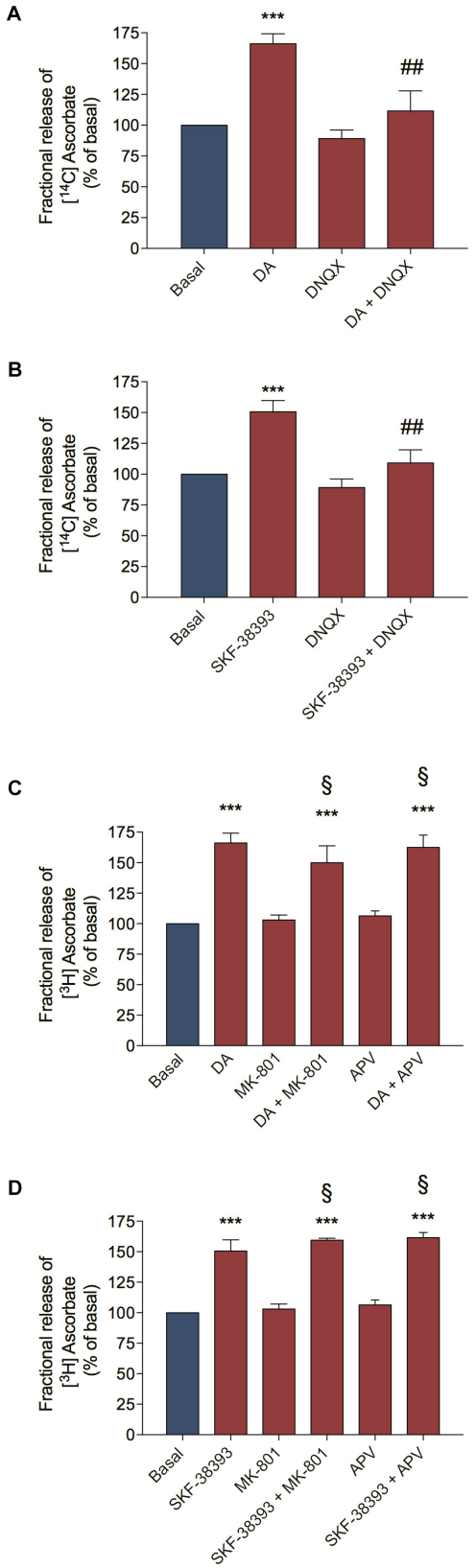

FIGURE 3 | Continued

FIGURE 3 | Dopamine-induced ascorbate release was inhibited by AMPA/Kainate receptors antagonist. Retinal cultures were incubated for 40 min with [^{14}C] Ascorbate (0.3 μCi/mL), washed and processed for release experiments as described in section "Materials and Methods." Cultures were pre-incubated with the AMPA/Kainate receptors antagonist DNQX (200 μM; **A,B**) or with the NMDA receptors antagonists, MK-801 (10 μM; **C,D**) or APV (100 μM; **C,D**) for 10 min. Then, cultures were incubated with DA (50 μM; **A,C**) or SKF-38393 (10 μM; **B,D**) for an additional period of 10 min. The results represent the mean ± SEM of three independent experiments. Statistical analyses were performed using one-way ANOVA followed by the Bonferroni post-test. $^{***}p < 0.001$ (relative to basal); $^{\#\#}p < 0.01$ (relative to DA or SKF38393). § Not statistically different compared with DA. DA, Dopamine.

the DA-induced ascorbate release, we pre-incubated cultured retinal cells with DNQX, an AMPA/Kainate receptor antagonist and then stimulated cultures with DA or the D_1R agonist SKF-38393. We observed that DNQX completely blocked the DA/D_1R-induced ascorbate release (**Figures 3A,B**, respectively). Moreover, we also used NMDA receptor antagonists (MK-801 and APV) and analyzed if cooperation between AMPA/Kainate and NMDA receptors could control the DA/D_1R-induced release of ascorbate. We observed that inhibiting NMDA receptors with MK-801 or with APV could not block the release of ascorbate from cultures stimulated with DA (**Figure 3C**) or with the D_1R agonist SKF-38393 (**Figure 3D**). Overall, these data corroborate the hypothesis that DA induces EAA release followed by activation of AMPA/Kainate receptors to elicit ascorbate release from neuronal cells.

DA-Induced Ascorbate Release Occurs via ERK MAP Kinases and PI3K/AKT Pathways

As DA can induce some biological events via the PI3K/AKT pathway (Brami-Cherrier et al., 2002; Xu et al., 2013), we further evaluated PI3K involvement in the DA-induced ascorbate release. For this we treated cultured retinal cells with Ly294002, a PI3K inhibitor, and observed that this treatment blocked the DA, the SKF-38393 and the Me-cAMP-induced ascorbate release (**Figures 4A–C**), indicating that DA/D_1R/EPAC promotes ascorbate release in a PI3K-dependent manner. To corroborate this hypothesis, we analyzed AKT phosphorylation at Ser473 and observed that DA, SKF-38393, or Me-cAMP were capable of increasing AKT^{Ser473} phosphorylation (**Figures 4D–F**). Therefore, these data suggest that DA-induced ascorbate release is mediated by EPAC/PI3K/AKT signaling pathway downstream of D_1 receptors activation.

Because DA can also stimulate ERK phosphorylation in neuronal cells (Xue et al., 2015), we investigated MEK/ERK involvement in ascorbate release. Hence, we inhibited this signaling pathway with UO126 or PD98059 and observed that both treatments completely blocked the DA, the SKF-38393 and the Me-cAMP-induced ascorbate release (**Figures 5A–C**). As expected, DA, SKF-38393, or Me-cAMP elicited ERK phosphorylation (**Figures 5D–F**), further demonstrating that the DA signaling increased MAP kinase activation. These data suggest that the DA-induced ascorbate release is

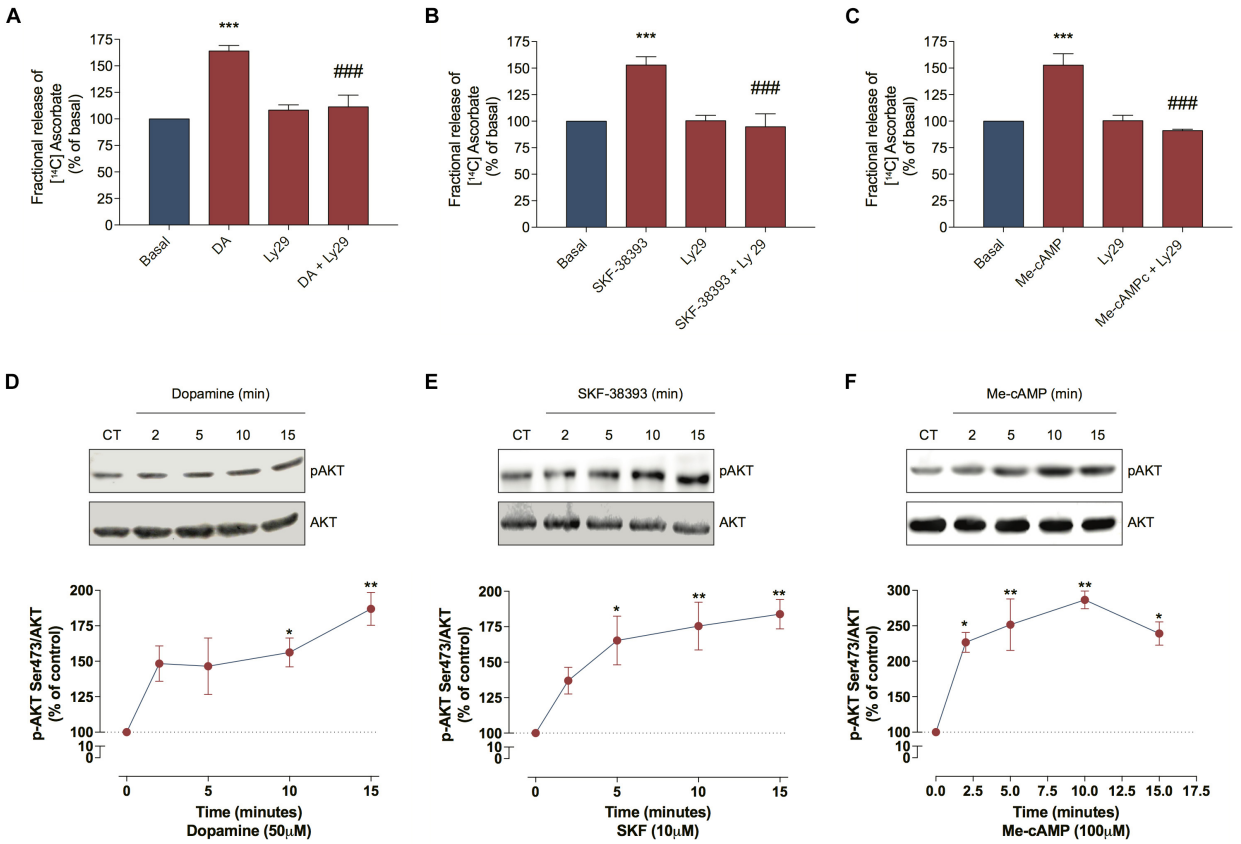

FIGURE 4 | Dopamine-induced ascorbate release is mediated by EPAC/PI3K/AKT signaling pathway. **(A–C)** Cultures were incubated with [^{14}C] ascorbate (0.3 μCi/mL) for 40 min, washed and processed for release experiments as described in section "Materials and Methods." Cultures were pre-incubated with the PI3K blocker Ly294002 (5 μM) for 10 min. Then, cells were incubated with DA (50 μM; **A**), SKF-38393 (10 μM; **B**), or Me-cAMP (100 μM; **C**) either in presence or absence of Ly294002 (5 μM) for additional 10 min. The results represent the mean ± SEM of three independent experiments. **(D–F)** Cultures were incubated with DA (50 μM; **D**), SKF-38393 (10 μM; **E**), or Me-cAMP (100 μM; **F**) for different time periods (2; 5; 10, and 15 min) and then prepared for western blotting as described in section "Materials and Methods." Data were quantified using ImageJ software and plotted as AKT^{S473}/AKT ratio normalized to the control. DA time-curve (n = 3); SKF-38393 time-curve (n = 3) and Me-cAMP time curve (n = 3). The results represent the mean ± SEM. Statistical analyses were performed using one-way ANOVA followed by the Bonferroni **(A–C)** or Dunnet **(D–F)** post-test. *$p < 0.05$ (relative to control); **$p < 0.01$ (relative to control); ***$p < 0.001$ (relative to basal); $^{###}p < 0.001$ (relative to DA, SKF38393 or Me-cAMP). DA, Dopamine; Me-cAMP, 8-pCPT-2′-O-Me-cAMP; Ly29, Ly294002; SKF, SKF-38393.

mediated by DA/D$_1$R/EPAC/MEK/ERK signaling pathway in cultured retinal cells.

ERK MAP Kinases and PI3K/AKT Pathways Are Not Involved in the DA-Induced [^{3}H] D-Aspartate Release

We characterize here that DA-induced ascorbate release is a mechanism dependent on EAA release, ERK MAP Kinases and PI3K/AKT signaling pathways. In order to better characterize the DA downstream signaling pathway involved in ascorbate release, we asked if the ERK MAP Kinases and the PI3K/AKT signaling pathways were upstream or downstream of the DA-induced [^{3}H] D-Aspartate release. For that, we blocked the MEK/ERK signaling pathway with UO126 and observed that this treatment did not inhibit the DA-induced [^{3}H] D-Aspartate release (**Figure 6A**). Also, we evaluated the PI3K/AKT signaling pathway by inhibiting PI3K with Ly294002 and observed that this treatment did not block the DA-induced [^{3}H] D-Aspartate release (**Figure 6B**). Altogether, these data demonstrate that ERK MAP Kinases and PI3K/AKT signaling pathways are not involved in the DA-induced D-Aspartate release, positioning these signaling pathways downstream of EAA release.

DISCUSSION

Here, we demonstrated that DA induces ascorbate release via a previous induction of [^{3}H] D-Aspartate release (that can serve as proxy for the release of other EAA, including glutamate) through a calcium-independent and EAAT-dependent mechanism. Then, we revealed that DA-induced ascorbate release depended on downstream glutamate-mediated activation of ERK and AKT signaling pathways. Using an illustrative model (**Figure 7**), we concatenated these data with our previous published works. DA, acting through the D$_1$R/cAMP/EPAC2 pathway

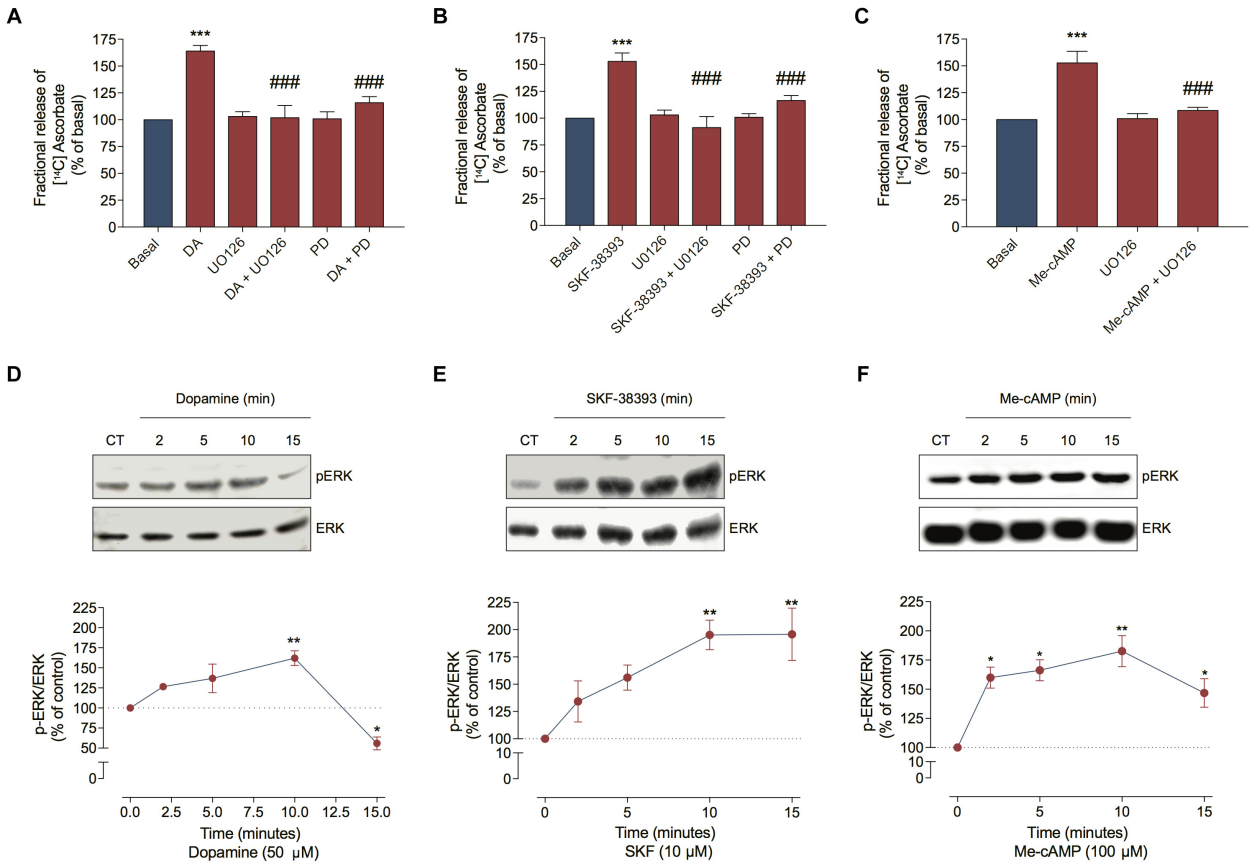

FIGURE 5 | Dopamine-induced ascorbate release is mediated by EPAC/MEK/ERK signaling pathway. **(A–C)** Cultures were incubated with [^{14}C] ascorbate (0.3 μCi/mL) for 40 min, washed and processed for release as described in section "Materials and Methods." Cultures were pre-incubated with the MEK blockers UO126 (10 μM) or PD98059 (25 μM) for 10 min. Then, cells were incubated with DA (50 μM; **A**), SKF-38393 (10 μM; **B**) or Me-cAMP (100 μM; **C**) either in presence or absence of UO126 (10 μM) or PD98059 (25 μM) for an additional period of 10 min. The results represent the mean ± SEM of three independent experiments. **(D–F)** Cultures were incubated with DA (50 μM; **D**); SKF-38393 (10 μM; **E**) or Me-cAMP (100 μM; **F**) for different time periods (2; 5; 10, and 15 min) and then prepared for western blotting as described in section "Materials and Methods." Data were quantified using ImageJ software and plotted as *p*-ERK/ERK ratio normalized to the control. DA time-curve (n = 3); SKF-38393 time-curve (n = 3) and Me-cAMP time curve (n = 3). The results represent the mean ± SEM. Statistical analyses were performed using one-way ANOVA followed by the Bonferroni **(A–C)** or Dunnet **(D–F)** post-test. $^{*}p < 0.05$ (relative to control); $^{**}p < 0.01$ (relative to control); $^{***}p < 0.001$ (relative to basal); $^{\#\#\#}$ $p < 0.001$ (relative to DA, SKF38393, or Me-cAMP). DA, Dopamine; Me-cAMP, 8-pCPT-2′-O-Me-cAMP; PD, PD98059; SKF, SKF-38393.

(da Encarnação et al., 2018), induces ascorbate release. Here, we demonstrated that DA/D_1R/EPAC2 signaling pathway induces ascorbate release mediated by a previous release of EAA, here represented by D-Aspartate and Glutamate. As D-Aspartate could not activate AMPA/Kainate receptors (Kubrusly et al., 1998), we represented just the glutamate interacting in these receptors, which is in accordance with our previous work demonstrating that glutamate induces ascorbate release via activation of AMPA/Kainate receptors (Portugal et al., 2009). By interacting with AMPA/kainate receptors, glutamate stimulates ERK MAP Kinases (Socodato et al., 2009, 2012) and the PI3K/AKT (Mejia-Garcia et al., 2013) signaling pathways, which were involved in the DA-induced ascorbate release. Finally, as we previously reported (Portugal et al., 2009; da Encarnação et al., 2018), the mechanism eliciting ascorbate release is mediated by the reversion of SVCT2 (**Figure 7**).

The retinal cell culture paradigm we used is composed by neurons and Müller glial cells in a well-known proportion (Portugal et al., 2012). Moreover, DL-TBOA-sensitive glutamate transporters are expressed in both neurons and Müller glia in these cultures as well as D_1Rs and EPAC2 (da Encarnação et al., 2018). Therefore, whether glutamate is being released from neurons, glial cells or both cells in response to DA needs further investigation. In any case, the ascorbate transporter SVCT2 is only present in neurons in retinal cultures (Portugal et al., 2009), thereby suggesting that ascorbate is released from neurons in response to DA/glutamate (**Figure 7**).

Ascorbate Bioavailability and Neurodegenerative Disorders

Reduced ascorbate levels in the brain not only induce changes in mood, behavior, and motor performance (Brown, 2015) but

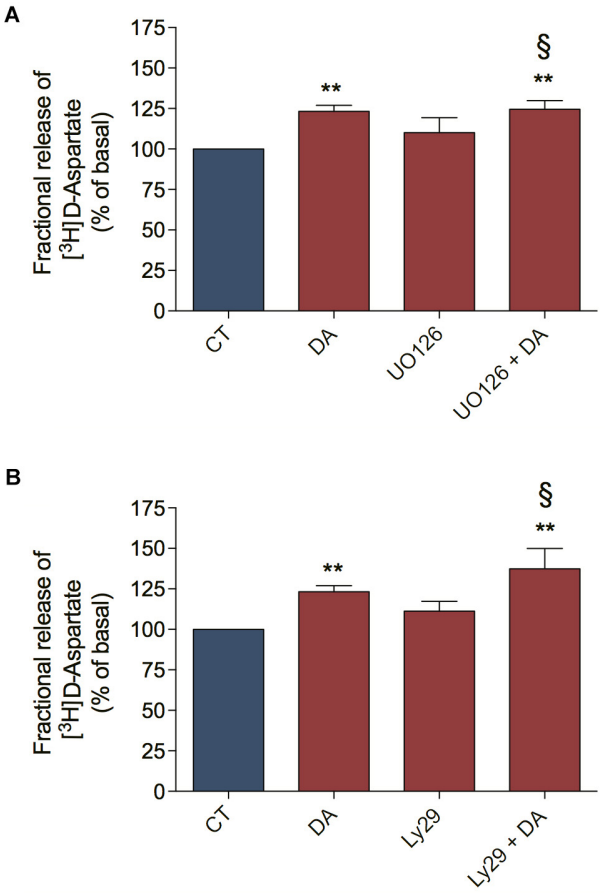

FIGURE 6 | Dopamine-induced D-aspartate release is independent of ERK and AKT signaling pathways. Retinal cultures were incubated for 90 min with [^{3}H] D-aspartate (1 μCi/mL), washed and processed for release experiments as described in section "Materials and Methods." Cultures were pre-incubated with the MEK blocker UO126 (10 μM; **A**) or the PI3K blocker Ly294002 (5 μM; **B**) for 10 min. Then, cultures were incubated with DA (50 μM; **A,B**) for an additional period of 10 min. The results represent the mean ± SEM of four independent experiments. Statistical analyses were performed using one-way ANOVA followed by the Bonferroni post-test. $^{**}p < 0.01$ (relative to basal). § Not statistically different compared with DA. DA, Dopamine; Ly29, Ly294002.

also are directly involved in some neurological disorders such as Alzheimer's and Huntington's disease (Harrison and May, 2009; Harrison et al., 2009; Acuna et al., 2013; Dixit et al., 2015). Because SVCT2 is the main ascorbate transporter present in the brain, heterozygosity for a null allele for Slc23a2, the gene coding for SVCT2 in mice, leads to a decrease in 30% in their brain ascorbate levels (Sotiriou et al., 2002; Dixit et al., 2015). When SVCT2 heterozygous mice are intercrossed with a mouse model of Alzheimer's disease, an aggravation of amyloid pathology and cognition impairment occurs (Dixit et al., 2015). Accordingly, ascorbate supplementation is capable of improving cognition in Alzheimer's mice (Harrison et al., 2009). Moreover, abnormal ascorbate transport is observed in the R6/2 transgenic mouse model of Huntington's disease (Dorner et al., 2009; Acuna et al., 2013). Reduction of ascorbate in the striatum is linked with the motor dysfunction observed in R6/2 Huntington's disease mice (Dorner et al., 2009). Because the regulation of ascorbate transport could potentially mitigate some neurological hallmarks in these disorders (Harrison and May, 2009), a better understanding of the mechanisms regulating ascorbate transport and bioavailability could lead to improved therapeutic approaches to manage the neuronal damage observed in the abovementioned pathologies.

Interaction Between DA and Glutamate Systems

Dopamine modulates glutamatergic signaling in different CNS regions (Scott et al., 2002; Tseng and O'Donnell, 2004; Fernandez et al., 2006; Surmeier et al., 2007), including the retina (Kolb, 2003). DA can alter the function of ionotropic glutamate receptors promoting exquisite control over neuronal and synaptic activity in different brain regions (Surmeier et al., 2007). However, much less is known about the control of EAA release by DA. For instance, DA acting via D_2R inhibits the release of aspartate/glutamate in the retinal tissue and in striatal nerve terminals (Maura et al., 1989; Kamisaki et al., 1991) while here we demonstrate that DA acting via D_1R/EPAC2 pathway increased the release of D-Aspartate in retinal cells. Interestingly, DA seems to be more efficient in inducing ascorbate than D-Aspartate release. As we demonstrated that aspartate/glutamate release is upstream of ascorbate release in retinal cultures, this difference could be explained by a signal amplification phenomenon, i.e., DA induces a small release of glutamate that, in turn, activates AMPA/Kainate receptors promoting a larger release of ascorbate.

EAA Release From Retinal Cells

In general, the release of neurotransmitters in the CNS is mediated by exocytosis, a conserved mechanism for transmitter release across species. However, neurotransmitters and neuromodulators can also be released by the reversion of their transporters. In the retina, the transporter-mediated neurotransmitter release is a very common process for retinal physiology and has been described for classical neurotransmitters and neuromodulators, such as, GABA (Duarte et al., 1993; do Nascimento et al., 1998; Calaza et al., 2006), dopamine (Bugnon et al., 1995), adenosine (Paes-de-Carvalho et al., 2005) and the EAA, including D-Aspartate and glutamate (Duarte et al., 1996; Santos et al., 1996; de Freitas et al., 2016).

Excitatory amino acid release from nerve cells occurs through several distinct mechanisms that can be calcium-dependent and calcium-independent, including exocytosis and transporter-mediated processes. For instance, NMDA-evoked exocytic D-Aspartate release depends on extracellular calcium influx through the NMDA receptor channel, whereas AMPA-evoked exocytic D-Aspartate release depends on calcium influx through voltage-gated calcium channels (Santos et al., 1996). Moreover, D-Aspartate can be released simultaneously in a calcium-dependent and exocytic-independent manner (Bradford and Nadler, 2004). Here, we describe that the release of D-Aspartate, triggered by DA, was independent of calcium mobilization and required EAATs.

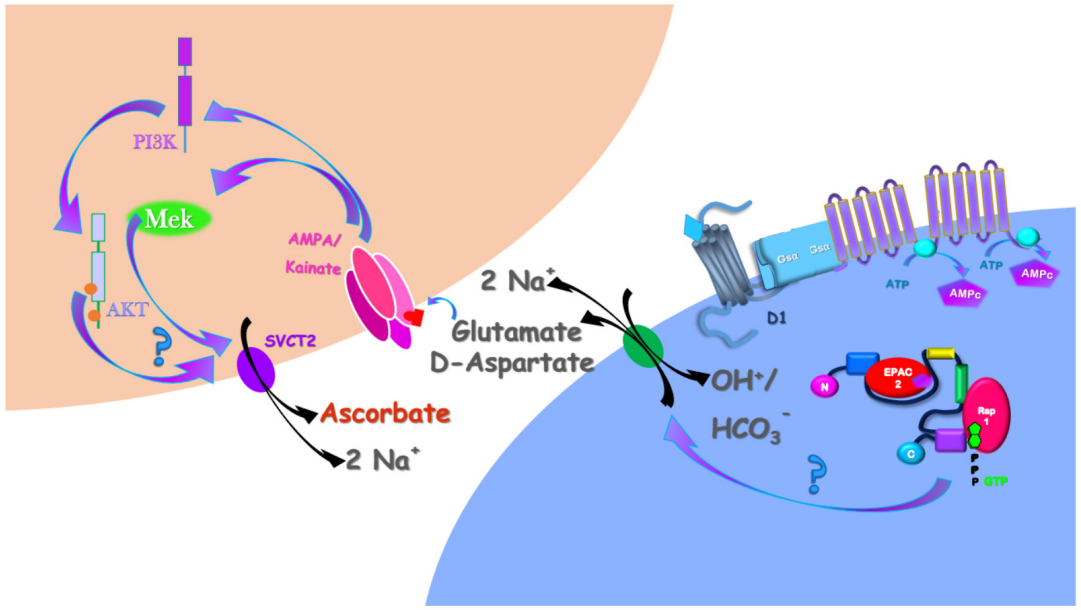

FIGURE 7 | Schematic representation of the DA-induced ascorbate release signaling pathway in the retina. DA, by interacting with the D_1R/cAMP/EPAC2 (da Encarnação et al., 2018) induces glutamate release by the EAAT reversion. At the extracellular milieu, glutamate interacts with AMPA/Kainate receptors that, in its turn, activates the MAP Kinases and the PI3K/AKT signaling pathways (Socodato et al., 2009, 2012; Mejia-Garcia et al., 2013). These signaling pathways, somehow, induces the SVCT2 reversion necessary to induce ascorbate release in the retina (Portugal et al., 2009; da Encarnação et al., 2018).

Glutamate-Induced Ascorbate Release and the Involvement of Glutamate Ionotropic Receptors

Glutamate increases ascorbate release in cultured retinal cells (Portugal et al., 2009). Direct activation of NMDA receptors stimulates the release of ascorbate from neuronal cells (Portugal et al., 2009). However, the release of ascorbate induced by glutamate (Portugal et al., 2009) or by DA (data herein) was not blocked by NMDA receptor antagonists, such as MK-801 and APV. At least two hypothesis could explain the DA-mediated and glutamate-dependent stimulation of ascorbate release from neuronal cells: One hypothesis could be that activation of NMDA receptors promotes the release of glutamate from a glutamatergic neuron that, in turn, leads to activation of AMPA/Kainate receptors culminating in ascorbate release (Portugal et al., 2009). Another hypothesis could be that D_1R and NMDA receptors are co-expressed in the same neuron in which D_1R activation by DA leads to the activation of the cAMP/Csk/Src tyrosine kinase pathway decreasing the gating of NMDA receptors (Socodato et al., 2017). The first hypothesis is further corroborated by the fact that (1) the release of ascorbate elicited by glutamate (Portugal et al., 2009) or by DA (data herein) is abrogated by blocking AMPA/Kainate receptors with DNQX and (2) the NMDA-induced ascorbate release is also blocked by DNQX (Portugal et al., 2009). Although these hypotheses are not mutually exclusive, our data do suggest that the main driver for ascorbate release from neuronal cells is the activation of AMPA/Kainate receptors triggered by glutamate, which release was previously elicited by DA acting on D_1R.

ERK MAP Kinases and PI3K/AKT Pathways Involvement in the DA-Induced Ascorbate Release

Dopamine-induced ascorbate release was mediated by ERK MAP Kinases and the PI3K/AKT signaling pathways as UO126, a MAP inhibitor, and Ly294002, a PI3Kinhibitor, completely blocked the DA-induced ascorbate release. To corroborate this involvement, we also demonstrated that DA, SKF-38393 or Me-cAMP stimulates ERK and AKT phosphorylation. Also, we demonstrated that these signaling pathways are not involved in DA-induced D-aspartate release, suggesting that MAP Kinases and the PI3K/AKT signaling pathway are downstream of EAA release. Because glutamate promotes the phosphorylation of both ERK and AKT in retinal cells (Socodato et al., 2009, 2012; Mejia-Garcia et al., 2013), these signaling pathways might be downstream of AMPA/Kainate receptors stimulation. However, linking MAP Kinases and PI3K/AKT signaling pathways with the reversion of SVCT2 requires further investigation (**Figure 7**).

Overall, we showed that DA regulation of ascorbate release requires glutamate release and activation of AMPA/Kainate receptors. Mechanistically, DA activated D_1R/EPAC2 to elicit an EAAT-dependent release of glutamate from retinal cells. Following DA-mediated glutamate release, glutamate promoted the activation of AMPA/Kainate receptors, increasing the activity of MAP Kinases and the PI3K/AKT, which coupled the reversion of SVCT2 to the release of ascorbate. Thus, our data suggest that a crosstalk between DA (the major catecholamine in the retina) and glutamate (the major excitatory neurotransmitter in the retina) controls the bioavailability of vitamin C in the retina.

AUTHOR CONTRIBUTIONS

CCP, TGE, and RPC conceived all the experiments. ID, ASR, and TGE performed [^{3}H] Aspartate release experiments. CCP and TGE performed [^{14}C] Ascorbate release experiments. CCP, TGE, RS, and NAO performed the western blots experiments. CCP, RS, ASR, and RPC wrote the manuscript. RPC provided the funding sources. All authors critically discussed the results and reviewed the final version of the manuscript.

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Broad Lipidomic and Transcriptional Changes of Prophylactic PEA Administration in Adult Mice

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Beside diverse therapeutic properties of palmitoylethanolamide (PEA) including: neuroprotection, inflammation and pain alleviation, prophylactic effects have also been reported in animal models of infections, inflammation, and neurological diseases. The availability of PEA as (ultra)micronized nutraceutical formulations with reportedly no side effects, renders it accordingly an appealing candidate in human preventive care, such as in population at high risk of disease development or for healthy aging. PEA's mode of action is multi-facetted. Consensus exists that PEA's effects are primarily modulated by the peroxisome proliferator-activated receptor alpha (PPARα) and that PEA-activated PPARα has a pleiotropic effect on lipid metabolism, inflammation gene networks, and host defense mechanisms. Yet, an exhaustive view of how the prophylactic PEA administration changes the lipid signaling in brain and periphery, thereby eliciting a beneficial response to various negative stimuli remains still elusive. We therefore, undertook a broad lipidomic and transcriptomic study in brain and spleen of adult mice to unravel the positive molecular phenotype rendered by prophylactic PEA. We applied a tissue lipidomic and transcriptomic approach based on simultaneous extraction and subsequent targeted liquid chromatography-multiple reaction monitoring (LC-MRM) and mRNA analysis by qPCR, respectively. We targeted lipids of COX-, LOX- and CYP450 pathways, respectively, membrane phospholipids, lipid products of $cPLA_2$, and free fatty acids, along with various genes involved in their biosynthesis and function. Additionally, plasma lipidomics was applied to reveal circulatory consequences and/or reflection of PEA's action. We found broad, distinct, and several previously unknown tissue transcriptional regulations of inflammatory pathways. In hippocampus also a PEA-induced transcriptional regulation of neuronal activity and excitability was evidenced. A massive downregulation of membrane lipid levels in the splenic tissue of the immune system with a consequent shift towards pro-resolving lipid environment was also detected. Plasma lipid pattern reflected to a large extent the hippocampal and splenic lipidome changes, highlighting the value of plasma lipidomics to monitor effects of nutraceutical PEA administration. Altogether, these findings contribute new insights into PEA's molecular mechanism and helps answering the questions, how PEA prepares the body for insults and what are the "good lipids" that underlie this action.

Keywords: PEA, PUFAs, inflammation, targeted lipidomics, phospholipids, endocannabinoids, mRNA

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INTRODUCTION

Since diet is an essential environmental factor to maintain health, it is important to advance research in the field of nutrition science and nutraceutical use in order to expedite disease prevention and/or hinder disease progression. Thus, we need more holistic biology approaches for nutrition research, including multi-omic strategies to investigate body's response to nutrition, and to validate and understand its beneficial effects (Allison et al., 2015). An advantage of nutrition-based approaches over, or in addition to, pharmacological ones for disease prevention and treatment is the opportunity for extended administration periods, due to the reduced number of possible side-effects. Palmitoylethanolamide (PEA), a saturated fatty acid (16:0) of the *N*-acylethanolamines family, is a food component found in egg yolk, soybeans and peanuts and was investigated for more than half a century and described in numerous studies to exert anti-inflammatory effects (Lambert et al., 2001; Conti et al., 2002; Keppel Hesselink et al., 2013; Mattace Raso et al., 2014; Rinne et al., 2018). PEA is also a natural own body compound found in most cell types, tissues, and bodily fluids. It is synthesized and metabolized via different enzymes, namely *N*-acyl phosphatidylethanolamine phospholipase D (NAPE-PLD), fatty acid amid hydrolase (FAAH) and/or *N*-acylethanolamine acid amidase (NAAA), thus sharing the same biosynthetic pathway with the endocannabinoid (eCB) anandamide (AEA) (Petrosino and Di Marzo, 2017). Several clinical trials proved that exogenous administration of PEA lacks side effects, and since 2008 it has been marketed in different countries as a nutraceutical food supplement (Keppel Hesselink et al., 2013; Artukoglu et al., 2017). Despite many clinical trials and a number of papers describing the therapeutic role of PEA in chronic pain, inflammation and neurodegenerative diseases, its mechanism of action is not yet clarified (Iannotti et al., 2016). PEA was evidenced to act through receptor binding, and was initially thought to bind to the cannabinoid 2 receptor (CB2) (Facci et al., 1995). Further research however, revealed that PEA, unlike AEA, exhibits only weak binding efficacy on the CB2 receptor, but possesses the capability to affect AEA signaling by acting as a competing substrate (Di Marzo et al., 2001). In recent years compelling evidence accumulated on the peroxisome proliferator-activated receptor α (PPARα) as the main molecular target of PEA (LoVerme et al., 2006; Hansen, 2010). PPARα is an ubiquitous transcription factor exerting a major role in lipid metabolism, e.g., was recently described to protect against diet-induced obesity in mice (Araki et al., 2018). Gene networks regulated by PPARα-PEA signaling lead to the reduction of the transcription of pro-inflammatory genes (Lo Verme et al., 2005; D'Agostino et al., 2007, 2009; Hansen, 2010). Another described direct target of PEA is the orphan receptor GPR55 (Ryberg et al., 2007). PEA's actions were also attributed to effects upon ATP−sensitive K^+−channels (Romero and Duarte, 2012), TRP channels (Lowin et al., 2015), and NFkB (D'Agostino et al., 2009). The role of lipid amides including of PEA, on down-modulation of mast cell activation has been demonstrated in the seminal work of Nobel prize laureate Rita Levi-Montalcini (Aloe et al., 1993), and has since prompted a body of work evidencing the mast cells as targets for the various anti-inflammatory effects of PEA (Skaper, 2017). However, the various molecular mechanisms underlying the multi-facetted effects of PEA, e.g., neuroprotective, anti-inflammatory, anticonvulsant, pain killer warrant further investigation, especially considering the emerging interest into its use as a prophylactic and therapeutic adjuvant.

We recently demonstrated neuroprotective, anticonvulsant and anti-inflammatory effects of sub- chronic, prophylactic PEA administration in a mouse model for acute epileptic seizures and found that these effects were accompanied by alterations in peripheral and hippocampal eCB and prostaglandin levels (Post et al., 2018). Similarly, prophylactic PEA improved survival and decreased inflammation in mice models with bacterial meningitis and these effects were in part accompanied by eicosanoids (eiCs) modulation (Heide et al., 2018).

In this study, we aim to expand our understanding of how the PEA, as a saturated lipid with a relatively long fatty acyl chain (C16:0), alters the lipid tissue composition, metabolism and signaling upon prophylactic administration such that it actually positively influence the immune system, neuroprotective functions and improve symptomatic when the body faces various insults (neuroinjury, infections, etc.). For this purpose, we applied a recently developed tissue lipidomics and transcriptomic approach (Lerner et al., 2018) to investigate changes of multiple lipid categories, e.g., eCBs, eiCs, poly unsaturated fatty acids (PUFAs), PUFA oxidation products, phospholipids (PLs), and different sphingosine species, as well as related genes involved in lipid signaling and metabolism in hippocampus (HC) and spleen of control mice. The choice of hippocampus was guided by its known role in the on-set of neurological diseases including epilepsy (Heide et al., 2018; Lerner et al., 2018), and the previously demonstrated effect of PEA to alleviate hippocampal neuroinflammation and endocannabinoids elevation (Post et al., 2018) at acute seizure state. The spleen has been increasingly recognized to have a unique function in immune responses, including clearance of cell debris and antigens from the blood stream, as well as an early host response to

Abbreviations: 2-AG, 2-arachidonyl glycerol; AA, arachidonic acid; ACN, acetonitrile; AEA, arachidonoyl ethanolamide; ALA, alpha-linolenic acid; BBB, blood–brain barrier; Bdnf, brain-derived neurotrophic factor; BHT, butylhydroxytoluene; C1P, ceramide-1-phosphate; CB1R, cannabinoid receptor type 1; CER, ceramide; COX, cyclooxygenase; Crh, corticotropin releasing hormone; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; eCBs, endocannabinoids; eiCs, eicosanoids; EPA, eicosapentaenoic acid; FAAH, fatty acid amide hydrolase; GLA, gamma-linolenic acid; HC, hippocampus; HETE, hydroxyeicosatetraenoic acid; ip, intraperitoneal; ISTD, internal standard; LA, linoleic acid; LLE, liquid–liquid extraction; LPA, lysophosphatidic acid; LPC, lysophosphatidylcholine; MAPK, mitogen-activated protein kinase; MRM, multiple reaction monitoring; MTBE, methyl *tert*-butyl ether; NAAA, *N*-acylethanolamine acid amidase, NAPE-PLD, *N*-acyl phosphatidylethanolamine phospholipase D; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PEA, palmitoyl ethanolamide; PG, phosphatidylglycerol; PGD_2, 11β-$PGF_2α$, 11beta-prostaglandin F2alpha, prostaglandin D_2; PGE_2, prostaglandin E_2; PI, phosphatidylinositol; PL, phospholipid; $cPLA_2$, cytosolic phospholipase A_2; PPAR, peroxisome proliferator-activated receptor; PS, phosphatidylserine; PUFAs, polyunsaturated fatty acids; RvD1, resolvin D1; sal, saline; S1P, sphingosine-1-phosphate; SM, sphingomyelin; SPH, sphingosine; TEA, triethylamine; URB597, KDS-4103, 3′-(aminocarbonyl) [1, 1′-biphenyl]-3-yl –cyclohexylcarbamate.

infections (Mebius and Kraal, 2005; Wluka and Olszewski, 2006; Bronte and Pittet, 2013). As the body's most proximal and largest blood filtering organ, with ability to mount innate and adaptive immune responses, we rationalized that the prophylactic and particularly the immunomodulatory effects of PEA (Lowin et al., 2015; Skaper, 2017; Post et al., 2018) could be partly attributed to eliciting splenic molecular changes. Hence, targeted lipidomics and transcriptomics were applied on spleen of adult (wild type) mice with and without PEA administration. In addition, circulatory levels of the target lipids were assessed for their value to reflect brain and peripheral molecular effects of prophylactic PEA. To our knowledge, most studies on PEA's action focus on gene networks and inflammatory mediators, whereas no broad lipidomic studies are reported so far. Hence, this study is the first to give a more thorough insight into the transcriptome and lipidome plasticity underlying the prophylactic and nutritional benefits of PEA.

MATERIALS AND METHODS

Reagents and Chemicals

Calibration Standards: arachidonoyl ethanolamide (AEA), 2-arachidonoyl glycerol (2-AG), arachidonic acid (AA), resolvin D1 (RvD1), prostaglandin D2 (PGD_2), prostaglandin E2 (PGE_2), 11-beta-prostaglandin F2alpha (11β-$PGF_2\alpha$), 5S-hydroxyeicosatetraenoic acid (5(S)-HETE), 12(S)-hydroxyeicosatetraenoic acid (12(S)-HETE), 15(S)-hydroxyeicosatetraenoic acid (15(S)-HETE), and 20-hydroxyeicosatetraenoic acid (20-HETE) were obtained from BIOMOL Research Laboratories, Inc. (Plymouth Meeting, PA, United States). Linoleic acid (LA), alpha-linolenic acid (ALA), docosahexaenoic acid (DHA), docosapentaenoic acid (DPA), and eicosapentaenoic acid (EPA) were purchased from Cayman Chemical (Ann Arbor, MI, United States). Phosphatidylcholine 16:0/18:1 (PC 16:0/18:1), phosphatidylglycerol 16:0/18:1 (PG 16:0/18:1), phosphatidylethanolamine 16:0/18:1 (PE 16:0/18:1), phosphatidylserine 16:0/18:1 (PS 16:0/18:1), phosphatidic acid 16:0/18:1 (PA 16:0/18:1), phosphatidylinositol 16:0/18:1 (PI 16:0/18:1), lysophosphatidylcholine 18:0/0:0 (LPC 18:0), lysophosphatidic acid 16:0/0:0 (LPA 16:0), sphingomyelin d18:1/18:0 (SM d18:1/18:0), ceramide-1-phosphate d18:1/16:0 (C1P d18:1/16:0), sphingosine d18:1 (SPH d18:1) and sphingosine-1-phosphate d18:1 (S1P d18:1) were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL, United States).

Internal standards (ISTDs) for quantification: arachidonoyl ethanolamide-d_4 (AEA-d_4), 2-arachidonoyl glycerol-d_5 (2-AG-d_5), arachidonic acid-d_8 (AA-d_8), prostaglandin D2-d_4 (PGD_2-d_4), prostaglandin E2-d_9 (PGE_2-d_9), 5(S)-hydroxyeicosatetraenoic acid-d_8 (5(S)-HETE-d_8), 12(S) hydroxyeicosatetraenoic acid-d_8 (12(S)-HETE-d_8), 20-hydroxyeicosatetraenoic acid-d_6 and resolvin D1-d_5 (RvD1-d_5) were obtained from BIOMOL Research Laboratories, Inc. (Plymouth Meeting, PA, United States). Linoleic acid-d_4 (LA-d_4), alpha-linolenic acid-d_5 (ALA-d_5), docosahexaenoic acid-d_5 (DHA-d_5), docosapentaenoic acid-d_5 (DPA-d_5) and eicosapentaenoic acid-d_5 (EPA-d_5) were purchased from Cayman Chemical (Ann Arbor, MI, United States). Phosphatidylcholine 17:0/14:1 (PC 17:0/14:1), phosphatidylglycerol 17:0/14:1 (PG 17:0/14:1), phosphatidylethanolamine 17:0/14:1 (PE 17:0/14:1), phosphatidylserine 17:0/14:1 (PS 17:0/14:1), phosphatidic acid 17:0/14:1 (PA 17:0/14:1), phosphatidylinositol 17:0/14:1 (PI 17:0/14:1), lysophosphatidylcholine 17:0/0:0 (LPC 17:0), lysophosphatidic acid 17:0/0:0 (LPA 17:0), sphingomyelin d18:1/12:0 (SM d18:1/12:0), ceramide-1-phosphate d18:1/12:0 (C1P d18:1/12:0), and sphingosine d17:1 (SPH d17:1), sphingosine-1-phosphate d17:1 (S1P d17:1) were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL, United States).

Water, *n*-hexane, ethylacetate, methanol, 2-propanol, acetonitrile, chloroform, formic acid, and ammonium formate of liquid chromatography/mass spectrometry (LC/MS) grade were invariably used (Sigma-Aldrich, St. Louis, MO, United States) for extraction and LC/multiple reaction monitoring (MRM) analysis. HPLC-grade methyl tert-butyl ether (MTBE), Trizma® hydrochloride solution (Tris-HCl) (pH 7.4), triethylamine (TEA) and butylhydroxytoluene (BHT) were purchased from Sigma-Aldrich (St. Louis, MO, United States). URB597 (KDS-4103, 3′-(aminocarbonyl) [1, 1′-biphenyl]-3-yl -cyclohexylcarbamate) was purchased from Cayman Chemical (Ann Arbor, MI, United States), tetrahydrolipstatin (THL) was obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, United States), and β-mercaptoethanol was obtained from Carl Roth (Karlsruhe, Germany).

Animals and PEA Administration

Experiments were performed according to the European Community's Council Directive of 22 September 2010 (2010/63EU) and approved by the local animal care committee of the German Rhineland-Palatinate (file reference: 23 177-07/G16- 1-075). Experimental procedures were conducted using 8–10 weeks old C57BL/6N male mice obtained from Janvier Labs (Saint-Berthevin, France). Mice (n = 24) were group housed (3–4/cage) on a 12-h light/dark schedule under environmental conditions (20–22°C and 55–60% humidity) with water and food available *ad libitum* for at least 1 week prior to commencement of experiments. In order to unravel the benefits of prophylactic PEA administration and its impact on lipidome and transcriptome level, mice were sub-chronically PEA-treated (n = 12) at two time points, at 7.8 h and at 50 min prior to sacrificing, via intraperitoneal (ip) injection with a dose of 40 mg/kg in 10 mL/kg, respectively. PEA was freshly dissolved in DMSO/chremophore/saline (17:2:1) and kept in thermo-mixer (low speed) at 33°C until injection to avoid precipitation. Control mice (n = 12) were ip injected with 0.9% saline in 10 mL/kg. Mice treated with PEA or saline (Sal) 7 h prior to 2nd injection, respectively. Six mice per group (PEA/Sal) were sacrificed 50 min after the 2nd injection (time point 1) and 3.5 h after 2nd -injection (time point 2), respectively (**Figure 1**). The animal experiments were carried out by one trained scientist. Visual assessment of animal behavior upon PEA administration indicated no noticeable changes.

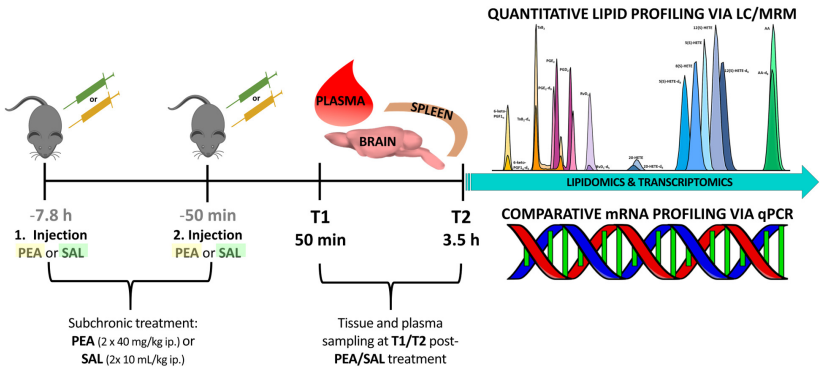

FIGURE 1 | Experimental timeline of PEA/Sal treatment: in total 24 mice were ip injected either with saline (n = 12) or with a dose of 40 mg/kg PEA (n = 12) each in a total volume of 10 mL/kg **(1st injection, at 7.8 h prior to sacrificing)**. After 7 h the same procedure was repeated **(2nd injection, at 50 min prior to sacrificing)**. At **time point 1** (50 min post – 2nd injection) 12 mice were sacrificed and plasma, brain, and spleen samples from PEA-treated (n = 6) and saline-treated (n = 6) mice were collected for further analysis. This procedure was repeated with remaining 12 mice, e.g., PEA-treated (n = 6) and saline-treated (n = 6) mice, at **time point 2** (3.5 h post – 2nd injection).

Spleen, Hippocampus, and Plasma Sampling

At each of the two time points 12 mice were shortly anesthetized with isoflurane and sacrificed by decapitation. Brains and spleens were isolated and immediately frozen on dry ice. Brain dissection was carried out according to the protocol previously described (Lerner et al., 2016). Spleens were stored at −80°C and pieces of 25–30 mg per spleen were cut and used for extraction and further analysis. Plasma sampling and handling prior to extraction was performed as described previously (Post et al., 2018).

Simultaneous RNA and Lipid Extraction From Spleen and Hippocampus

Simultaneous extraction of RNA and lipids from spleen and HC was conducted via the protocol established in Lerner et al. (2018). Briefly, 200 µL chloroform, ice-cold ceramic beads and 600 µL of RLT buffer (supplied with the RNeasy® Mini Kit) containing 1% β-mercaptoethanol, 5 µM THL/URB597 and 10 µg/mL BHT in final volume were added to the frozen tissue samples, obtained from the PEA- and Sal injected animals (n = 12 for each group). The aliquots were spiked with 10 µl ISTDs to a target concentration of 150 ng/mL PC 17:0/14:1; PE 17:0/14:1; PA 17:0/14:1, 100 ng/mL PG 17:0/14:1; PS 17:0/14:1; PI 17:0/14:1; LPC 17:0; LPA 17:0; SM d18:1/12:0; EPA-d_5; DPA-d_5; DHA-d_5; 2-AG-d_5, 1000 ng/ml LA-d_4; ALA-d_5, 500 ng/ml AA-d_8; C1P d18:1/12:0, 200 ng/ml SPH d17:1; S1P d17:1 and 0.5 ng/ml AEA-d_4 respectively, in the final volume. After homogenization via Precellys 24 (Peqlab, Erlangen, Germany) (6000 rpm; 20 s) and subsequent centrifugation for 5 min at full speed and 4°C, the upper phase was used for further RNA extraction via the RNeasy® Mini Kit according to the manufacturer's instructions (RNase-Free DNase Set, Qiagen, Hilden, Germany) and the lower chloroform-containing phase was further used for lipid extraction (Lerner et al., 2018).

In our study, we used a previously established protocol for lipidomic and transcriptomic profiling, due to its amenability for simultaneous lipid and RNA extraction from the same tissue sample, and pertaining the imperative standard operating procedure in regard to tissue/plasma sampling, sample handling and storage conditions, in order to reduce artificial analyte alterations (Lerner et al., 2016, 2018). Quantification of the lipid analytes via LC/MRM with on-line polarity switching enabled investigation of several lipid species encompassing 9 PL classes as well as eCBs in single experiments, respectively. In order to attain a broader view of lipid plasticity related to PEA's action, we adapted the MRM parameters to allow additional analysis of PUFAs and additional sphingolipid species (**Table 1**).

An additional lipid extraction was carried out from the same tissue origin (see above for PLs and eCBs) followed by the LC/MRM analysis to target lipids of the COX- LOX and CYP450 pathways namely RvD1, HETEs, PGD_2, 11β-$PGF_2\alpha$ and PGE_2. Analysis of LOX/CYP450/COX-derived lipids was carried out in this case in negative ion mode (Post et al., 2018).

Assessment of lipid/transcriptional analyte plasticity in spleen, plasma and brain was conducted to unravel interrelations between brain and periphery after PEA injection. The obtained values of lipid levels from spleen and plasma are normalized to saline values and presented as percentage and depicted in **Figures 2**, **3** as obtained after 50 min of PEA injection, 3.5 h and pooled time points, respectively. The changes for LPC 20:4, which was the only lipid to be changed in every investigated region, are shown in **Figure 4**. Changes for HETEs are depicted in **Figure 5**. Splenic concentrations of RvD1 after PEA administration are shown in **Figure 6**. The changes in mRNA levels for spleen and HC for the different time points are shown in **Figures 7**, **8**. A simplified signaling scheme for all investigated analytes is depicted in **Figure 9**.

Lipid Extraction From Plasma

All lipids, except LOX/CYP450/COX-derived ones, were extracted using an adapted LLE method as described in Lerner et al. (2016). Briefly, 1000 µL MTBE/methanol (10:3; v/v), containing the spiking solution (concentrations of internal

TABLE 1 | Targeted ion transitions.

Positive ion mode					
Calibration standards and quantified PLs			**Corresponding internal standards**		
Analyte Name	**Precursor ion *m/z***	**Product ion *m/z***	**Analyte name**	**Precursor ion *m/z***	**Product ion *m/z***
2-AG	379.1	287.2	2-AG-d_5	384.2	287.2
AEA	348.3	62.1	AEA-d_4	352.3	66.1
LPC 18:0	524.37	184.07	LPC 17:0	510.36	184.07
LPC 20:4	544.34	184.07			
PC 16:0/18:1	760.59	184.07	PC 17:0/14:1	718.54	184.07
PC 38:6	806.67	184.07			
PC 38:4	810.66	184.07			
SM d18:1/18:0	731.61	184.07	SM d18:1/12:0	647.51	184.07
SM 34:1	703.57	184.07			
SPH d18:1	300.28	282.2	SPH d17:1	286.47	268.2
Negative ion mode					
Calibration standards and quantified PLs			**Corresponding internal standards**		
Analyte Name	**Precursor ion *m/z***	**Product ion *m/z***	**Analyte name**	**Precursor ion *m/z***	**Product ion *m/z***
AA	303.05	259.1	AA-d_8	311.04	267.0
LA	279.23	261.1	LPA 17:0	423.25	153.00
ALA	277.22	259.1	ALA-d_5	282.2	238.1
GLA	277.22	259.1			
EPA	301.22	257.2	EPA-d_5	306.25	262.2
18-HEPE	317.22	259.0			
DPA	329.25	285.2	DPA-d_5	334.3	290.2
DHA	327.23	283.2	DHA-d_5	332.26	288.2
17(S)-HDHA	343.24	201.0			
LPA 16:0	409.24	153.00	LPA 17:0	423.25	153.00
LPA 20:4	457.24	153.00			
PA 16:0/18:1	673.48	255.23	PA 17:0/14:1	631.43	269.25
PS 16:0/18:1	760.51	255.23	PS 17:0/14:1	718.47	269.25
PS 36:4	782.49	303.23			
PS 38:4	810.53	303.23			
PI 16:0/18:1	835.53	281.25	PI 17:0/14:1	793.49	269.25
PI 36:4	857.52	303.23			
PI 38:4	885.55	303.23			
LPI 20:4	619.29	303.23			
PG 16:0/18:1	747.52	281.25	PG 17:0/14:1	705.47	225.19
PG 36:5	767.49	303.23			
PG 38:5	795.52	303.23			
PE 16:0/18:1	716.52	281.25	PE 17:0/14:1	674.48	225.19
PE 38:6	762.51	303.23			
PE 38:4	766.54	303.23			
PE 40:6	790.54	303.23			
PE 40:4	794.57	303.23			
C1P d18:1/16:0	616.47	78.9	C1P d18:1/12:0	560.41	78.9
S1P d18:1	378.24	78.9	S1P d17:1	364.23	78.9
5(S)-HETE	319.23	115.0	5(S)-HETE-d_8	327.23	116.0
12(S)-HETE	319.23	179.0	12(S)-HETE-d_8	327.23	184.0
15(S)-HETE	319.23	219.0	5(S)-HETE-d_8	327.23	116.0
20-HETE	319.23	289.0	20-HETE-d_6	325.23	295.0
PGE_2	351.23	315.3	PGE_2-d_9	360.25	351.23
PGD_2	351.23	315.3	PGD_2-d_4	355.25	275.3
RvD1	375.22	215.1	RvD_1-d_5	380.22	180.2
11β-$PGF_2\alpha$	353.24	193.0	PGD_2-d_4	355.25	275.3

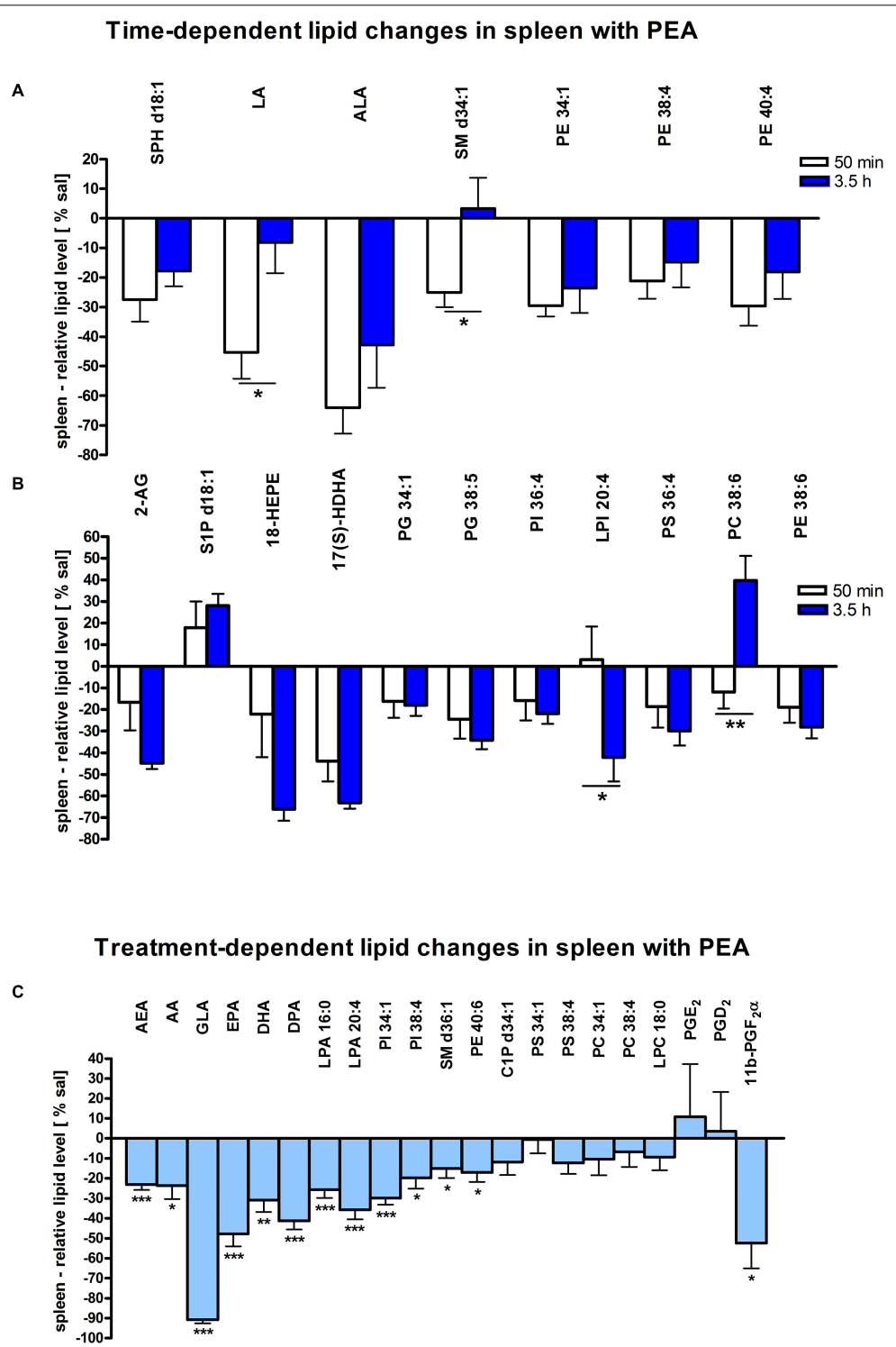

FIGURE 2 | Lipid levels of spleen after PEA treatment: relative lipid concentrations [normalized to the tissue weight (spleen, approximately 20 mg)] after PEA treatment as percentage of saline treated mice are shown and presented as mean value ± SEM. **(A) Time point 1:** time-specific lipid plasticity after 50 min of PEA treatment. Relative lipid levels for the two different time points are shown for reference, but only lipids significantly changing after 50 min of PEA treatment are depicted. **(B) Time point 2:** time specific lipid plasticity after 3.5 h of sub chronic PEA treatment. Relative lipid level for two different time points are shown and only lipids significantly changing after 3.5 h of PEA treatment are depicted. Significant differences between the time points were also detected and as they underline time specificity for these lipids, they were marked on the figure with *. **(C) Merged time points:** PEA treatment effect on lipid plasticity. Changes for AA, PI 38:4, SM d36:1, PE 40:6 and 11β-$PGF_2\alpha$ are significant when pooling the time points. The remaining significantly changed lipids show significant changes for both time points, respectively. Data were considered significant at a p-value < 0.05, e.g., $^{***}p < 0.001$, $^{**}p = 0.001$ to 0.01; $^{*}p = 0.01$ to 0.05.

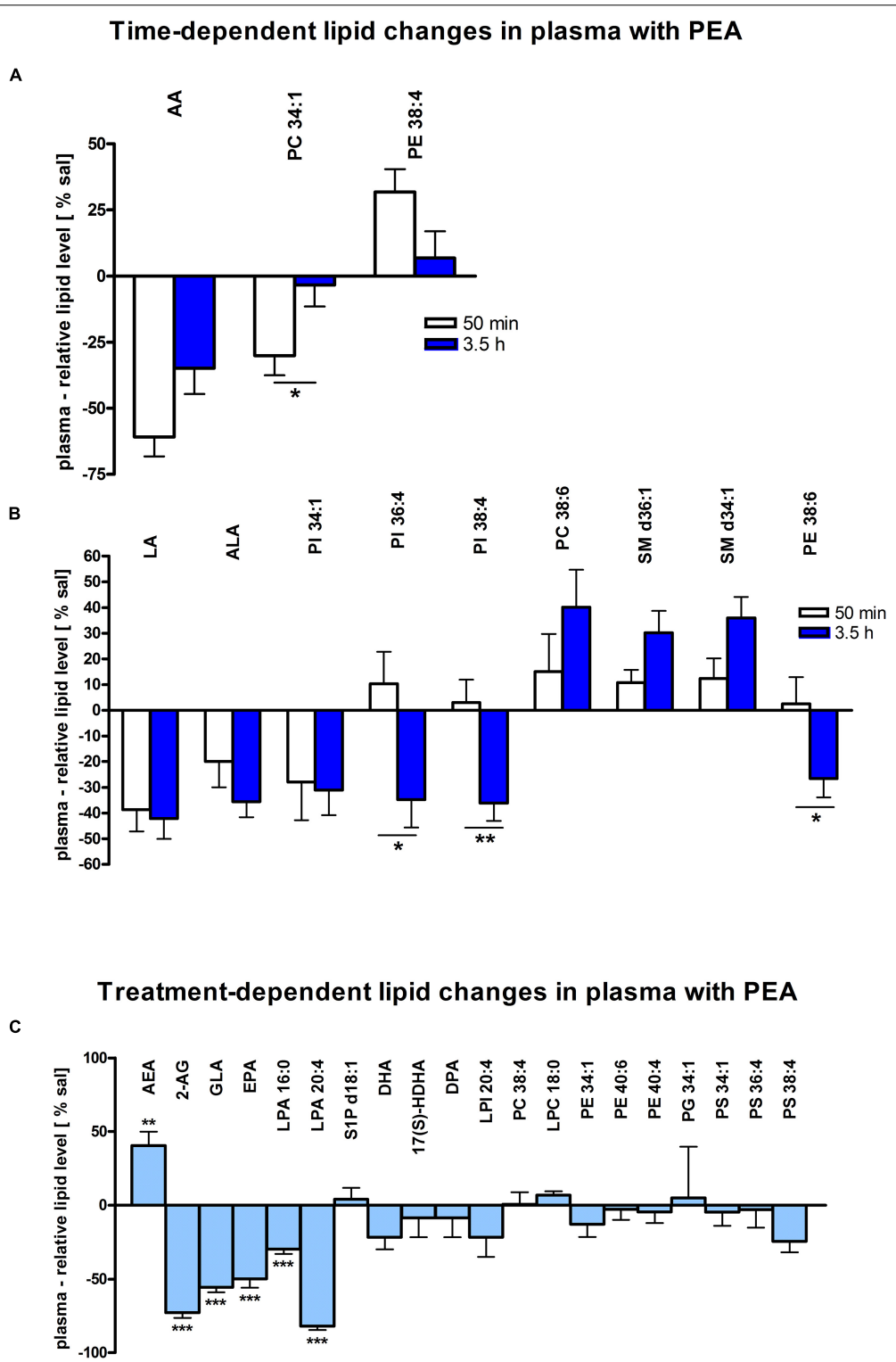

FIGURE 3 | Lipid levels of plasma after PEA treatment. Relative lipid concentrations (normalized to ml plasma: 40 μl) after PEA treatment as percentage of saline treated mice are shown and presented as mean value ± SEM. **(A) Time point 1:** time-specific lipid plasticity after 50 min of PEA treatment. Relative lipid levels for two different time points are shown and only lipids significantly changing after 50 min of PEA treatment are depicted. **(B) Time point 2:** time specific lipid plasticity after 3.5 h of sub-chronic PEA treatment. Relative lipid level for two different time points are shown and only lipids significantly changing after 3.5 h of PEA treatment are depicted. Significant differences between the time points were also detected and as they underline time specificity for these lipids, they were marked on the figure with *. **(C) Merged time points:** PEA treatment effect on lipid plasticity. Changes for AA, PI 38:4, SM d36:1 and PE 40:6 are significant when pooling the time points. The remaining significantly changed lipids show significant changes for both time points, respectively. Data were considered significant at a p-value < 0.05, e.g., ***$p < 0.001$, **$p = 0.001$ to 0.01; *$p = 0.01$ to 0.05.

standards were analog to those used for tissue extraction), as well as 250 µL water containing 5 µM THL/URB597 and 10 µg/mL BHT in final volume were used for lipid extraction out of 40 µL plasma, respectively. Extraction and LC/MRM analysis to target lipids of the LOX/CYP450/COX pathway namely RvD1, HETEs, PGD_2, 11β-$PGF_2\alpha$ and PGE_2 was carried out as previously described, with the exception of using solely negative ion mode analysis (Post et al., 2018).

Except the RNA extraction steps (see RNeasy® Mini Kit protocol), all extraction procedure steps, were carried out at 4°C to minimize *ex vivo* alterations of the endogenous lipid levels.

Reverse Transcription and Real-Time PCR Analysis

Approximately 120–800 ng isolated RNA per brain sample and 1000 ng per spleen sample were reverse-transcribed in order to generate complementary DNA (cDNA) using the High Capacity cDNA Reverse Transcription Kit with random primer hexamers (Applied Biosystems/Life Technologies, Darmstadt, Germany). The cDNA was diluted 1:10 in H_2O and amplified in the quantitative PCR (qPCR) using commercial FAM dye-labeled TaqMan assays (Applied Biosystems/Life Technologies, Darmstadt, Germany). The primers used for cDNA detection were specific for the exonic regions of the genes: *Bdnf* (Mm04230607_s1), PLA_2 (Mm00447040_m1), *ALOX5* (Mm01182747_m1), *ALOX15* (Mm00507789_m1), *COX-2* (Mm03294838_g1), *PPARα* (Mm00440939_m1), *ALOX12* (Mm00545833_m1), *MAPK11* (Mm00440955_m1), *IL1*-β (Mm00434228_m1), *Enpp2* (Mm00516572_m1), *PPARγ* (Mm00440940_m1). The gene *Crh* (PPM04632A-200) was analyzed using SYBR green primers from QIAGEN instead of TaqMan probes. The reference genes for the TaqMan and SYBR green assays were *GusB* (Mm01197698_m1) and *GAPDH* (F: CTCTGCTCCTCCCTGTTCC/R: TCCCTAGACCCGTACAGTGC), respectively.

The qPCR reactions were performed in duplicates using either TaqMan Gene Expression Mastermix or PowerSYBR Green PCR Mastermix (Applied Biosystems/Life Technologies, Darmstadt, Germany) and analyzed with an ABI 7300 Real-Time PCR cycler (Applied Biosystems/Life Technologies, Darmstadt, Germany).

LC/MS Qualitative and Quantitative Profiling

Targeted quantitative LC/MRM experiments were carried out with polarity switching using an SCIEX 5500 QTrap triple-quadrupole linear ion trap mass spectrometer (Concord, ON, Canada), as previously described (Lerner et al., 2016, 2018; Post et al., 2018). Via manual tuning, declustering potential, collision cell exit potential, entrance potential, and collision energy of the additionally analyzed lipids S1P, SPH, C1P, PUFAs, and PUFA oxidation products were individually optimized using their calibration standards. The MRM transitions of calibration standards and their corresponding ISTDs, as well as the transitions for the quantification of additionally targeted lipid molecules are depicted in **Table 1**. For ALA and LA the fragments produced by loss of water exhibited the highest intensity; additional transitions have been used for molecule confirmation. For S1P and C1P, transitions in negative ion mode to their phosphate group were used for quantification, while additional transitions in positive ion mode served as qualifiers. The LC conditions for eCBs and PLs were set as recently described (Bindila and Lutz, 2016; Lerner et al., 2016, 2018). The additional analysis of 7 PUFAs and 2 of their oxidation products 18-HEPE and 17S-DHA was carried out, using the LC conditions for eCB analysis. Chromatographic separation of the sphingosine and ceramide species was carried out together with the PL species. Therefore, the same LC conditions as for PL analysis have been used except of one minor change. To achieve a better ionization, 0.2% formic acid have been added to the mobile phase A, consisting of methanol/water (1:1; v/v) containing 7.5 mM ammonium formate and 0.1% TEA and to the mobile phase B, consisting of methanol/isopropanol (2:8; v/v) containing 7.5 mM ammonium formate and 0.1% TEA, respectively. LOX-, CYP450, and COX-derived lipids were extracted and analyzed using the protocol described in Post et al. (2018), but without the inclusion of eCBs, hence only using the negative ion polarity. MRM transitions and conditions were inferred by manual tuning and are depicted in **Table 1**.

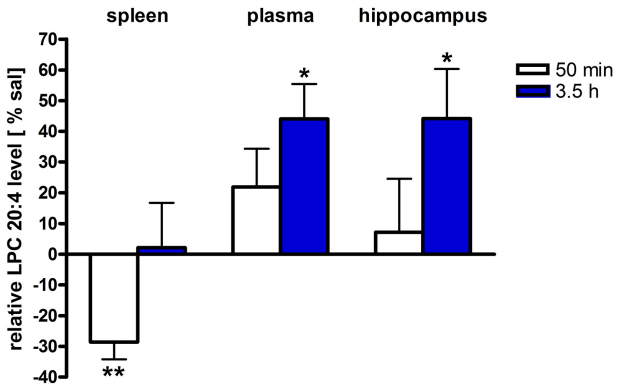

FIGURE 4 | LPC 20:4 levels of brain and periphery after PEA treatment. Relative LPC 20:4 concentrations (normalized to ml plasma/tissue weight) after PEA treatment as percentage of saline treated mice are shown and presented as mean value ± SEM. Relative lipid level for two different time points are depicted. Time specific changes in LPC 20:4 levels occur in spleen at 50 min while in plasma and hippocampus at 3.5 h after PEA treatment. Data were considered significant at a p-value < 0.05, e.g., $^{***}p < 0.001$, $^{**}p = 0.001$ to 0.01; $^{*}p = 0.01$ to 0.05.

Data Processing and Statistical Analysis

Lipids were quantified by Analyst 1.6.2 software (AB SCIEX, Darmstadt, Germany) and MultiQuant 3.0 quantitation package. The obtained values were normalized to the tissue weight/ ml plasma. The analysis of the relative gene expression (RGE) data received from the qPCR was performed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Target genes were normalized to the reference genes, and the normalized expression levels of the target genes then to that of the control mice. Data were analyzed with GraphPad Prism 4.0 and 8.0 software package (GraphPad Software, San Diego, CA, United States), presented as

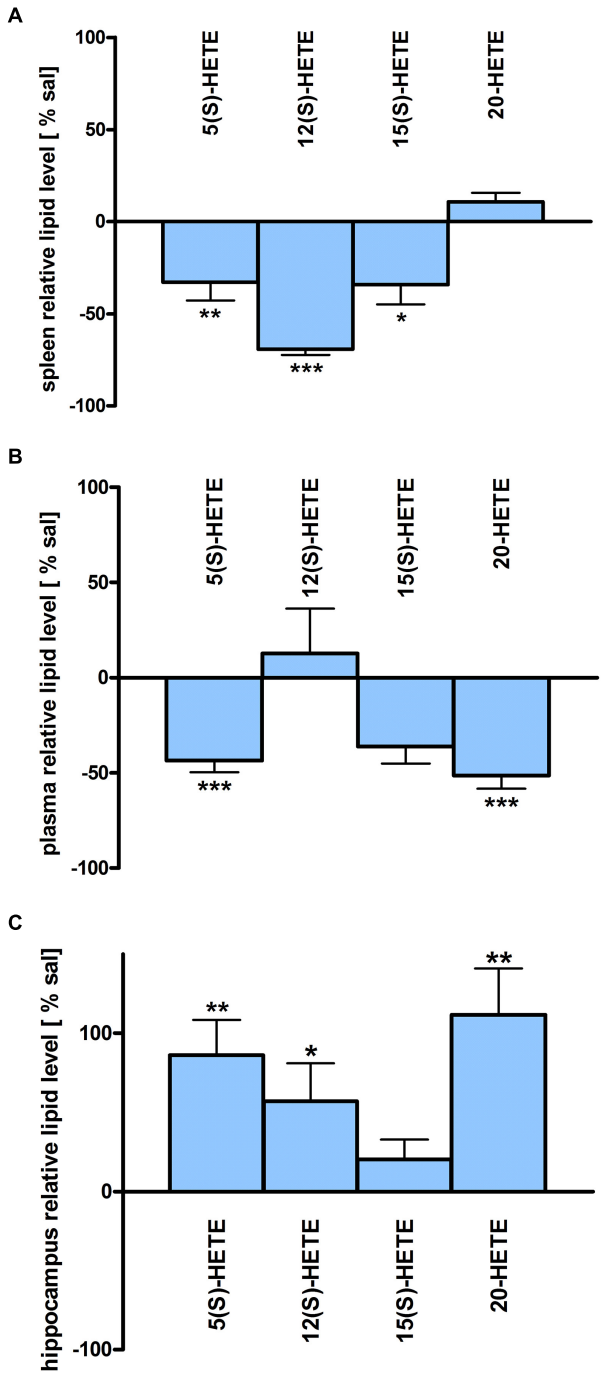

FIGURE 5 | HETE lipid levels in brain and periphery after PEA treatment: relative HETE concentrations (normalized to ml plasma/tissue weight) after PEA treatment as percentage of saline treated mice are shown and presented as mean value ± SEM. **(A) Spleen:** HETE alterations with sub-chronic PEA treatment and unchanged HETE levels in spleen. Only significant reductions of HETE levels can be found in spleen. **(B) Plasma** HETE alterations with sub-chronic PEA treatment and unchanged HETE levels in plasma. Only significant reductions of HETE levels can be found in plasma. **(C) Hippocampus:** HETE alterations with sub chronic PEA treatment and unchanged HETE levels in hippocampus. Only significant increments of HETE levels can be found in hippocampus. Data were considered significant at a p-value < 0.05, e.g., $^{***}p < 0.001$, $^{**}p = 0.001$ to 0.01; $^{*}p = 0.01$ to 0.05.

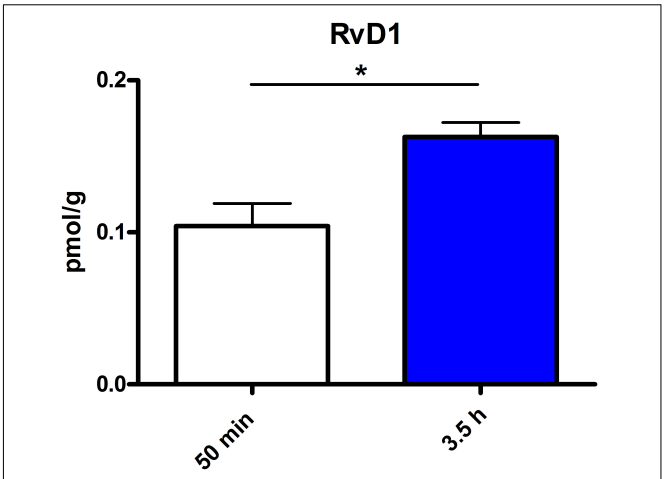

FIGURE 6 | RvD1 concentration in spleen after different time points of PEA treatment: absolute concentrations of RvD1 [normalized to the tissue weight (spleen ≙ 20 mg)] after two time points of PEA treatment presented as mean value ± SEM. RvD1 is significantly increased after 3.5 h of PEA treatment as compared to 50 min. Of note, RvD1 was not detected in saline-treated control mice, likely because they are at basal level under limit of detection/ quantification or not biosynthesized. Data were considered significant at a p-value < 0.05, e.g., $^{***}p < 0.001$, $^{**}p = 0.001$ to 0.01; $^{*}p = 0.01$ to 0.05.

mean ± SEM and considered significant at a p-value < 0.05, e.g., $^{***}p < 0.001$, $^{**}p = 0.001$ to 0.01; $^{*}p = 0.01$ to 0.05. Statistical analyses of the difference between group means were carried out using two-tailed unpaired Student's t-test.

RESULTS

Rationale of Study Design, Data Provision, and Biological Matrix Choice

Using the tissue lipidomics and transcriptomic method, as well as plasma lipidomics we investigated molecular effects of sub-chronic, prophylactic PEA administration compared to Sal in both time-dependent manner, e.g., 50 min and 3.5 h post-administration, respectively, and in a treatment-dependent manner, whereby the molecular levels from the PEA and Sal groups at the two time points were summed up and statistically evaluated independent of the time point. We thus, aimed to understand on one hand, a lipid and genomic temporal dynamic upon PEA administration, and nonetheless a cumulative effect of the treatment itself. We chose these time points because of the previously proven molecular effects of PEA (between 50 min and 3.5 h post PEA administration) at an acute symptomatic phase in mice models of epilepsy (Post et al., 2018). This inter-study reference will help elucidate how PEA prepares the body in the face of insults.

Exogenous PEA has been shown to act on both, brain and periphery, and putatively across brain-periphery axis in modulating not only anti-inflammatory but also neuroprotective or symptomatic effects (pain reduction, anticonvulsant) (Mattace Raso et al., 2014; Petrosino and Di Marzo, 2017). The choice

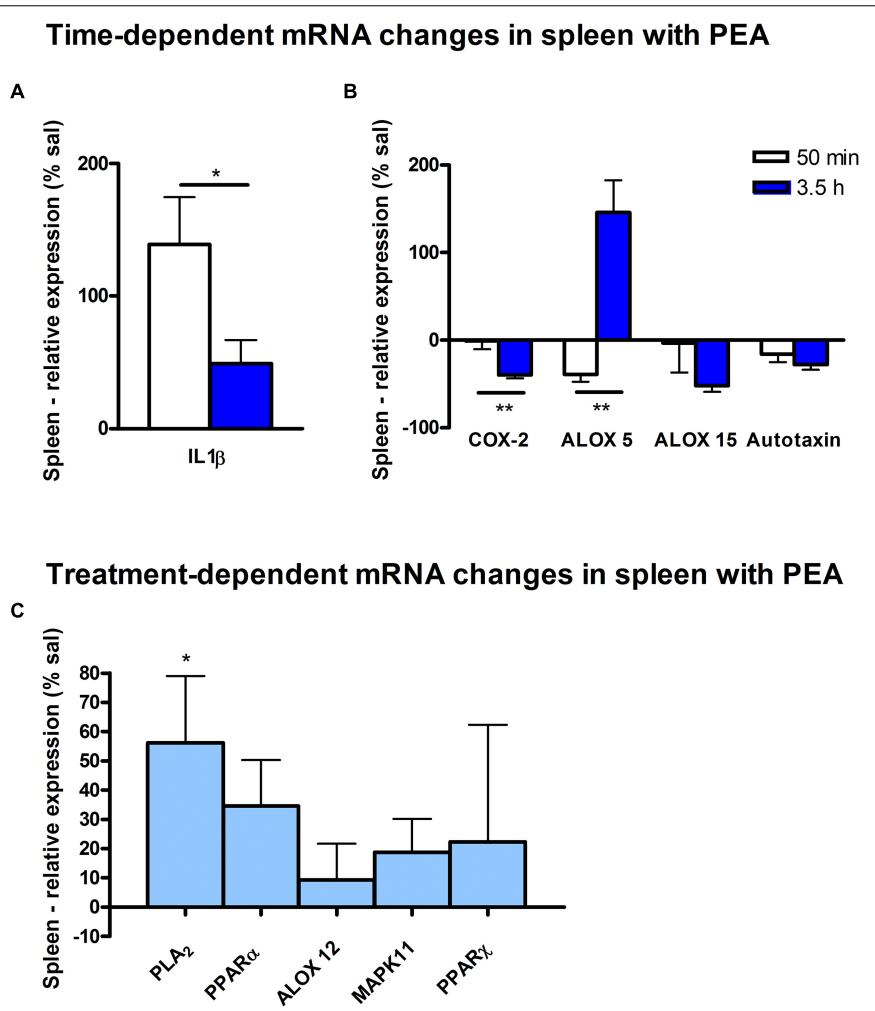

FIGURE 7 | mRNA levels of spleen after PEA treatment: relative mRNA values after PEA treatment as percentage of saline-treated mice, presented as mean value ± SEM. **(A) Time point 1:** time specific mRNA changes after 50 min of sub-chronic PEA treatment. Relative mRNA levels for two different time points are shown and only genes significantly changing after 50 min of PEA treatment are depicted. **(B) Time point 2:** time-specific mRNA changes after 3.5 h of PEA treatment. Relative mRNA levels for two different time points are shown and only genes significantly changing after 3.5 h of PEA treatment are depicted. Significant differences between the time points underline time specificity for these mRNAs and are depicted on the graph when occurring. **(C) Merged time points:** relative mRNA levels for merged time points. Changes for $cPLA_2$ are significant when pooling the time points. Data were considered significant at a p-value < 0.05, e.g., $^{***}p < 0.001$, $^{**}p = 0.001$ to 0.01; $^{*}p = 0.01$ to 0.05.

of hippocampus was guided by its pivotal function in the on-set of neuroinflammation and neuronal hyperexcitability, which are common features of several neurological diseases (epilepsy, stroke, brain injuries, etc.), and have been shown to be positively affected by PEA (Small et al., 2011; Post et al., 2018). Spleen was chosen as a main organ of the immune system mounting both innate and adaptive immune responses. Splenic immune responses are also modulated in part by a fascinating lipid signaling, for example of the S1P and LPA, involved in immune trafficking and response (Goetzl et al., 2004; Mebius and Kraal, 2005; Bronte and Pittet, 2013). Since, spleen is interposed in the blood stream and bodily's largest blood filter, we rationalized that administration of a nutraceutical such as PEA would render molecular changes in the spleen, and that PEA's immunomodulatory properties are partly occurring in spleen. Therefore, PEA's effect on the splenic lipidome and transcriptome is expected to contribute new aspects of the PEA prophylactic mechanism. Finally, plasma lipidomics can provide both, readout of the PEA's actions in brain and periphery, and a complementary source to elucidate the PEA's prophylactic mechanism.

We analyzed changes in the levels of representative mRNAs involved in: (i) inflammation and breakdown of membrane lipids and synthesis of pro- and anti-inflammatory signaling lipids: IL1β, $cPLA_2$, COX-2, ALOX 5, ALOX 12, ALOX 15, and autotaxin; (ii) neuronal activity: Bdnf, Crh, and (iii) PEA signaling: PPARα, PPARγ, and MAPK. We have previously showed that a transcriptional over-activation of the Bdnf gene occur specifically in the hippocampal regions of the brain (Lerner et al., 2018) at acute seizure state, underscoring an increased neuronal activity in the onset and progression of seizures

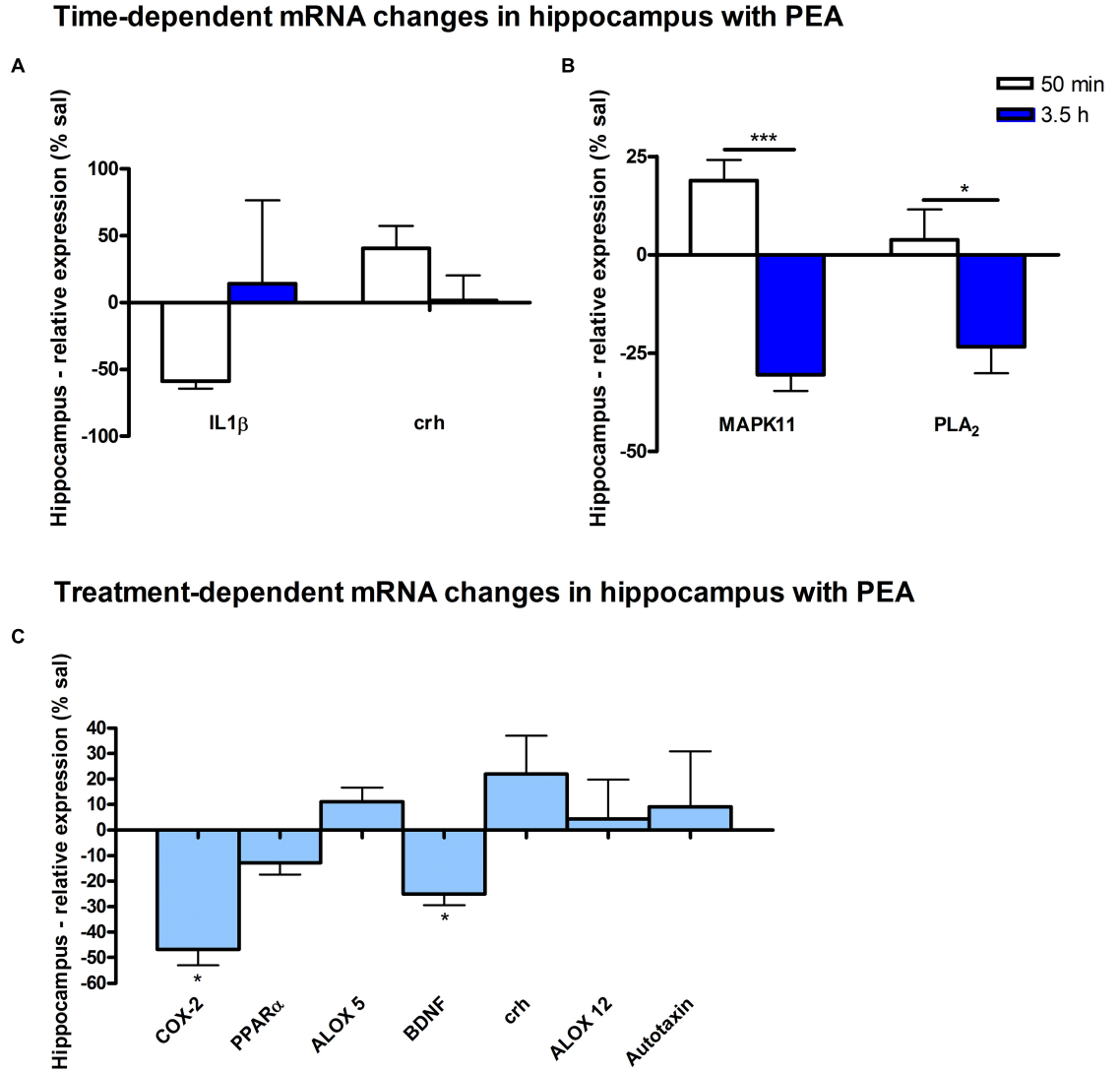

FIGURE 8 | mRNA levels of hippocampus after PEA treatment: relative mRNA values after PEA treatment as percentage of saline treated mice, presented as mean value ± SEM. **(A) Time point 1:** time specific mRNA changes after 50 min of sub-chronic PEA treatment. Relative mRNA levels for two different time points are shown and only genes significantly changing after 50 min of PEA treatment are depicted. **(B) Time point 2:** time-specific mRNA changes after 3.5 h of PEA treatment. Relative mRNA levels for two different time points are shown and only genes significantly changing after 3.5 h of PEA treatment are depicted. Significant differences between the time points underline time specificity for these mRNAs and are depicted on the graph when occurring. **(C) Merged time points:** relative mRNA levels for merged time points. Changes for COX-2 and Bdnf are significant when pooling the time points. Data were considered significant at a p-value < 0.05, e.g., $^{***}p < 0.001$, $^{**}p = 0.001$ to 0.01; $^{*}p = 0.01$ to 0.05.

(Binder et al., 2001). Hippocampal Crh-expressing neurons have more recently been shown to modulate excitability of the CA3 neurons in response to stress and even affect locomotor activity (Hooper et al., 2018). Due to the reported neuroprotective and anticonvulsant properties of PEA, a transcriptional modulation of Crh and Bdnf upon PEA prophylactic administration was reasoned to be of interest to investigate.

Spleen

The most significant changes in lipid levels could be detected in the spleen, whereby the great majority of analytes underwent a significant reduction. At 50 min, SPH (**), LA (*), ALA (**), LPC 20:4 (**), SM 34:1 (*), PE 34:1 (***), PE 38:4 (*), and PE 40:4 (*) were significantly decreased. Thereby, changes of LA and SM 34:1 were significantly stronger as compared to 3.5 h (**Figures 2A**, **4**). At 3.5 h, 2-AG (***), 18-HEPE (**), 17(S)-HDHA (***), PG 34:1 (*), PG 38:5 (***), PI 36:4 (**), LPI 20:4 (*), PS 36:4 (*), and PE 38:6 (*) underwent significant reduction, while S1P (**) and PC 38:6 (*) were significantly enhanced. The LPI 20:4 and PC 38:6 levels were significantly stronger altered at 3.5 h as compared to 50 min (**Figure 2B**). When pooling both time points, a significant decrease for AA (*), PI 38:4 (*), SM d36:1 (*), PE 40:6 (*) and 11β-$PGF_2\alpha$ could be detected. The AEA (***), GLA

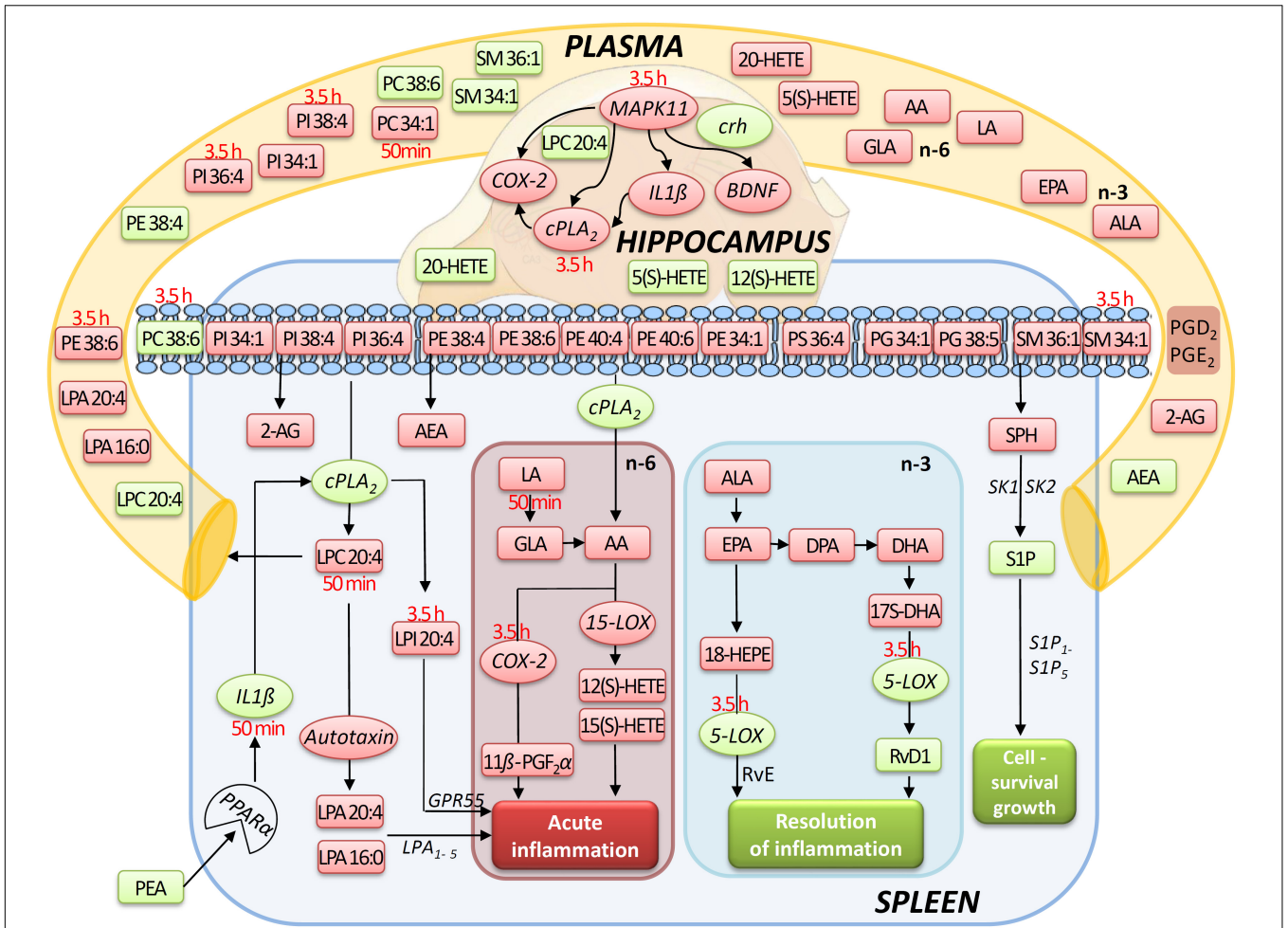

FIGURE 9 | Simplified signaling pathway: this simplified signaling cartoon encompasses all significant molecular changes detected in this study in spleen, hippocampus, and plasma after PEA treatment (PEA/sal). Decreased molecular levels compared to Sal are displayed in red while increased ones in green, respectively. Where lipid level alteration where restricted to a time point, they were specified in red color. The proposed pathways are inferred from published studies that show correlations between the targeted molecules under defined physiological or pathophysiological contexts. This graphic gives also an idea how the PEA-derived splenic molecular phenotype leads to a general state of decreased acute inflammation and increased resolution of inflammation and cell survival/growth. Similarly, possible interrelation between the transcriptional changes (mRNA) by PEA is depicted.

(***), EPA (***), DHA (**), DPA (**), LPA 16:0 (***), LPA 20:4 (***), and PI 34:1 (***) were significantly decreased at both time points (**Figure 2C**). A significant downregulation of 5(S)-HETE (**), 12(S)-HETE (***), and 15(S)-HETE (*) was detected with PEA treatment (**Figure 5A**). Absolute concentrations of RvD1 significantly increased after 3.5 h of PEA treatment as compared to 50 min. Of note, RvD1 was not detected in saline-treated control mice, likely because they are at basal level under limit of detection/quantification or not biosynthesized (**Figure 6**).

At transcriptomic level after 50 min, only significant changes for IL1-β (**) were detected (**Figure 7A**). At 3.5 h COX-2 (*), ALOX 15 (*), and autotaxin (*) were significantly decreased, while ALOX 5 (*) was significantly increased. The COX-2 and ALOX 5 levels were significantly more altered at 3.5 h compared to 50 min (**Figure 7B**). Fitting to the general PL breakdown seen in the spleen, mRNA encoding $cPLA_2$ was significantly (*) enhanced with the PEA treatment, but not at individual time points (**Figure 7C**).

Plasma

At 50 min, AA (***) and PC 34:1 (*) were significantly decreased, while PE 38:4 (*) was significantly enhanced. PC 34:1 was significantly more decreased as compared to 3.5 h (**Figure 3A**).

In plasma, more significant changes could be detected at 3.5 h. Two PUFAs; LA (*) and ALA (**), as well as four PLs; PI 34:1 (*), PI 36:4 (*), PI 38:4 (**), and PE 38:6 (*) showed significantly decreased levels, while four PLs; LPC 20:4 (*), PC 38:6 (*), SM 36:1 (*), and SM 34:1 (**) were significantly increased (**Figures 3B**, **4**). The changes in PI 36:4 (*), PI 38:4 (**), and PE 38:6 (*) were significantly stronger at 3.5 h as compared to 50 min after PEA injection, thus underlying the time specificity of their significant reduction at 3.5 h (**Figure 3B**). The eCBs, PUFAs and signaling PLs: AEA (**), 2-AG (***), GLA (***),

EPA (***), LPA 16:0 (***), and LPA 20:4 (***) showed significant changes with treatment and with time. While PEA injection led to enhanced levels of the eCB AEA, all other lipids underwent a significant reduction (**Figure 3C**). A significant downregulation of 5(S)-HETE (***) and 20-HETE (***) was detected with PEA treatment (**Figure 5C**).

Hippocampus

The only alteration in hippocampal PL levels was found for LPC 20:4 (*), which was increased 3.5 h after PEA injection (**Figure 4**). A significant upregulation of the 5(S)-HETE (**), 12(S)-HETE (*), and 20-HETE (**) was detected with PEA treatment (**Figure 5C**). PEA administration was previously shown to increase the PEA levels in the brain of control mice (Post et al., 2018) when administered at a dosis of 40 mg/kg, but not at 0.1 mg/kg (Heide et al., 2018).

At transcriptomic level after 50 min, significant changes for IL1-β (*) and Crh (*) were detected (**Figure 8A**). At 3.5 h after PEA treatment, significant changes for MAPK11 (**) and $cPLA_2$ (*) were detected, both being significantly stronger as compared to time point 1 (**Figure 8B**). Treatment-specific significant decrease for COX-2 (*) and Bdnf could be detected (**Figure 8C**).

DISCUSSION

Our data evidence that prophylactic sub-chronic PEA administration distinctly effects peripheral immune system and hippocampus, at both transcriptional and lipid molecular level-the exception is COX-2 pathway, which is downregulated in both tissues. Moreover, the downregulation of the COX-2 mRNA is time-point specific (3.5 h post PEA-injection) for both regions. This finding supports the hypothesis of PEA's action across brain-periphery axis to decrease pro-inflammatory environment via transcriptional modulation of COX-2 pathway. Hence, splenic and hippocampal transcriptional downregulation of COX-2 pathway upon prophylactic PEA administration could balance the hippocampal over-expression of COX-2 upon brain insult (Lerner et al., 2018) and thus prevent development of neuroinflammation (Post et al., 2018). Similarly, PEA's downregulation of COX-2 pathway in brain and periphery could contribute to building up resistance to bacterial infections and sepsis development (Heide et al., 2018). The transcriptional regulation of splenic and hippocampal $cPLA_2$ is divergent (increase in spleen and decrease in HC) and, interestingly, with different consequences on the lipidome in the two regions: in spleen with the exception of PC 38:6, all other phospholipids were downregulated, while, in HC no change of the membrane PLs was detected, except the LPA 20:4 (**Figure 9**). Even though an upregulation of $cPLA_2$ and breakdown of membrane PLs is a hallmark of many diseases, in such cases it is accompanied by the increased production of pro-inflammatory lipids, lipid peroxidation and free fatty acids (Farooqui et al., 2004). This is not the case here in spleen (**Figures 2**, **5**, **9**), where it is evident that the transcriptional upregulation of $cPLA_2$ is accompanied by a downregulation of COX-2 and 15-LOX with a concurrent upregulation of the 5-LOX mRNA (**Figures 7**, **9**). The increase of resolvin D1 (**Figure 6**), which is reportedly an anti-inflammatory lipid, along with concurrent decrease of AA and downstream COX and LOX- lipid derivatives (11β-$PGF_2\alpha$, 12(S)- and 15(S)-HETE, respectively) (**Figures 2C**, **5**) evidence a shift towards provision of a pro-resolving lipid environment in the spleen via transcriptional activation of the $cPLA_2$, 5-LOX, with 5-LOX rendering conversion of omega-3 fatty acids in pro-resolving lipid environment (**Figure 9**). In line with this mechanism, the omega-6 fatty acids are not used to increase the AA pool (**Figure 9**) for inflammatory signals, but more likely to remodel/resynthesize splenic membrane PLs, indicated by an increase of PC 38:6 (3.5 h post-injection). The plasma omega 3- and omega-6 fatty acids, as well as PC 38:6 have the same alteration pattern as spleen, which could explain the PEA's role in positively influencing lipid metabolism in obesity and associated inflammatory consequences (Hoareau et al., 2009).

The downregulation of the splenic pro-inflammatory signaling in favor of pro-resolution signaling is also supported by the transcriptional downregulation of the autotaxin, a major modulator of acute and chronic inflammation (Knowlden and Georas, 2014; Valdés-Rives and González-Arenas, 2017), and by the subsequent decrease of its extracellularly produced LPA lipids (LPA 20:4 and C16:0, respectively) (**Figures 2C**, **7B**). LPA is one of the main signaling lipids in spleen that is multi-functional in modulating immune response (Saba, 2004). Recently, autotaxin-LPA pathway has been implicated as a main player in aberrant immune responses and development of inflammatory responses. The PEA's effect on this pathway is therefore supporting its role as a positive immunomodulatory and warrants further investigation. PEA prophylactic administration modulates (increases) the level of S1P which is a vital lipid signal in the splenic immune function. The increase of S1P likely occurs through the breakdown of SM 36:1 (**Figure 9**) and conversion through SPH to S1P. S1P, via binding to its S1P1R receptors, is the bioactive lipid regulator of both innate and adaptive immune system and serves a plethora of biological functions in the splenic immune system. From our data it is impossible to reveal the role of PEA-derived S1P increase. However, considering the reported immunomodulatory properties of PEA it is pertinent to conclude that PEA-derived S1P increase must be part of a beneficial immune system boosting, which certainly needs further investigation. A time-specific (50 min) increase of IL1-β was also detected in spleen. Even though this molecule is mainly described as a pro-inflammatory cytokine that activates the $cPLA_2$ leading to pro-inflammatory cascades, it is obvious that this was not the case in spleen. In contrast, IL1-β and $cPLA_2$ activation is accompanied by a shift in signaling towards decreasing inflammatory cascade and boosting pro-resolving lipid environment (**Figures 6**, **9**). This is also supported by the fact that IL1-β increase is terminated after 50 min. It is therefore, more likely that (short) activation of IL1-β boosts in this case immune defense and functions as an immunoadjuvant

upon PEA prophylaxis (Dinarello, 1998). In contrast, in HC mRNA levels of both, IL1-β and $cPLA_2$ are downregulated along with those of MPAK11 and COX-2. These indicate a distinct regulation by PEA of neuroinflammatory pathway in HC than inflammatory pathway in spleen. The concerted decrease of these molecules in hippocampus point towards a MAPK11-induced downregulation of cytokine production and cytosolic cPLA2 with a downstream regulation of COX-2 as well (Wang et al., 2008). Nevertheless, this broad downregulation of the hippocampal pro-inflammatory tone explains the reported anti neuro-inflammatory properties of the PEA in various brain injuries, recently demonstrated for multiple sclerosis as well (Orefice et al., 2016). LPC 20:4 has reportedly been implicated in triggering inflammation via activation of COX-2 (Brkić et al., 2012). Because in hippocampus, the mRNA of the COX-2 is down-regulated, but LPC 20:4 up-regulated, modulation of hippocampal COX-2 is independent of LPC 20:4. It follows then that MAPK- induced $cPLA_2$/ IL1-β /COX-2 pathway is the more likely venue for PEA modulation of hippocampal inflammation. What is the source and function of hippocampal upregulation of LPC 20:4 upon PEA administration is not apparent from our data and remains to be determined. Similarly, increase and function of 5(S)-, 12(S)-, and 20-HETE in HC remains an open question to be investigated, especially, since none of the investigated LOX- mRNAs are altered, and provision of more data on CYP450 pathway is required. Indeed, in our study neither the translation of genes nor enzymatic activities were investigated which would answer and guide the interpretation of such lipid changes, e.g., hippocampal increase of LPC 20:4 and 5(S)-, 12(S)- and 20-HETE and/or plasma increase of PE 38:4, PC 38:6, LPC 20:4, SM 34:1, and SM 36:1.

Of general note is that the biochemical processes leading to the significant changes of the targeted lipidome upon PEA administration are time-wise diverse, as evident from the time-resolution lipid analysis. A broader lipidome change occurs at 3.5 h than at 50 min post-administration (**Figures 2–4**). We, therefore consider that generally, investigating a temporal dynamic of lipidome is advantageous to exhaustively reveal lipids involved in a particular biological context such as here described.

A particular feature of prophylactic PEA effect in the HC is the transcriptional downregulation of Bdnf gene and upregulation of Crh gene. Bdnf-mRNA downregulation by PEA could counterbalance aberrant increase elicited by brain insults such as the case in epilepsy (Lerner et al., 2018), hence downregulating the underlying increased neuronal activity rendering decreased seizure intensity (Post et al., 2018). More recently, a hippocampal neuronal subpopulation was shown to express Crh which modulates hippocampal excitability and maintain adaptive network excitability. Inhibition of Crh-expressing neurons increased locomotor activity while selective ablation of Crh-neurons in HC led to increased seizure susceptibility (Hooper et al., 2018). In view of this line of evidence, the transcriptional upregulation of hippocampal Crh gene upon PEA prophylactic administration is really interesting, and opens new venue of research to explain anticonvulsant effects of PEA administration. Collectively, hippocampal decreased neuroinflammatory pathways MAPK11/IL1-β/$cPLA_2$/COX-2 and decreased hippocampal excitability as indicated by decreased Bdnf mRNA along with increased Crh mRNA expression could contribute to the neuroprotective, anti-neuroinflammatory, and anticonvulsant properties described for PEA in epilepsy and other brain insults. A transcriptional activation of PPARα gene by PEA at the time points investigated here in control mice was not detected, which certainly does not exclude its activation.

CONCLUSION

Prophylactic PEA administration generated a complex molecular phenotype at transcriptomic and lipid level in spleen and brain and blood. Because, the PEA prophylaxis is reportedly effective it can be concluded that the induced molecular phenotype is beneficial to the body in facing various negative insults (infections, neuro injuries, etc.).

Finally, the resulting new insight into molecular plasticity is a step forward in understanding what can be targeted by PEA in prospective preventive care measures and hence help guide prophylactic or adjuvant approaches for example for groups at high risk of inflammation (through bacterial exposure) or neuro injury (epilepsy, stroke brain injuries, etc.). Concurrently, our findings open new questions into the multi-facetted mechanisms of PEA and new inroads of research into the prophylactic effects of PEA on immune system and central nervous system.

Future studies focusing on gender and age-dependent response to PEA administration should clarify mechanistic aspects of PEA and its applicability in general health care. Of note is that the methodology presented here is of general applicability in studying nutrition and nutraceutical effects on tissue molecular composition and consequential affected pathways.

The circulatory lipid profile reflects major molecular events in the brain and peripheral immune system upon PEA administration, so that plasma lipidomics can be a promising clinical tool to monitor and possibly predict response to nutraceutical/nutrition based therapy and prophylaxis.

ETHICS STATEMENT

Experiments were performed according to the European Community's Council Directive of 22 September 2010 (2010/63EU) and approved by the local animal care committee of the German Rhineland-Palatinate (file reference: 23 177-07/G16- 1-075).

AUTHOR CONTRIBUTIONS

RL carried out the main lipidomic experiments, prepared, interpreted and processed the data, contributed to manuscript writing. DPC performed the transcriptomic experiments and processing of the mRNA data. JMP performed the animal experiments and part of the lipidomic experiments.

BL contributed to data interpretation. LB coordinated the study contributed to data interpretation and manuscript writing.

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frontiers in Neuroscience

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Taurine/Pilocarpine Interaction in the Malnourished Rat Brain: A Behavioral, Electrophysiological, and Immunohistochemical Analysis

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This study aimed to evaluate the possible protective role of taurine on anxiety-like behavior, brain electrical activity and glial cell immunoreactivity in well-nourished and malnourished rats that were treated with a subconvulsing dose of pilocarpine. Newborn Wistar rats were subjected to normal or unfavorable lactation conditions, represented by the suckling of litters with 9 or 15 pups, resulting in well-nourished and malnourished animals, respectively. Each nutritional group was split into five subgroups that were treated from postnatal day (PND) 35 to 55 with 300 mg/kg/day of taurine + 45 mg/kg/day of pilocarpine (group T + P), taurine only (group T), pilocarpine only (group P), vehicle control (group V), or not treated control (group naïve; Nv). At PND56-58, the groups were subjected to the elevated plus-maze behavioral tests. Glycemia was measured on PND59. Between PND60 and PND65, the cortical spreading depression (CSD) was recorded in the cerebral cortex, and the levels of malondialdehyde and microglial and astrocyte immunoreactivity were evaluated in the cortex and hippocampus. Our data indicate that treatment with taurine and pilocarpine resulted in anxiolytic-like and anxiogenic behavior, respectively, and that nutritional deficiency modulated these effects. Both treatments decelerated CSD propagation and modulated GFAP- and Iba1-containing glial cells. Pilocarpine reduced body weight and glycemia, and administration of taurine was not able to attenuate the effects of pilocarpine. The molecular mechanisms underlying taurine action on behavioral and electrophysiological parameters in the normal and altered brain remain to be further explored.

Keywords: taurine, pilocarpine, anxiety-like behavior, blood glucose, brain excitability, glial cells, nutritional deficiency, spreading depression

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INTRODUCTION

Taurine is an amino sulfonic acid that is found abundantly in several areas of the mammalian central nervous system. It is a structural analog of the inhibitory transmitter gamma-aminobutyric acid (GABA), which is recognized as one of the most important inhibitory amino acids distributed in the brain tissue (Barbeau et al., 1975; Yakimova et al., 1996; Ripps and Shen, 2012). Taurine

mediates a myriad of physiological processes in the nervous system, including neuromodulation, maintenance of calcium homeostasis, and neuronal proliferation and differentiation (Chen et al., 1998). Administration of exogenous taurine improves glucose homeostasis in genetically obese animals (Santos-Silva et al., 2015), promotes an anxiolytic-like behavioral profile (Murakami and Furuse, 2010; Francisco and Guedes, 2015), acts as an antioxidant and anti-inflammatory molecule (Oliveira et al., 2010; Marcinkiewicz and Kontny, 2014) and rescues hippocampal long-term potentiation (LTP) from ammonia-induced impairment (Chepkova et al., 2006). Furthermore, taurine reportedly acts as a neuroprotectant in epilepsy, reducing or abolishing seizures (Junyent et al., 2011; Oja and Saransaari, 2013; see a recent review in Hrnčić et al., 2018).

The acute systemic injection of pilocarpine in rodents constitutes an effective, experimental model largely used to study the pathophysiology of seizures and to identify potential therapeutic agents for the treatment of epilepsy (Santos et al., 2000). This model was first described by Turski et al. (1983a,b)); it consists of the single administration of a high dose (300–380 mg/kg; Guedes and Cavalheiro, 1997) or various consecutive low doses of pilocarpine until induction of *status epilepticus* (Glien et al., 2001). This acute phase is followed by a condition of permanent recurrent spontaneous seizures, altering the central nervous system structure and function (Turski et al., 1989) with behavioral and electroencephalographic changes that are similar to those observed in human temporal lobe epilepsy. In the last decade, some studies had given special attention to possible effects of subconvulsing doses of pilocarpine when administered acutely. Under this condition, no behavioral or electrocorticographic changes indicative of seizures were observed (Guedes and Vasconcelos, 2008). However, under subconvulsing paradigms several reports have described anxiety-like behavioral profiles (Duarte et al., 2014; Francisco and Guedes, 2018), reductions in glycemia (Francisco and Guedes, 2018), increases in brain oxidative stress (Mendes-da-Silva et al., 2018) and impairment of propagation of the excitability-related phenomenon known as cortical spreading depression (CSD) along the cortical rodent tissue (Francisco and Guedes, 2018; Mendes-da-Silva et al., 2018).

Cortical spreading depression is a brain phenomenon that is based on neuronal and glial depolarization and is influenced by conditions that modify neural excitability, including cholinergic agonists (Guedes and Cavalheiro, 1997). CSD has been related to excitability-associated diseases such as migraine with aura (Lauritzen, 1994); CSD in the injured human brain was first shown by Mayevsky et al. (1998) and Strong et al. (2002). CSD has been also associated with ischemic stroke (Dohmen et al., 2008), traumatic brain injury (Hartings et al., 2009), subarachnoid hemorrhage (Dreier, 2011), multiple sclerosis (Pusic et al., 2015), and epilepsy (Vinogradova et al., 2006; Dreier et al., 2012). Evidence suggests that chemicals such as potassium (Grafstein, 1956) or glutamate (Van Harreveld, 1959; Marrannes et al., 1988; Pietrobon and Moskowitz, 2014; Hertelendy et al., 2018), as well as various other neurotransmitters and neuromodulators (Ayata and Lauritzen, 2015; Guedes et al., 2017) might be involved in CSD, either eliciting, or modulating the phenomenon. Experimental evidence demonstrated that CSD can potentiate the brain's spontaneous and evoked electrical activity, both *in vitro* (Footitt and Newberry, 1998) and *in vivo* (Guedes et al., 2005; Souza et al., 2015). Under conditions of environmental, nutritional and pharmacological manipulations, our group has extensively employed the CSD model to evaluate the proper functioning of the brain in health and disease. When occurring early in life, such conditions can affect brain development and functioning, and can substantially alter the ability of the brain to produce and propagate CSD (see Guedes, 2011 for an overview). Recently, we demonstrated that the chronic administration (21 days) of a very low, subconvulsive dose of pilocarpine (45 mg/kg/day) is able to counteract CSD, and this effect is modulated by nutritional deficiency (Francisco and Guedes, 2018).

In the present study we tested the hypothesis that taurine modulates the CSD effects of pilocarpine, in association, or not, with early malnutrition. In addition, we investigated the taurine/pilocarpine/malnutrition interaction on anxiety-like behavior, fasting glycemia and oxidative stress. Finally, some structural correlates of this interaction were investigated by correlating the experimental treatments with the astrocytic and microglial immunostaining pattern in the cerebral cortex and hippocampus.

MATERIALS AND METHODS

Animals

All experimental procedures were previously approved by the Institutional Ethics Committee for Animal Research of the Federal University of Pernambuco (approval protocol no. 23076.015655/2015-99), whose norms comply with the norms established by the National Institutes of Health Guide for Care and Use of Laboratory Animals (Bethesda, MD, United States). Newborn Wistar rats of both sexes, born from different dams, were assigned to be suckled under normal or unfavorable lactation conditions, represented respectively by litters with nine pups (L_9 groups) and litters with 15 pups (L_{15} groups), as previously described (Francisco and Guedes, 2015). Weaning occurred on postnatal day (PND) 21, when pups were separated by sex and housed in polypropylene cages (51 cm × 35.5 cm × 18.5 cm; three rats per cage) under a 12-h light:12-h dark cycle (lights on at 6:00 a.m.), controlled temperature (23 ± 1°C), and with free access to water and the same commercial lab chow, with 23% protein, that was offered to their dams during the lactation period (Purina, Ltd.). In this study, we analyzed data from male pups only, i.e., 46 L_9 and 45 L_{15} rats.

Administration of Pilocarpine and/or Taurine

Pilocarpine hydrochloride, scopolamine methyl nitrate, and taurine were purchased from Sigma-Aldrich (St. Louis, MO, United States). All solutions were prepared daily, shortly before the injections, dissolved in 0.9% saline and administered from

PND35 to PND55. Each lactation condition gave rise to the following five subgroups: (1) Naïve (control group, without any treatment; n = 10 L_9 and 7 L_{15} rats); (2) Vehicle (second control group; treated with saline via i.p. and gavage; n = 10 L_9 and 8 L_{15} rats); (3) Taurine (300 mg/kg/day via gavage; n = 9 L_9 and 9 L_{15} rats), as previously described (Francisco and Guedes, 2015); (4) Pilocarpine (45 mg/kg/day via i.p.; n = 9 L_9 and 10 L_{15} rats), following Mendes-da-Silva et al. (2018); (5) Taurine plus pilocarpine (300 mg/kg/day via gavage and 45 mg/kg/day via i.p, respectively; n = 8 L_9 and 11 L_{15} rats). Scopolamine methyl nitrate, a muscarinic receptor antagonist, was administered (1 mg/kg/day via i.p.) in all groups, with the exception of the naïve group, 30 min before pilocarpine or saline administration to prevent the peripheral cholinergic effects elicited by pilocarpine (Peixinho-Pena et al., 2012). Immediately following pilocarpine administration, the animals were observed over 1 h to confirm the absence of seizures, as evaluated by the Racine scale (Racine, 1972). At this low dose of pilocarpine, 45 mg/kg/d, no behavioral signs of epilepsy were observed in the animals (all rats presented with Racine's score of zero).

Body and Brain Weights

Body weight was measured using a Filizola MF-3/1 electronic scale (3.0 kg capacity and precision of 0.5 g) at PND7, PND21, PND35, PND49, and PND60. The brain weights were obtained using an analytical balance (Shimadzu, model AUY220, with a sensitivity of up to 0.1 mg) at the end of the transcardiac perfusion procedure (see item 2.8).

Elevated Plus-Maze Test

The elevated plus-maze test (EPM) was conducted at PND56-58. The cross-shaped EPM apparatus was made of varnished wood and consisted of four arms (49 cm × 10 cm each) elevated 55 cm above the ground. Two of the arms were opened and the other two arms were closed (lateral walls of 50 cm of height), arranged perpendicular to the open ones. The arms of the apparatus were joined by a central 10 cm × 10 cm square platform. At the beginning of the test, each animal was placed individually in the central area of the labyrinth, with the head directed toward one of the open arms. Each animal was allowed to freely explore the labyrinth for 5 min, under dim light and in a sound-attenuated room. Before each test, the EPM apparatus was wiped with a paper cloth soaked in 70:30 ethanol:water solution. The animal's behavioral activity was recorded by a video camera. The video-recorded activity was stored in a computer and subsequently analyzed with the aid of the software ANY maze^{TM} (version 4.99 m), as previously described (Lima et al., 2017). The following parameters were considered: number of expelled fecal boluses, total distance traveled, total immobility time, number of entries into the open arms and the time spent in the open arms.

Analysis of Blood Glucose

As reported formerly (Francisco and Guedes, 2015), on PND59 the animals were fasted for 6 h and a drop of blood was collected from the animal's tail and used for measuring the blood glucose level using a portable glucose meter (G-TECH free).

CSD Recording

On the day of the electrophysiological recording (PND60–PND65), animals were anesthetized with a mixture of 1000 mg/kg urethane plus 40 mg/kg chloralose injected intraperitoneally. The level of anesthesia was monitored as previously described (Souza et al., 2015). Three trephine holes were drilled on the right side of the skull, aligned in the frontal-to-occipital direction and parallel to the midline. One hole was positioned on the frontal bone (2 mm in diameter) and used to apply the stimulus (KCl) to elicit CSD. The other two holes were positioned on the parietal bone (3–4 mm in diameter) and used to record the propagating CSD wave. Rectal temperature was continuously monitored and maintained at 37 ± 1°C by means of a heating blanket. The electrophysiological recording session lasted 6 h. We used two Ag–AgCl agar–Ringer electrodes (one in each hole) against a common reference electrode of the same type, placed on the nasal bones. The two initial recording hours constituted the baseline period, during which no KCl stimulus was applied and, consequently, no CSD was elicited. In the remaining four recording hours, CSD episodes were elicited at 30-min intervals by a 1-min application of a cotton ball (1–2 mm in diameter) soaked with 2% KCl solution (approximately 270 mM) to the anterior hole drilled at the frontal region. The ECoG and the DC (direct current) slow potential variation that is typical of CSD were continuously recorded on the cortical surface (on the intact dura mater) through a digital recording system (Biopac MP 150, Goleta, CA, United States). For each animal, the amplifier's gain was kept constant over the entire recording session, as previously reported (Lopes-de-Morais et al., 2014).

We calculated the CSD velocity of propagation from the time required for a CSD wave to pass the distance between the two cortical electrodes. In the two recording locations, we used the initial point of each DC-negative rising phase as the reference point to calculate the CSD velocities. In addition, we calculated the amplitude and duration of the CSD waves, as previously reported (Lima et al., 2017). As the basis for assessing the occurrence of CSD-dependent potentiation of spontaneous electrical activity, in each animal we compared the amplitudes of the ECoG before (baseline recording) and after starting to regularly elicit CSD. For this comparison, we analyzed six 10-min samples of the record at six time points of the ECoG, i.e., two samples from the baseline period and four samples from the CSD period. These samples were analyzed offline with the aid of an algorithm implemented in MATLAB^{TM} software (The Mathworks, Natick, MA, United States), version R2011B. This algorithm calculates the average amplitude of the ECoG waves. For each animal, the averaged ECoG amplitude was normalized in relation to the lowest sample value, which was considered equal to 1, expressed in relative units and compared before and after the episodes of CSD, as a basis for analyzing the occurrence of potentiation of the spontaneous electrical activity, as reported by our group previously (Lopes-de-Morais et al., 2014; Souza et al., 2015).

Lipid Peroxidation Analysis

After the CSD recording session, 50 of the still-anesthetized animals (26 L_9 and 24 L_{15} rats) were decapitated; their brains were rapidly removed and frozen. The cortical tissue was homogenized in a cold Tris buffer solution and centrifuged for 10 min at 1000 *g* at 4°C. Supernatants were used to estimate the lipid peroxidation by measuring malondialdehyde (MDA) levels using a thiobarbituric acid-reactive substances-based method (Ohkawa et al., 1979), which is a parameter to evaluate the lipid peroxidation. The reaction was developed by the sequential addition of 40 μl 8.1% sodium dodecyl sulfate, 300 μl 20% acetic acid (pH 3.5), and 300 μl 0.8% thiobarbituric acid solutions to the 300 μl homogenate aliquot in a boiling water bath for 50 min. After cooling the tubes with tap water, 300 μl of n-butanol was added to the sample. The tubes were centrifuged at 2500 *g* for 10 min, and the organic phase was read at 532 nm using a plate reader. Measurements were carried out in triplicate. Total protein concentrations were determined based on the Bradford protein assay; bovine serum albumin was used as a standard. MDA concentrations were determined by using 1,1,3,3-tetraethoxypropane as the standard and expressed as μg/mg protein, as previously reported (Mendes-da-Silva et al., 2014). All measurements were performed in triplicate.

Immunohistochemistry

After the CSD recording session, forty-one of the still-anesthetized animals were perfused with 0.9% saline solution followed by 4% paraformaldehyde diluted in 0.1M phosphate-buffered saline (pH 7.4). After being immersed in the fixative for 4 h, the brains were subjected to cryoprotection in sucrose solutions of increasing concentrations of 10%, 20% and 30%. Longitudinal serial sections (40-μm thickness) from the left (CSD-free) hemisphere were obtained at −20°C using a cryoslicer (Leica, 1850). One of the 41 animals was processed as a negative control for the immunolabeling. The remaining 40 rats included 20 from the L_9 and 20 from the L_{15} condition. In each lactation condition, 4 rats were from each one of the five treatment groups. Their brains were processed for microglia immunolabeling with anti- ionized calcium binding adapter molecule 1 (Iba1) antibody and astrocyte immunolabeling with anti-GFAP antibody.

For microglia immunostaining, sections were immunolabeled with a polyclonal antibody against Iba-1 (1:1500; anti-Iba-1, #019-19741; Wako Pure Chemical Industries, Ltd., Osaka, Japan). Free-floating sections were subjected to endogenous peroxidase blocking (2% H_2O_2 in 70% methanol for 10 min) and the sections were incubated for 1 h in blocking buffer (BB) solution containing 0.05M Tris-buffered saline (TBS; pH 7.4), 10% fetal calf serum, 3% bovine serum albumin, and 1% Triton X-100. The sections were then incubated overnight at 4°C with rabbit anti-Iba-1 (1:1500 diluted in BB solution). After three washes with TBS + 1% Triton X-100, sections were incubated at room temperature for 1 h with biotinylated anti-rabbit (1:500) secondary antibodies. Sections were then rinsed in TBS + 1% Triton X-100 and incubated with horseradish peroxidase streptavidin (1:500). The peroxidase reaction was visualized by incubating the sections in Tris buffer containing 0.5 mg/ml 3,3′-diaminobenzidine) and 0.33 μl/ml H_2O_2. The experimental protocol used for astrocyte immunostaining was similar to that applied for microglial labeling, mentioned above, with the following change: the primary antibody that was used (polyclonal rabbit anti-GFAP-D1F4Q-XP RABBIT MAB; Dako, Denmark) was specific for astrocyte labeling at the ratio of 1:2400.

Finally, the sections were mounted, dehydrated in graded alcohols, and coverslipped with Entellan® after xylene treatment. Densitometric analysis was performed on four parallel longitudinal sections for each animal. A Leica DMLS microscope coupled to a Samsung high-level color camera (model SHC-410NAD) was used to obtain digital images from the brain sections. Images from selected regions of interest (**Figure 1**) of the parietal cortex and CA1 hippocampus stained for Iba1 and GFAP were obtained using a 20 × microscope objective. In **Figure 1**, the protocol for double labeling was as follows: the sections were incubated simultaneously with mouse monoclonal anti-GFAP (Sigma-Aldrich; United States, #G3893, 1:1000) and rabbit polyclonal anti-neuN (Novus Biologicals, United States, #NBP1-77686, 1:200) for 18 h. Then, they were rinsed in phosphate buffer 0.1M (PB), pH 7.4 followed by incubation for 4 h with Cy-3 conjugated 546 labeled anti-mouse IgG and FITC-conjugated 488 anti-rabbit IgG (1:500; Jackson ImmunoResearch Labs, United States). After washing twice in PB, they were mounted onto gelatin coated slides and dried at 50°C for 5 min, cleared in xylene for 1 min and coverslipped with Entellan (Merck-Millipore, United States). Digital images were obtained using an epifluorescence microscope (Nikon coupled to a high-level color camera Model SHC-410NAD). The CA1 area and the parietal neocortex were chosen based on morphological and pharmacological studies in pilocarpine-treated animals (Curia et al., 2008; Rossi et al., 2013; Arisi et al., 2015; García-García et al., 2017). In each section, photomicrographs of four fields within the parietal cortex (layers 2 and 3) and three fields of the CA1 hippocampal region (including Stratum Oriens, Stratum Pyramidalis, Stratum Radiatum, and Stratum Lacunosum Moleculare) were analyzed, using the ImageJ software (National Institutes of Health, United States, version 1.46r). Care was taken to obtain the digital images using the same light intensity. The color images were first converted into a gray scale. Based on the color difference, an algorithm of the program, devoted to area selection, identified the darker areas (marked cells) in relation to the lighter areas (background), and the total marked area was calculated. The threshold for selection was manually adjusted such that the background was not marked. All sections that were photographed at the same magnification displayed the same total area in the photographs; therefore, the ratios between the labeled cells' area and the total picture area could be directly compared. The labeled area was expressed as percentage of the total area in the picture. The immunoreactivity intensity was obtained in the program by calculating the mean gray value (MGV) within the selected area. The MGV can vary numerically from 0 (darkest) to 255 (lightest). Therefore, the reactivity intensity was given by the difference (255-MGV). By multiplying this value by the marked area, we came to the figure (arbitrary unit) that indicated how

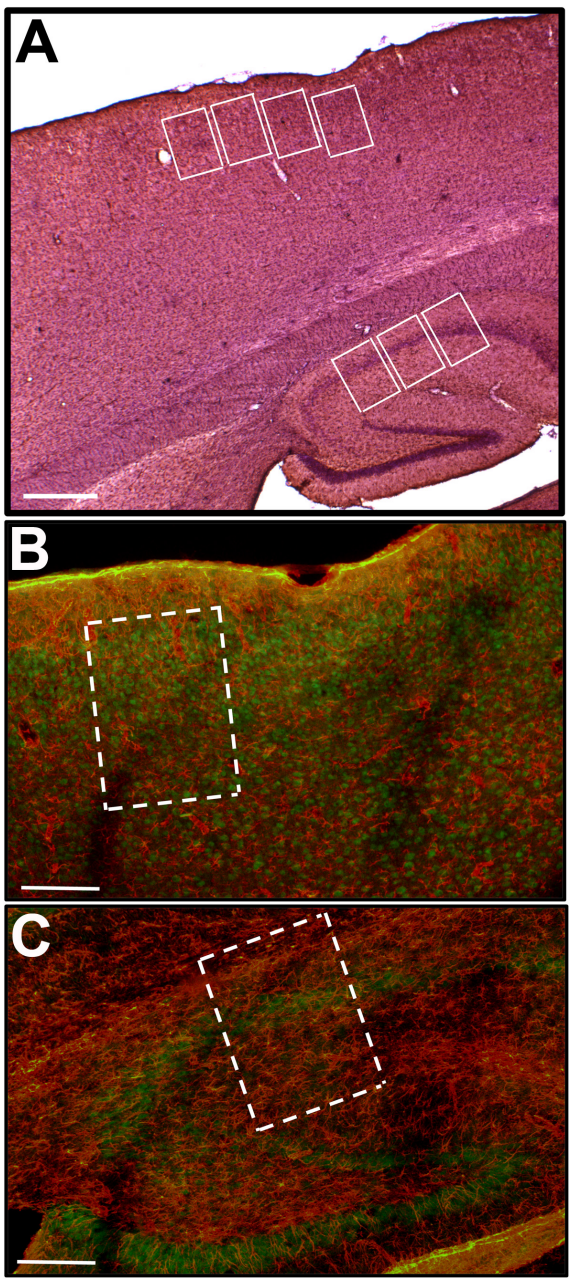

FIGURE 1 | (A) Shows a low magnification (2.5×) image of a representative parasagittal section immunostained for GFAP and counterstained with 5% Hematoxylin. The white rectangles illustrate the position of the sampling windows (470 μm × 340 μm) in the cerebral cortex and CA1 region of hippocampus. Double staining for GFAP (red) and Neu-N (green) was performed in order to better define the cytoarchitectonic limits of layers 2 and 3 in the cerebral cortex (**B**; 10×) as well as the four layers (Stratum Oriens, Stratum Pyramidalis, Stratum Radiatum, and Stratum Lacunosum Moleculare) in the hippocampus (**C**; 10×), where the quantitative analysis was carried out. Scale bars equal 500 μm in **(A)**, and 200 μm in **(B,C)**.

much of the gray area in the image was due to cell labeling, i.e., mathematically, the more intense the labeling, the greater is the arbitrary unit value. Total immunoreactivity expressed as arbitrary units as well as the percentage of the area occupied by the immunolabeled cells were analyzed, as previously reported (Lima et al., 2017).

Statistical Analysis

Results in all groups are expressed as the means ± standard deviations (SD). Intergroup differences and interactions were first analyzed using a two-way ANOVA and thereafter a MANOVA. In the ANOVA analysis, we consider, as factors, nutritional status (L9 and L15) and treatment (naïve, vehicle, taurine, pilocarpine, and taurine + pilocarpine). In the MANOVA analysis we included nutritional status (L_9 and L_{15}), pilocarpine administration and taurine treatment as factors. This was followed by a *post hoc* test (Holm–Sidak) where indicated. ECoG amplitude values before and after CSD for each animal were normalized and expressed in relative units. Differences in these amplitudes, before and after CSD, were analyzed with the paired *t*-test, using ANOVA followed by the Holm–Sidak test for intergroup comparisons when indicated. Differences with $p < 0.05$ were accepted as significant. Since MANOVA confirmed the differences that had been previously indicated by two-way ANOVA, we kept the ANOVA statistics figures in the description of results.

RESULTS

The two control groups – naïve (no treatment) and vehicle (that received gavage and intraperitoneal injection of saline and scopolamine) presented comparable values.

Body and Brain Weights

As shown in **Figure 2A**, in all treatment groups (Nv, V, T, P, and T + P) ANOVA showed a main effect of the lactation condition on body weight ($p < 0.001$). The L_{15} animals presented with lower body weights compared with the corresponding L_9 groups. In the control condition (naïve and vehicle groups) the weight reduction ranged from 11.9 to 32.5%. In the L_9 condition, intergroup differences were observed at PND49 only [$F(4,85) = 15.048$; $p < 0.001$]. At that age, the treatment with pilocarpine was associated with a weight reduction compared to the respective L_9 control groups. In the unfavorable (L_{15}) lactation condition, pilocarpine and taurine + pilocarpine treatments reduced body weight at PND49 and PND60 [$F(4,79) = 11.993$; $p < 0.001$].

For the brain weight data (**Figure 2B**), ANOVA showed a main effect of the lactation condition on brain weight [$F(1,41) = 51.659$, $p < 0.001$]. L_{15} animals presented with a lower brain weight compared to the respective L_9 groups. The average weight reduction was 17.03% and was independent of the treatment.

Blood Glucose Level

In the L_{15} condition, the Nv, V, and T groups displayed significantly lower glycemia than the corresponding L_9 groups [$F(1,77) = 33.484$; $p < 0.001$]. Treatment with P and T + P reduced blood glucose levels in the L_9, but not in the L_{15} groups

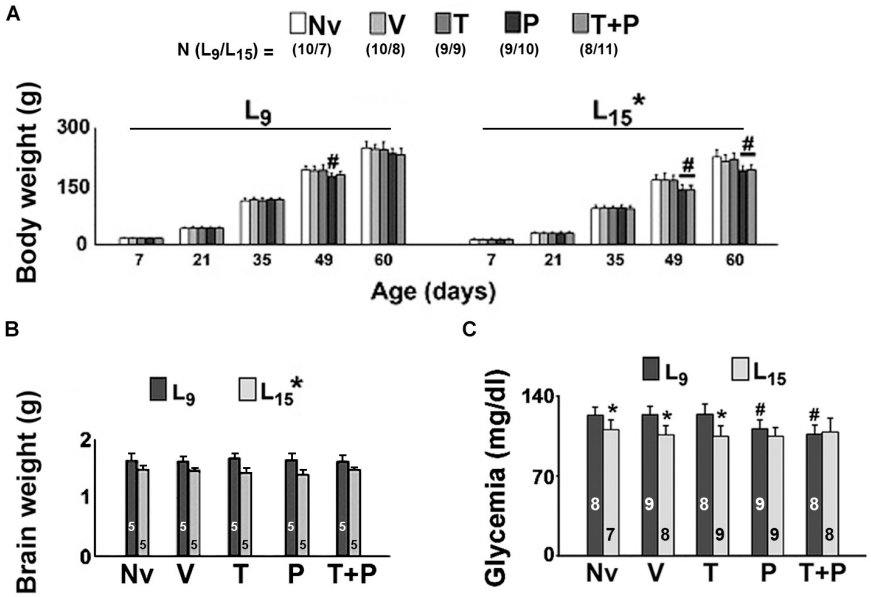

FIGURE 2 | Body **(A)** and brain weights **(B)**, and glycemia **(C)** of male rats previously suckled in litters with 9 and 15 pups (respectively, L_9 and L_{15} condition). Naïve (Nv) = no treatment; Vehicle (V) = scopolamine methyl nitrate 1 mg/kg/day + saline; Taurine (T) = 1 mg/kg/day scopolamine methyl nitrate + 300 mg/kg/day of taurine; Pilocarpine (P) = 1 mg/kg/day scopolamine methyl nitrate + 45 mg/kg/day of pilocarpine; Taurine + pilocarpine (T + P) = 1 mg/kg/day scopolamine methyl nitrate + 300 mg/kg/day of taurine + 45 mg/kg/day of pilocarpine. All drugs were dissolved in 0.9% saline. Administration occurred from postnatal days 35 to 55. Data are mean ± standard deviation. $^*p < 0.001$ compared with the corresponding L_9 condition. $^\#p < 0.001$ compared with the control groups in the same lactation condition (ANOVA plus Holm–Sidak test). In panels **(B,C)**, the numbers in each bar indicate the sample size of each measurement.

[$F(4,77) = 4.122$; $p = 0.004$] compared with the corresponding Nv, V, and T groups. Data on glycemia are illustrated in **Figure 2C**.

Behavioral Activity in the Elevated Plus-Maze

The effect of administration of taurine and/or pilocarpine on behavioral activity in the EPM test is shown in **Figure 3**. Regarding the time spent in the open arms, ANOVA indicated a main effect of treatment [$F(4,76) = 10.653$, $p < 0.001$]. In the L_9 condition, the Holm–Sidak test revealed that the group treated with taurine remained longer in the open arms compared to the other groups in the same lactation condition. In the L_{15} condition, the taurine-treated rats remained significantly longer in the EPM open arms, compared to the Nv, V and P groups, but not to the T + P groups.

Regarding the number of entries into the open arms, ANOVA revealed a main effect of treatment [$F(4,81) = 6.259$; $p < 0.001$] in the L_{15} condition only; the Holm–Sidak test indicated that the groups P and T + P entered less frequently into the open arms, compared to the Nv, V, and T groups. Statistical analysis also detected interactions between the treatment and lactation condition [$F(4,81) = 3.339$, $p = 0.014$], revealing that the group T-L_{15} entered the open arms a higher number of times and the group T + P-L_{15} entered a lower number of times when compared to their respective L_9 groups.

In relation to the total distance traveled by the animals in the EPM, ANOVA identified a main effect of the lactation condition [$F(1,80) = 5.445$, $p = 0.022$], treatment [$F(4,80) = 19.010$, $p < 0.001$] and an interaction between these two factors [$F(4,80) = 2.697$, $p = 0.036$]. The T-L_9 group traveled a greater distance compared to the groups Nv, V, P, and T + P of the same lactation condition and the group P-L_9, a smaller distance compared to Nv, V, and T-L_9. In the L_{15} condition the groups P and T + P ran a smaller distance compared to the groups Nv, V, and T. The group Nv-L_{15} ran a greater distance when compared to the group Nv-L_9.

Analysis of variance (ANOVA) identified a main effect of treatment for the ***total immobility*** of the animals [$F(4,81) = 8.137$; $p < 0.001$] and the Holm–Sidak test revealed that the P group had the longest immobility compared to the Nv, V, and T groups in both lactation conditions.

Regarding the number of fecal boluses expelled by each animal during the behavioral test, there was a main effect of treatment [$F(4.81) = 6.498$, $p < 0.001$] and the Holm–Sidak test revealed that the P-L_9 group expelled a higher number of fecal boluses compared to the corresponding Nv, V, and T groups. In the L_{15} condition, the P group expelled a higher number of fecal boluses compared with the corresponding T and T + P groups.

CSD Parameters

CSD-Induced ECoG Potentiation

Examples of ECoG amplitude at recording points 1 (E1) and 2 (E2), before and after CSD, are shown in **Figure 4A**. In all groups, the paired *t*-test showed that for each animal the ECoG amplitude in the CSD period was significantly ($p < 0.005$) higher than in the baseline period (potentiation). This effect was observed for

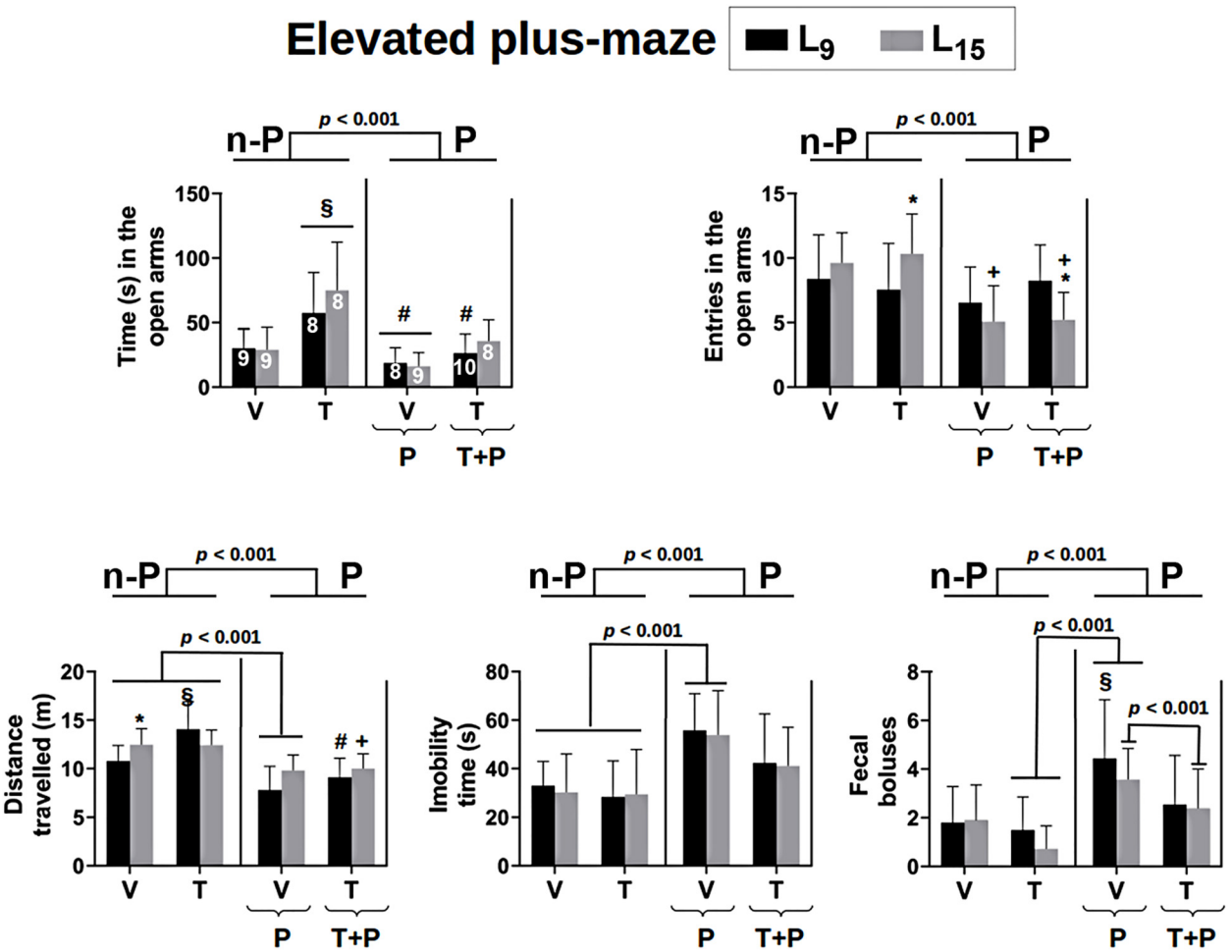

FIGURE 3 | Behavioral activity in the elevated plus-maze test of 56-day-old male rats previously suckled in litters with 9 and 15 pups (respectively, L_9 and L_{15} condition). Group description is as in **Figure 2**. n-P = no pilocarpine; P = pilocarpine; T = taurine; V = vehicle. Data are mean ± standard deviation. $^{*}p < 0.001$ compared to the corresponding L_9 condition. § $p < 0.001$ in comparison to groups Nv and V in the same lactation condition. $^{\#}p < 0.001$ compared to group T of the same lactation condition. $^{+}p < 0.001$ compared to groups V and T of the same lactation condition (ANOVA plus Holm–Sidak test). The number in each bar of the upper-left graphic indicates the sample size, which applies to all graphics.

both recording points E1 and E2. The data on CSD-related ECoG potentiation are presented in **Table 1**.

CSD Velocity of Propagation

In the L_9 animals, CSD velocities (mean ± SD in mm/min) in the Nv, V, T, P, and T + P groups were respectively 3.71 ± 0.12, 3.73 ± 0.12, 3.15 ± 0.17, 3.12 ± 0.15, and 3.02 ± 0.20. ***In the L_{15} animals***, the CSD velocities for the Nv, V, T, P, and T + P groups were respectively 4.20 ± 0.16, 4.17 ± 0.15, 3.40 ± 0.11, 3.04 ± 0.19, and 2.94 ± 0.22. ANOVA indicated a main effect of the lactation condition [$F(1,81) = 34.876$; $p < 0.001$], and *post hoc* (Holm–Sidak) test comparisons showed that the velocities were higher in the L_{15} groups compared to the L_9 for the Nv, V, and T groups. ANOVA also detected a main effect of treatment [$F(4,81) = 150.675$; $p < 0.001$], and *post hoc* testing revealed that T, P, and T + P treatment significantly lowered the CSD propagation velocity compared with the corresponding Nv and V controls. The ANOVA revealed interactions between nutritional status and treatment, in which the L_{15} P and T + P groups had lower CSD propagation velocities compared to the Nv, V, and T groups [$F(4,81) = 12.739$, $p < 0.001$], and no difference was observed between the L_9 and L_{15} animals treated with P and T + P. Data on the CSD propagation velocity are given in **Figure 4B**.

Amplitude and Duration of the CSD Negative Slow Potential Change

Table 2 shows data on the amplitude and duration of the negative slow potential change, which is the hallmark of CSD. ANOVA indicated a main effect of the lactation condition ***on the CSD amplitude*** [$F(1,81) = 11.408$; $p < 0.001$], and a *post hoc* (Holm–Sidak) test comparison showed that the amplitudes were higher in the naïve, vehicle, and taurine L_{15} groups compared to the corresponding L_9 groups. The factor treatment also affected the

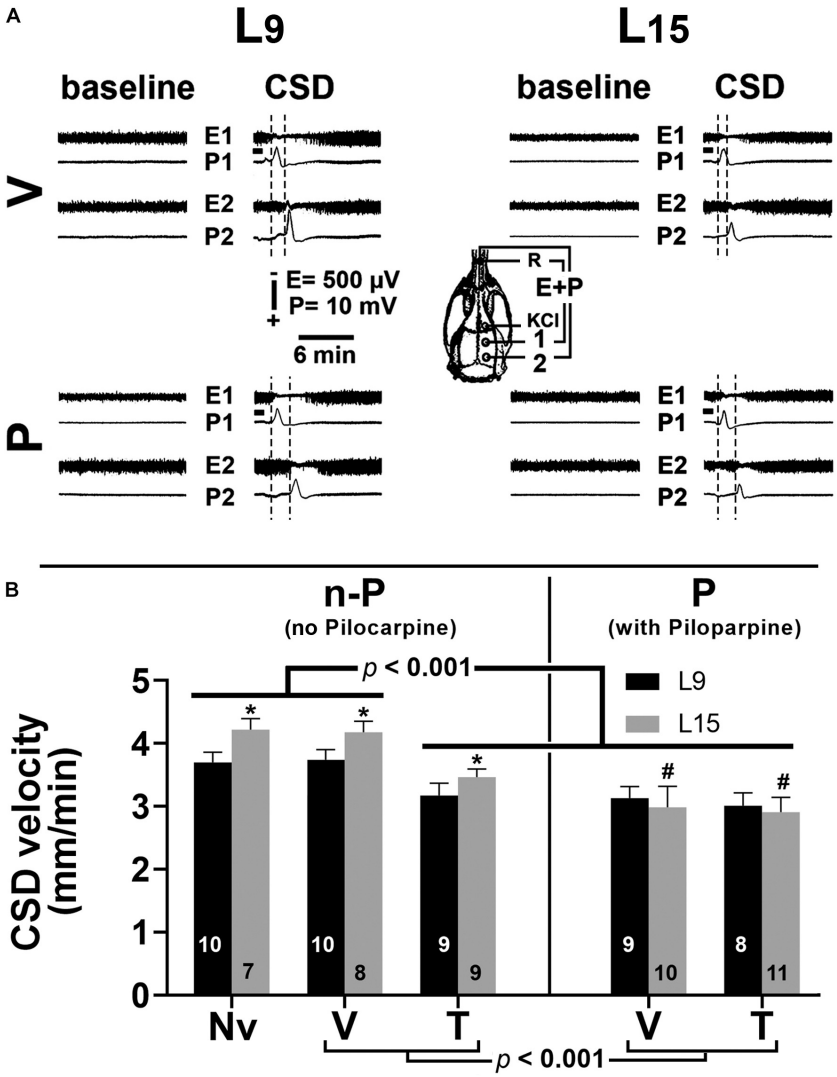

FIGURE 4 | (A) Electrocorticogram (E) and slow potential change (P) on two points of the surface of the right hemisphere before (baseline period) and after the passage of cortical spreading depression (CSD period) in four 60–65-day-old male rats previously suckled in litters with 9 and 15 pups (respectively, L_9 and L_{15} condition). Traces are from two vehicle and two pilocarpine-treated rats in the L_9 (left) and L_{15} nutritional condition (right). Group description is as in **Figure 2**. The skull diagram shows the recording positions 1 and 2, from which the traces marked with the same numbers were obtained. The position of the common reference electrode (R) on the nasal bones and the application point of the CSD-eliciting stimulus (KCl) are also shown. CSD was elicited in the frontal cortex by chemical stimulation (a 1- to 2-mm diameter cotton ball soaked with 2% KCl) applied for 1 min on the intact dura mater, as indicated by the horizontal bars. ECoG amplitude in the CSD period is higher (potentiation), in comparison with the baseline amplitude for the same animal. **(B)** Cortical spreading depression velocity (mm/min) of 60–65-day-old male rats previously suckled in litters with 9 and 15 pups (respectively, L_9 and L_{15} condition). Administration of vehicle, taurine and pilocarpine occurred from postnatal day 35 to 55. Data are mean ± standard deviation. $^*p < 0.001$ compared with the corresponding L_9 condition. $^{\#}p < 0.001$ compared to groups Nv (naïve), V (vehicle), and T (taurine) of the same lactation condition (ANOVA plus Holm–Sidak test). The number in each bar indicates the sample size.

amplitude [$F(4,81) = 3.274$; $p = 0.015$], and a *post hoc* test showed that the amplitude was lower in the pilocarpine-treated L_{15}, but not in the L_9 group, compared with the corresponding naïve, vehicle, and taurine of the same lactation condition. ANOVA also confirmed an interaction between both factors [$F(4,81) = 2.934$; $p = 0.026$].

Analysis of ***CSD duration*** indicated a main effect of the lactation condition [$F(1,81) = 115.827$; $p < 0.001$] and treatment [$F(4,81) = 5.291$; $p < 0.001$]. The Holm–Sidak test indicated a shorter duration in the L_{15} groups compared with the corresponding L_9 groups and a longer duration in the L_{15} pilocarpine-treated animals compared with the corresponding naïve, vehicle, taurine, and taurine + pilocarpine of the same lactation condition.

MDA Levels in the Cortex and Hippocampus

Measurements of MDA levels in the cerebral cortex and hippocampus are shown in **Figure 5**. ANOVA revealed no significant differences. MDA levels ***in the cortex*** in the L_9 animals (mean ± SD in nmol/mg of protein) in the naïve, vehicle, taurine,

TABLE 1 | Amplitude of ECoG, at the recording points 1 and 2, before (baseline period) and during the period of cortical spreading depression elicitation (CSD period), in rats previously suckled in litters with 9 and 15 pups (respectively, condition L_9 and L_{15}).

Group	ECoG 1		ECoG 2	
	Baseline	CSD	Baseline	CSD
L_9				
Naïve	1.01 ± 0.01(10)	1.63 ± 0.25*	1.04 ± 0.04(10)	1.28 ± 0.21*
Vehicle	1.04 ± 0.04(10)	1.67 ± 0.22*	1.04 ± 0.04(10)	1.33 ± 0.14*
Taurine	1.00 ± 0.00(9)	1.40 ± 0.11*#	1.02 ± 0.04(9)	1.32 ± 0.13*
Pilocarpine	1.01 ± 0.03(9)	1.30 ± 0.08*#	1.02 ± 0.03(9)	1.22 ± 0.13*
Taurine + pilocarpine	1.02 ± 0.04(8)	1.43 ± 0.16*#	1.05 ± 0.08(8)	1.35 ± 0.09*
L_{15}				
Naïve	1.02 ± 0.04(7)	1.43 ± 0.22* +	1.03 ± 0.05(7)	1.25 ± 0.15*
Vehicle	1.05 ± 0.05(8)	1.46 ± 0.11* +	1.01 ± 0.02(8)	1.25 ± 0.14*
Taurine	1.08 ± 0.10(9)	1.47 ± 0.25*	1.04 ± 0.05(9)	1.24 ± 0.09*
Pilocarpine	1.03 ± 0.10(8)	1.21 ± 0.12*§	1.03 ± 0.06(8)	1.16 ± 0.11*
Taurine + pilocarpine	1.03 ± 0.05(11)	1.39 ± 0.10*	1.03 ± 0.06(11)	1.27 ± 0.09*

*The groups received no treatment (naïve; Nv), or vehicle (saline; V), or taurine (T), or pilocarpine (P), or taurine + pilocarpine (T + P). These treatments occurred from PND35 to PND55, and a 6-h long ECoG recording session occurred at PND60-65. In the initial 2 h of the recording session, no CSD was elicited (baseline period). This was followed by 4 h recording, during which CSD was evoked at 30 min intervals (CSD period). From each hour of recording, a 10-min ECoG sample was analyzed by an algorithm implemented in MATLAB™ software, which calculated the average ECoG amplitude. Data are expressed as the mean ± SD and presented as relative units (values of normalized amplitudes with respect to the lowest value, which was considered equal to 1). *$p < 0.005$ compared to the baseline amplitude in the same lactation condition (paired t-test). #$p < 0.001$ compared to the naïve and vehicle groups in the same lactation condition. §$p < 0.001$ compared to the other four groups in the same lactation condition. +$p < 0.001$ compared to the corresponding L_9 condition (ANOVA followed by the Holm–Sidak test).*

TABLE 2 | Amplitude and duration of the negative slow potential change of CSD in male rats previously suckled in litters with 9 and 15 pups (respectively, L_9 and L_{15} condition).

Group	Amplitude (mV)	Duration (s)
L_9		
Naïve	8.5 ± 1.6(10)	69.2 ± 2.6(10)
Vehicle	8.6 ± 1.0(10)	69.2 ± 2.6(10)
Taurine	8.6 ± 1.2(9)	69.3 ± 2.2(9)
Pilocarpine	8.4 ± 1.1(9)	70.9 ± 2.7(9)
Taurine + pilocarpine	9.3 ± 1.5(8)	70.3 ± 1.9(9)
L_{15}		
Naïve	10.6 ± 3.1(7)*	64.7 ± 0.6(7)*
Vehicle	10.8 ± 1.8(8)*	65.1 ± 0.8(11)*
Taurine	10.7 ± 1.6(9)*	65.1 ± 0.5(9)*
Pilocarpine	7.5 ± 1.9(10) +	67.9 ± 1.0(10)*#
Taurine + pilocarpine	9.6 ± 2.2(11)	65.2 ± 1.3(11)*

*Naïve = no treatment; Vehicle = scopolamine methyl nitrate 1 mg/kg/day + saline; Taurine = scopolamine methyl nitrate 1 + 300 mg/kg/day of taurine; Pilocarpine = scopolamine methyl nitrate 1 + 45 mg/kg/day of pilocarpine; Taurine + pilocarpine = scopolamine methyl nitrate 1 + 300 mg/kg/day of taurine + 45 mg/kg/day of pilocarpine. All drugs were dissolved in 0.9% saline. Data are expressed as the mean ± standard deviation, with the number of animals in parentheses. *$p < 0.001$ compared with the corresponding L_9 condition. #$p = 0.015$ compared with naïve, vehicle, and taurine groups in the same suckling condition. +$p < 0.001$ compared with all other groups in the same suckling condition (ANOVA plus Holm–Sidak test).*

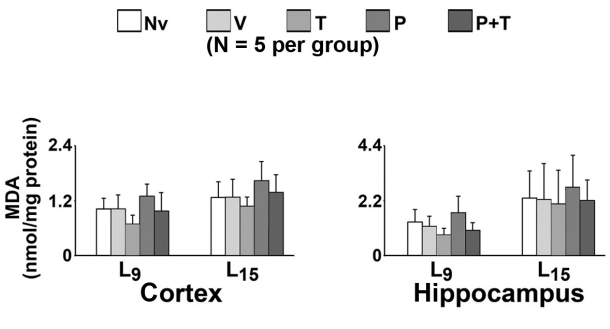

FIGURE 5 | Malondialdehyde (MDA) levels (nmol/mg protein) of 60–65-day-old male rats previously suckled in litters with 9 and 15 pups (respectively, L_9 and L_{15} condition). Group description as in **Figure 2**. Data are mean ± standard deviation of 5 rats per group. Measurements were performed in triplicate. No significant differences were observed.

pilocarpine, and taurine + pilocarpine groups were respectively 1.02 ± 0.23, 1.02 ± 0.30, 0.68 ± 0.20, 1.30 ± 0.26, and 0.97 ± 0.42. In the L_{15} animals, the MDA levels were respectively 1.27 ± 0.34, 1.27 ± 0.39, 1.07 ± 0.20, 1.63 ± 0.42, and 1.36 ± 0.39.

Malondialdehyde levels ***in the hippocampus*** in the L_9 animals (mean ± SD in nmol/mg of protein) in the naïve, vehicle, taurine, pilocarpine, and taurine + pilocarpine groups were respectively 1.34 ± 0.50, 1.16 ± 0.41, 0.84 ± 0.25, 1.72 ± 0.66 and 1.00 ± 0.31. In the L_{15} animals, the MDA levels were respectively 2.30 ± 1.08, 2.24 ± 1.44, 2.06 ± 1.36, 2.74 ± 1.26, and 2.21 ± 0.80.

Immunohistochemistry for Microglia and Astrocytes

The effect of administration of taurine and/or pilocarpine on the percentage of labeled area and immunoreactivity of astrocyte and microglia cells in the parietal cortex and CA1 hippocampus is shown in the **Figure 6** for Iba1, and 7 for GFAP. All data are described as the mean ± standard deviation from four animals per group.

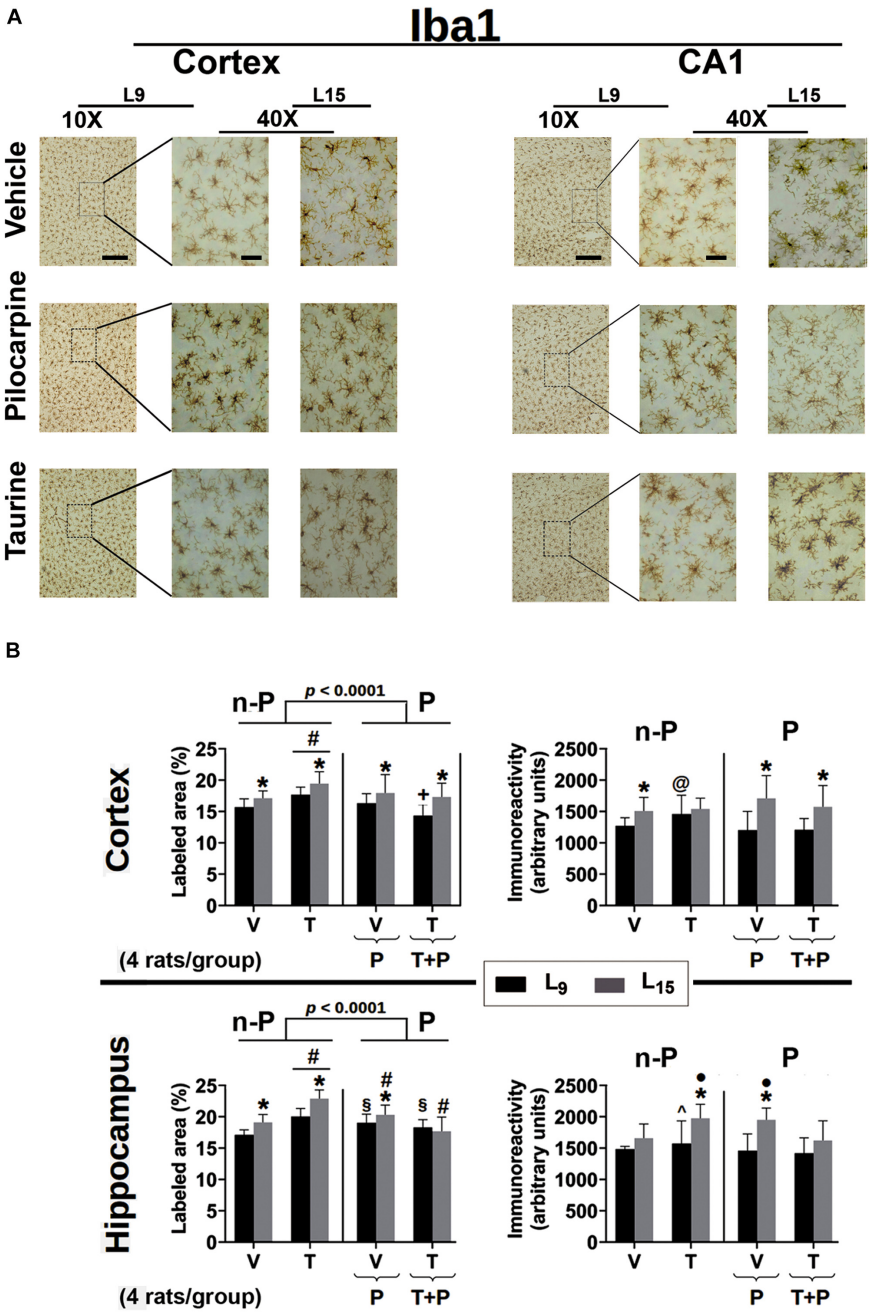

FIGURE 6 | (A) Photomicrographs of immunolabeled Iba1-positive cells in the left cortex and hippocampus of 60–65-day-old male rats previously suckled in litters with 9 and 15 pups (respectively, L_9 and L_{15} condition). Group description is as in **Figure 2**. Scale bars = 50 and 10 μm for the low (10×) and high magnification pictures (40×), respectively. **(B)** Percent labeled area (left panels) and immunoreactivity as arbitrary units (right panels) of immunolabeled Iba1-positive cells in the cortex (upper panels) and hippocampus (lower panels) of 60–65-day-old male rats previously suckled in litters with 9 and 15 pups (respectively, L_9 and L_{15} condition). Group description is as in **Figure 2**. n-P = no pilocarpine; P = with pilocarpine. Data are mean ± standard deviation of four rats per group. $^{*}p < 0.001$ compared to the corresponding L_9 condition. $^{§}p < 0.001$ compared to groups V in the same lactation condition. $^{\#}p < 0.001$ compared to the other three groups in the same lactation condition. $+p < 0.001$ compared to groups V and P of the same lactation condition. $^{\bullet}p < 0.001$ compared to groups V and T + P of the same lactation condition. $@p < 0.001$ compared to groups P and T + P of the same lactation condition. $\hat{}p < 0.001$ compared to group Nv of the same lactation condition (ANOVA followed by the Holm–Sidak test).

Iba-1 Immunohistochemistry

Data from Iba1 immunohistochemistry are presented in **Figure 6**. In the ***cerebral cortex***, ANOVA showed in all groups a main effect of the lactation condition on the ***percentage of Iba1-labeled area*** [$F(1,371) = 5.074$; $p < 0.001$] and *post hoc* (Holm–Sidak) test comparisons showed that the animals of the

L_{15} condition had a greater percentage of labeled area when compared to the corresponding L_9 groups. ANOVA also detected a main effect of treatment [$F(4,371) = 23.681$; $p < 0.001$], and *post hoc* testing revealed in the two lactation conditions that taurine treatment significantly increased the percentage of labeled area, compared to the other four treatment groups. On the other hand, in the L_9 condition, the taurine + pilocarpine treated group presented with a lower percentage of the labeled area when compared with the naïve, vehicle, and pilocarpine-treated groups. Regarding ***the Iba1 immunoreactivity in the cerebral cortex***, ANOVA indicated a main effect of the lactation condition [$F(1,355) = 59.454$; $p < 0.001$] and *post hoc* testing comparisons showed that the animals of the L_{15} condition had greater immunoreactivity when compared to the corresponding L_9 condition, ***except for the taurine-treated groups***. ANOVA also revealed interactions between nutritional status and treatment, in which the pilocarpine and taurine + pilocarpine groups ***in the L_{15}, but not in the L_9 condition*** had higher immunoreactivity compared to the other groups [$F(4,355) = 5.954$; $p < 0.001$].

In the hippocampus, ANOVA revealed a main effect of the lactation condition on the percentage of the Iba-1 labeled area [$F(1,376) = 64.536$; $p < 0.001$]; the *post hoc* (Holm–Sidak) test comparisons showed that the groups naïve, vehicle, taurine, and pilocarpine in the L_{15} condition had a greater percentage of labeled area when compared to the corresponding L_9 groups. ANOVA revealed a main effect of treatment [$F(4,376) = 65.946$; $p < 0.001$], and *post hoc* testing detected that in the two lactation conditions taurine treatment significantly increased the percentage of Iba1 labeled area compared to the other four groups.

Furthermore, in the L_9 condition, pilocarpine and taurine + pilocarpine treatments resulted in a higher percentage of labeled area when compared with the L_9 naïve and vehicle. In the L_{15} condition, the pilocarpine group displayed a percentage of labeled area that was greater than in the naïve, vehicle and taurine + pilocarpine groups, but was lower than in the taurine group; the L_{15} taurine + pilocarpine group had the lowest percentage of Iba1-labeled area. ANOVA also revealed interactions between nutritional status and treatment [$F(4,376) = 11.742$; $p < 0.001$], in which the L_{15} taurine group had a higher percentage of Iba1 labeled cortical area compared to the other four groups.

Regarding the ***Iba-1 immunoreactivity in the hippocampus***, ANOVA indicated a main effect of the lactation condition [$F(1,350) = 59.217$; $p < 0.001$] and *post hoc* testing comparisons showed that the animals of the groups naïve, taurine, and pilocarpine in the L_{15} condition had greater immunoreactivity when compared to the corresponding L_9 groups. ANOVA revealed a main effect of taurine treatment [$F(4,350) = 14.626$; $p < 0.001$], and *post hoc* testing showed in the L_9 condition a significantly greater immunoreactivity of the taurine-treated animals when compared to the corresponding naïve group. ***Among the L_{15} animals***, the taurine- and pilocarpine-treated groups had higher Iba1 immunoreactivity compared with the naïve, vehicle, and taurine + pilocarpine groups. ANOVA also identified interactions between nutritional status and treatment, in which the L_{15} taurine and pilocarpine groups had higher immunoreactivity compared to the other L_{15} groups [$F(4,350) = 4.545$; $p < 0.001$].

GFAP Immunohistochemistry

Data on GFAP immunohistochemistry are shown in **Figure 7**. ANOVA showed a main effect of the lactation condition on the ***percentage of GFAP-labeled areas in the cerebral cortex*** [$F(1,434) = 63.276$; $p < 0.001$] and *post hoc* (Holm–Sidak) test comparisons showed that the naïve, vehicle, and pilocarpine-treated groups of the L_{15} condition had a lower percentage of labeled area when compared to the corresponding L_9 groups. ANOVA also indicated a main effect of treatment [$F(4,434) = 6.988$; $p < 0.001$], and *post hoc* testing revealed that, in the L_9 condition, taurine + pilocarpine treatment significantly lowered the percentage of labeled area compared to the naïve, vehicle and pilocarpine groups, and in the L_{15} condition, taurine treatment resulted in a lower percentage of labeled area when compared with the other four groups in the L_{15} condition. ANOVA also revealed interactions between nutritional status and treatment [$F(4,434) = 5.744$; $p < 0.001$], in which the L_{15} taurine group had a lower percentage of labeled area in comparison with all other L_{15} groups.

In relation to GFAP ***immunoreactivity quantification in the cerebral cortex***, in the L_9 condition ANOVA revealed a main effect of treatment [$F(4,434) = 6.147$; $p < 0.001$], and *post hoc* testing showed greater immunoreactivity in the groups treated with taurine, pilocarpine, and taurine + pilocarpine when compared to the naïve and vehicle-treated controls. Among ***the L_{15} groups***, taurine animals presented with lower immunoreactivity compared to the other four groups. ANOVA also demonstrated interactions between nutritional status and treatment, in which the L_{15} taurine group had lower immunoreactivity compared to the corresponding naïve, vehicle groups, and with the taurine L_9 group [$F(4,434) = 10.551$; $p < 0.001$].

Regarding ***GFAP-labeled cells in the hippocampus***, in the L_9 condition ANOVA showed a main effect of treatment [$F(4,401) = 14.819$; $p < 0.001$], and *post hoc* testing revealed that taurine and pilocarpine treatments significantly lowered the percentage of labeled area compared to the naïve, vehicle, and taurine + pilocarpine groups. ***In the L_{15} condition,*** taurine and taurine + pilocarpine treatments resulted in a higher percentage of the labeled area when compared with the naïve, vehicle, and pilocarpine groups. ANOVA also revealed interactions between nutritional status and treatment [$F(4,401) = 15.918$; $p < 0.001$], in which the taurine treatment resulted in a greater percentage of labeled area in the L_{15} but not in the L_9 condition. Regarding the ***GFAP immunoreactivity in the hippocampus,*** ANOVA detected a main effect of the lactation condition [$F(1,359) = 107.184$; $p < 0.001$], and the *post hoc* testing comparisons showed that the groups naïve, vehicle, taurine, and pilocarpine in the L15 condition had greater immunoreactivity when compared to the corresponding L9 groups. The ANOVA detected a main effect of treatment [$F(4,359) = 53.366$; $p < 0.001$], and *post hoc* testing showed a significantly lower immunoreactivity in the L_9 pilocarpine group compared to the other L_9 groups. *In the L_{15} condition*, taurine animals presented with greater

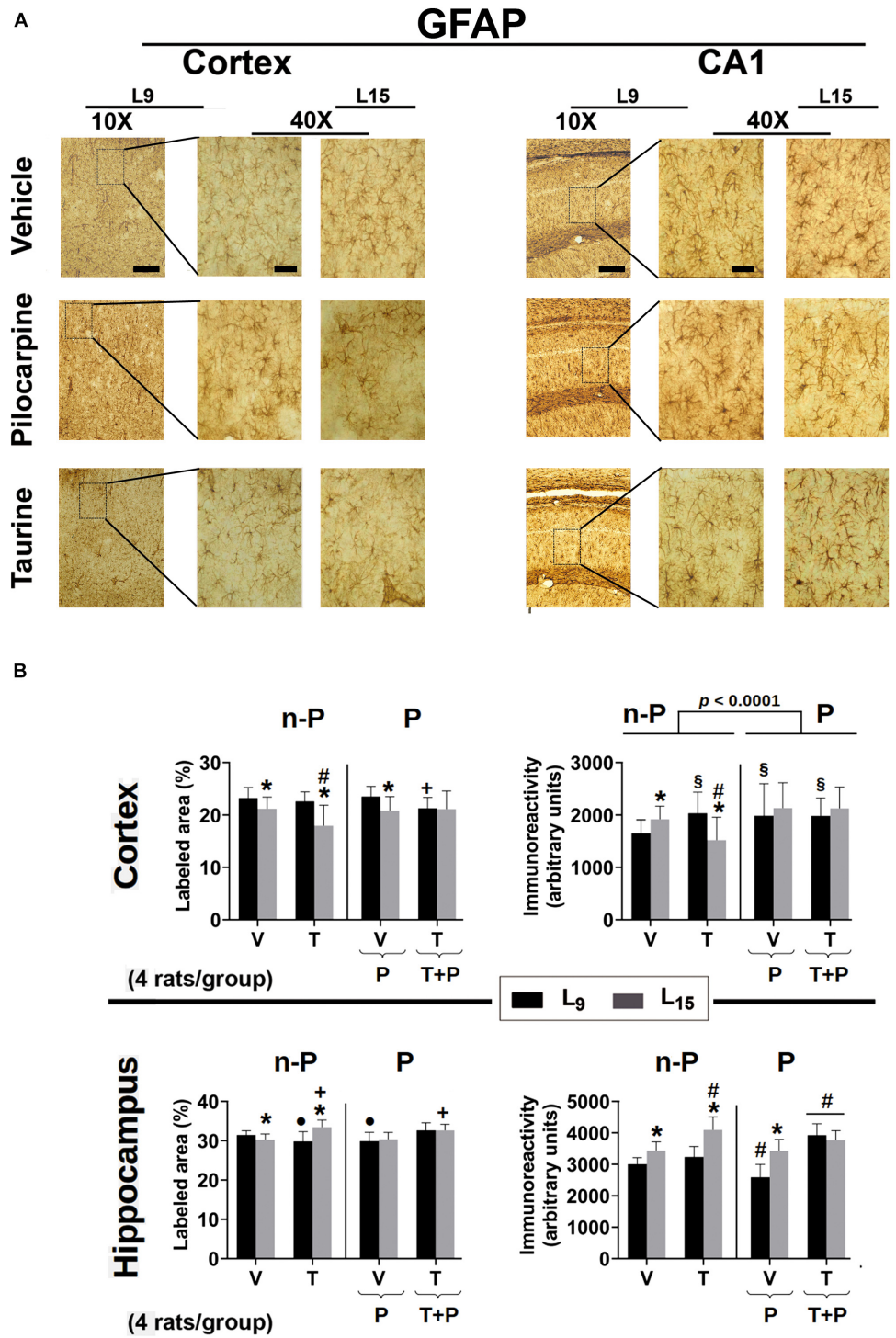

FIGURE 7 | (A) Photomicrographs of immunolabeled GFAP-positive cells in the left cortex and hippocampus of 60–65-day-old male rats previously suckled in litters with 9 and 15 pups (respectively, L_9 and L_{15} condition). Group description is as in **Figure 2**. Scale bars = 50 and 10 μm for the low (10×) and high magnification pictures (40×), respectively. **(B)** Percent labeled area (left panels) and immunoreactivity as arbitrary units (right panels) of immunolabeled GFAP-positive cells in the cortex (upper panels) and hippocampus (lower panels) of 60–65-day-old male rats previously suckled in litters with 9 and 15 pups (respectively, L_9 and L_{15} condition). Group description is as in **Figure 2**. n-P = no pilocarpine; P = with pilocarpine. Data are mean ± standard deviation of four rats per group. $^{*}p < 0.001$ compared to the corresponding L_9 condition. $^{§}p < 0.001$ in comparison to group V in the same lactation condition. $^{\#}p < 0.001$ compared to the other three groups in the same lactation condition. $^{+}p < 0.001$ compared to groups V and P of the same lactation condition. $^{\bullet}p < 0.001$ compared to groups V and T + P of the same lactation condition (ANOVA followed by the Holm–Sidak test).

immunoreactivity compared to the other four L_{15} groups. In both lactation conditions, taurine + pilocarpine treatment resulted in greater immunoreactivity compared to the other nutrition-matched treatment groups. ANOVA also detected interactions between nutritional status and treatment, in which the L_{15} taurine group had greater immunoreactivity compared to the corresponding L_9 group [$F(4,359) = 21.074$; $p < 0.001$].

DISCUSSION

The present study extends the previous observations of our group on the effects of taurine (Francisco and Guedes, 2015) and pilocarpine (Mendes-da-Silva et al., 2018) in rats of a younger age (PND7-27) than our rats (PND35-55). Analyzing such effects at another age might be of importance, regarding the time course of drug actions (Guedes and Abadie-Guedes, 2019). Treatment with taurine and pilocarpine resulted in anxiolytic-like and anxiogenic behavior, respectively. In addition, chronic pilocarpine, which was administered on a subconvulsing basis, reduced body weight and glycemia; both taurine and pilocarpine treatment decelerated CSD propagation. Furthermore, these data extend our knowledge about the actions of taurine and pilocarpine on electrophysiological responses and glial cell reactions, as demonstrated by the CSD-related potentiation of ECoG amplitude and microglial and astrocyte immunoreactivity. The previously described *in vivo* CSD-induced ECoG potentiation (Lopes-de-Morais et al., 2014; Mendes-da-Silva et al., 2018) has been confirmed in this paper, and its modulation by pilocarpine and taurine has been described (**Table 1**). Data on anxiety-like behavior, MDA measurements and brain immunohistochemical effects that were produced by taurine and/or pilocarpine treatment represent novel evidence of the action of these compounds on 35–55-day-old rats, whereas data on weight gain, glycemia, and CSD confirm the results of a previous study (Francisco and Guedes, 2018). The stress that is associated with drug administration cannot be the cause of the reported alterations because – as first mentioned in the results section – the groups that received gavage and intraperitoneal injection of vehicle (saline and scopolamine) presented values similar to the naïve controls that received no gavage or intraperitoneal injection. The results collectively emphasize the effectiveness of taurine and pilocarpine treatment in modulating behavioral and electrophysiological parameters of the brain, as well as glial cell reactivity.

The ponderal reduction of body and brain weights in the animals under the L_{15} lactation condition (**Figure 2**) are consistent with our previous data (Francisco and Guedes, 2015, 2018), confirming the effectiveness of increasing litter size in producing malnutrition during the suckling period (Rocha-de-Melo et al., 2006). In early life, the brain is more vulnerable to environmental challenges, such as unfavorable lactation conditions (Morgane et al., 1978; Rocha-de-Melo et al., 2006). As previously pointed out, the evidence indicates that a body weight reduction implies weight diminution in the brain and other organs, which is usually associated with alterations in the organs' function (Morgane et al., 2002). In animal models, nutritional deficiency early in life can permanently (or at least in a long-lasting manner) reduce the number of synapses, diminish myelin production, decrease the size and/or number of brain cells, and alter neurotransmitter systems (Morgane et al., 2002). As previously suggested (Francisco and Guedes, 2015), alterations in these processes may interfere with behavioral, electrophysiological and immunohistochemical parameters such as those presently investigated.

Experimental alteration of the blood glucose levels has been shown to correlate inversely with the brain's ability to produce and propagate CSD (Ximenes-da-Silva and Guedes, 1991; Hoffmann et al., 2013), and the present data are in line with the literature. Our interpretation is that an adequate glucose supply is crucial in providing the energy necessary for the glial mechanisms that counteract CSD propagation, as CSD is an energy-demanding phenomenon (Back et al., 1994; Dohmen et al., 2008).

Elevated plus-maze test is one of the most commonly used behavioral tests to evaluate aspects of anxiety-like reactions in rodents (Ramos, 2008). Our findings indicated that treatment with pilocarpine and taurine were associated with anxiogenic and anxiolytic behavior, respectively, as judged by the frequency and duration of the animals' visiting the open arms of the EPM (**Figure 3**). Our EPM data on taurine and pilocarpine administration are consistent with previous reports that showed an anxiolytic effect of taurine administration on animals tested in the EPM (Kong et al., 2006; Murakami and Furuse, 2010; Francisco and Guedes, 2015), and pilocarpine-associated anxiogenic behavior in rats tested in the EPM (Castelhano et al., 2015) and open field apparatus (Francisco and Guedes, 2018). Therefore, we think that it is possible that taurine's action in the brain counteracts anxiety, as suggested by others (Murakami and Furuse, 2010; Yu et al., 2015). By the same logic, chronic pilocarpine at a subconvulsing dose seems to be anxiogenic (present data). Considering that the pilocarpine dose in this study (45 mg/kg/day) was very low (corresponding to 12–15% of the convulsing dose), we suggest that pilocarpine-induced anxiogenesis does not necessarily require the generation of convulsive episodes, as previously indicated (Duarte et al., 2014).

Nutrient intake in insufficient quantity and/or quality in early life can negatively modify structural, biochemical, and electrophysiological parameters of the brain and these effects may last into adult life (Morgane et al., 1978; Guedes, 2011). In rats, it is well-established that early-in-life malnutrition accelerates CSD propagation, both when malnutrition was induced by maternal diet manipulation (Mendes-da-Silva et al., 2014, 2018) and in an unfavorable lactation condition (Lima et al., 2017; Francisco and Guedes, 2018; present study). The mechanisms by which malnutrition facilitates CSD propagation are still a matter of debate. While a larger volume of brain extracellular space hinders CSD elicitation and propagation (Mazel et al., 2002), malnutrition in early life may increase cell-packing density and reduce the extracellular space in the brain (Morgane et al., 1978, 2002), which may facilitate CSD propagation (Lima et al., 2017). Furthermore, malnutrition reduces brain myelination (Morgane et al., 1978). It is important to note that CSD propagation velocity is

increased in the myelin-deficient cortex and decreased in hypermyelinated knock-in animals (Merkler et al., 2009). In addition, early malnutrition may induce impairment of glial function (Morgane et al., 1978), and this condition has been shown to accelerate CSD (Largo et al., 1997). Malnutrition can also enhance the brain levels of the enzyme glutamic acid decarboxylase (Díaz-Cintra et al., 2007) and reduce brain glutamate uptake (Feoli et al., 2006), constituting a scenario that is favorable to CSD elicitation and propagation (Marrannes et al., 1988).

Regarding the redox balance in the brain, previous data suggested that CSD can induce oxidative stress (Viggiano et al., 2011; Shatillo et al., 2013). In the other direction, the accumulation of reactive oxygen species in the nervous system can trigger CSD (Netto and Martins-Ferreira, 1989; El-Bachá et al., 1998; Malkov et al., 2014). Interestingly, various antioxidant agents have been shown to counteract CSD (Abadie-Guedes et al., 2012, 2016; Lopes-de-Morais et al., 2014). As referred above (see introduction), taurine exhibits antioxidant properties and plays a neuroprotective role in epilepsy (Junyent et al., 2011). Because taurine is able to cross the blood–brain barrier, its level in the brain is increased when taurine is administered systemically (Menzie et al., 2013). Taken together, these pieces of evidence could explain the decelerating action of taurine on CSD, as taurine is considered a molecule with antioxidant properties (El-Maraghi et al., 2018; Jafri et al., 2019; Ommati et al., 2019). When administered to pregnant rat dams, taurine is capable of attenuating the impact of maternal food restriction on the progeny (Wang et al., 2017). Our data reinforce a recent suggestion that, in an excitability imbalance condition, taurine plays an inhibitory and neuroprotective role (Hrnčić et al., 2018). As a molecular structure that is very similar to the neurotransmitter GABA, taurine affects the opening of chloride channels, preferably by interactions with $GABA_A$ receptors and with lower affinity to the glycine and $GABA_B$ receptors in the adult brain (Oja and Saransaari, 2013; Hrnčić et al., 2018). Interestingly, extracellular chloride imbalance has been shown to affect CSD propagation *in vitro* in the isolated retina (Martins-Ferreira et al., 1974) and *in vivo* in the rabbit cortex (Guedes and Do Carmo, 1980).

Pilocarpine-induced seizures are correlated with elevated production of reactive oxygen species and by cerebral amino acid level changes, with increased glutamate content and decreased taurine levels in the rat hippocampus (Santos et al., 2011). On the other hand, chronic treatment with a subconvulsive dose of pilocarpine (45 mg/kg/day) did not increase MDA levels in the brain tissue (Mendes-da-Silva et al., 2018), suggesting that under those conditions oxidative stress does not increase, and the present data reinforces this suggestion. Taken together, the findings support the hypothesis that, after pilocarpine administration, brain levels of MDA increase only if pilocarpine induces convulsive seizures (Hamed and Abdellah, 2004; Freitas et al., 2010; Carmona-Aparicio et al., 2016; McElroy et al., 2017).

Microglial cells have the ability to react in response to CNS demands, such as those in epileptic disorders (Eyo et al., 2017) and nutritional imbalance (Rideau Batista Novais et al., 2016). In this study, unfavorable suckling (in large litters) increased microglia activation in the cortex and hippocampus, in addition to the behavioral (anxiety) and electrophysiological (CSD) effects, corroborating the findings of previous studies (Viana et al., 2013; Lima et al., 2017). Furthermore, in line with taurine's effects on traumatic brain injury (Su et al., 2014), our taurine treatment decreased glial immunoreactivity in the cortex and hippocampus. Interestingly, both the cortex and hippocampus are pathologically affected regions in human temporal lobe epilepsy (Gonçalves Pereira et al., 2005; Das et al., 2009). In the pathogenesis of this disease, astrocyte and microglial cell activation is an important link (Clasadonte et al., 2016; Zhao et al., 2018).

Microglia cells may be activated, not merely by delayed neuronal degeneration, but by neuronal hyperactivity that precedes neuronal demise (Eyo et al., 2017). These cells are the resident immune cells of the CNS. They continuously survey the microenvironment with their highly dynamic processes, thus being able to become activated in response to tissue requirements associated with deleterious conditions, such as those involved in brain inflammatory and behavioral responses (Viana et al., 2013). It is interesting to note that Iba1 immunolabeling also labels macrophages, which may infiltrate the CNS; therefore, it is not a specific marker for microglia, and macrophages may become involved in inflammatory responses in the brain, as well as in behavioral responses (Wohleb et al., 2015).

Similar to microglia, astrocyte activation occurs following nutrition imbalance and seizures, and plays an important role in epileptogenesis. A recent study showed that early in life nutritional imbalance can have a lasting impact on astrocytes. It seems that protein deprivation decreased GFAP expression, whereas other undernutrition models (e.g., large litter size) increased GFAP expression (Abbink et al., 2019). In the temporal lobe epilepsy, hippocampal astrocytes undergo dramatic morphological and molecular changes, similar to the glial morphological changes in the hippocampus seen in the pilocarpine-induced epilepsy model (Shapiro et al., 2008). These hippocampal changes occur during the early latent stage of the pilocarpine model. Regarding the cortical astrocytes however, it has been suggested that molecular changes could be a consequence of the repetitive occurrence of seizures (Clasadonte et al., 2016).

In our study, in the L_{15} condition taurine increased GFAP immunoreactivity compared to the L_{15} control groups. It appears that this effect was not harmful to the animals, since in this group the behavioral and electrophysiological parameters were not impaired. Increased immunoreactivity for GFAP has been observed in healthy animals that were subjected to an enriched environment and treated with taurine, suggesting an interaction between these two factors (Rahmeier et al., 2016). Behaviorally, taurine supplementation in rats can improve stress-induced cognitive dysfunction (Jia et al., 2016). Surprisingly, we found lower immunoreactivity for GFAP in the hippocampus of L_9 pilocarpine-treated rats. Borges et al. (2006) reported transient loss of astrocytes in mice dentate hilus early after pilocarpine-induced status epilepticus. In rats, they observed about 50% reduction in hilar neurons but no change in astrocyte number (Borges et al., 2006). Another study reported that a sub-threshold dose of pilocarpine (15 mg/kg), following a lithium chloride administration (127 mg/kg), increases GFAP immunoreactivity

in the hippocampus (Sun et al., 2016). In view of the controversial reports, we think that further research is necessary for the complete understanding of the effect of non-convulsing dose of pilocarpine on astrocytes.

CONCLUSION

The results of this study suggest a brain effect of taurine in pilocarpine-treated rats, which is expressed in body weight, glycemia, behavioral (anxiety), electrophysiological parameters related with CSD and glial alterations. For some of these parameters, the data suggest taurine/pilocarpine/malnutrition interactions, whose mechanisms deserve further exploration.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the supplementary files.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Ethics Committee for Animal Research of the Federal University of Pernambuco, Brazil. The protocol was approved by the same committee (approval protocol no. 23076.015655/2015-99).

AUTHOR CONTRIBUTIONS

EF and RG conceived the study, performed the experiments, analyzed the data, and wrote the manuscript. RM conducted the biochemical analysis and helped in manuscript revision. GS and CC conducted the immunohistochemical analysis, and helped in manuscript revision and in preparing some illustrations.

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REVIEW
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Ketone Administration for Seizure Disorders: History and Rationale for Ketone Esters and Metabolic Alternatives

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The ketogenic diet (KD) is a high-fat, low-carbohydrate treatment for medically intractable epilepsy. One of the hallmark features of the KD is the production of ketone bodies which have long been believed, but not yet proven, to exert direct anti-seizure effects. The prevailing view has been that ketosis is an epiphenomenon during KD treatment, mostly due to clinical observations that blood ketone levels do not correlate well with seizure control. Nevertheless, there is increasing experimental evidence that ketone bodies alone can exert anti-seizure properties through a multiplicity of mechanisms, including but not limited to: (1) activation of inhibitory adenosine and ATP-sensitive potassium channels; (2) enhancement of mitochondrial function and reduction in oxidative stress; (3) attenuation of excitatory neurotransmission; and (4) enhancement of central γ-aminobutyric acid (GABA) synthesis. Other novel actions more recently reported include inhibition of inflammasome assembly and activation of peripheral immune cells, and epigenetic effects by decreasing the activity of histone deacetylases (HDACs). Collectively, the preclinical evidence to date suggests that ketone administration alone might afford anti-seizure benefits for patients with epilepsy. There are, however, pragmatic challenges in administering ketone bodies in humans, but prior concerns may largely be mitigated through the use of ketone esters or balanced ketone electrolyte formulations that can be given orally and induce elevated and sustained hyperketonemia to achieve therapeutic effects.

KEY POINTS

- Cellular metabolism plays a key role in the modulation of neuronal excitability.
- The high-fat, low-carbohydrate ketogenic diet (KD) is a validated treatment for persons with epilepsy. and is also effective in preventing seizures in animal models.

- Beta-hydroxybutyrate (βHB) and acetoacetate (AcAc), the ketone bodies that increase during KD treatment, exert anti-seizure effects in animal models of epilepsy and neurometabolic disorders.
- Although human clinical trials are still needed, therapeutic ketosis with ketone esters represents a clinically viable formulation for the potential treatment of epilepsy and other seizure disorders.

Keywords: ketogenic diet, metabolic therapy, beta-hydroxybutyrate, acetoacetate, ketosis, exogenous ketones, ketone esters, epilepsy

INTRODUCTION

The traditional paradigm for discovery of new anti-seizure drugs (ASDs, also referred to as antiepileptic drugs or AEDs) has involved the assessment of agents blocking acutely provoked or kindled seizures, and which has led to the development of medications that largely influence cellular membrane-bound ion channels and transporters localized to synapses in the central nervous system (CNS) (Rogawski et al., 2016). More recently, however, it has become clear that metabolic factors – whether substrates or enzymes involved in cellular bioenergetics and metabolism – can profoundly influence neuronal excitability (Rogawski, 2016). Research linking brain metabolic changes to neuronal excitability has been driven by efforts to understand how the high-fat, low-carbohydrate ketogenic diet (KD) exerts its anti-seizure – and potential neuroprotective – effects in persons with epilepsy (Neal et al., 2008; Tanner et al., 2011; Rho and Stafstrom, 2012; Stafstrom and Rho, 2012; Gano et al., 2014; Rogawski et al., 2016).

While the efficacy of the KD in the clinical arena is clearly established (Freeman et al., 1998; Neal et al., 2008, 2009; Lambrechts et al., 2017), the mechanisms underlying its beneficial effects remain incompletely understood. Of the many hypotheses proposed (Rogawski et al., 2016), – a historical and unresolved question – is whether ketone bodies (i.e., β-hydroxybutyrate [βHB], acetoacetate [AcAc] and acetone [ACE]) are direct mediators or whether they are epiphenomena, instead indicative of a shift from glycolysis to fatty acid oxidation. Certainly, the current human clinical data do not yet strongly support the view that ketone bodies possess anti-seizure properties independent of their serving as fuel for ATP production, mostly because clinical and a few experimental studies have shown that blood ketone levels do not correlate directly with seizure control (Gilbert et al., 2000; Thavendiranathan et al., 2000; van Delft et al., 2010; Dallerac et al., 2017), despite increasing evidence in the preclinical literature (Kim et al., 2015; Simeone et al., 2018) and more recent clinical evidence to the contrary (Buchhalter et al., 2017). And all three major ketones (βHB, AcAc and ACE) have been shown to have anti-seizure effects in prior animal studies (Keith, 1931; Rho et al., 2002; Likhodii et al., 2003; Kim et al., 2015). For a comprehensive review of ketone bodies as anti-seizure agents, see Simeone et al. (2018). In this manuscript, we review the literature surrounding exogenous administration of ketogenic agents as a potential anti-seizure therapy. As the field is in its infancy, there is little published clinical data available; therefore, we place particular focus on the scientific rationale and pre-clinical evidence which support the translation of these therapies into currently ongoing and future human trials.

KETONE METABOLISM

The pathways involved with ketone body synthesis and metabolism have been firmly established for decades. Fatty acids are converted to acetyl-CoA which then enters the tricarboxylic acid (TCA) cycle. Under conditions where fatty acid levels increase and exceed maximal TCA cycle function, such as during fasting or treatment with the KD, acetyl-CoA is diverted to ketogenesis. Two molecules of acetyl-CoA are used to form acetoacetyl-CoA via acetoacetyl-CoA thiolase. Acetoacetyl-CoA is then condensed with another molecule of acetyl-CoA to form 3-hydroxy-3-methylglutaryl CoA (HMGCoA) in a non-reversible step catalyzed by the rate-limiting enzyme HMG-CoA synthase 2 (HMG-CoAS2). The ketone body AcAc is then produced via the breakdown of HMG-CoA, which releases a molecule of acetyl-CoA. AcAc in turn can either be interconverted to βHB through the βHB-dehydrogenase enzyme or can be spontaneously decarboxylated to acetone and released primarily through the kidneys or lungs. Ketone bodies can then pass through the blood-brain-barrier through monocarboxylic acid transporters (MCTs) and enter the brain interstitial space. After being transported into mitochondria, ketone bodies can be converted back through several enzymatic steps to acetyl-CoA which enters the TCA cycle in neurons or glia to produce ATP. Alternatively, ketone bodies may exert other biological effects such as those described below.

EVIDENCE FOR THE ANTI-SEIZURE EFFECTS OF KETONE BODIES

Not surprisingly, given the key hallmark feature of the KD is systemic ketosis, investigators focused on ketone bodies as possible mediators of anti-seizure effects. Indeed, ketone bodies were shown as early as the 1930's to protect against acutely provoked seizures in rabbits (Keith, 1931, 1932, 1933, 1935), findings that were replicated and expanded decades later in multiple rodent models of seizures and epilepsy (Likhodii and Burnham, 2002; Rho et al., 2002; Likhodii et al., 2003; Minlebaev and Khazipov, 2011; Kim et al., 2015; Yum et al., 2015). Notably,

in vivo anti-seizure effects were reported for either BHB, AcAc or ACE. However, the question of whether one or a combination of these ketone bodies affords even greater efficacy has not been answered. Taken together, these and other studies provide compelling evidence that ketone bodies can induce significant anti-seizure effects, and thus one cannot readily dismiss the possibility that these metabolic substrates contribute directly to the clinical effects of the KD.

In contrast to preclinical data referenced above, ketone bodies (when administered *in vitro* at low millimolar concentrations) were unable to affect either excitatory or inhibitory hippocampal synaptic transmission (Thio et al., 2000) and did not affect voltage-gated sodium channels (Yang et al., 2007) in normal hippocampus, unlike how current anti-seizure drugs are believed to generally work (Rogawski et al., 2016). Notwithstanding these observations, there are two aspects of ketone body action that overlap with synaptic function, but in distinct ways. First, ketone bodies (notably, AcAc) were shown to block neuronal excitability and seizures by inhibiting the presynaptic release of glutamate by modulating vesicular glutamate transporters or VGLUTs (Juge et al., 2010). Second, BHB was shown to alter the aspartate-to-glutamate ratio by driving the aspartate aminotransferase reaction (specifically, by decreasing the transamination of glutamate to aspartate) such that glutamate decarboxylation to GABA is increased (Erecinska et al., 1996; Daikhin and Yudkoff, 1998). The increase in GABA production would then be expected to enhance inhibitory neurotransmission and dampen seizure activity. Despite the rational neurochemical data, more direct evidence for this mechanism has not emerged (Yudkoff et al., 2001; Lund et al., 2009, 2011; Valente-Silva et al., 2015; Zhang et al., 2015), and this GABAergic hypothesis of ketone body action has not been reconciled with the fact that patients with epilepsy who were refractory to GABAergic medications often respond to the KD (Freeman et al., 2006).

The central challenge within the field of diet-based treatments for epilepsy has been to demonstrate clear causal links between cellular metabolism and plasmalemmal membrane excitability. A strong candidate molecular target was discussed nearly 20 years ago, i.e., ATP-sensitive potassium channels that, when activated by reduced ATP-to-ADP ratios, cause membrane hyperpolarization (Schwartzkroin, 1999). Using brain slices from normal and genetically engineered mice, Yellen and colleagues (Ma et al., 2007) showed that ketone bodies decreased the spontaneous firing of GABAergic interneurons in the substantia nigra pars reticulata (which is a known subcortical modulator of seizure propagation in the brain). Moreover, they demonstrated that this action required K_{ATP} channels and $GABA_B$ receptors (Ma et al., 2007). However, it remains unclear whether K_{ATP} channels can be directly activated by ketone bodies, as other investigators have shown that both the KD and ketone bodies increase cellular levels of ATP, which would inhibit K_{ATP} channel opening (DeVivo et al., 1978; Bough et al., 2006; Kim et al., 2010). One potential mechanism reconciling these discrepant observations was provided by Kawamura et al. (2010) a few years later. These investigators showed that under low-glucose conditions (observed during KD treatment), ATP efflux from pyramidal neurons in CA3 hippocampus leads to conversion of ATP to adenosine by ectonucleotidase enzymes and subsequent activation of inhibitory adenosine receptors (A1Rs) which are coupled to K_{ATP} channel activation (Kawamura et al., 2010).

In more recent years, other novel targets for ketone body action have been reported. Kim et al. (2015) reported that BHB blocks spontaneous recurrent seizures in the *Kcna1*-null mouse model of epilepsy, and does so by inhibiting mitochondrial permeability transition – a critical death switch for the cell (Izzo et al., 2016). Further, while other studies have revealed ever increasing complexity of ketone body action on biological targets, they involved non-epileptic and/or extra-CNS tissues. Among the most intriguing are the following: (1) systemic anti-inflammatory effects induced by BHB via inhibition of nucleotide-binding domain (NOD)-like receptor protein 3 (NLRP3) inflammasome assembly (Youm et al., 2015); (2) neuroprotective and anti-inflammatory effects of BHB through an interaction with the hydroxycarboxylic acid 2 (HCA2) receptor (Rahman et al., 2014); and (3) inhibition of histone deacetylases (HDACs) and anti-oxidant effects in renal tissue by BHB (Shimazu et al., 2013). All of these mechanisms, although incompletely understood in the context of epileptic brain, expand the biological profile of BHB and provide further evidence that a strategy based on ketone body administration or inducing prominent ketosis might yield significant and measurable anti-seizure effects in the clinical setting.

HISTORY AND PRAGMATIC CHALLENGES OF IMPLEMENTING THERAPEUTIC KETOSIS – RATIONALE FOR KETONE ESTERS

Administering the KD to implement therapeutic ketosis for seizure disorders is not without its challenges. The restrictive and precise macronutrient composition required to maintain and sustain nutritional ketosis can be difficult to implement. So while fundamental research may be spurred by the intrinsic curiosity and appeal of understanding how a dietary treatment can control epileptic seizures, a longstanding goal has been to determine whether a "KD in a pill" could be developed (Rho and Sankar, 2008). Indeed, investigators have sought ways to circumvent conventional means to administer the KD, one through more liberal and less restrictive diets such as the modified Atkins diet (Eric Kossoff et al., 2016) or the Low-Glycemic Index Therapy (LGIT) (Muzykewicz et al., 2009). Experimentally, other researchers have focused on ketone bodies and pragmatic formulations that could eventually be administered to humans to safely induce a dose-dependent and therapeutic hyperketonemia (Veech, 2004; D'Agostino et al., 2013; Hashim and VanItallie, 2014).

The idea of administering a ketogenic agent to induce and sustain therapeutic ketosis for parenteral and oral nutrition has been around for decades (Miller and Dymsza, 1967). Researchers in the 1950s at Massachusetts Institute of Technology, in

collaboration with the Air Force Research Laboratory (AFRL), focused their efforts on high energy-dense compounds that had the greatest nutritional potential for long-duration manned spaceflight (Bornmann, 1954). Numerous agents were tested, but the ketogenic compound R,S-1,3-butanediol (BD; also known as R,S-1,3-butylene glycol) was selected as the most promising energy source, leading to further studies to determine its safety, stability, and potential as a food additive and preservative (Dymsza, 1975). Data were collected on rodents, dogs, pigs, and humans given this ketogenic compound, and although it induced hypoglycemia concomitant with ketonemia, it was deemed remarkably safe. R,S-1,3-butanediol met the criteria needed for the optimal synthetic "space food", but the "unpleasant taste problem" and lack of FDA approval prevented its use for military or space flight applications.

Despite the palatability challenges, investigators remained intrigued with the potential applications of BD given its metabolic characteristics that mimicked fasting – mild hypoglycemia and safe and predictable hyperketonemia. When ingested orally, BD is metabolized by the liver via alcohol dehydrogenase (ADH) to β-hydroxybutyraldehyde, which is then rapidly oxidized to BHB by aldehyde dehydrogenase (Tate et al., 1971). BD contributes approximately 6 kcal/gm of energy and can produce dose-dependent millimolar concentrations of ketones in the blood at a ratio of 6:1 of BHB to AcAc (Tobin et al., 1972; Desrochers et al., 1992; D'Agostino et al., 2013). Published studies and a number of unpublished reports pertaining to the nutritional and metabolic effects of BD, including a human clinical study feeding study (young male and female subjects given 250 mg/kg body weight per day in bread for four separate 7-day periods), reported a blood glucose lowering effect as well (12% lower relative to controls) (Tobin et al., 1975). This was presumably due to a redox shift in the liver suppressing gluconeogenesis (Ciraolo and Previs, 1995). Although the mild hypoglycemic effect was a potential concern, extensive toxicology studies concluded that BD is safe with very few adverse health effects in animals and humans (Scala and Paynter, 1967; Dymsza, 1975; Hess et al., 1981). Consequently, it was given the status of being Generally Recognized As Safe (GRAS) in May 1997 by the FDA (Docket No. 87G-0351).

The early extensive safety and feasibility studies of BD, its FDA GRAS status, and high stability (i.e., shelf-life) inspired chemists and researchers to use BD as a backbone for synthesizing ketone esters (Brunengraber, 1997; Veech et al., 2001). Chemical synthesis by adding ketones (either BHB or ACAC) to this ketogenic diol through transesterification makes the resulting ketone esters the most energy-dense ketogenic supplements on a per gram basis. In addition to BD-derived ketone esters, there also exist glycerol-derived ketone esters of BHB. The diol BD and triol glycerol contain two or three hydroxyl groups, respectively, and through transesterification, these functional groups can pair with ketone bodies to make mono-esters, di-esters, or such as in the case of glycerol, a tri-ester compound known as glyceryl-tris-3-hydroxybutyrate (Hashim and VanItallie, 2014). Although deriving ketone esters utilizing glycerol as a backbone is feasible (Birkhahn and Border, 1978), the simultaneous elevation of glucose (glycerol is a gluconeogenic precursor) upon hydrolysis and subsequent increase in glycolysis can be unfavorable in the context of inducing anti-seizure effects. The advantage of BD as a backbone is that it delivers ketones upon esterase hydrolysis (in both gut and liver) and also metabolizes completely to BHB to further elevate and sustain ketosis in a predictable manner. Furthermore, dietary interventions that reduce glucose availability, Muzykewicz et al. (2009) and drugs targeting glycolysis such as 2-deoxyglucose (2-DG), Stafstrom et al. (2009) induce anti-seizure effects independent of ketone elevation, so mild hypoglycemia as a "side-effect" is theoretically advantageous for choosing ketogenic supplements that can be effective in controlling epileptic seizures.

The BD-derived ketone esters have been shown to induce a dose-dependent hyperketonemia (1–7 mM) in mice, rats, dogs, pigs, and humans (Desrochers et al., 1995; Clarke et al., 2012; Pascual et al., 2014; Newport et al., 2015). The emerging data indicate that these compounds produce no negative health effects when given acutely or chronically, aside from an aversive taste and the potential for dose-dependent gastrointestinal side effects. There are a growing number of promising metabolic alternatives to ketone esters that have improved or neutral taste and are considerably less expensive to produce. Emerging ketogenic supplements and formulas are being evaluated for their therapeutic efficacy (Borges and Sonnewald, 2012; Kesl et al., 2016) and their anti-seizure potential is discussed below (**Table 1**).

EVIDENCE FOR THE EFFICACY OF KETOGENIC AGENT-BASED THERAPIES FOR EPILEPSY

The science and clinical applications of therapeutic ketosis for neurological applications is growing rapidly, but work evaluating exogenous ketogenic agents remains largely in the pre-clinical space (Stafstrom and Rho, 2012). In addition to ketone esters, there are numerous alternative sources of ketones and ketogenic precursors being developed and shown to produce dose-dependent elevations in blood BHB and AcAc in animals, human case report and pilot studies (Puchowicz et al., 2000; Clarke et al., 2012; D'Agostino et al., 2013; Kesl et al., 2016). Ketone supplemental therapies allow for a calculated, rapid induction and maintenance of physiologic ketosis that mimics levels associated with KD treatment for epileptic seizures (**Figure 1**). In humans it is likely that 2–3 doses/day would be needed to maintain therapeutic hyperketonemia. Ketone supplementation also appears to fundamentally shift metabolic physiology and fuel utilization (Cox et al., 2016), so its potential for supporting physical endurance and military applications is emerging. Since metabolic shifts can affect so many cellular and molecular processes simultaneously, it is not surprising that there is a growing list of mechanisms that have been implicated for exogenous ketones, as previously discussed. However, efficacy may vary depending on the model and endpoints utilized, as well as the physicochemical and pharmacological properties of the individual ketogenic agents and formulations.

TABLE 1 | Studies evaluating anticonvulsant efficacy of ketogenic agents in pre-clinical models.

Authors	Ketogenic agent	Route of admin	Species	Model System	Result	References
Rho et al., 2002	AcAc, ACE, L-BHB	i.p.	Mice	Frings audiogenic-induced Sz	↑Latency to Sz(D-BHIB no efect)	Rho et al., 2002
Likhodii et al., 2003	ACE	i.p. (acute) Oral in H20 (chronic)	Rat	PTZ-induced Sz	↑Sz threshold ↓Sz activity	Likhodii and Burnham, 2002
Likhodii et al., 2003	ACE	i.p.	Rat	Maximal Electroshock Test Amygdala Kindling Test AY-9944Test	↑Sz threshold ↓Sz activity	Likhodii et al., 2003
Minlebaev and Khazipov, 2011	DL-βHB	i.p.	Suckling Rat	Fluorthyl-induced Sz	↓Sz activity	Minlebaev and Khazipov, 2011
Wlaz et al., 2012	Caprylic Acid (C8)	Oral	Mice	i.v. PTZ-induced Sz 6Hz Psychomotor Sz Maximal Electroshock Test	↑Sz threshold (6Hz& i.v. PTZ Sz) No effect MEST	Wlaz et al., 2012
D'Agostino et al., 2013	BD-$AcAc_2$	Oral	Rat	Hyperbaric Hyperoxia-induced CNS-OTSz	↑Latency to Sz	D'Agostino et al., 2013
Wlaz et al., 2015	Capric Acid (C10)	Oral	Mice	6Hz Psychomotor Sz Maximal Electroshock Test i.v. PTZ-induced Sz	↑Sz threshold (6Hz & MEST) No effect i.v. PTZSz	Wlaz et al., 2015
Viggiano et al., 2015	BD-$AcAL_2$	Oral	Rat	PTZ-induced Sz	↑TPTZ threshold for Sz induction	Viggiano et al., 2015
Yum et al., 2015	D-βHB	i.p. (acute and chronic)	Rat	NMDA-induced Sz	No effect (acute) ↓Sz frequency (chronic)	Yum et al., 2015
Kim et al., 2015	BHB	s.c. (osmotic pump)	Mice	Kcna-null Mutant Mice	↓Sz frequency (*in vivo*) ↓Spontaneous Sz-like events (hippocampal slice)	Kim et al., 2015
Chang et al., 2015	4-ethyloctanoid acid (4-EOA)	Oral	Mice	6Hz Psychomotor Sz Maximal Electroshock Test S.c. Metrazol Sz Threshold Test Corneal Kindled Mouse Model	↑Sz control ↑Sz threshold	Chang et al., 2015
Kovacs et al., 2017	BD-$AcAc_2$ KS-MCT	Oral	Rat	WAG/Rjj rats, absent Sz	↓Spike Wave Discharges	Kovacs et al., 2017
Ciarlone et al., 2016	BD-$AcAc_2$	Oral	Mice	Ube3a m-/p+ and WT mice Audiogenic-induced Sz Kanic acid-induced Sz	↑Latency to Sz ↓Sz Activity ↓Sz Severity	Ciarlone et al., 2016

A number of pre-clinical studies in varied seizure models have revealed anticonvulsant effects of exogenous ketogenic agents, including ketone salts, MCTs, and ketone esters.

KETONE ESTERS

Currently, it appears that certain ketone esters hold great anti-seizure potential based upon previous work and on their pharmacokinetic profiles, although the current limitation is that research to date has largely been performed in pre-clinical animal models. When ketone esters are administered, gastric esterases liberate ketones (BHB or AcAc) as a free acid from a backbone molecule. This varies depending upon the specific formulation, but a ketogenic precursor such as BD would be ideal. As previously discussed, BD is subsequently metabolized by the liver to produce BHB (D'Agostino et al., 2013). Thus, the ketone esters currently available are unique in that they can directly elevate ketones and supply ketogenic precursors that can favorably change metabolic parameters like the glucose-ketone index (GKI) (Meidenbauer et al., 2015). Additionally, synthetically derived ketone esters are currently the most potent form of exogenous ketones available, but their potency also necessitates a thorough investigation of their long-term safety and toxicity which is currently lacking. When given at tolerable doses, the potential for side-effects like ketoacidosis are theoretically possible, so human studies are needed to assess at what dose and time interval exogenous ketones should be administered.

The first ketone esters appeared in the late 1970s. Birkhahn and colleagues synthesized a monoester of glycerol and AcAc (monoacetoacetin) for parenteral nutrition. These studies demonstrated that monoacetoacetin induced hyperketonemia comparable to fasted rats at a high dose of 50 g/kg per day (Birkhahn et al., 1977, 1979; Birkhahn and Border, 1978). In an attempt to increase the caloric density of monoacetoacetin, they synthesized both a monoester and triester of glycerol and BHB. Later, Desrochers and colleagues synthesized monoesters and diesters of AcAc with BD, and these had distinctly different pharmacokinetic profiles – i.e., they elevated both AcAc and BHB (Desrochers et al., 1992, 1995). Pigs given oral boluses of the ketone ester R,S-1,3-butanediol acetoacetate diester (BD-AcAc2), at 15% of the daily caloric requirement exhibited a peak total ketone level of 5 mM within 30 min, before slowly returning to baseline after several hours (Desrochers et al., 1995). No deleterious side-effects were observed through

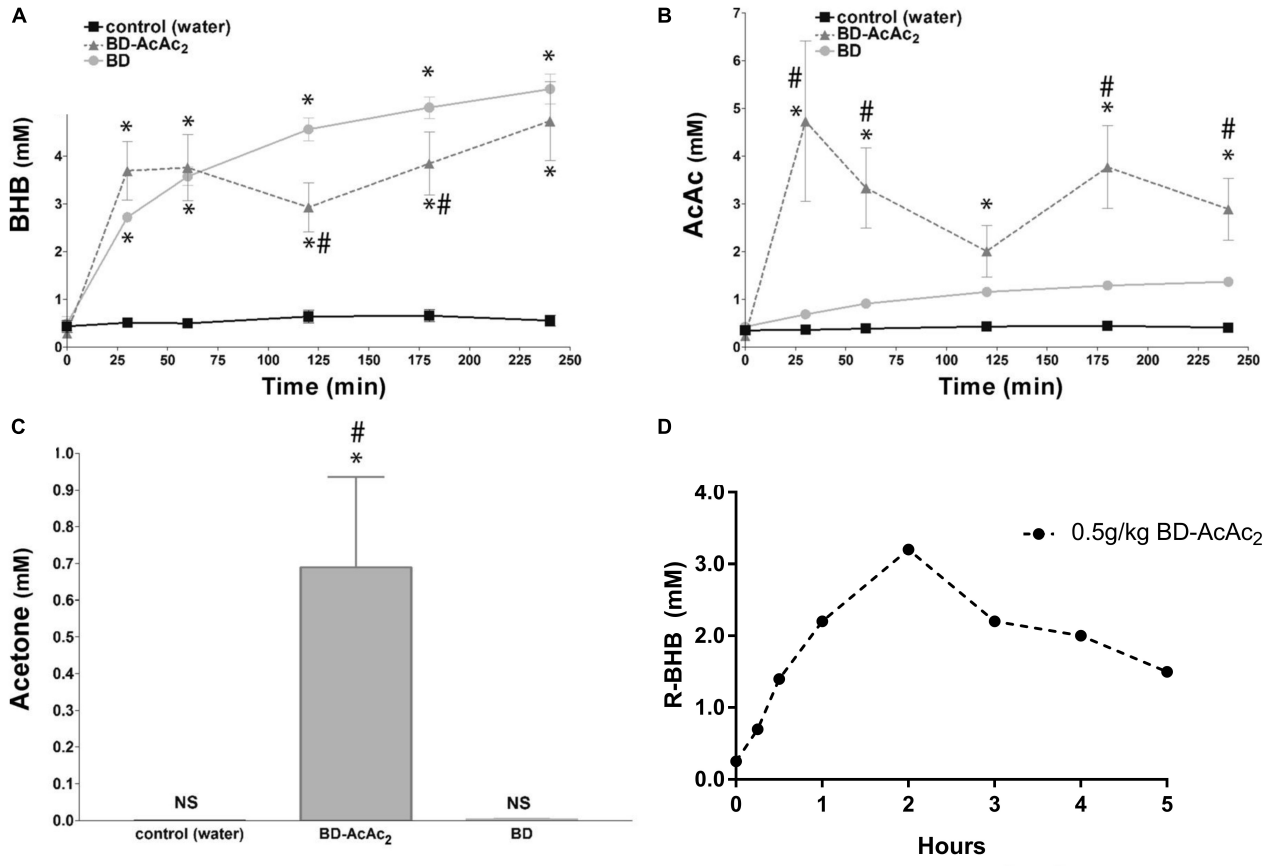

FIGURE 1 | Exogenous ketogenic agents induce therapeutic ketosis within hours of oral ingestion. **(A–C)** Blood ketones following intragastric administration (time 0) of water, R,S-1,3-butanediol acetoacetate diester (BD-AcAc2), and 1,3-butanediol (BD) in Sprague Dawley rats. **(A)** β-hydroxybutyrate (βHB) level was elevated compared with control after either ketogenic compound ($P < 0.001$). **(B)** Acetoacetate (AcAc) level was increased significantly by BD-AcAc2 ($P < 0.001$) compared with water or BD. **(C)** Acetone level increased significantly more after treatment with BD-AcAc2 ($P < 0.001$). **(D)** Blood ketones (R-BHB only) following intragastric administration (time 0) of BD-AcAc2 in male human ($n = 1$). Figure adapted with permission from D'Agostino et al. (2013), American Physiological Society.
*Significant difference from control and #significant difference from BD.

intragastric or high-dose IV administration, including an absence of pathological hypoglycemia and acidosis. These and other ketone esters have demonstrated an ability to induce a dose-dependent hyperketonemia (1–7 mM) in mice, rats, dogs, and humans (**Figure 1**; Ciraolo and Previs, 1995; Sylvain et al., 1995; Brunengraber, 1997; Puchowicz et al., 2000; Srivastava et al., 2012). In a 28-day study, a daily intragastric gavage (5 gm/kg body weight) of BD-AcAc2 induced significantly elevated blood ketone levels and significantly reduced blood glucose levels without significantly altering blood triglyceride or lipoprotein levels (Kesl et al., 2015). In a 15-week chronic feeding study, the BD-AcAc2 was administered to Sprague Dawley rats in low-dose (10 gm/kg/day) (LKE) and high-dose (25 g/kg/day) (HKE) *ad libitum* protocols. Serum clinical chemistry of both LKE and HKE did not reveal any alterations in markers of kidney and liver function compared to rats fed standard chow (Poff et al., 2016), suggesting that chronic high-dose feeding was without overt toxicity. Similarly, Clarke et al. (2012) demonstrated the safety of a BD-backed BHB monoester in rats and humans, and this has also been documented in a recent case study of Alzheimer's disease, where the subject consumed a relatively high dose (20–30 g) thrice daily over 20 months (Srivastava et al., 2012; Newport et al., 2015).

The anti-seizure effects of ketone esters were first reported in a unique seizure model which uses hyperbaric hyperoxia (HBO) to reliably induce epileptic-like (i.e., tonic-clonic) seizures in normal rats, a condition known as CNS oxygen toxicity (CNS-OT). The rats in this study were eating standard rodent chow with abundant carbohydrate (>60%) before induced into hyperketonemia. A single oral dose of the ketone ester BD-AcAc2 induced rapid (within 30 min) and sustained (>4 h) ketosis (>3 mM BHB and >3 mM AcAc, 0.5 mM ACE) and prolonged the latency to seizures by 574% (**Figure 1**) (D'Agostino et al., 2013). Elevations in AcAc and ACE levels were necessary for producing the anti-seizure effects in this particular model of tonic-clonic seizures. BD alone (not in ester form) elevated blood BHB levels (>5 mM) but did not significantly alter AcAc or ACE levels, nor did it prolong the latency to seizure induction. This encouraging response prompted preliminary investigations into preventing or delaying seizures with ketogenic

supplements in a variety of transgenic rodent and chemical-induced seizure models.

Pentylenetetrazol (PTZ) is a $GABA_A$ receptor antagonist and epileptogenic agent that is used to induce seizures in rodents for preclinical development of anti-seizure therapies. In a study by Coppola and colleagues, the dosage threshold for seizure induction by PTZ was assessed in control (water) and KE-treated rats (Viggiano et al., 2015). A single oral dose (4 gm/kg body weight) of BD-AcAc2 elevated blood BHB to 2.7 mM and increased the threshold of PTZ-evoked seizures from 122 ± 6 mg/kg to 140 ± 11 mg/kg. Although AcAc was not measured in this study, the KE was BD-AcAc2 which produces an approximate 1:1 ratio of BHB and AcAc in the blood. Thus, it can be assumed based on PK data (D'Agostino et al., 2013) that the concentration of AcAc was >2 mM.

More recently, the anti-seizure effect of ketone ester treatment has been evaluated in other Sz models. For example, it has been demonstrated that intragastric administration (gavage) of ketone supplements, such as KE, decreased the absence epileptic activity (spike-wave discharges: SWDs) in WAG/Rij rats (Kovacs et al., 2017). The increase in BHB may exert its therapeutic effects on neurological diseases via modulation of inflammatory systems (Newman and Verdin, 2014; Youm et al., 2015; Yamanashi et al., 2017), which are implicated in the pathophysiology of absence epilepsy (Kovacs et al., 2006, 2011; Tolmacheva et al., 2012; Russo et al., 2014). For example, BHB decreased the expression of NLRP3, ASC, caspase-1 and IL-1β (Bae et al., 2016), attenuated release of IL-1β in human monocytes (Youm et al., 2015) and mitigated stress-induced increase in TNF-α and IL-1β in the hippocampus (Yamanashi et al., 2017). Moreover, BHB attenuates the LPS-evoked increase in IL-1β and TNF-α level, as well as LPS-generated increase in COX-2, IL-1β, and TNF-α mRNA expression in BV-2 cells, likely via inhibition of NF-κB signaling (Fu et al., 2014). It was also demonstrated that BHB may decrease inflammatory processes (e.g., expression of COX and IL-1β) via its G-protein-coupled receptor 109A (GPR109A), which evoked inhibitory influence on NF-κB signaling pathway in microglial cells (Fu et al., 2015; Graff et al., 2016). Thus, ketosis suppresses inflammatory signaling (e.g., NLRP3/TLR4/IL-1R/NF-κB) signaling pathways and proinflammatory cytokines/enzymes (e.g., IL-1β and COX-2) that are linked pathophysiologically to epilepsy and other seizure disorders. Interestingly, BHB induced suppression of inflammation was independent of TCA cycle oxidation, and is thus independent of its function as an energy metabolite. Furthermore, the anti-inflammatory changes associated with BHB were not dependent on AMPK signaling, reactive oxygen species (ROS), glycolytic inhibition, UCP, or SIRT2 signaling, further validating its function as a signaling metabolite with potential anti-seizure function. Indeed, other research has shown that inhibition of the NLRP3 inflammasome mitigates the severity of numerous inflammatory diseases, including atherosclerosis, type 2 diabetes, Alzheimer's disease, and gout, among others (Martinon et al., 2006; Duewell et al., 2010; Vandanmagsar et al., 2011; Heneka et al., 2013; Youm et al., 2013, 2015). It is also well established that proinflammatory mediators evoke epileptogenic and ictogenic properties following traumatic brain injury (Webster et al., 2017), and thus ketogenic supplementation like BD-AcAc2 that target these inflammatory pathways hold potential for treating post traumatic epilepsy, especially penetrating brain injuries where neuroinflammation is thought to trigger seizure occurrence.

In addition to classical seizure models, the seizure-prone Ube3a m-/p+ mouse model of Angelman Syndrome was studied by supplementing BD-AcAc2 in the food *ad libitum* for 8 weeks (Ciarlone et al., 2016). The KE therapy improved motor coordination, learning and memory, and synaptic plasticity and in AS mice, as well as suppressed Sz frequency and severity. The kainic acid-induced mouse seizure model was also studied. KE increased latency to Sz, decreased Sz activity, and decreased Sz severity. Interestingly, the KE altered brain amino acid metabolism in AS treated animals by increasing levels of glutamic acid decarboxylase (GAD) 65 and 67 (Ciarlone et al., 2016), thus shifting the neuropharmacology of the brain to favor a higher GABA/glutamate ratio. These pre-clinical findings suggested that KE supplementation produces sustained ketosis and ameliorates many symptoms of AS, including seizure activity. Pre-clinical animal studies with exogenous ketone supplementation therapy have inspired human clinical trials in patients with Angelman syndrome (ClinicalTrials.gov Identifier: NCT03644693) and a wide variety of other neurological and metabolic disorders (e.g., NCT03659825, NCT03531554, NCT03226197, NCT03011203, NCT03889210 NCT03878225).

MEDIUM CHAIN TRIGLYCERIDES

Ketogenic fatty acids such medium-chain triglycerides (MCTs) have been a therapy for intractable childhood epilepsy since the early 1970s (Huttenlocher et al., 1971). MCTs are rapidly absorbed, energy dense (8.3 calories/gram), water-miscible, tasteless, and have a much greater ketogenic potential than long chain fatty acids, Huttenlocher et al. (1971) making them an ideal alternative fat source for the KD. Commercial MCT oil is comprised primarily of caprylic acid (C8:0, octanoic acid) and capric acid (C10:0, decanoic acid), and these are absorbed directly into the bloodstream via the hepatic portal vein without the need for bile or pancreatic enzymes for degradation. MCT-induced ketosis (up to 1 mM βHB) occurs independent of carbohydrate or protein consumption, but is currently limited in clinical usage due to gastrointestinal (GI) side effects associated with the dose needed to produce therapeutic ketosis (approximately 40 g/day) (Huttenlocher, 1976). Similarly, the original MCT-based KD allowed 60% of its energy from MCTs, but the reported GI distress in some children (Huttenlocher, 1976; Trauner, 1985; Sills et al., 1986; Mak et al., 1999) lead to a modified MCT-based (30%) KD that induced lower levels of ketosis (Neal et al., 2009).

Several pre-clinical studies have demonstrated that specific MCTs (e.g., C10:0) may have anti-seizure properties through a mechanism of action independent of ketone metabolism and signaling (Chang et al., 2015; Augustin et al., 2018). Oral administration of 4-ethyloctanoic acid (4-EOA) increased Sz control and Sz threshold in several murine Sz models, including the 6 Hz psychomotor Sz model, the maximal electroshock test

(MEST), the s.c. metrazol Sz threshold test, and the corneal kindled mouse model (Chang et al., 2015). Capric acid (C10 MCFA) increased Sz threshold in the 6 Hz psychomotor and MEST Sz models, but did not affect outcome in i.v. PTZ-induced Sz (Wlaz et al., 2015). And caprylic acid (C8 MCFA) increased Sz threshold in the 6 Hz psychomotor and i.v. PTZ-induced Sz models, but not in the MEST model (Wlaz et al., 2012).

The addition of MCTs to ketone esters or ketone salts may offer a novel way to improve or further augment their anti-seizure/neuroprotective potential (Ari et al., 2018). A combination of BHB salts and MCT oil has been administered in ratios of 1:1 to 1:2 mixtures. Formulating in this way allows for rapid and sustained elevation of ketosis by delivering exogenous ketones while simultaneously stimulating endogenous ketogenesis with MCTs. In addition, the combination formulation allows for a lower dosing of the components as compared to administering the individual supplements, thus reducing potential for side effects (gastric hyperosmolality) and resulting in a distinct blood ketone profile that is sustained over a longer period of time (D'Agostino et al., 2015). In a 28-day study in rats, the combination of MCT with a 50% Na^+/K^+ βHB salt mixture (in 1:1 solution) significantly elevated and sustained blood ketone levels and reduced blood glucose levels in a dose-dependent manner (Kesl et al., 2016). In a 15-week study, Sprague Dawley rats were administered a 1:1 mixture of Na^+/Ca^{+2} ketone salt + MCT oil (20% by weight which resulted in approximately ∼25 g/kg/day) in their food fed *ad libitum*. The combination-supplemented rats had significantly sustained and elevated blood ketone levels at weeks 3, 4, 8, 10, and 13 (Kesl et al., 2014). Exogenous ketone supplements have typically been studied as a single stand-alone supplement, but the unique combination MCT added to ketone salts or ketone esters appears to have pharmacokinetic advantages and favorable behavior effects (Ari et al., 2016; Kesl et al., 2016). Formulating specific supplements will likely enhance the tolerability, absorption, peak and sustained levels of ketones in the blood, which may also translate to greater therapeutic potency and anti-seizure efficacy (Kovacs et al., 2017; Ari et al., 2018).

KETONE SALTS

The recent commercialization of ketone salt supplements has fueled interest in these formulations for general health and wellness, but their clinical efficacy for seizure disorders remains largely unknown. Originally, researchers attempted to administer oral BHB or AcAc in their free acid forms; however, this was prohibitively expensive and ineffective. Subsequently, it was suggested to buffer the free acid of BHB with sodium, but it was feared that sodium overload would occur at therapeutic levels of ketosis. Furthermore, the existing data does not support elevating BHB alone will effectively prevent seizures in animal models (Bough and Rho, 2007). A study showed that oral administration D,LBHB (racemic BHB) treatment for multiple acyl-CoA dehydrogenase deficiency (MADD) was remarkably therapeutic for cerebral and cardiac complication in doses from 80 to 900 mg/kg/day (BHB levels 0.19-0.36 mM) in children with the disease (Hove et al., 2003). Similarly, a successful treatment of severe cardiomyopathy in a pediatric patient with glycogen storage disease type III with the KD and racemic ketone (D/L-BHB) sodium salts was achieved (Valayannopoulos et al., 2011). Although these results are compelling, these protocols would require ingesting between 5.6 and 6.3 g sodium/day for a 70 kg man. Considering the potential safety effects of such a large sodium load, the costs of the administration of Na^+/βHB salts to achieve ketosis made this approach unrealistic (Veech, 2004). Since any physiological electrolyte (Na^+, K^+, $Ca2^+$, Mg^{2+}) readily ionically bonds with BHB, it was determined that a balanced ketone electrolyte formulation would be safer and more feasible for sustaining therapeutic ketosis. Over the last few years chemists have synthesized these balanced ketone electrolyte formulations and numerous studies have been published in animal models (Kephart et al., 2017) and humans (Stubbs et al., 2017, 2018a). A few pre-clinical studies have evaluated the anti-convulsant effects of ketone salts delivered exogenously by i.p. injection (**Figure 1**). D-BHB i.p. increased latency to Sz in the Frings audiogenic-induced Sz mouse model (Rho et al., 2002). I.p. D/L-BHB decreased Sz activity in suckling rats given flurothyl (Minlebaev and Khazipov, 2011), and chronic, but not acute, i.p. administration of D-BHB decreased Sz frequency in NMDA-induced rat Sz model (Yum et al., 2015). Similarly, s.c. delivery of BHB via osmotic pump decreased Sz frequency *in vivo*, and decreased spontaneous Sz-like events (SLE) in hippocampal slices, from *Kcna1*-null mutant mice (Kim et al., 2015). At the time of this writing, millions of doses of commercially available ketone salt products have been purchased and consumed, and no severe adverse reactions have been reported on the FDA website. Widespread use of these products, and better formulations for palatability and tolerability, may help to advance their clinical acceptance and implementation as a means to induce and sustain therapeutic ketosis. Regardless, significant clinical evaluation of the safety and efficacy of chronic ketone salt consumption for seizure disorders has yet to be published.

LIMITATIONS

There are a number of studies that highlight the limitations to ketone salt and ketone esters that are available commercially or for research applications. These limitations are primarily due to gastrointestinal symptoms associated with aversive taste or osmotic load in the GI tract (Leckey et al., 2017; Fischer et al., 2018). Future studies need to assure that the ketone supplement formulations are well tolerated and provide an ideal pharmacokinetic profile of sustained ketone elevation before such supplements are evaluated in humans (Stubbs et al., 2018b). Of relevance to this review, it is important to highlight that while pre-clinical studies have demonstrated that ketone supplements offer promising anti-convulsant effects in a variety of animal models, very little work to date has been published evaluating their potential anti-seizure efficacy or utility in humans.

Historically, issues of palatability and tolerability have limited the clinical investigation of exogenous ketone supplements. More recently, commercialization of ketone salt and MCT oil products

have resulted in formulations that are pleasant to the taste and unlikely to elicit significant gastrointestinal distress unless overconsumed. For more potent formulations, such as ketone esters, it has proven more difficult to mask these flavoring issues and GI effects. In fact, in a recent study evaluating the potential utility of BD-AcAc2 as an ergogenic agent in cyclists, performance was impaired in the group receiving the ketone ester (Leckey et al., 2017). However, all of the study participants experienced notable gastrointestinal discomfort from consuming the supplement, confounding interpretation of the results (Stubbs et al., 2018b). Still, some ketone ester formulations have overcome these major obstacles, resulting in commercially viable products with a much improved taste and GI effect profile, such as the beta-hydroxybutyrate monoester (Stubbs et al., 2018a). Ongoing efforts to optimize other ketone esters such as BD-AcAc2 is promising, and likely to result in a similar commercialized product soon. Combining multiple ketogenic agents in a controlled-release formulation appears to be a promising direction (Meidenbauer et al., 2015).

Another major issue that will need to be addressed to move ketogenic agents into the clinic is establishing an understanding of their appropriate method of administration and dosing regimen. As described, different formulations of ketone supplements elicit markedly different pharmacokinetic profiles, with variable concentrations and durations of blood BHB and/or AcAc produced. Currently, a single dose of most commercially available ketone salt formulations can elevate blood BHB by approximately 1 mM for 1–3 h (O'Malley et al., 2017). If sustained ketosis is required for therapeutic effects, numerous daily doses of these agents would be needed, which may prove to be logistically difficult. Furthermore, as ketone salt formulations often contain large quantities of electrolytes, namely sodium, frequent dosing may present challenges in complying with the recommended maximum daily intake guidelines for these minerals. MCT oil alone can elevate blood ketones modestly (∼0.5 mM) (Courchesne-Loyer et al., 2015), but can also produce significant GI distress at large or frequent doses, especially in naïve patients. Pre-clinical work in rats suggest that adding MCTs to a ketone salt formulation may provide a method to improve the sustained elevation of ketosis with less side effects (Kesl et al., 2016), and therefore may provide a viable option for clinical use. Ketone esters appear to be able to elevate blood ketones to higher concentrations and for longer periods of time than any other currently available ketone formulation (Stubbs et al., 2017), but carry with them a greater need for safety testing and a higher risk of inducing hyperketonemia if overconsumed. In addition to these obstacles, the background diet of the patient would need to be considered, as it may affect the clinical profile of exogenous ketone therapy. If an individual is consuming a KD, exogenous ketone supplements may increase levels of ketosis overall, but also could potentially reduce rates of endogenous fatty acid oxidation and ketogenesis which may play a role in the diet's therapeutic efficacy. Thus, these and other details regarding dosing protocols will need to be established for individual clinical applications. We expect that optimal protocols will depend on the type of ketone formulation being utilized and the specific condition being treated, similar to the use of any pharmaceutical agent. Efforts to optimize the composition and delivery of exogenous ketone supplements are ongoing – such as efforts to improve palatability, reduce GI side effects, and prolong sustained ketosis – and will likely improve tolerability and utility of these agents with time.

CONCLUSION

Considering the multifaceted therapeutic effects and success of the KD for seizure disorders, the goal of many ketone supplement researchers has often been described as creating "the KD in a pill." As such, exogenous ketone supplements are being developed as an alternative or adjuvant method of inducing therapeutic ketosis without the need for a strict dietary regimen. Considering the promising results of the recent pre-clinical studies described here, along with advancements in optimizing ketone supplement formulations, it is possible that many of the seizure conditions which are known to benefit from the KD could receive some benefit from exogenous ketone supplementation by elevating blood ketones and lowering blood glucose. Importantly, if ketone supplements prove safe and efficacious in human trials, they may provide a tool for achieving ketosis in patients who are unable, unwilling, or uninterested in consuming a classic KD, modified Atkins diet, or LGIT. Ketone supplementation may also help circumvent some of the difficulties associated with dietary therapy, as it allows for a rapid dose-dependent induction of ketosis, which can be sustained with prolonged consumption and monitored precisely with commercially available technologies (e.g., blood ketone meters). Simultaneously, it could provide patients with the opportunity to reap the benefits of therapeutic ketosis without the practical and social difficulties of a highly restrictive diet. Moreover, these agents may represent a means to further enhance or optimize existing ketogenic therapies by supplying a form of non-glycemic calories that improves parameters (e.g., GKI) that are associated with therapeutic benefits.

Research on the potential applications of ketone supplementation is rapidly growing, and there are currently several registered clinical trials evaluating their safety and efficacy in a variety of conditions, including healthy adults, athletes, and patients with various diseases including Alzheimer's, Parkinson's, Type 2 Diabetes Mellitus, and more[1]. Encouragingly, clinical studies evaluating these agents in seizure disorders are beginning to emerge. An ongoing trial in Angelman syndrome – a genetic neurodevelopmental disorder characterized intellectual and developmental disability and seizures – is evaluating the use of a fat-based nutritional formulation containing exogenous ketones to support nutritional needs of this patient population (NCT03644693). As a secondary outcome measure, the investigators will also be tracking changes in EEG and seizure activity. Anecdotal reports of individuals consuming commercially available ketone supplements have suggested that some individuals experience a subjective improvement in seizure activity with their use, despite the fact that some of

[1] www.clinicaltrials.gov

the more potent formulations, such as BD-AcAc2, are not yet commercially available. Regardless, it is important to highlight that there is a lack of published clinical work demonstrating efficacy of such agents in patients with seizure disorders, and the relationship between blood ketone elevation and the protective effects of ketosis on seizures is unclear. Thus, further research is needed to fully investigate the molecular mechanisms, clinical utility, and feasibility of exogenous ketone supplements as a method of inducing therapeutic ketosis for managing seizures.

DISCLOSURE

We confirm that we have read the journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines. JR has served as a paid consultant for Accera Pharma, Xenon Pharmaceuticals, Danone Nutricia, and Ajinomoto United States. DD'A and AP receive travel reimbursement and honoraria for speaking at scientific and clinical conferences. AP has served as a paid consultant for Pruvit Ventures, LLC.

AUTHOR CONTRIBUTIONS

AP, JR, and DD'A contributed to the literature review, figure and table design, and writing of the manuscript. All authors read and approved the submitted version.

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An Age-Adjusted EEG Source Classifier Accurately Detects School-Aged Barbadian Children That Had Protein Energy Malnutrition in the First Year of Life

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We have identified an electroencephalographic (EEG) based statistical classifier that correctly distinguishes children with histories of Protein Energy Malnutrition (PEM) in the first year of life from healthy controls with 0.82% accuracy (area under the ROC curve). Our previous study achieved similar accuracy but was based on scalp quantitative EEG features that precluded anatomical interpretation. We have now employed BC-VARETA, a novel high-resolution EEG source imaging method with minimal leakage and maximal sparseness, which allowed us to identify a classifier in the source space. The EEGs were recorded in 1978 in a sample of 108 children who were 5–11 years old and were participants in the 45+ year longitudinal Barbados Nutrition Study. The PEM cohort experienced moderate-severe PEM limited to the first year of life and were age, handedness and gender-matched with healthy classmates who served as controls. In the current study, we utilized a machine learning approach based on the elastic net to create a stable sparse classifier. Interestingly, the classifier was driven predominantly by nutrition group differences in alpha activity in the lingual gyrus. This structure is part of the pathway associated with generating alpha rhythms that increase with normal maturation. Our findings indicate that the PEM group showed a significant decrease in alpha activity, suggestive of a delay in brain development. Childhood malnutrition is still a serious worldwide public health problem and its consequences are particularly severe when present during early life. Deficits during this critical period are permanent and predict impaired cognitive and behavioral functioning later in life. Our EEG source classifier may provide a functionally interpretable diagnostic technology to study the effects of early childhood malnutrition on the brain, and may have far-reaching applicability in low resource settings.

Keywords: protein energy malnutrition PEM, children, EEG, source analysis, classifiers

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INTRODUCTION

Childhood malnutrition continues to be a serious health problem worldwide and is the primary cause of morbidity and mortality in children under 5 years of age (UNICEF et al., 2017). Protein Energy Malnutrition (PEM) in particular is prevalent among infants aged 6 months to 5 years old and critically impacts brain and cognitive development (Morgane et al., 1993; Galler et al., 1996; Black et al., 2017). The Barbados Nutrition Study (BNS) is a unique 45+ year longitudinal cohort study that has followed individuals with histories of moderate-severe PEM limited to the first year of life and healthy controls who were classmates of the PEM participants. This study has documented cognitive and behavioral problems (Galler et al., 1983a,b, 2010) including poor attention, impaired school performance and increased conduct disorder as well as depressive symptoms over the lifespan. Recent evidence suggests that early childhood malnutrition leads to epigenetic changes that impact the next generation (Peter et al., 2016).

We recently recovered EEG data that was collected in BNS participants in 1977–1978 at ages 5–11 years. EEG analyses using qEEGt (Taboada-Crispi et al., 2018) showed the following results in study participants with histories of childhood malnutrition: (1) increased theta activity (3.91–5.86 Hz) in electrodes T4, O2, Pz and in the supplementary motor area (SMA); (2) decreased alpha1 (8.59–8.98 Hz) in fronto-central electrodes and sources of widespread bilateral prefrontal area; (3) increased alpha2 (11.33–12.50 Hz) in temporo-parietal electrodes as well as in sources in central-parietal areas of the right hemisphere; and (4) increased beta (13.67–18.36 Hz), in T4, T5, and P4 electrodes and decreased in bilateral occipital-temporal regions of PEM versus control groups. Earlier EEG studies in children with histories of childhood malnutrition (e.g., Bartel et al., 1979) similarly found increased slow wave rhythms (theta band), as well as decreased alpha. The effect of early malnutrition on brain maturation and delayed brain development has also been reported in animal models of early malnutrition (Bronzino et al., 1999).

Studies directly examining brain function in individuals with histories of childhood malnutrition are limited. Although neuroimaging techniques such as MRI have been used to identify a neural signature of early PEM (Ivanovic et al., 2002), these techniques are not feasible for the development of scalable screening programs in low resource settings where PEM is most prevalent. They are also costly and have the disadvantage of low throughput.

Consequently, attention has shifted to EEG studies for identifying brain signatures of malnutrition. A prime candidate for this purpose is tomographic quantitative electroencephalography (qEEGT), which quantifies the EEG rhythms via its frequency spectrum—the power in the signal at each frequency bin and each channel. However, standard EEG studies have limitations, especially because of their reliance on scalp recording which is problematic for pinpointing anatomical substrates, physio-pathological explanations and the relationship to the animal literature. It is therefore preferable to carry out EEG Source Imaging (ESI) (He et al., 2019) for increased biological validity. Our earlier report (Taboada-Crispi et al., 2018) partially addressed these issues by:

1. Employing z spectra: each log spectral value has the age appropriate mean and divided by the age appropriate standard deviation (as encoded in regression equations obtained from the first wave of the Cuban Human Brain Mapping Project) (Szava et al., 1994). This ensured correction for normal brain age related variance.
2. Utilizing our novel machine learning technique (Bosch-Bayard et al., 2018) to identify a stable neural signature with high classification accuracy of EEGs to distinguish children with PEM versus controls.
3. Interpreting the neural signature by identifying their anatomical substrate differences with our ESI method Variable Resolution Electrical Tomography (VARETA) (Bosch-Bayard et al., 2001).

However, Taboada-Crispi et al. (2018) were not able to develop a classification procedure for malnutrition based on ESI measures. This was due to the extremely large number of highly correlated variables produced by VARETA. This method is based on a Bayesian inference structure which does not have a built-in variable selection procedure. To overcome this type of limitation we developed a new ESI method that guarantees sparse sets of active sources: Brain Connectivity Variable Resolution Electromagnetic Tomographic Analysis (BC-VARETA) (Paz-Linares et al., 2017, 2018, 2019; Gonzalez-Moreira et al., 2018). By leveraging the graphical lasso procedure (Friedman et al., 2010), we simultaneously estimate source activity and connectivity, thus producing a much sparser and decorrelated set of measures. This research paves the way for classification procedures based on EEG source spectra. For a recent review on ESI and connectivity see He et al. (2019).

In the current paper, we report the results of using BC-VARETA ESI to document the effects of early childhood PEM on brain function. We used BNS archival EEG data collected at ages 5–11 years (Ahn et al., 1980; Galler et al., 1983a,b) to identify a stable machine learning classification scheme to distinguish children with PEM versus controls based on EEG source spectra.

MATERIALS AND METHODS

Barbados Nutrition Study Sample

The two nutrition groups were selected as follows:

1. PEM: Children born between 1967 and 1972 in Barbados and diagnosed with protein-energy malnutrition (PEM) in the first year of life (n = 129, 52 females, 77 males);
2. Controls: Healthy classmates (n = 129, 52 females, 77 males), matched by age ± 3 months, gender and handedness.

Inclusion criteria for PEM and control children were, as follows: (1) birth weight > 2500 g; (2) Apgar score > 8 at birth; (3) no birth complications; and (4) no encephalopathic events in childhood. The PEM group experienced a single episode of Grade

II or III PEM (Gomez et al., 2000) in the first year of life based on clinical diagnosis at the time of admission to the Queen Elizabeth Hospital. The control group met the same inclusion criteria as the PEM group but did not have a history of PEM. Final selection was based on parental consent and access to birth and preschool health records. All PEM children were enrolled in a national program (NIP- Nutrition Intervention Program)- that provided subsidized food, maternal nutrition education, regular home visits, a pre-school nursery, and health care from the time of hospital discharge until 12 years of age (Ramsey, 1979), ensuring that no child had further episodes of malnutrition.

Written informed consent was obtained from all participants. Approval for this study was granted by the Ethics Committee of the Ministry of Health, Barbados, the Judge Baker Children's Centre Human Research Review Committee (Assurance No. FWA 00001811) and the Massachusetts General Hospital IRB (2015P000329/MGH). Participants were compensated for their time and travel to and from the BNS research center.

EEG Data Acquisition and Preprocessing

A complete description of the EEG procedures has been previously reported in Taboada-Crispi et al. (2018). Briefly, EEGs were recorded in 1977–1978 when the BNS children were 5–11 years of age by trained staff at the Barbados Nutrition Centre, who were blinded to the child's nutritional history. A designated room was available for EEG recording. All participants were instructed to sit in a comfortable half recliner chair and to close their eyes but not to fall asleep. A custom-designed digital electrophysiological data acquisition and analysis system (DEDAAS) was constructed by Prof. E. Roy John at the Brain Research Labs, NYU (Thatcher and John, 1977) and was used to acquire the EEG data. The DEDAAS front-end consisted of 24 solid-state EEG amplifiers. The output of the amplifiers was fed through a 12-bit A/D converter with a sampling frequency (f_s) of 100 Hz into a PDP-11 minicomputer that calibrated the amplifiers and checked the electrode impedances automatically. Simultaneous monopolar recordings were obtained of the 10/20 International Electrode System (Fp1, Fp2, F3, F4, C3, C4, P3, P4, O1, O2, F7, F8, T3, T4, T5, T6, FZ, CZ, and PZ) system, all referenced to linked earlobes. Data was stored on digital tape at the Brain Research Lab, New York University until 2016 when it was shared with our group (courtesy of Prof. Leslie Prichep). A total of 258 digital resting state EEG recordings were collected but only 137 raw EEG files were recovered in 2016 for the analysis. The original raw dataset was converted to EEGLAB (Delorme and Makeig, 2004) and PLG[1] format for further processing.

Two neurophysiologists carried out quality control using visual inspection via time and frequency domain tools. Artifacts derived from oculo-motor and facial movements were eliminated use the AAR plug-in from the EEGLAB 13.6.5b toolbox described by De Clercq et al. (2006) and Gomez-Herrero et al. (2006). In sum, 29 recordings displayed somnolence and were excluded from this study, leaving a final dataset of 108 recordings (of the original 258).

[1]http://www.neuronicsa.com

Source Space Analysis Using BC_VARETA

Source Imaging Technique

For the usable data, 1 min of artifact free EEG was obtained for all channels and was divided into quasi-stationary segments that were 2.56 s long. This yielded a total of $k = 1, \cdots, T = 24$ windows. Each window was subjected to a Fourier transform each frequency ω, and segment $k_{\text{andyielded}}$ a vector of complex Fourier coefficients $V_k(\omega)$. In the current report, we attempt to estimate the sources of these vectors.

As noted in the introduction, we used Variable Resolution Electrical Tomography (VARETA) as the Electrophysiological Source Imaging (ESI) technique in our previous study on malnutrition (Taboada-Crispi et al., 2018). VARETA has been extensively used in clinical studies (Bosch-Bayard et al., 2001) but has two main disadvantages for using its features as variables for classifiers: lack of sparseness and also "activation leakage"– the spillover of estimated activity from the actual active cortical voxels to other sites due to unavoidable ESI reconstruction errors.

We overcame these problems in the current report using another ESI, the Brain Connectivity VARETA or BC-VARETA (in place of VARETA), which consisted of two stages:

1. "Screening" of the entire voxel space to retain only those voxels with possible activation. This is carried out with our new method for activation detection via model evidence maximization of the non-linear-univariate ENET-SSBL model (Paz-Linares et al., 2017). In the cited paper we reported that this new technique produced activation maps with the highest sparsity and least leakage of many state-of-the-art ESI methods.
2. The second stage consisted of an improved estimation of activation, achieved with simultaneous estimation of source connectivity (Paz-Linares et al., 2018, 2019). This approach substantially improves the connectivity estimation as well as source activity due to their mutual interdependence.

We now formally summarize this second stage for ease of reference. Scalar quantities are denoted by lower case capital letters (x), vectors by lowercase bold ones ($\mathbf{x}$), and matrices are indicated by bold upper-case notation ($\mathbf{X}$). Observed quantities will be denoted by Latin script and latent variables by Greek script. The usual conventions are followed: e.g., x^T, X^{-1} are, respectively the transpose of a vector and the inverse of a matrix. Since we are working in the frequency domain we assume that all vectors $\mathbf{x}$ are considered as complex ($\mathbf{x} \in \mathbb{C}^p$), and distributed as independent random vectors with a Circularly Symmetric Complex Multivariate Normal probability density function, that is $\mathbf{x} \sim N_q^{\mathbb{C}}(\mu, \Sigma)$ with dimension q, mean μ, and covariance matrix Σ

The frequency domain resting state EEG is modeled as:

$$\mathbf{v}_k(\omega) = L\,\boldsymbol{\iota}_k(\omega) + \mathbf{e}_k(\omega) \tag{1}$$

where $\mathbf{v}_k(\omega) \in \mathbb{C}^m$ is the complex EEG Fourier coefficient vector of dimension m, frequency ω and the k^{th} segment. Also $\mathbf{e}_k(\omega)$ is

the corresponding sensor noise while L is the lead field matrix defined on the cortical surface for q. Finally, the sources of the EEG are denoted by the vector $\iota_k(\omega) \in \mathbb{C}^q$, where q is the number of sources. Since processing of each frequency ω is independent this argument will be dropped henceforth. The model is additionally specified by the following hierarchical Bayesian model for each subject:

$$\begin{aligned} &a)\ v_k \sim N_q^{\mathbb{C}}\left(L\,\iota_k, \sigma_e^2 R\right) \\ &b)\ e_k \sim N_q^{\mathbb{C}}\left(0, \sigma_e^2 R\right) \\ &c)\ \iota_k \sim N_q^{\mathbb{C}}\left(0, \Theta_{\iota\iota}^{-1}\right) \\ &d)\ \Theta_{\iota\iota} \sim e^{-\lambda\|\Theta_{\iota\iota}\|_1} \end{aligned} \tag{2}$$

In Equation [2 (a) and b] codify the observation equation (1) with the sensor error variance $\sigma_e^2 R$ and R known. Line c) specifies a prior distribution for the sources with a covariance matrix $\Theta_{\iota\iota}^{-1} = \Sigma_{\iota\iota}$ with the inverse covariance (or Precision matrix$\Theta_{\iota\iota}$ and cross-spectral matrix $\Sigma_{\iota\iota}$. $\Theta_{\iota\iota}$is also known as the matrix of partial covariances and is usually assumed independent in most current. The novel feature of BC-VARETA is that it estimates $\Theta_{\iota\iota}$in a data-driven fashion by assuming it is, in turn, a sample from a Gibbs distribution with general penalty function $P\left(\Theta_{\iota\iota}\right)$, here an L1 norm. All parameters are estimated via Expectation Maximization optimization of the model evidence. In summary BC-VARETA finds estimators of the Estimators of the cross-spectra $\widehat{\Sigma}_{\iota\iota}(\omega)$ for each frequency. The diagonals of these matrices are the power spectra in the sources. Further technical details are in the cited papers and the software is available at https://github.com/CCC-members/BC-VARETA_Toolbox.

Specific Source Analysis

BC-VARETA was used to analyze the artifact-free EEG dataset for each participant to obtain the log source spectra at each of 6003 cortical sites and all 48 frequency components within a range of 0.1–19 Hz. These were then summarized by averaging over the areas of the AAL atlas of the MNI (Mazziotta et al., 2001). An approximate Lead Field matrix used for the source-space analysis for all subjects was obtained with Brainstorm software[2] for 19 sensors defined on the 10–20 system and co-registered using the MNI Average Brain template subject anatomy. This approach of using an average lead field (across the sample) was experimentally tested (Valdés-Hernández et al., 2009) resulting in the most convenient tool in research studies demanding EEG source localization when MRI are unavailable. The atlas employed by us to identify the neural structures is available as a toolbox for SPM at http://www.gin.cnrs.fr/AAL2. To further summarize the number of frequencies and reduce the final number of variables, we grouped the bins of frequencies using the broad band parameter, according to the IFCN Guidelines (Nuwer, 1997) and proposed the following frequency bands: delta (1.5–3.9 Hz), low theta (4–5.4 Hz), high theta (5.8–7.4 Hz), low alpha (7.5–9.4 Hz), high alpha (9.5–12.5 Hz), low beta (12.8–14.9 Hz), and high beta (15–19.14 Hz).

[2]https://neuroimage.usc.edu/brainstorm/

Stable Sparse Biomarkers Detection (SSB)

The EEG spectral signatures obtained from BC-VARETA are used here to identify biomarkers that discriminate between the two nutrition groups using the EEG activity. In what follows we denote the spectral estimators for each frequency band/location as $s_{i,j}\ i = 1, \cdots N; j = 1, \cdots p$, where N is the total number of subjects and p is the total number of features (potential biomarkers) to be explored. For this purpose, we use the SSB methodology (Bosch-Bayard et al., 2018; Chiarenza et al., 2018) which is a classification procedure that extract a minimal set of features, in a high dimensional problem, by providing a classification equation with a high predictive power and stability. SSB specially deals with the case where the number of variables is high (p = 294,147 in our case) and the number of observations is relatively small (n = 108). In such $p > > \mathrm{N}$ situations it is possible, by chance, to achieve classification equations with spuriously high accuracy. Nevertheless, slight changes in the training set can lead to quite different feature selection and classification rates.

To protect against this problem, the sparse stable biomarker (SSB) proceeds in two steps.

First Step: Selection of a Stable Set of Predictors

This is done by a resampling methodology that repeatedly and randomly splits the data (in our case 500 times) with 70% of the data in a training set and 30% in a test set. With the generation of each random pair of training and testing sets the following operations are carried out:

(A) The indfeat procedure (Weiss and Indurkhya, 1998) winnows out promising classification variables in the training set.

(B) An even smaller set of predictors is selected from the training set by means of the elastic net regression (GLMNet) (Hastie et al., 2016) to select a classification equation (Zou and Hastie, 2005; Friedman et al., 2010). The model is described by the equation (3):

$$\min_{\varphi_0 \in \Re, \varphi \in \Re}\left[\frac{1}{2N}\sum_{i=1}^{N}\left(y_i - \varphi_0 - x_i^T\varphi\right)^2 + \lambda P_\gamma(\varphi)\right] \tag{3}$$

Here N is the number of subjects, $x_i \in \Re\,.\, x_{i,j} = \log\left(s_{i,j}\right)\ x_i \in \Re$ observations of subject i, and $y_i \in \Re$ is the label group of subject i; $\varphi_0 \in \Re, \varphi \in \Re$ are the model parameters; γ is the regularization parameter; p is the number of variables in the model; and

$$P_\gamma(\varphi) = (1-\gamma)\frac{1}{2}\|\varphi\|_2^2 + \gamma\|\varphi\|_1 \tag{4}$$

The penalty P_γ in equation (4) is known as the elastic-net norm (Zou and Hastie, 2005). To understand its behavior, note that the $\|\varphi\|_2^2$ norm induces regressions that behaves well for high dimensional regressions but that tend to spread out coefficient weights among highly correlated variables. On the contrary, the $\|\varphi\|_1$ norm produces the "lasso regression" which is indifferent to highly correlated predictors and tries to select only one thus inducing sparsity. The elastic-net reaches a compromise between the ridge and the lasso, the relative contributions being determined by the γ and λ parameters. Since these parameters are

selected by cross-validation (based on the test set), in any specific case, the sparsity of the solution will be data-driven. The set of predictor variables selected at this repetition is recorded.

Finally, after all repetitions, a stable set of predictor variables is obtained by retaining only those variables that are selected in at least 50% of repetitions.

Second Step: Evaluation of the Stable Predictor Set

In a totally independent set of resampling experiments, the sensitivity and specificity of the classification equation is evaluated. In an earlier report (Bosch-Bayard et al., 2018), a ROC methodology was introduced which also guarantees stability and robustness. Again, the total sample is repeatedly and randomly split into two samples 70% of the data for the training set and 30% for the out of sample test set. For each repetition the following operations are carried out:

(A) A GLMENT classifier is obtained from the training set as using a similar procedure as in the first step (B) above.
(B) The area (AUC) and partial areas under the ROC curve (pAUC) is calculated on the training set and stored.

The AUC and pAUC values are used to generate kernel empirical distribution functions for these measures. We use the median of these distributions as an estimate of the true underlying measures accuracies of the classification procedure. In the case of the pAUC, these are evaluated for the False Positive Ratios 0.1 and 0.2 and transformed to the standardized partial Area under the ROC curve (spAUC) as described by McClish (1989) to facilitate comparisons. Note that SSB described in this section is not to be confused with the ENET-SSBL procedure (Paz-Linares et al., 2018).

Age Adjusted Classifier and Interpretation

Note that in our previous report in the BNS participants (Taboada-Crispi et al., 2018), gender and age were included as covariates in the EEG analyses. However, only age was found to be significantly correlated with the EEG and is therefore the primary covariate included in the analysis described below. In our previous work with VARETA we pre-process the log source spectra by the z-transformation: to partial out the variability due to normal age changes. Normative data is not yet available for BC-VARETA. Instead, we obtained an age adjusted classifier by introducing both age and the interaction (product) of age and log source activity as potential biomarkers.

$$\min_{\varphi_0\in\Re,\varphi\in\Re}\left[\frac{1}{2N}\sum_{i=1}^{N}\left(y_i-\varphi_0-x_i^T\varphi-age_i\,x_i^T\;\psi\right)^2+\lambda P_\gamma(\varphi,\psi)\right] \quad (5)$$

Where the notation is the same as in equation (3) with the additional parameter vector ψ which represents the slope of the age-dependent classifier, with age_i the age of subject i.

Since the SSB procedure provides the biomarkers and their coefficients in the classification equation, we performed an additional t-test analysis between the two groups, using the contrast malnutrition (PEM) versus Control group in order to determine the direction of the group differences. In this case, the negative sign indicated lower activation of PEM and the positive indicated higher activation of the PEM group. This last analysis in included for illustrative purposes only and is not part of the classification procedure.

RESULTS

Demographic Characteristics of the Sample

Table 1 summarizes the demographic characteristics of the study participants. There were no nutrition group differences in gender, age or handedness. The table shows significant differences between the PEM and control groups in IQ, academic performance and ecology at 5–11 years, as previously reported in the full sample (Galler et al., 1983a,b). This subsample retains the age/sex balance of the original 1977-1978 cohort.

Age Adjusted Classification of PEM vs. Control Children Using BC-VARETA Sources

A linear mixed-effects model testing influence of age and sex on the source variables (Chung et al., 2010) showed no effect of sex but did show a significant effect of age. To deal with this an age-adjusted classifier was developed and is described below. The regions and frequency bands selected as stable classifiers are listed in **Table 2**. The third and fourth column show the % of times selected as a classifier during the randomization procedure for the age-independent coefficients. The fifth and sixth column show the same information for the age-dependent coefficients ψ .

Figure 1 shows the ROC analysis of the age-adjusted classification procedure based on these coefficients BC VARETA to distinguish between the EEGs of both groups (PEM vs. Control). Note that this is the same EEG dataset reported (Taboada-Crispi et al., 2018). As can be seen the classification accuracy is quite high with the using EEG sources calculated with the VARETA procedure. As can be seen the classification

TABLE 1 | Demographic characteristics of the sample.

	PEM	Control	*t*-test/χ2	*p*-value
N	46	62		
Males [*N* (%)]	28 (60.9)	34 (54.8)	0.39	0.531
Age (years)				
- Males	8.5 ± 1.9	8.6 ± 1.7	0.14	0.888
- Females	7.9 ± 2.0	8.5 ± 1.9	1.01	0.318
Handedness [N left (%)]	4 (8.7)	3 (4.8)	0.65	0.456
Childhood Ecology	−1.14 ± 0.89	−0.14 ± 0.81	6.01	<0.0001
WISC Full-Scale IQ	88.9 ± 12.9	105.2 ± 11.9	6.62	<0.0001
School Performance (1–5)	3.1 ± 1.06	4.3 ± 0.88	6.20	<0.0001

Data are presented in mean ± SD or %; Age (in years at the moment of the EEG recordings). The differences between groups were tested using Chi-square and t-test.

TABLE 2 | Regions and frequency bands selected as stable classifiers.

Region of AAL atlas	EEG Frequency Band	% times selected as classifier		Regression Coefficients	
		ϕ	ψ	ϕ	ψ
Middle Temporal Gyrus Left (MT.L)	High Theta θ_H	78.72	75	−0.28	−0.02
Inferior Frontal Gyrus Orbital Right (IFO.R)	Low Alpha α_L		54.55		−0.006
Lingual Gyrus Right (LING.R)	Low Alpha α_L	50.6		228.26	
Cuneus Right (CUN.R)	High Alpha α_H	75	81.71	0.18	0.03
Pre-Central Gyrus Right (PRECG.R)	High Alpha α_H	60.82	51.22	−1.18	−0.06
Lingual Gyrus Right (LING.R)	High Alpha α_H	52.44	53.66	198.02	31.29
Superior Temporal Gyrus Left (ST.L)	Low Beta β_L	50.59		−0.20	
Middle Occipital Gyrus Right (MO.R)	Low Beta β_L	75.42	53.33	−0.094	−0.006
Superior Medial Gyrus Left (SMG.L)	Low Beta β_L	57.83	53.57	−0.19	−0.013
Inferior Temporal Gyrus Left (ITG.L)	High Beta β_H	76.4	56.25	−0.19	0.009

From left to right: anatomical region, frequency band% times selected as a classifier, and the actual regression coefficients of the elastic net classifier. ϕ Are the coefficients for each variable for those independent of age, and $\psi$for those that reflect the interaction with age.

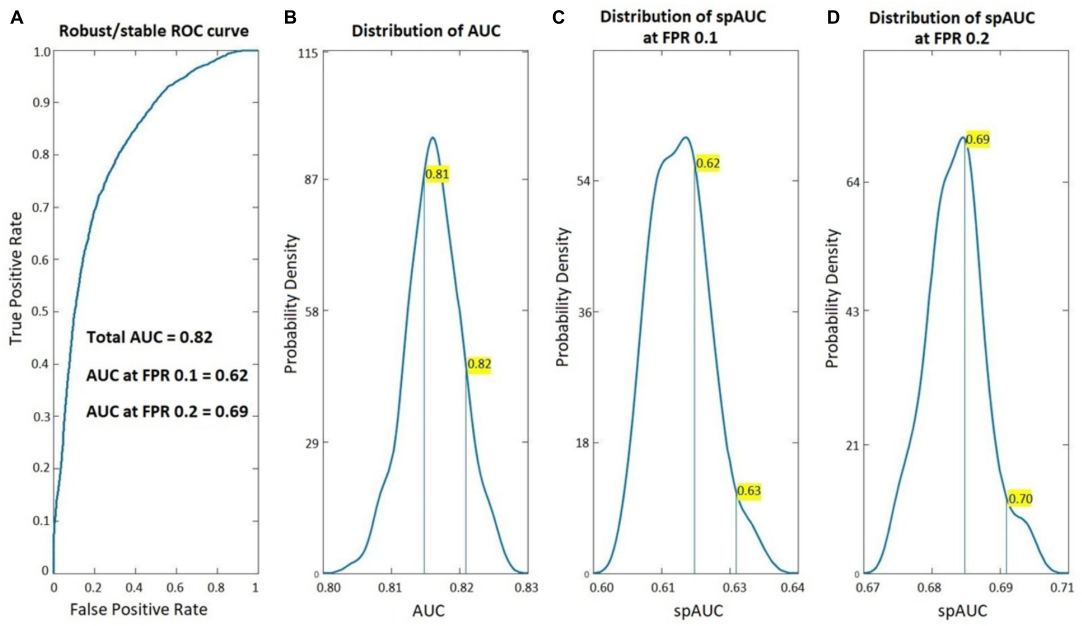

FIGURE 1 | Classification accuracy of the age-adjusted PEM vs. Control Classifier. ROC analysis of the age-adjusted classification between Children with PEM and Controls based on the BC-VARETA. Electrophysiological Source Imaging techniques. On the left, **(A)** the full ROC curve, on the right **(B–D)** the probability density functions (estimated from the resampling cross validation) of the area under the ROC curve (AUC) for the full curve **(B–D)** the standardized partial AUC (spAUC) at 0.1, and 0.2 false positive ratio cut-off points respectively.

accuracy is quite high with the area under the curve 0.82. The figure also shows that the estimated probability density for the AUC values from the randomized subsamples is quite far away from 0.5 (chance classification). This is also true for the standardized partial area under the ROC curve (spAUC) at both the 0.1 and 0.2 false positive rate cut-off points.

Figure 2 compares the resampling-based probability densities for the AUC and spAUC as in **Figure 1** for the age adjusted classification based on BC-VARETA sources and superimposed, for purposes of comparison, the same curves for the classifier based on scalp qEEG measures previously presented in Taboada-Crispi et al. (2018). Both are very high, with a slight advantage for the scalp-based qEEG measures.

The actual effectiveness of the classification is shown in **Figure 3** with the boxplot of the individual classification scores

$$t_i = \varphi_0 + x_i^T \varphi + age_i\, x_i^T \;\; \psi = \sum_{j=1}^{p} x_{i,j}\, d_j\left(age_i\right) \qquad (6)$$

Which is based on the age adjusted elastic net classifier (5). Note that $d_j\left(age_i\right)$ is the age-adjusted regression coefficient.

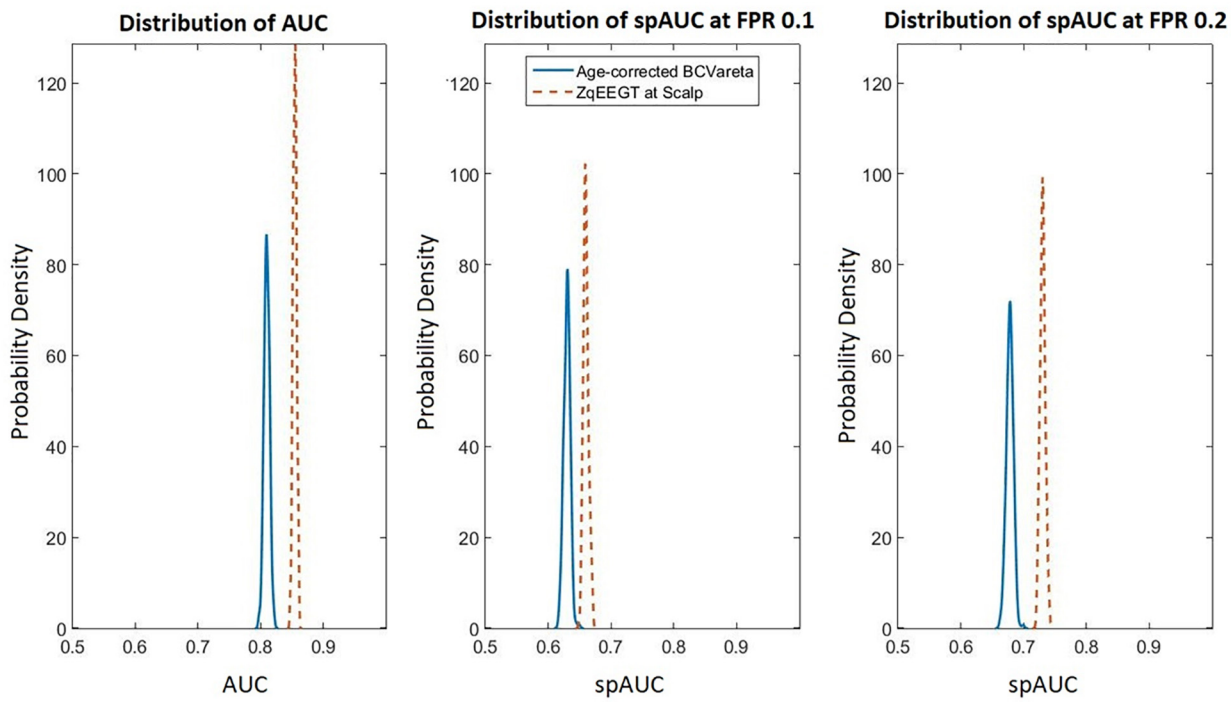

FIGURE 2 | Comparison of scalp based and source-based BC-VARETA classification accuracy. As in **Figure 1**, the probability density functions of the AUC are shown for the full curve **(left)**, the spAUC at 0.1 **(center)**, and at 0.2 **(right)** false positive probability cut-off points. The blue (solid) lines correspond to the age-adjusted BC-VARETA classifier, while the red (dashed) lines correspond to the scalp qEEG based classifier. The scalp-based classification performed slightly better than the classifiers at the sources.

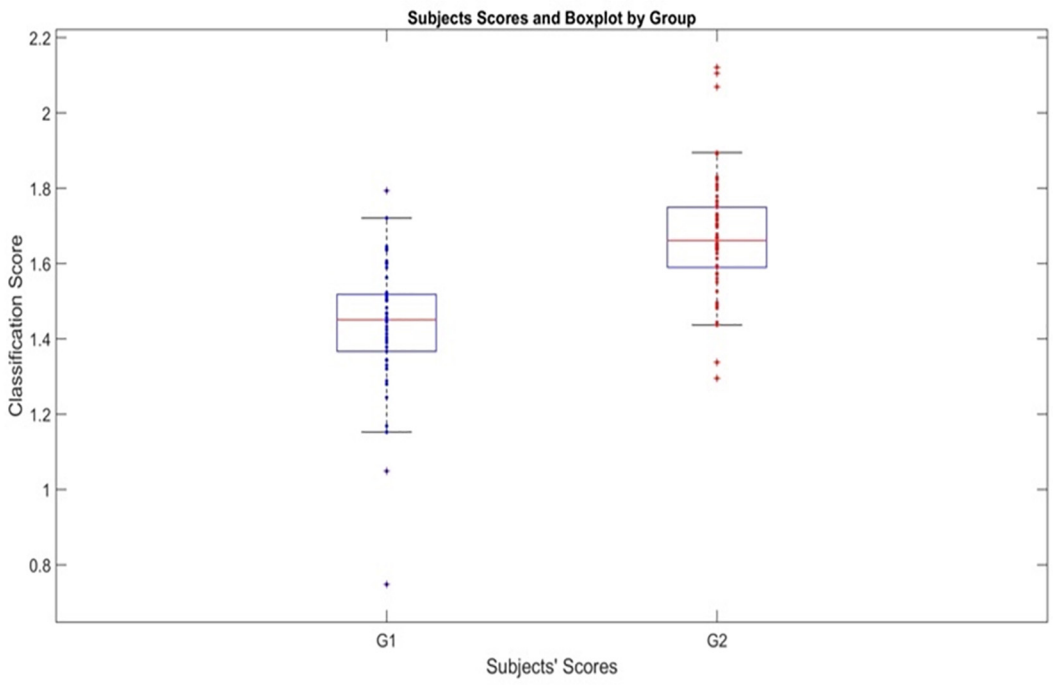

FIGURE 3 | Classification scores produced by the age-adjusted classifier for PEM and Control Groups. Boxplot showing the scores t_i of subjects for both groups using the individual classification scores. G1 is Malnutrition group (PEM) and G2 Control group.

There is a clear separation provided by the stable age-adjusted classifier. Note that this classification is based on the test set, not the training set for the median value of the AUC curve density.

Figure 4 shows the values of the coefficients (also in **Table 2**). In this figure positive for both coefficients indicate that increased activity (taking into consideration age or not) drives the score toward the control group. Due to the disparity in the values of the coefficients a square root transformation and rescaling of the alpha activity in the right lingual gyrus were applied. Thus, classifier is dominantly driven by low and high alpha activity in the right lingual gyrus, which is part of the occipital lobe.

Figure 5 (two upper rows) displays t-test comparisons (threshold selected by permutations) between the source activity of PEM vs. control groups. Areas colored in blue indicate negative values, for those frequency bands and structures in which PEM has significantly less activation than control. Red indicates significant positive t statistics, where PEM has excess activation when compared with controls. This figure is only shown to allow comparison with similar results using VARETA (Taboada-Crispi et al., 2018).

The areas selected by the age-adjusted classifier are shown in the lower two rows of the figure. Note that all the areas included in the age-adjusted classifier are significant other than the lingual gyrus hi-alpha which is just below the permutation threshold.

DISCUSSION

Classification Accuracy

In this paper we report a new classification procedure that uses source activity estimated using the BC-VARETA procedure, to delineate neural effects of exposure to protein energy malnutrition (PEM) in the first year of life. As seen in **Figure 1**, the area under the ROC curve is well above chance level. Importantly, it is necessary to limit the False Positive Rate (FPR) to either 0.1 or 0.2 to obtain consistent and high classification rates. Such low FPRs are of practical importance, since in screening programs an excess of false positives might overload health systems. The good performance of our classifiers at low FPR protects against this.

An Alternative View of Frequency Bands and Source Locations

The regression coefficients in **Table 2** are interpreted, not in terms of the log transformed power at the sources but in terms of the power. If one takes the exponent of the classification equation (6) then the classification score is the product of terms:

$$\exp(t_i) = \prod_{j=1:p} s_{i,j}^{d_j(age_i)} \tag{7}$$

with t_i the classification score for subject i, $s_{i,j}$ the spectrum for i in frequency band/anatomical location j. Note that this (7) is a product of the source activations with the age-dependent regression coefficients $d_j(age_i)$ as exponents. This formula provides a data-driven generalization of previous power ratios that were previously popular. For example, the α/ϑ ratio for any given sources is obtained by setting the $d_\alpha(age_i) = 1$, and $d_\theta(age_i) = -1$, irrespective of the data.

The resulting classifier is mainly driven by alpha activity in the right lingual gyrus. The signs of the age dependent regression coefficients indicate that low and high alpha activity in this area contribute to classification of nutrition status in the first year

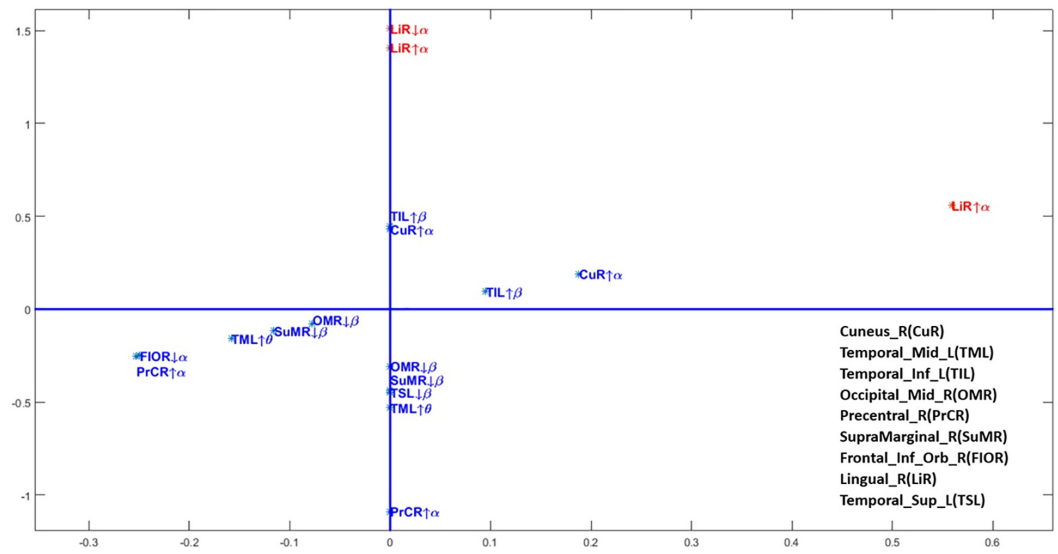

FIGURE 4 | Scatterplot of the coefficients of the age-adjusted classification regression equation. The regression coefficients for each frequency band and source anatomical region included in the age-adjusted classifier. Axis transformed by a sqrt root function to improve visualization. On the horizontal axis the coefficient ϕ_j for the interaction with age and on the vertical axis the age intendent coefficients ψ_j. This is the same information as in **Table 2**. Note that due to the disparity of scales the alpha activities in the lingual gyrus were also further divided by a factor of 100.

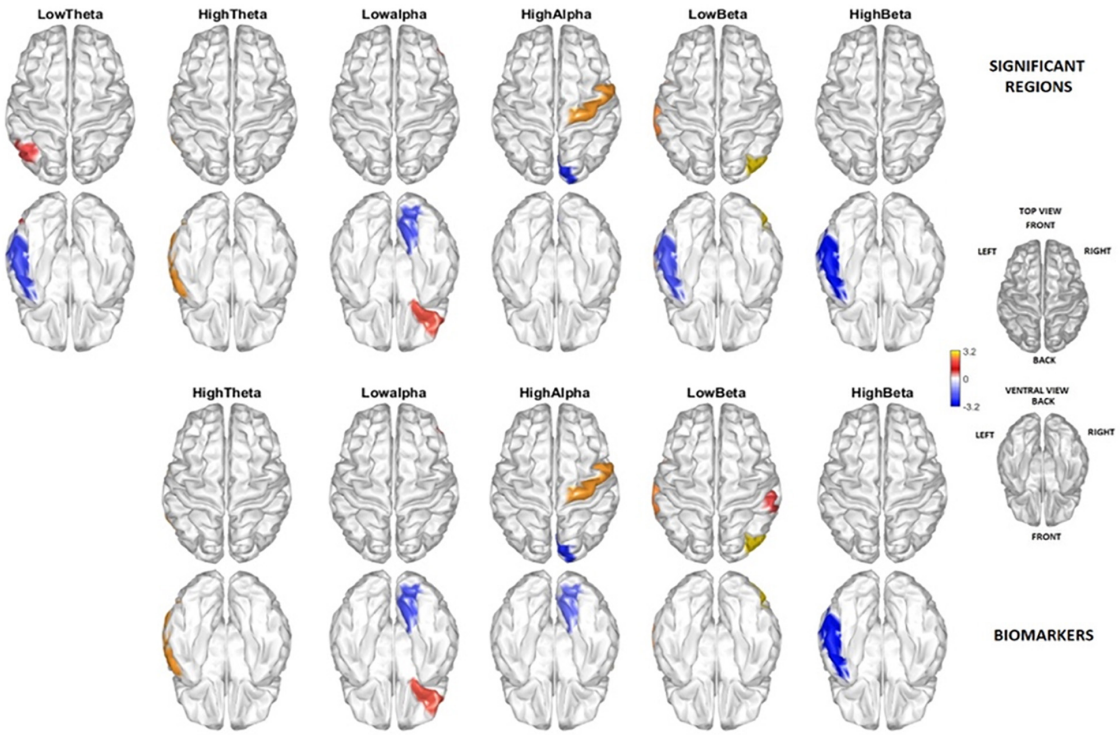

FIGURE 5 | Brain areas and frequencies contributing to the discrimination between PEM and Control. The two rows above show the regions by frequency bands with significant *t*-test group differences (threshold corrected by permutations) contrasting PEM vs. Control. Highlighted are those areas with a *t*-test in a range of exceeding (red) and below (blue) the permutation selected threshold for $p < 0.05$. The two lower rows show the brain regions and frequencies selected as biomarkers. There is a quite good correspondence between the two independent results, except that no biomarker was selected in the Delta band by the classification procedure.

of life. Specifically, low alpha values in the lingual gyrus are an indicator of a history of PEM. This is in agreement with the significant differences between the two nutrition groups, as confirmed by *t*-tests (**Figure 5**).

Comparison With the Previous Classifier Using Scalp qEEG Variables

We previously used the Sparse Stable Biomarker (SSB) selection method to obtain a classifier to differentiate between PEM/Control for this same data set (Taboada-Crispi et al., 2018). In our earlier paper, the potential biomarkers were the log EEG spectra at the scalp (topographical level) which yielded an AUC of 0.83, nearly equivalent to our current result. Due to the use of a randomization sampling scheme, the estimates vary somewhat, ranging from 0.81 to 0.86 for the qEEG classifier.

As mentioned previously, it was impossible to use a source-based classifier in Taboada-Crispi et al. (2018) due to the high dimensionality and correlation of VARETA sources, a limitation which we have overcome in the present paper. The new classifier achieves similar accuracy compared to that reported previously, allowing us to now identify the areas that most differentiate PEM from controls with high confidence. However, our classifier selects the minimal subset needed to accurately identify children with early malnutrition and this is only a small portion of the widespread areas affected by PEM. As such, a more complete analysis follows.

Physiological Interpretation of PEM-Control Differences

The neurophysiological impairments associated with early PEM can be permanent, often accompanied by widespread neurological disturbances involving sensory-motor activity, learning, memory, consciousness, cognition and emotion (Guedes, 2011). The BNS study is unique because the participants experienced a single episode of malnutrition limited to the first year of life. In order to identify all areas affected by malnutrition, *t*-tests at the source level (threshold corrected by permutations) compared participants with PEM with Controls (**Figure 5**). Many frequency bands/areas identified in this study were previously reported (Taboada-Crispi et al., 2018).

Notably, there are widespread changes in alpha activity; alpha is decreased in previously malnourished participants in the right inferior fronto-orbital area, and increased in the right precentral gyrus. However, a novel finding is the marked decrease of alpha activity in the right lingual gyrus for the PEM group made possible only by the improved localization capability of BC-VARETA. This is the single feature which predominantly accounts for individual classification of participants. These

findings lend further support to our hypothesis (Taboada-Crispi et al., 2018) that early PEM impacts timely cortical myelination thereby causing a delay in the development of the alpha rhythm. This conclusion is supported by several lines of evidence:

1. Cortical rhythmic activity depends critically on thalamo-cortical pathways and the inhibitory feedback of the thalamic reticular nucleus (Llinás and Steriade, 2006). This has been modeled in detail previously (Valdes et al., 1999; Valdes-Sosa et al., 2009). These models predict an increase in the peak alpha frequency dependent on the set of parameters defining the thalamo-cortical loop. The specific role of axonal delays is also considered in Douglas and Douglas (2019).
2. A previous study of 300 normal subjects from the Cuban Human Brain Mapping Project database showed that the peak alpha frequency in normal subjects depends on the microstructure of thalamo-cortical pathways in the optic radiation, thus supporting our neural mass model explanation (Valdés-Hernández et al., 2010).
3. Extensive normative studies by our group have shown that the peak alpha frequency increases from the high theta band to the typical alpha band over the lifespan (5–97 years). This is valid both for scalp EEG (Alvarez Amador et al., 1989) and sources (Bosch-Bayard et al., 2001). These changes in alpha are most pronounced in the first years of life.
4. Studies of myelin development resulting from longitudinal studies have demonstrated that normal brain development involves a linear increase in white matter from childhood to adulthood especially in the optic radiation (Almli et al., 2007; Laule et al., 2007; Dean et al., 2014). See especially **Figure 5** FA changes for the OR in Dubois et al. (2014). For a review of related studies see Tau and Peterson (2010).
5. In the current study, PEM took place during the first year of life, the most significant period of myelination. This may have life-long lasting effects on alpha rhythm development, as evidenced by a slower peak alpha frequency.

Limitations

This study has several limitations:

1. The variables explored as classifiers are the source log Spectra estimated by BC-VARETA. An essential, but still difficult problem is the full incorporation of partial coherences (brain network information) in addition to source activations as classification variables. This will conceivably improve classification accuracy greatly. While the calculation of partial coherences is inherent to BC-VARETA, their use is challenging due to the need for Riemannian classification procedures mandated by the estimated quantities (Li et al., 2009).
2. Due to the novelty of the BC -VARETA technique and the statistical challenges involved with this procedure, a comprehensive calculation of multinational age dependent norms is being developed and will be tested in the future.

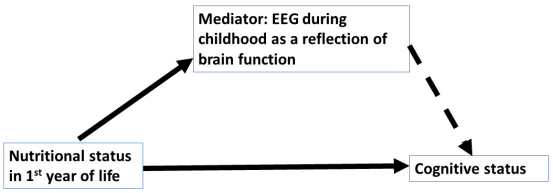

FIGURE 6 | Conceptual model to study EEG sources as mediators. The current paper is part of a program to determine EEG biomarkers signaling neural mediators on the long-term effect of PEM in the first year of life on childhood cognitive performance. The solid arrows indicate confirmed paths: Nutrition- > cognitive variables (Galler et al., 1983a,b); Nutrition - > EEG (this paper). Dashed arrow indicates paths to be confirmed.

3. Importantly we are analyzing data in fixed frequency bands. Methods based on individualized moments of the spectra or decompositions into peaks (Pascual-Marqui et al., 1988; Valdés et al., 1992) could further serve to enhance the accuracy of the classification scheme since it would focus on peak alpha frequency. However, we have already shown elsewhere that the less computer intensive approach using fixed spectral bins can serve as a screening procedure for further analysis.
4. In Taboada-Crispi et al. (2018) we showed that visual inspection of the EEG by experts provided additional information about brain states in both PEM and control groups. These evaluations were based on "grapho-elements" (such as "sharp waves" whose shape exhibits non-linearly determined time/frequency phase relationships). A non-linear set of features obtained at the source level also needs to be explored in order to quantify this type of assessment (Valdes et al., 1999).
5. This study is only the first step in building a disease progression model in which EEG variables are explored as potential mediators of the long-term cognitive and behavioral outcomes of childhood malnutrition. Such a model could incorporate other environmental factors to identify individual trajectories of the evolution of brain states. Our immediate research agenda is shown in **Figure 6** for detecting the mediation (through altered brain function) of the effect of early malnutrition on cognitive outcomes. With this paper we have identified the link: Nutritional status - > Functional alteration in brain structures. The analysis of the full model will be carried out in future studies.

CONCLUSION

The effects of Protein Energy Malnutrition in the first year of life can be detected by an age-adjusted classifier based on the logarithms of the EEG source spectra. Basing the classifier on the source spectra allows for an anatomic interpretation of the classifier's variables—in this case alpha activity in the lingual gyrus. Thus, we provide evidence that EEG sources can be an important component to consider as mediators in

disease progression models that ultimately could provide lifelong predictions of cognitive development to optimize cost/effective health interventions.

DATA AVAILABILITY STATEMENT

The EEG datasets employed for this study can be obtained by request to the corresponding author (pedro.valdes@neuroinformatics-collaboratory.org) and the dataset regarding social, demographic, and psychological assessment to JG (Janina.galler@gmail.com) who is the co-founder and co-director of the Barbados Nutrition Study.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of the Ministry of Health, Barbados, the Judge Baker Children's Centre Human Research Review Committee (Assurance No. FWA 00001811), and the Massachusetts General Hospital IRB (2015P000329/MGH). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

PV conceived, organized and executed this project with the collaboration of MB and JG who is the co-founder and director of the 45+ year Barbados Nutrition Study. MB, JG, and PV were involved with the recovery of the EEG archival data. The initial EEG pre-processing was performed by AC and the sensors and EEG source analysis was performed by YG and QT under supervision of PR, FR, and DP. The statistical analysis was designed by PV together with JB-B and LG. The manuscript was prepared by MB, PV, and JG. AR contributed to the writing and final editing of this report.

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Comparison of Visual Functions of Two Amazonian Populations: Possible Consequences of Different Mercury Exposure

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The present study investigated the visual perimetry and color vision of two Amazonian populations differently exposed to mercury. Ten riverines environmentally exposed to mercury by fish eating and 34 gold-miners occupationally exposed to mercury vapor. The visual perimetry was estimated using the Förster perimeter and the color vision was evaluated using a computerized version of Farnsworth–Munsell test. Riverine and gold-miners' hair mercury concentrations were quantified. Mercury hair concentration of the riverines was significantly higher than that from gold-miners. Riverines had lower perimetric area than the gold-miners. The errors in the hue ordering test of both Amazonian populations were larger than the controls (non-exposed subjects), but there was no difference between themselves. Riverines had significant multiple association between the visual function and hair mercury concentration, while the gold-miners has no significant association with the exposure. We concluded that the different ways of mercury exposure led to similar visual outcomes, with greater impairment in riverines (organic mercury exposed subjects).

Keywords: neurotoxicology, mercury vapor, organic mercury, visual field, color vision, Amazon region, psychophysics

INTRODUCTION

Since the end of the 1980s, there has been a growing concern with the environmental contamination of the Amazon region by mercury. The "gold rush" in the Amazon region brought about the unselective use of mercury in the process of mineral extraction, resulting in a large deposition of mercury in the Amazon rivers (Malm et al., 1995). This happens because of a high natural mercury concentration in the soil, to which deforestation, erosion, and spray of anthropogenic mercury contamination through rainfall are added (Roulet et al., 1998). Amazon rainforest soil retains mercury as a result of continuous input of mercury from the atmosphere and weathering processes on the rocks (Figueiredo et al., 2018). Deforestation can significatively increases the

soil-atmosphere mercury exchanges and the leaching process that increases the mercury release from the soil to the rivers (Magarelli and Fostier, 2005).

Human exposure in the Amazon may occur in miners, directly by inhalation of metallic mercury vapor in the process of gold extraction through occupational exposure, or in riverines, indirectly by consumption of fish containing methylmercury accumulated in the food chain (Kershaw et al., 1980; WHO, International Program on Chemical Safety, 1989; Malm et al., 1995; Akagi and Naganuma, 2000). After the incidents of high mercury exposure in Minamata, Japan, the brain was the main target of the mercury toxicity (Amin-Zaki et al., 1976), mercury neurotoxicity involves neuronal destruction, beginning with effects on the occipital cortex and cerebellum (Takeuchi, 1968), and the clinical manifestations include loss of vision and hearing, mental disturbances, impairment of verbal learning and memory, paresthesia, ataxia, neurasthenia, spasticity, tremor, and reduction of concentration, tendon reflex, manual dexterity, fine motor speed and dexterity, salivation, and even coma and death (Takeuchi, 1977; Ekino et al., 2007).

Our knowledge about the visual consequences associated with mercury vapor exposure came from investigations with workers of factories that manipulated mercury in some stage of the productive process (Ventura et al., 2004, 2005; Feitosa-Santana et al., 2007, 2008). Few studies have reported repercussions of the occupational mercury exposure in Amazonian gold-miners that inhaled mercury vapor (Rodrigues et al., 2007; da Costa et al., 2008). Other studies have examined fish or seafood consumers to detect visual sequels associated with environmental exposure to mercury. Specifically, in the Brazilian Amazon region, most of the investigation has been done in riverside populations dependent on fishery (Lebel et al., 1996, 1998; Rodrigues et al., 2007; Lacerda, 1997; Passos and Mergler, 2008; Fillion et al., 2011, 2013; Dos Santos Freitas et al., 2018; Feitosa-Santana et al., 2018). The literature describes that mercury exposure is associated with several visual impairments – decreased color vision, reduced contrast sensitivity, visual field constriction (Lebel et al., 1996, 1998; Rodrigues et al., 2007; Fillion et al., 2011, 2013).

As the mercury biochemical pathways in the human body are different for mercury vapor and methylmercury from food, the comparison of visual functions between two populations with similar genetic background, but different exposure type could help to understand the visual function changes associated with mercury exposure. In the present study we compared the visual field perimetry and the hue ordering of Amazonian populations that had mercury exposure to methylmercury through fish consumption or to mercury vapor through occupational exposure.

MATERIALS AND METHODS

Study and Ethics Statements

This is a cross-sectional descriptive and analytical observational study. All subjects gave written and informed consent to participate in this study. All procedures were evaluated and approved by the Ethics Committee in Research in Humans of the Tropical Medicine Center of the Federal University of Pará (Protocol #021/2009-CEP-NMT/UFPA).

Population

The sample is comprised of 44 total subjects, forming two groups according to the type of mercury exposure: 10 riverines (all males, 44.8 ± 14.3 year old) environmentally exposed to mercury by fish eating who lived in the Tapajós River basin, Pará, Brazil; and 34 gold-miners (all males, 46.2 ± 8.6 year old) occupationally exposed to mercury vapor from Serra Pelada mining, Pará, Brazil. **Figure 1** shows the location of the communities studied in the present investigation.

The riverside communities from the Tapajós River basin are located near Itaituba a medium sized port city in the West of Pará State, Brazil. These villages are located around regions historically known as mineral extraction regions that used mercury in the artisanal process of gold extraction, an important source of environmental contamination. Moreover, there is important deforestation around the city of Itaituba that should be considered as source of mercury in the region. No participant from Tapajós river communities inhaled mercury vapor, and mercury exposure was mainly dependent on fish consumption. Serra Pelada village is located near the small city called Curionópolis, in the Southeast of the state of Pará, Brazil. It is a region that constituted one of most important sites of gold extraction mining in the 1980's and was still active at the moment of the study. The workers involved in this activity were strongly exposed to mercury vapor originated from amalgamation of gold in the metal extraction process.

These communities shared the same culture, language, and socio-environmental conditions and they are in the same state of Brazil. They also had little education, poor sanitation, and low-paying jobs. All the participants from both communities reported a non-systematic smoking habit or even alcoholic beverage drinking habit.

On our arrival to each village, the invitation procedure to join this study was similar: a meeting was called by the local community health nursing aides to explain the study purposes to the villagers and to invite them to participate. The study took place at the Community Public Health Post and a questionnaire including socio-demographic information, smoking and drinking habits, fish weekly intake, and medical and work history was given by interview; the entire procedure took approximately 1 h. We tested a different number of participants for each visual test, depending on their availability.

Hair Mercury Exposure Quantification

Hair samples were analyzed using cold vapor atomic absorption spectrometry (Mercury Analyzer HG-201, Sanso Seisakusho, Tokyo, Japan) according to a previously published protocol (Suzuki et al., 2004). Analytical quality control was warranted by International Atomic Energy Agency certification (IAEA-085) and the measurements were performed in duplicate. All the results were expressed as μg Hg per g hair (μg/g).

Human hair material (IAEA-086) was the reference to validate the mercury concentration.

FIGURE 1 | Location of the mercury-exposed communities studied in the present investigation. Both communities shared similar geographical, economical, environmental conditions, but differed in mercury exposure.

Visual Tests

All the subjects had visual acuity of 20/40 or better in both eyes. We chose the eye with better visual acuity to be tested.

Visual Perimetry

All tests were carried out monocularly. The visual perimetry was estimated using the Förster perimeter. Foster perimeter is a broad semicircular arc that can be rotated manually on its axis. There is a fixation point at the arc center and a white dot at the arc that can be moved along the arc. The patient was instructed to fixate on the center of the equipment. The experimenter moved the white dot from the border of the arc toward to the center. The patient was instructed to inform the moment that the white dot is detect. The test is performed in different rotating angles of the arc, and the experimenter recorded the angle at the arc which was detected the white dot. At the end of the experiment, we quantified the perimetric area.

Hue Ordering Test

The hue ordering test was a home-made computerized version of the Farnsworth–Munsell 100 hue test (Bento-Torres et al., 2016). It consisted of 85 circular stimuli (1° of visual angle, mean luminance of 41.75 cd/m^2) of different hues and same saturation. Four sets of caps were shown separately in the same order of the conventional test. Initially, the correct sequence of the caps was presented to the participant, followed by its disarrangement. The participants were instructed to reorder as close as possible in the original hue sequence. We measured the arrangement errors for each cap position and the total error score (TES) such as done in Bento-Torres et al. (2016) and Feitosa-Santana et al. (2018). The error calculation considered that the caps had values from 1 to 85. After the participant completed the hue ordering task, we calculated the partial error score (PES) for each cap (Eq. 1), as the sum of the absolute difference between the cap value in the position i and in the neighbor positions i+1 and i-1. For each cap, the correct ordering resulted in a PES value of 2.

$$\mathrm{PES} = |n_i - n_{i-1}| + |n_i - n_{i+1}| \quad (1)$$

Total error score was considered as the sum of all 85 PES minus 170. The perfect performance resulted in a TES

of 0. The hue ordering results were transformed to square root values.

Statistics

The hue ordering score of the exposed groups were compared to the database of an age-matched control group that lived in an urban region without relevant contact to Hg contamination sources (n = 41 male volunteers). All the controls had no history of systemic or neurological diseases that influenced the visual function and had normal or corrected to 20/20 visual acuity. We considered the perimetric area of 57.07 cm^2 as reference value for normative results of the visual perimetry as informed by the device manufacturer (American Optical Company, United States).

We compared the hair mercury concentration and the visual perimetric area obtained from each group using the t-test with Welch's correction. We used G-test to compare the fish weekly intake of the groups. We used Welch one-way ANOVA followed by Tukey test *post hoc* to compare the color vision outcomes among the control, riverines and gold-miners. We evaluated the linear correlation between both visual outcomes from each community using the Pearson product-moment correlation. For all comparisons, we considered the significance level of 0.05.

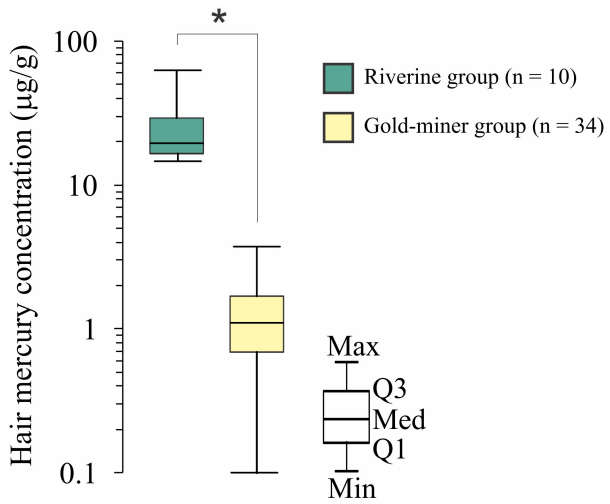

FIGURE 2 | Hair mercury concentration comparison between riverines and gold-miners. Riverines has significant higher hair mercury concentration than the gold-miners. *Significant difference, $p < 0.05$. Box-plots are composed by first (Q1), second (Q2), and third (Q3) quartiles, and whiskers represent the maximum (Max) and minimum (Min) values.

RESULTS

Mercury Exposure

The **Table 1** shows the age and fish weekly consumption from both groups. Both groups were age matched. The riverines had a higher fish weekly intake compared to the gold-miners. All the gold-miners used to have fish less than twice in a week, while the riverines have more than two meals including fish during the week. **Figure 2** shows the mercury exposure of each community. We observed that the riverine population had higher mercury concentration compared to the gold-miners [$t(9.02) = 5.159$, $p = 0.0006$].

Visual Function Evaluation

Figure 3 shows an example of the perimetry obtained from the right eye of a standard observer (in blue) and a mercury exposed subject (in red). All riverine subjects had a perimetric area smaller than the reference value, while 61.8% of the gold-miner group (21/34 subjects) were below the reference level. We found that the riverine population had smaller perimetric area compared to the gold-miners results [$t(31.57) = 3.613$, $p = 0.001$].

Figure 4 shows the comparison among control, riverines and gold-miners results obtained in the hue ordering test. Gold-miners had larger amounts of error than the controls [One-way ANOVA, $F(2,10.69) = 5.764$, $p = 0.02$], but there was no difference between the results obtained from the two mercury-exposed groups and between riverines and controls. Although, we have observed no difference between controls and riverines, the riverines had error closer to the gold-miners performance than to the controls.

The results of the multiple linear correlation among visual outcomes, age and hair mercury concentration are shown in the **Table 2**. We observed that both mercury exposed groups had no significant multiple correlation, but only the riverines had significant partial correlation between mercury exposure and visual outcome (visual perimetry). The visual outcomes of the gold-miners was not significantly associated to the hair mercury concentration and age. **Figure 5** shows the multiple correlation between the visual outcomes and the independent variables of age and hair mercury concentration.

We correlated the visual outcomes from each group. We found a significant linear correlation between the visual evaluation results obtained from the riverine population ($p = 0.004$), that was described by a negative correlation with linear coefficient of 0.97. For the gold-miners, no significant correlation was observed for their visual evaluation ($r = -0.24$, $p = 1755$).

TABLE 1 | Demographic characteristics and fish weekly intake of both communities.

Variable	Riverines	Gold-miners	*p*-value
Age (year old)	40.86 ± 13.2	45.9 ± 8.5	0.11[a]
Fish weekly intake			
<2	–	34	0.001[b]
2–4	4	–	
>4	6	–	

[a]*t-test;* [b]*G-test.*

DISCUSSION

The present investigation was the first comparison of visual performance between different groups of mercury-exposed

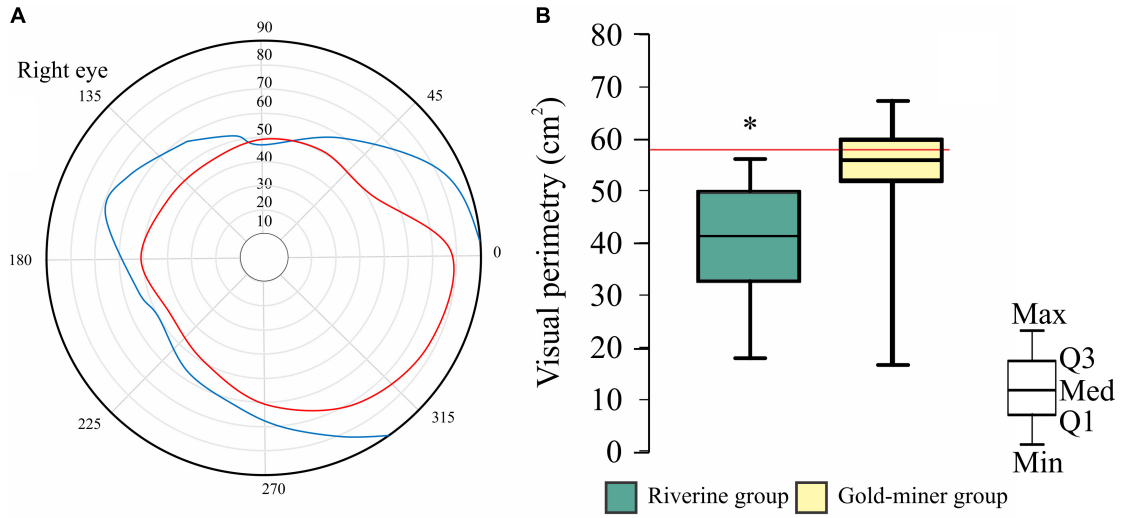

FIGURE 3 | Visual perimetric results. **(A)** Visual perimetry of a standard observer (blue line) and of a mercury exposed observer (red line). **(B)** Visual perimetric area distribution of the riverine (green box-plot) and gold-miner (yellow box-plot) group. *Significant difference, $p < 0.05$. Box-plots are composed by first (Q1), second (Q2), and third (Q3) quartiles, and whiskers represent the maximum (Max) and minimum (Min) values.

subjects living in the same region but with different types of exposure. Our main result was that both mercury exposed groups had visual deficits, but riverines showed greater visual impairment than gold miners.

The association between the mercury exposure and the severity of the decreased visual function is indicated by three findings: (i) the riverines had higher exposure to the mercury than gold-miners; (ii) riverines had worse performance for the visual perimetry than the gold-miners, but gold-miners had smaller performance in the color vision test than riverines; (iii) riverines' visual outcomes were highly correlated, the poor color vision, the smaller perimetric area, but the same was not found for the gold-miners what can be indicative of lower influence of the mercury exposure on the visual system in this participants.

The comparison between the mercury exposure suffering by riverines and gold-miners should be made carefully, because both groups have distinct types of mercury exposure and the metabolism of the metal in their bodies is completely different. We observed that the riverines had higher hair mercury exposure than the gold-miners. Our results showed that the riverines had higher fish weekly intake than the gold-miners. The fish intake is positively associated to the hair mercury concentration (Passos et al., 2008).

The visual function has been used as biomarker of the mercury exposure. No specific mechanism of visual loss has been fully described, but probably involves alterations since from the optical apparatus of the eye up to visual cortex. Mercury has a cataractogenic potential because it can induce aggregation of human lens proteins (Domínguez-Calva et al., 2018),

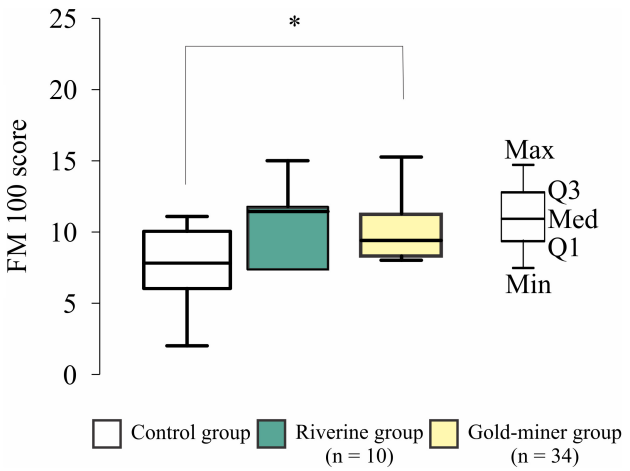

FIGURE 4 | Hue ordering results. Distribution of control (white box-plot), riverine (green box-plot), and gold-miner (yellow box-plot) groups. Both mercury exposed groups had higher error scores than the controls (*$p < 0.05$). Box-plots are composed by first (Q1), second (Q2), and third (Q3) quartiles, and whiskers represent the maximum (Max) and minimum (Min) values.

TABLE 2 | Multiple linear regression results.

Dependent variable	Partial r (Hg concentration)/ *p*-value	Partial r (age)/ *p*-value	R^2/*p*-value
Riverines			
Visual perimetry	0.56/0.04	0.04/0.87	0.46/0.1
Color vision	−0.08/0.33	−0.09/0.47	0.82/0.06
Gold-miners			
Visual perimetry	0.06/0.97	−0.25/0.16	0.06/0.12
Color vision	0.53/0.21	0.08/0.09	0.05/0.16

R^2 = coefficient of multiple determination, r = coefficient of regression.

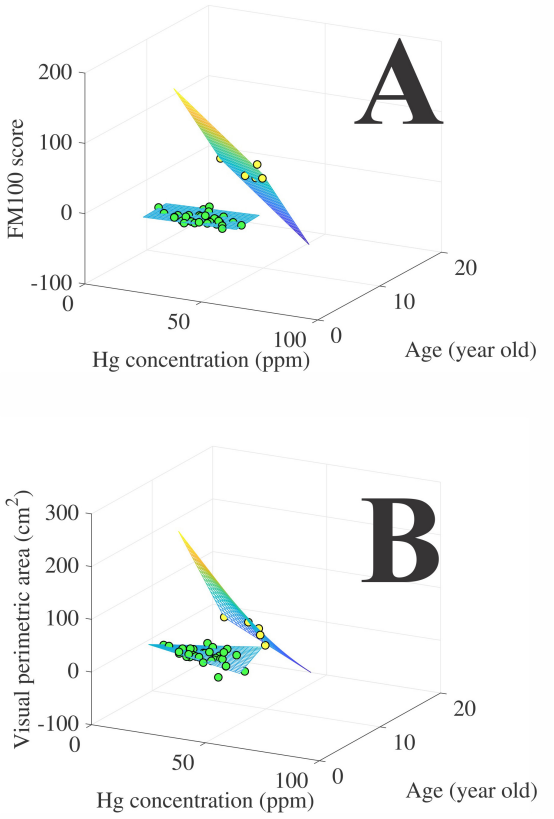

FIGURE 5 | Multiple regression between the visual outcomes (**A**, hue ordering test performance; **B**, visual perimetric area) and the independent variables (age and hair mercury concentration). Green circles represent the riverines data points (n = 5) and yellow circles represent gold-miners data points (n = 34). The grid represents the best three-dimensional model that fitted the visual outcomes to the independent variables.

electroretinographic and cortical recordings in mercury exposed humans suggest impairment of the retinal and cortical activity (Ventura et al., 2004, 2005; Saldana et al., 2006; da Costa et al., 2008; Yorifuji et al., 2013). The influence of mercury in the visual perception has been investigated by many psychophysical tests for hue ordering test, chromatic discrimination, visual acuity, and visual field sensitivity (Cavalleri et al., 1995; Cavalleri and Gobba, 1998; Rodrigues et al., 2007; Fillion et al., 2011, 2013; Dos Santos Freitas et al., 2018; Feitosa-Santana et al., 2018).

Other investigations evaluated the visual field of subjects exposed to mercury vapor (Barboni et al., 2008). Exposed subjects were factory workers that inhaled the mercury vapor during the manufacturing of fluorescent lamps. A constriction of the visual field was observed in these subjects. In the present study, we observed that the gold-miners, despite being associated with mercury vapor exposure, had normal visual fields. This difference could be explained by the factory workers that had a constant exposure to mercury vapor in the work environment (constant exposure), while the gold-miners only had exposure during the process of gold extraction (intermittent exposure). Additionally, the present study and Barboni et al. (2008) differed in the method to evaluate the visual field. In the factory workers evaluation, static Humphrey visual perimetry – that measured the luminance threshold for different locations of the visual field – was used, while we used the dynamic perimetry to identify the boundaries of the visual field. The test we chose is less sensitive than the static visual field analyzer, but it is portable, which makes it possible to take it for field work in the Amazonian villages.

Previously, other investigations have shown color vision loss in fish consuming riverine populations (Rodrigues et al., 2007; Dos Santos Freitas et al., 2018; Feitosa-Santana et al., 2018) in the Amazon region. Most of them have used hue ordering tests to evaluate the color vision of the exposure subjects (Dos Santos Freitas et al., 2018; Feitosa-Santana et al., 2018). We confirmed the previous findings showing that color vision is altered in subjects with no history of mercury vapor exposure, but have fish as their main source of proteins. We also confirmed that color vision is altered in mercury vapor exposure subjects, as observed in the factory workers. Our new contribution about color vision in Amazonian populations is that there was no difference in the color vision deficits shown by both groups of mercury exposed subjects.

We cannot assert that all the visual disturbances that we found are caused by mercury, but as both communities have similar socioeconomical profile (low paying-jobs, poor sanitation, infectious diseases), age, environmental condition (sunlight exposure and climate), we consider that the difference of mercury exposure is associated to the visual differences between the communities. The inhalation of the mercury vapor is the primary route of entry into the body for inorganic mercury. Its absorption is fast by diffusion in the lungs and the half-life estimated in the body is about 60 days. Its excretion is mainly by the feces and urine elimination (Sandborgh-Englund et al., 1998; Park and Zheng, 2012). In the body, the elemental mercury is converted to an oxidized form, which does not effectively cross the blood-brain barrier (Friberg and Mottet, 1989). Methylmercury is separated from the food by the gastric acid and is absorbed in the duodenum. It has high affinity with lipophilic tissues and easily crosses the blood-brain barrier and accumulates in the nervous tissue (Lee et al., 2006, Korean Food and Drug Administration, 2007).

We concluded that the Amazonian population exposed to different mercury forms showed similar visual deficits, with greater impairment in riverine communities. Our findings indicated that these different populations need specific health and education programs to become conscious of the dangers of exposure to mercury. People could very well be aware of the dangers of mercury, but avoiding it could be a greater challenge as fish is a dietary mainstay for the riverine population and gold extraction is one of the few economical activities in the Serra Pelada region. Larger scale interventions should aim at reducing mercury at the source, and the delivery of health services should also be improved.

ETHICS STATEMENT

All procedures were evaluated and approved by the Ethics Committee in Research in Humans of the Tropical Medicine Center of the Federal University of Pará (Protocol #021/2009-CEP-NMT/UFPA).

AUTHOR CONTRIBUTIONS

All authors contributed to the conception of the work, drafting and revising the manuscript, approved the final version, and agree to be accountable for all aspects of the work. LS, MP, and DV designed the experiments. MC, EL, and AR carried out the psychophysical experiments. GS and EL analyzed the data. EL, GS, and DV wrote the components of the manuscript.

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Antidepressant, Anxiolytic and Neuroprotective Activities of Two Zinc Compounds in Diabetic Rats

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Behavioral disorders affect most diabetic patients and Zinc (Zn) has been used among adjuvant therapies for involvement in the etiology of depression and anxiety, however, the results are still controversial. The objective of this study was to compare the antidepressant, anxiolytic and neuroprotective activity of the supplementation of two Zn compounds in an animal model of Diabetes Mellitus type 1 (DM1). Thirty-eight (38) adult rats were randomized into four groups: Control (C; n = 8); Diabetic (D; n = 10); Diabetic Zn Sulfate Supplement (DSZ; n = 10) and Diabetic Zn Gluconate Supplement (DGZ; n = 10). The DSZ group received Zn sulfate supplementation and the DGZ group received Zn gluconate supplementation at a dose of 15 mg/kg for 4 weeks. Data (mean ±SEM) were analyzed by the Mann–Whitney test with a significance level of $p < 0.05$. The results indicate that Zn gluconate supplementation in diabetic animals presented an antidepressant effect demonstrated through the results obtained in the Forced Swim Test, and neuroprotective effect by attenuating alterations in the cerebral cortex; while Zn sulfate supplementation in diabetic animals showed an anxiolytic effect demonstrated by the results obtained in the open field test and the elevated plus maze test. Considering the set of results, supplementation with both zinc compounds showed neurobehavioral benefits in diabetic animals with different effects depending on the type of anion associated with Zn.

Keywords: anxiety, depression, supplementation, zinc, diabetic rats

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INTRODUCTION

Diabetes mellitus type 1 (T1DM) is a chronic disease characterized by insulin deficiency due to pancreatic β-cell loss and leads to hyperglycemia (American Diabetes Association, 2017). Diabetes Mellitus (DM) is a public health problem due to the large number of people affected and the consequences of the disease, that contribute to decreasing the quality of life of patients (American Diabetes Association, 2017). Studies have shown that DM is related to an increased prevalence of psychiatric disorders, among which depression and anxiety (Rotella and Mannucci, 2013; Petrak et al., 2015) are noteworthy.

Depression is a common, chronic, and disabling psychiatric disorder, strongly related to an anonymous state (Strandberg et al., 2014) resulting in social and economic burden, as well as

enormous personal suffering and increased risk of mortality (Lépine and Briley, 2011). This disorder has been postulated to play a causal role in DM, and an increased risk has been reported in 60% of depressive individuals in developing this disease (Balhara, 2011). Similarly, the prevalence of anxiety disorders among diabetic patients is considerably higher compared to the general population (Huang et al., 2012).

In this context, it is imperative to seek adjuvants in treating diabetes and in preventing these disorders. Studies have related diabetes to zinc deficiency (Fernando and Zhou, 2015; Ranasinghe et al., 2015), even though this essential trace element is often present in the diets of diabetics in dietary sources such as eggs, cheese, meat, vegetables, whole grains, nuts and cereals, in addition to others such as several chemical form factors, the presence of absorption inhibitors or promoters, age and nutritional status of the individual may compromise their bioavailability in the body (Gibson, 2012). Zinc (Zn) plays substantial roles in inflammation suppression, oxidative stress reduction and in the correct functioning of lipid and glucose metabolism (Olechnowicz et al., 2018), especially in the brain, with higher concentrations being found in the hippocampus and amygdala regions (Grønli J. et al., 2013). Moreover, 300 enzymes are dependent on this trace element, many of them expressed in the central nervous system (Hagmeyer et al., 2015).

Zn deprivation affects brain homeostasis, leading to behavioral, cognitive and mental changes (Partyka et al., 2011; Szewczyk et al., 2011). In this sense, experimental and clinical observations have suggested that Zn is involved in the pathophysiology of depression and anxiety (Szewczyk et al., 2008; Mlyniec et al., 2014).

In fact, some studies suggest Zn as a protective agent against brain damage (Szewczyk, 2013), although it's excess may produce cytotoxic effects (Sensi and Jeng, 2004; Plum et al., 2010). However, in clinical studies, the association between the prevalence of psychiatric disorders and zinc deficiency is controversial (Nguyen et al., 2009; Irmisch et al., 2010; Grønli O. et al., 2013).

Some studies with animals have shown that Zn deficiency increases symptoms which are similar to those of depression (Tassabehji et al., 2008; Mlyniec et al., 2012), while others have shown that Zn deficiency-like symptoms similar to depression appear to be reversed by antidepressant treatment (Whittle et al., 2008; Mlyniec and Nowak, 2012; Mlyniec et al., 2012). Other non-clinical studies have demonstrated a more expressive type of depressant behavior in animals with induced diabetes (Wayhs et al., 2013; De Morais et al., 2014).

Different Zn compounds such as oxides, hydroaspartate, chloride were intraperitoneally administered at varying doses (Joshi et al., 2012; Torabi et al., 2013; Satała et al., 2016) to test antidepressant properties in several behavioral tests, suggesting that Zn may potentiate the action of antidepressants, in addition to reducing their side effects so that their supplementation has a therapeutic effect (Siwek et al., 2010; Ranjbar et al., 2013). Zn sulfate and gluconate are the most common compounds administered orally (Capdor et al., 2013; Roohani et al., 2013), with the choice of this route being important in T1DM since patients already use injectable insulin. However, their actions so far have been poorly elucidated in behavioral tests in diabetic animals.

In this context, experimental studies involving Zn supplementation in diabetic rats substantially contribute to better understanding behavioral disorders, specifically depression and anxiety, as well as being essential for the safe prescription of Zn supplementation as an adjuvant treatment alternative. Thus, the present study aimed to compare the antidepressant, anxiolytic and neuroprotective activity of supplementing two Zn compounds in an animal model of Diabetes Mellitus Type 1 (T1DM).

MATERIALS AND METHODS

Experimental Protocol

The experiments and protocols performed with the animals are in accordance with the principles recommended by Institute of Laboratory Animal Resources (2011). The protocols were approved by the Ethics Committee on Animal Use (CEUA) of the Federal University of Paraiba (UFPB), under no. 045/2015.

A total of 38 male adult Wistar (*Rattus norvegicus*, variety *albinus*) rats weighing 250 ± 30 g from Thomas George (UFPB) were used. The animals were housed in individual metabolic cages and maintained under standard lighting conditions (light/dark cycle, 12/12 h) and temperature (22 ± 2°C). Commercial ration (Presence, Paulínea, São Paulo) and filtered water were offered *ad libitum*. After 1 week of acclimatization, the animals were randomized into four groups: Control (C; $n = 8$); Diabetic (D; $n = 10$); Diabetic Zn sulfate supplemented (DSZ; $n = 10$) and Diabetic Zn gluconate supplemented (DGZ; $n = 10$) (**Supplementary Figure S1**). Data from healthy groups supplemented with zinc sulfate (SZ, $n = 8$) and zinc gluconate (GZ, $n = 8$) were presented as **Supplementary Figures S2, S3**.

Induction of Diabetes Mellitus Type 1 (T1DM)

The procedure for chemical induction of diabetes in the animals of the D, DSZ and DGZ groups was performed after a 12-h fast. A solution of streptozotocin (STZ, Sigma-Aldrich, St. Louis, MO, United States) dissolved in citrate buffer (0.1 M, pH = 4.5) was intraperitoneally administered in a single dose of 50 mg/kg body weight (Santos et al., 2014). Group C intraperitoneally received equivalent doses of citrate buffer (0.1 M, pH = 4.5), without the addition of STZ. Three days after induction, animals that received glycemia greater than 250 mg/dL were considered diabetic and included in the experimental groups (Santos et al., 2014).

Zinc Supplementation

The animals in the DSZ group received Zn sulfate supplementation and the animals in the DGZ group received Zn gluconate supplementation, both compounds orogastrically administered at a dose of 15 mg/kg body weight/day once daily for 4 weeks (Sapota et al., 2014). The dose choice used complied with the recommended maximum limit for rodents (National Academy of Sciences, 2001) and humans

(Institute of Medicine, 2002) and it was calculated for each compound as a whole. The animals of groups C and D underwent the stress of a gavage with filtered water during the same period of the other groups.

Behavioral Parameters

The animals of all groups were submitted to behavioral tests at the end of the 4 weeks of supplementation, and each test was performed on subsequent days. Ideal conditions were maintained for these tests: attenuation of noise levels, low illumination intensity and controlled temperature (±25°C). The animals were taken to the test room for acclimatization and adaptation half an hour before the experiments were performed.

Evaluation of Anxiolytic Activity

Anxiolytic activity was evaluated by the Elevated Plus Maze (EPM), in which behavioral conflict is based on the animal's need to explore the environment and the potential danger it poses (Pellow et al., 1985).

The apparatus (Insight brand, Madeira, EP 151, Ribeirão Preto, São Paulo, Brazil) consists of a platform with two open arms (50 cm× 10 cm) perpendicular to two closed arms (50 cm× 10 cm× 50 cm), forming a cross and raised 50 cm from the ground. There is an acrylic border of 1 cm in height surrounding the open arms in order to avoid falls by the animals. Each animal was positioned in the center of the apparatus facing one of the closed arms and allowed to operate the apparatus for 5 min. Animal preference for open or closed arms was evaluated. An observer made the annotations regarding the percentage of entries in the open arms and closed arms, length of stay in each arms and total number of arm entries (open arm entries+close arm entries). The apparatus was cleaned with 10% ethyl alcohol and paper towels before and after the exposure of each animal, allowing it to dry naturally.

Motor Activity Assessment

The exploratory activity was evaluated by the Open Field (OF) test (Candland and Campbell, 1962), used for evaluating stimulant compounds or CNS depressants. The apparatus (Insight brand, EP 154, Ribeirão Preto, São Paulo, Brazil) consists of a circular, transparent acrylic box measuring 50 cm in height and 60 cm in diameter. The white acrylic floor (100 × 80 cm) is divided into concentric circles (15, 34, and 55 cm radius) and black radial lines forming 12 quadrants of similar areas. The animals were individually placed in the center of the device and the following behaviors were then observed during 5 min: a manual counter was used for recording ambulation (number of entries by the animal with four legs into any of the squares), rearing (number of times the animal is raised on its hind legs, perpendicular to the ground), grooming (number of times the animal self-cleans), immobility time (complete immobility of the animal) and number of fecal cakes. There was a specific observer for each evaluated behavior, totaling four properly trained observers. The apparatus was cleaned with 10% ethyl alcohol and paper towels before and after the exposure of each animal, allowing it to dry naturally.

Evaluation of Antidepressant Activity

Antidepressant activity was evaluated by the Forced Swim Test (FST) through observation of animal behavior in the face of an unconditioned stress situation (Porsolt, 1979).

A cylinder (30 cm in diameter and 50 cm in height) was used with water at a depth of 30 cm and temperature (25 ± 2°C). The animals were taken to the antechamber, where they remained for 30 min. After that time, the animals were individually placed in the cylinder with water and submitted to the test for 5 min in a single session, with their active (swimming and climbing) and passive behavior (immobility) evaluated (Slattery and Cryan, 2012). The evaluations were performed by two observers and filmed, then later transferred to a computer and analyzed in detail. At the end of the test the animals were dried with cloth towels. The water was exchanged before and after exposure of each animal, and the apparatus was cleaned with 10% ethyl alcohol, allowing it to dry naturally.

Euthanasia

After 8 h fasting, the animals were intraperitoneally anesthetized with ketamine (25 mg/kg) associated with xylazine (25 mg/kg). The absence of reflexes was confirmed after anesthesia and the brains were collected for histological analysis.

Histological Analysis of the Brain

The brains (right hemisphere) of each animal were sanitized with saline, fixed in 10% buffered formalin and stored in coded containers. The organs were processed according to the routine histopathological technique. Ten semi-serial cuts of 5 μm thick were obtained from the paraffin embedded material, following a cross-sectional plane to the analyzed organ of each animal. The obtained slides were stained using Hematoxylin and Eosin (H and E) technique, and the assembly was performed between lamina and laminula with synthetic resin (Entellan-Merck) for analysis in increasing lenses and photographed at 100x total magnification under an optical microscope (Motic BA 200, Kowloon, Hong Kong). The structural architectures of the organs and the presence, characteristic and intensity of possible inflammatory infiltrates were evaluated in these analyzes.

Statistical Analysis

Data sets were tested for normality and homogeneity using tests of variance. Behavioral data (mean ±SEM) were analyzed by the Mann–Whitney test. The accepted level of significance was $p < 0.05$. The results were expressed as mean and standard error of the mean and were analyzed in Prism 6.0 software (GraphPad, San Diego, CA, United States).

RESULTS

Behavioral Parameters of Diabetic Rats Supplemented With Two Different Zinc Compounds

The study highlights the importance of zinc supplementation in the attenuated of common behavioral disorders, depression and

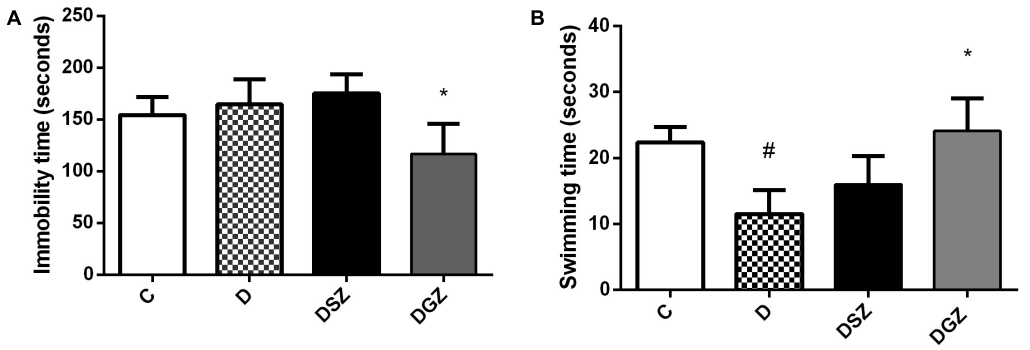

FIGURE 1 | Evaluation of antidepressant activity using the Forced Swim Test (FST) in diabetic rats treated with two different zinc compounds (15 mg/kg). **(A)** Immobility time and **(B)** Swimming time. C, Control Group (n = 8); D, Diabetic Group (n = 10); DSZ (n = 10): Diabetic Group Supplemented with Zn Sulfate and DGZ (n = 10): Diabetic Group Supplemented with Zn Gluconate (n = 10). Mann–Whitney test ($p < 0.05$). *Significant difference compared with D group; #significant difference compared with C group.

anxiety in diabetes. Zn gluconate supplementation in diabetic animals reduced the time of passive behavior characterized by the animal's immobility (116.6 ± 10.29 versus 164.4 ± 8.63 s, $p < 0.05$) and increased swimming time (24.12 ± 4.91 versus 11.5 ± 3.62 s, $p < 0.05$), both compared to group D (**Figure 1**). The active swimming behavior was similar between groups D and DSZ ($p > 0.05$).

Regarding the anxious type behavior measured in the Elevated Labyrinth Plus test (**Figure 2**), the diabetic group supplemented with Zn sulfate (DSZ) had longer length of stay in the open arms (3.12 ± 0.39 versus 1.12 ± 0.39, $p < 0.05$), higher frequency of open arm entries (22.13 ± 2.99 versus 7.87 ± 2.96 s, $p < 0.05$) and greater total number of open plus closed arm entries compared to group D (5.50 ± 0.56 versus 4.00 ± 0.53, $p < 0.05$). Whereas the DGZ group presented shorter length of stay in the open arms and higher frequency of open arms entry than the group D ($p < 0.05$).

The motor activity was measured using the Open Field (**Figure 3**). The DSZ group presented better responses as evidenced by greater ambulation (29.25 ± 0.90 versus 18.50 ± 2.24, $p < 0.05$) and shorter immobility time (3.12 ± 0.35 versus 6.87 ± 0.63 s, $p < 0.05$) compared to group D. The DGZ group had a shorter immobility time (3.87 ± 0.42 versus 6.87 ± 0.63 s, $p < 0.05$) compared to group D. In relation to the other evaluated parameters (rearing, grooming and number of fecal cakes) there were no differences ($p > 0.05$), so that these data were not included in the graphs (**Supplementary Table S1**).

Histological Analysis of the Brain of Diabetic Rats Supplemented With Two Different Zinc Compounds

Histology of the cerebral cortex of diabetic rats treated with two different zinc compounds is show in **Figure 4**, in order to associate these results with those of behavioral tests. It was observed that the group D animals presented cerebral degeneration characterized by the presence of ischemic neurons, hemorrhage and dilated vessels, whereas the DSZ group presented the presence of ischemic neurons, and animals from the DGZ group only had dilated vessels.

DISCUSSION

Diabetic animals had longer immobility time on the FST compared to diabetic and Zn Gluconate supplemented animals, thus reflecting their called "despair behavior" (Wayhs et al., 2010). The existence of DM is associated with anxiety disorders and increases the probability depression occurring (Golden et al., 2008; Huang et al., 2012). Our results suggest potential Zn Gluconate antidepressant activity evidenced by the shorter immobility time ($p < 0.05$) compared to the D and DSZ groups, considering that immobility time is used as an index of depressive behavior (Slattery and Cryan, 2012).

The swimming time parameter was used for evaluating locomotor activity and the results also showed a significant increase in the DGZ ($p < 0.05$) compared to the D group. The other active behavior parameter (climbing) did not present a statistically significant difference between groups ($p > 0.05$). In this sense, it is important to note that healthy animals supplemented with Zn did not obtain significant changes in behavior during the behavioral tests in our study, and therefore we can suggest that the beneficial effect of Zn supplementation only occurred in diabetic animals (**Supplementary Figure S2**).

A previous study (Szewczyk et al., 2011) found that immobility time on the FST was significantly higher in Zn-deficient mice, suggesting that a deficiency of this trace element causes an increase in behavior similar to depression. Hyperactivity of the glutamatergic system occurs in situations of Zn deficiency, thus generating depressive behavior, as demonstrated by Slattery and Cryan (2012). Animals using an antidepressant allied to an adequate or supplemented with Zn diet for 3 weeks showed reduced behavioral despair as measured by the FST (Tassabehji et al., 2008).

In recent decades, the Elevated Plus Maze (EPM) has established itself as the most classic animal model of exploratory behavior used in the laboratory. The animal explores both the

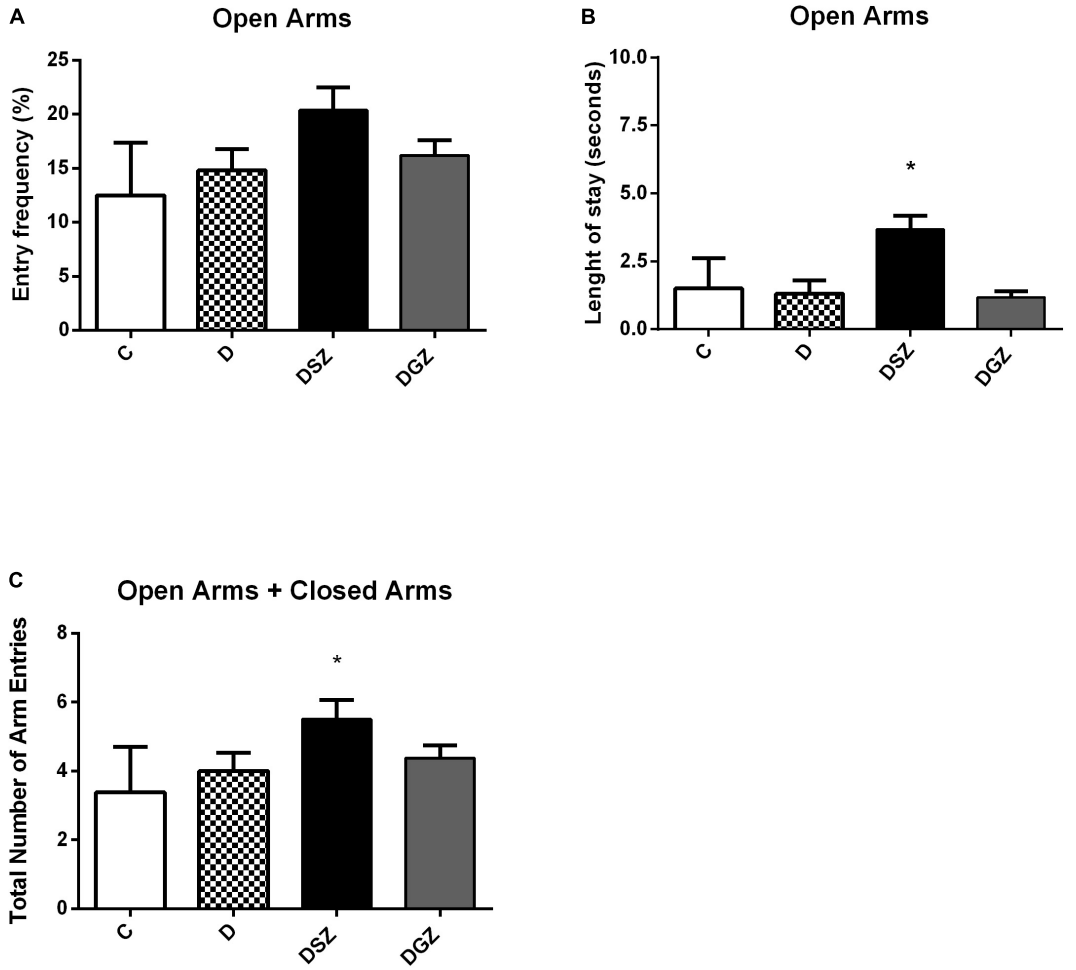

FIGURE 2 | Evaluation of the anxiolytic activity using the Elevated Plus Maze (EPM) considering the percentage of length of stay, the percentage of number of entries in the open arms and the total number of arm entries (open arm entries + close arm entries) in diabetic rats treated with two different zinc compounds (15 mg/kg). **(A)** Entry frequency in open arms, **(B)** Length of stay in open arms and **(C)** Total number of arm entries. C, Control Group (n = 8); D, Diabetic Group (n = 10); DSZ (n = 10): Diabetic Group Supplemented with Zn Sulfate and DGZ (n = 10): Diabetic Group Supplemented with Zn Gluconate (n = 10). Mann–Whitney test ($p < 0.05$). *Significant difference compared with D group.

open and closed arms of the labyrinth, but typically it will more frequently enter and will remain longer in the closed arms. However, a higher intensity of anxiety behavior equates to a lower preference for open arms and thus a higher predilection for closed arms (Ahn et al., 2013).

Our results demonstrated an increase in the length of stay and in the number of open arms ($p < 0.05$) in diabetic animals supplemented with Zn sulfate compared to the animals in groups D and DGZ, suggesting that this compound had an anxiolytic effect as confirmed by the longer stay in the open arms, and that the ambulation ability in these animals was preserved, indicating the ability of Zn to reduce anxiety without causing sedation.

In using the EPM to predict the potential anxiolytic activity of Zn hydroaspartate in rats and mice (Partyka et al., 2011) found an increase in the percentage of open arms entries in both species without significantly altering the length of stay in these arms. Zn chloride at different doses of Zn (15 and 20 mg/kg) promoted anxiolytic effects in supplemented rats, evidenced by the increase in the residence time and the greater number of open arms entries in the EPM (Joshi et al., 2012). Zn oxide supplementation at different doses (5 mg/kg and 10 mg/kg) resulted in an anxiolytic effect in the EPM test in rats (Torabi et al., 2013). However, a study on rats supplemented with Zn oxide at a higher dose (25 mg/kg) did not find a significant difference in the anxiety indexes assessed by the EPM (Amara et al., 2013).

In our study we evaluated five parameters: ambulation, latency time, rearing, grooming and fecal cakes. We realized that the abilities to explore the surrounding environment and spontaneous ability were severely impaired in group D, representing the behavioral and emotional changes that could mimic the clinical symptoms of depression. The increase in anxiety in OF is related to a decrease in locomotion and an increase in the peripheral area; an increased permanence

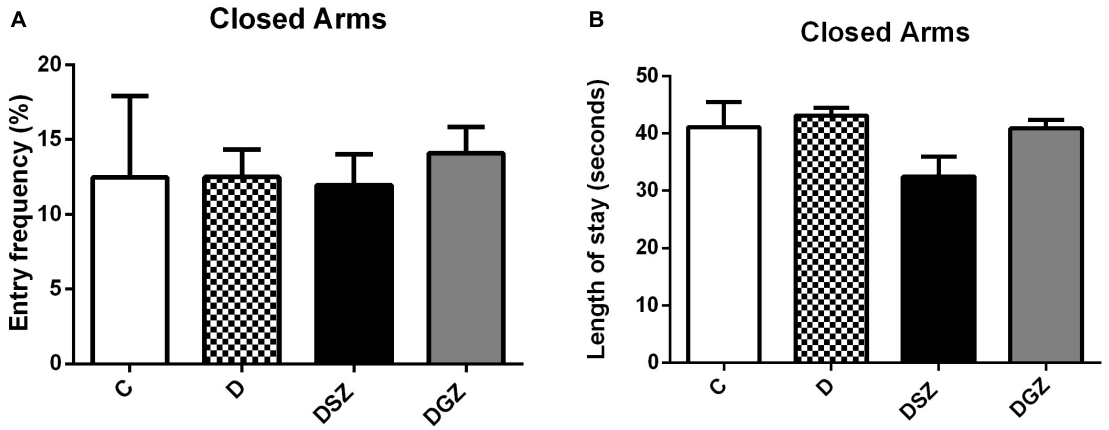

FIGURE 3 | Evaluation of motor activity using the Open Field Test in diabetic rats treated with two different zinc compounds (15 mg/kg). **(A)** Entry frequency and **(B)** Length of stay in closed arms. C, Control Group (n = 8); D, Diabetic Group (n = 10); DSZ (n = 10): Diabetic Group Supplemented with Zn Sulfate and DGZ (n = 10): Diabetic Group Supplemented with Zn Gluconate (n = 10). Mann–Whitney test ($p < 0.05$).

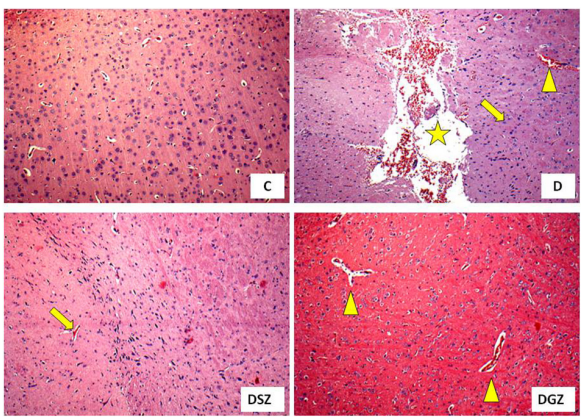

FIGURE 4 | Histology of the cerebral cortex of diabetic rats treated with two different zinc compounds (H and E, × 100) C, control group (n = 8); D, diabetic group (n = 10), the arrow indicates ischemic neurons, the star indicates hemorrhage and the triangle indicates dilated vessels; DSZ, diabetic group supplemented with Zn Sulpfate (n = 10), the arrow indicates ischemic neurons; DGZ: Diabetic Group Supplemented with Zn Gluconate (n = 10), the triangle indicates dilated vessels.

in the central portion of the field indicates anxiolytic effect, whereas a decrease in the permanence in the central part of the device can be interpreted as an anxiogenic effect (Ahn et al., 2013).

The OF arena is considered a stressful environment for fear. More anxious and emotional animals tend to wander less and stay away from the central part of the arena in such conditions (Takeda et al., 2007; Strandberg et al., 2014). Regarding the evaluated parameters, we noticed that Zn sulfate supplementation in the DSZ group animals improved their locomotor activity in the OF test, increased the number of crossed quadrants and decreased their immobility time ($p < 0.05$), indicating a lower anxiety level compared to the D and DGZ groups. These results allow for suggesting that the locomotor activity was preserved after administrating Zn sulfate, so this Zn compound did not present sedative effect.

Were observed an increase in anxious behavior in Zn-deficient mice through less ambulation and less immobility during the OF test (Takeda et al., 2012). Rats fed a Zn-deficient diet for 2 weeks showed decreased ambulation and grooming, suggesting that anxiety-like behavior is increased in Zn deficiency (Xie et al., 2012). Behavioral changes may especially represent an effect of Zn deficiency and may be informative about the various pathways in which this trace element is an important functional factor in brain function (Hagmeyer et al., 2015).

In DM animal models, including the streptozotocin (STZ)-induced diabetic rodent model, abnormalities in the regulation of several neurotransmitters have been reported (Suzuki et al., 2012). Persisting hyperglycemia leads to impaired neurogenesis, decreased synaptic plasticity, undesired neuro-anatomical alterations, neurochemical deficits, and reduced neurotransmitter activity (Prabhakar et al., 2015). Zinc sulfate was not effective in improving the parameters evaluated in the FST and was effective in EPM and OF. While zinc gluconate was active in improving the parameters evaluated in FST and was not effective in EPM and OF. These data may indicate that the anion associated with Zn determines its antidepressant and anxiolytic activity. The antidepressant effect shown by Zn may be related to attenuation of the glutamatergic system by inhibition of N-methyl-D-aspartate (NMDA) receptor activity, as demonstrated in previous studies (Szewczyk et al., 2008, 2011; Mlyniec et al., 2012). Zn antidepressant and anxiolytic activities can also occur via modulating the serotonergic system through a complex mechanism not yet fully elucidated involving the participation of pre- and postsynaptic 5-HT_{1A}Rs (Satała et al., 2016). However, the mechanisms of how zinc-associated anions work in DM are not yet well understood.

Stress and anxiety have some parallels, as they appear to be genetically linked by sharing neurocircuits and common

brain areas, including the prefrontal cortex, hippocampus, and amygdale (Sartori et al., 2011). In this sense, it is interesting to analyze some brain structures in search of connections between the role of Zn and the brain. In our study, we investigated the cerebral cortex of the animals and verified that the diabetic animals presented alterations characterized by the presence of ischemic neurons, hemorrhaging and dilated vessels, characterizing the oxidative stress of DM1. It is known that DM is associated with learning deficits and oxidative imbalances, neurophysiological and structural changes in cerebral cortex (Ates et al., 2014; Kim et al., 2015).

In our study, supplementation with the two Zn compounds showed beneficial effects on the cerebral cortex of diabetic animals, since these organs had more preserved structures with a reduction in ischemic neurons and hemorrhaging, suggesting a potential neuroprotective effect; especially of the Zn Gluconate compound at doses within the recommended maximum limit for rodents (National Academy of Sciences, 2001). Were found beneficial effects in the brains of healthy rats supplemented with Zn chloride (5 mg/kg) demonstrated by decreasing the degeneration and preservation of neuronal cells (Brocardo et al., 2007).

We have a limitation related to the absence of the plasma Zn dosage for verifying the deficiency in the animals. However, in view of the obtained only in diabetic animals and those supplemented with the two Zn compounds, it can be suggested that the resulting benefits may be due to the Zn deficiency in these animals, which may confirm a possible relation between DM1, Zn deficiency and behavioral changes.

CONCLUSION

Considering the set of results, supplementation with both zinc compounds showed neurobehavioral benefits in diabetic animals with different effects depending on the type of anion associated with Zn: Zn gluconate had antidepressant and neuroprotective effect, while Zn sulfate had an anxiolytic effect, and further studies are needed to clarify how these mechanisms of action occur.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by the Ethics Committee on Animal Use (CEUA) of the Federal University of Paraiba (UFPB), under no. 045/2015.

AUTHOR CONTRIBUTIONS

CC, MG, LP, and JA designed the study and the experiments. CC, AA, EA, JC, RA, PL, and NS carried out the experiments. CC, LP, and JA analyzed the data. CC, AA, and JA implemented the reagents, materials, and analysis tools. CC and JA wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2019.01411/full#supplementary-material

FIGURE S1 | Experimental assay design.

FIGURE S2 | Evaluation of antidepressant activity using the Forced Swim Test (FST) in healthy and diabetic rats treated with two different zinc compounds (15 mg/kg). C, control group (n = 8); SZ, healthy group supplemented with Zn Sulfate (n = 8); GZ, healthy group supplemented with Zn Gluconate (n = 8); D, diabetic group (n = 10); DSZ, diabetic group supplemented with Zn Sulfate and DGZ (n = 10): Diabetic Group Supplemented with Zn Gluconate (n = 10). Mann–Whitney test ($p < 0.05$). *Significant difference compared with D group; #significant difference compared with C group.

FIGURE S3 | Evaluation of motor activity using the Open Field (OF) Test in healthy and diabetic rats treated with two different zinc compounds (15 mg/kg). C, control group (n = 8); SZ, healthy group supplemented with Zn Sulfate (n = 8); GZ, healthy group supplemented with Zn Gluconate (n = 8); D, diabetic group (n = 10); DSZ, diabetic group supplemented with Zn Sulfate and DGZ (n = 10): Diabetic Group Supplemented with Zn Gluconate (n = 10). Mann-Whitney test ($p < 0.05$). *Significant difference compared with D group; #significant difference compared with C group.

TABLE S1 | Rearing, grooming and number of fecal cakes quantified in Open Field (OP) Test in healthy and diabetic rats treated with two different zinc compounds (15 mg/kg).

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frontiers in Neuroscience

ORIGINAL RESEARCH
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Ketogenic Diet Provided During Three Months Increases KCC2 Expression but Not NKCC1 in the Rat Dentate Gyrus

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Ketogenic diet, a high fat and low carbohydrate diet, has been used as a non-pharmacological treatment in refractory epilepsy since 1920. In recent years, it has demonstrated to be effective in the treatment of numerous neurological and non-neurological diseases. Some neurological and neuropsychiatric disorders are known to be caused by gamma-aminobutyric acid (GABA)-mediated neurotransmission dysfunction. The strength and polarity of GABA-mediated neurotransmission are determined by the intracellular chloride concentration, which in turn is regulated by cation-chloride cotransporters NKCC1 and KCC2. Currently, it is unknown if the effect of ketogenic diet is due to the modulation of these cotransporters. Thus, we analyzed the effect of a ketogenic diet on the cation-chloride cotransporters expression in the dentate gyrus. We estimated the total number of NKCC1 immunoreactive (NKCC1-IR) neuronal and glial cells by stereology and determined KCC2 labeling intensity by densitometry in the molecular and granule layers as well as in the hilus of dentate gyrus of rats fed with normal or ketogenic diet for 3 months. The results indicated that ketogenic diet provided during 3 months increased KCC2 expression, but not NKCC1 in the dentate gyrus of the rat. The significant increase of KCC2 expression could explain, at least in part, the beneficial effect of ketogenic diet in the diseases where the GABAergic system is altered by increasing its inhibitory efficiency.

Keywords: ketogenic diet, NKCC1, KCC2, dentate gyrus, optical fractionator, optical density, rat

INTRODUCTION

Ketogenic diet (KD) is a high-fat, low-carbohydrate and adequate-protein diet characterized by producing a state of ketosis in the organism. The term ketogenic diet was first coined at Mayo Clinic in 1921 and derives its name from the fact that it increases the circulating concentration of the ketone bodies β-hydroxybutyrate, acetoacetate and acetone (Wheless, 2008). These ketone bodies,

particularly β-hydroxybutyrate, can replace glucose as fuel for cells. The synthesis of ketone bodies begins once glycogen stores have depleted in the liver. Thus, the KD is a biochemical model of fasting, where cells use ketone bodies as energy substrate (Fedorovich et al., 2018). KD was initially employed as an effective non-pharmacological treatment for refractory epilepsy with beneficial results (Henderson et al., 2006; Levy et al., 2012). However, in recent years, it has demonstrated to be effective in the treatment of numerous neurological disorders such as traumatic brain injury (Appelberg et al., 2009; Hu et al., 2009); neuropsychiatric disorders as schizophrenia (Kraft and Westman, 2009; Kraeuter et al., 2015), autism spectrum disorder (Ahn et al., 2014; Lee et al., 2018), depression (Murphy et al., 2004; Sussman et al., 2015), anxiety (Sussman et al., 2015; Ari et al., 2016) and bipolar disorder (Phelps et al., 2013); as well as mitochondrial dysfunctions (Kang et al., 2007; Kim et al., 2010), cancer (Seyfried et al., 2012; Cohen et al., 2018), aging (Balietti et al., 2010a,b) and obesity (Paoli, 2014; Zhang et al., 2018).

Numerous neurological and neuropsychiatric disorders including autism spectrum disorder (Huberfeld et al., 2007; Di Cristo et al., 2018), schizophrenia (Gonzalez-Burgos and Lewis, 2008; Hashimoto et al., 2008), stress (Kwon et al., 2018), traumatic brain injury (Bonislawski et al., 2007) and epilepsy (Ben-Ari et al., 2012) are known to be caused by the dysfunction of the gamma-aminobutyric acid (GABA)-mediated neurotransmission (Kwon et al., 2018). It is well known that the strength and polarity of GABA-mediated neurotransmission are determined by the intracellular chloride concentration, which in turn is regulated by cation-chloride cotransporters and is indeed essential for neuronal homeostasis activity in the brain. The main chloride "exporter" is the K^+/Cl^- cotransporter (KCC2), which can extrude chloride from the neuron against its concentration gradient. In opposite direction, the $Na^+/K^+/Cl^-$ cotransporter (NKCC1) is regarded as the most active chloride "importer". Together, KCC2 and NKCC1 are the two main transporters responsible for regulating intracellular chloride concentration (Schulte et al., 2018).

Recent studies have reported alterations of NKCC1 or KCC2 cotransporters in multiple models of neurological and psychiatric diseases, including schizophrenia (Hyde et al., 2011; Merner et al., 2016), autism (Cellot and Cherubini, 2014; Merner et al., 2015), Down syndrome (Deidda et al., 2015), epilepsy (Hübner et al., 2001; Huberfeld et al., 2007), cerebral ischemia (Jaenisch et al., 2010), tuberous sclerosis complex (Ruffolo et al., 2016), traumatic brain injury (Bonislawski et al., 2007), neuropathic pain (Cramer et al., 2008) and stress (Tsukahara et al., 2015; Kwon et al., 2018). KCC2 is down-regulated while NKCC1 is up-regulated under certain pathophysiological conditions, such as epilepsy and trauma (Wang et al., 2016). These studies underpin the importance of NKCC1 and KCC2 regulation for the homeostasis of neuronal intracellular chloride concentration and appropriate function of GABA signaling.

Some studies have shown that the efficacy of KD is manifested after 1 month of treatment in animal models. In a previous study of our group (Gómez-Lira et al., 2011), we reported that KD *per se* does not alter the expression of the cotransporters NKCC1 and KCC2 in the hippocampus after 1 month of treatment, however, Wang et al. (2016) reported that KD increases the expression of KCC2 cotransporter in the cerebral cortex after a month of diet.

The mechanism by which KD acts is not clearly understood. However, it is important to note that KD has a beneficial effect in several diseases or disorders where GABAergic system failure is involved, probably by modifying the cation-chloride cotransporters NKCC1 and KCC2 as a common mechanism. However, so far, there are no studies that have analyzed the long-term effect of KD *per se* on the expression of the cation-chloride cotransporters in the dentate gyrus. Hence, the present work was focused to analyzing the long-term effect of KD on the expression of cation-chloride cotransporters, particularly in the dentate gyrus. In view of this, the total number of NKCC1 immunoreactive (NKCC1-IR) neuronal and glial cells was estimated by stereology, while KCC2 labeling intensity was determined by optical densitometry in the molecular and granule layers and in hilus of dentate gyrus of rats after 3 months of normal diet or KD administration.

MATERIALS AND METHODS

Animals and Diets

Male Sprague-Dawley rats were bred and maintained in controlled conditions of temperature (22–24°C), light:dark cycle (12:12 h) and relative humidity (40%). This research was performed according to the guidelines of the Official Mexican Norm (NOM-062-ZOO-1999) and are part of project 085-2010, approved by the Research Board of the National Institute of Pediatrics, registered at the Office for Human Research Protection of the NIH[1] with number IRB00008065; the project was also approved by the Institutional Committee for the Care and Use of Laboratory Animals (CICUAL).

At postnatal day 21 (P21), rats from 8 litters were weaned and randomly divided into two groups: (1) control group (ND, $n = 8$), animals fed with a normal diet (2018S, Envigo Teklad, United States) (**Table 1**) and (2) experimental group (KD, $n = 8$), animals fed with a ketogenic diet (TD.96355, Envigo Teklad, United States) (**Table 1**). Both diets were started at weaning and maintained during 3 months. The animals had *ad libitum* access to water and food. The rats were fasted for 1 day prior to the dietary treatment. Body weight, glucose and β-hydroxybutyrate blood levels (in tail blood samples) of the animals were measured at the beginning (P21) and at the end of the treatments (P112). The glucose and the β-hydroxybutyrate concentrations were determined using a FreeStyle Optium system and glucose or β-hydroxybutyrate test strips (Abbott Laboratories).

Tissue Processing and Sample Collection

At the end of treatment, rats were anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneally) and transcardially perfused with saline followed by 4% paraformaldehyde in phosphate buffer, 0.1 M, pH 7.4 (PFA). Brains were removed, post-fixed in PFA overnight and serially cryo-protected in 10,

[1] http://ohrp.cit.nih.gov/search/search.aspx

TABLE 1 | Nutritional composition of diets.

Macronutrients (% by weight)	Normal diet 2018S Envigo Teklad	Ketogenic diet TD.96355 Envigo Teklad
Protein	18.60	15.30
Fat	6.20	67.40
Carbohydrate	44.20	0.50
Minerals		
Calcium	1.00	0.90
Phosphorus	0.70	0.56
Potassium	0.60	0.54
Sodium	0.20	0.15
Chloride	0.40	0.24
Magnesium	0.20	0.10
Copper (mg/kg)	15.00	8.90
Iron (mg/kg)	200.00	54.60
Zinc (mg/kg)	70.00	56.80
Manganese (mg/kg)	100.00	87.60
Iodine (mg/kg)	6.00	0.31
Selenium (mg/kg)	0.23	0.16
Energy (kcal/g)	3.10	6.70

2018S diet contains vitamins A, D3, E, K3, B1, B2, B6, B12, niacin, pantothenic acid, biotin, folate and choline. TD.96355 diet is supplemented with vitamin mix (Teklad, 40060).

20, and 30% sucrose at 4°C. Afterward, 50 μm thick coronal serial sections were obtained of all rat dentate gyrus (−1.72 to −6.84 mm posterior to Bregma, Paxinos and Watson, 2007) using a cryostat (Leica, Germany). Ventral and dorsal dentate gyrus were considered, and both hemispheres were inspected. Sections were stored in a cryoprotectant solution (25% glycerol, 25% ethylene glycol, 50% phosphate buffer 0.1 M, pH 7.4) at −20°C in 24 well plates until use. To select the sections from the serial slides per animal, we use a systematic random procedure consisting of choosing one of every eight sections that resulted in eight series of 12–15 sections of all rat dentate gyrus. One of the series was immunohistochemically processed for immunodetection of NKCC1 and other for KCC2 in each rat.

Immunohistochemical Staining

To evaluate the expression of the cation-chloride cotransporters NKCC1 and KCC2 in ND and KD rats, an immunohistochemistry protocol was carried out using a secondary biotinylated antibody according to Brandt et al. (2010). Brain tissue sections from ND and KD rats were processed in parallel free-floating at room temperature in constant motion on a shaker. Sections were initially subjected to three-time 10-min washes with PBS, between the change of each solution and at the end. After washing with PBS, sections were subjected to 1% hydrogen peroxide in PBS during 10 min. Tissues were then incubated with 20X ImmunoDNA retriever buffer (Bio SB, United States) at 65°C for 60 min. After that, they were incubated overnight with the primary rabbit polyclonal antibodies anti-NKCC1 (1:500; Merck-Millipore, Germany, Cat. # AB3560P), or anti-KCC2 (1:2000; Merck Millipore, Germany, Cat. # 07-432), diluted in 5% horse serum (Gibco) and 3% Triton X-100 (Merck, Germany) in PBS. Both antibodies recognize their respective total protein. The next day, sections were washed and incubated with a secondary biotinylated goat anti-rabbit biotinylated IgG antibody (1:500; Vector Laboratories, United States, Cat. # BA-1000) for 2 h and subsequently incubated with avidin peroxidase complex (ABC kit; Vectastain; Vector Laboratories, United States, Cat. # Pk-4000) for 1 h. To reveal peroxidase activity, we used a nickel-intensified 3,3′-diaminobenzidine (DAB; Vector Laboratories, United States, Cat. # SK-4100) solution for 2 1/2 min. Finally, the sections were mounted on poly-L-lysine-coated slides, entellan (Merck, Germany) was added and slides covered with a glass coverslip. In additional sections, the primary antibody NKCC1 or KCC2 as well as the secondary biotinylated antibody were omitted as negative controls to assess non-specific binding. The same amount of horse serum used to replace the primary or secondary antibody resulted in lack of any staining. Evaluation of sections was performed in a blind fashion, i.e., the researcher was not aware whether sections were from ND or KD rats.

Immunofluorescence

To further investigate the cell lineage of NKCC1-IR and KCC2-IR cells, co-staining of NKCC1 or KCC2 was realized with NeuN (a neuron-specific marker), GFAP (a marker for astrocytes) and DAPI (nuclear staining). Free floating brain sections were post-fixed in 4% PFA for 10 min. After three washes (10 min each) with PBT, the sections were incubated with 2X SSC during 60 min at 70°C and then at room temperature for 30 min. Next, sections were washed three times with PBT and then simultaneously incubated with mouse anti-NeuN (1:500; Chemicon, Merck, Germany Cat. # MAB377), chicken anti-GFAP (1:500, Merck Millipore, Germany, Cat. # AB5541), rabbit anti-NKCC1 (1:100; Merck Millipore, Germany, Cat. # AB3560P) or anti-KCC2 (1:200; Merck Millipore, Germany, Cat. # 07-432), diluted in PBT added with 3% horse serum at room temperature overnight. After this, sections were washed three times with PB and incubated during 2 h at room temperature with the following secondary antibodies: AlexaFluor 488 donkey anti-mouse (1:200, Thermo Fisher Scientific Inc., Waltham, MA, United States, Cat. # A-21202), AlexaFluor 647 donkey anti-rabbit (1:200, Thermo Fisher Scientific Inc., Waltham, MA, United States, Cat. # A-31573) and biotinylated donkey anti-chicken (1:400, Merck Millipore, Germany, Cat. # AP1948) diluted in PB. After three washes, they were incubated with Texas Red avidin D (1:200, Vector Laboratories, United States, Cat. # A-2006) and then counterstained with DAPI (Merck, Germany, Cat. # 10236276001). Finally, sections were mounted onto slides and coverslipped with anti-fading medium (Dako Fluorescence Mounting Medium, Denmark). Negative controls were done on a slide with all primary omitted but incubated with secondary antibodies; no signal was detected on this. Fluorescence images were acquired with a Nikon A1R$^+$ laser scanning confocal scanning head coupled to an Eclipse Ti-E inverted microscope (Nikon Corporation, Tokyo, Japan) equipped with a motorized stage (TI-S-E, Nikon). For XY imaging, samples were sequentially excited with 647 (2.1 mW), 561 (1.05 mW), 488 (1.05 mW) and 405 (2.4 mW) laser, imaged through a CFI Plan Apo VC 60X N.A. 1.2 water

immersion objective (Nikon), and evaluated with galvanometric scanner, 660LP, 600/50, 525/50, 450/50 emission filters, and GaAsP/standard detectors. Pinhole value was set at 12.77 μm. All images were captured with NIS Elements C software v. 5.00 (Nikon), and processed with Fiji software (v.1.52p) (Linkert et al., 2010; Schindelin et al., 2012).

Stereology for NKCC1

A stereological, systematic random procedure, *optical fractionator*, (West et al., 1991) was employed for counting the number of NKCC1-IR neuronal and glial cells in dentate gyrus of both ND and KD fed rats. To achieve this, we used Stereo Investigator 9 software in a semi-automatic stereological system (MBF Bioscience, VT, United States). The counting frame size was set at 70 × 45 μm, height dissector 13 μm, and guard zones were defined at 1.5 μm from the upper and lower borders of the counting frame. Grid size was set at 300 × 300 μm, except for the granular layer, for which was set at 250 × 250 μm. Cell counting was done at 60× and the coefficient of error (Gundersen, $m = 1$) was <0.1. NKCC1-IR neuronal and glial cell number was counted in the three layers of dentate gyrus: molecular, granule and hilus. NKCC1-IR neuronal or glial cells were identified according to Brandt et al. (2010), following the criteria of size and morphologic appearance. Thus, we considered NKCC1-IR glial cells as those with small-sized profiles (<8 μm) and intensely staining, and as NKCC1-IR neural cells, those with large-sized profiles (>8 μm) and staining from slightly to considerably darker than background.

Optical Density of KCC2

The determination of KCC2 cotransporter expression in the dentate gyrus was done through digital densitometrical analysis of the image color intensities. All images were taken with identical characteristics of acquisition (objective lens, aperture condenser, light intensity, exposure time and white balance) with a MBF-CX9000 RGB CCD camera (MBF Bioscience, VT, United States) coupled to a BX-51 microscope (Olympus Corporation, Tokyo, Japan) and Stereoinvestigator software (MBF Bioscience, VT, United States). ImageJ software (v 1.52e, Rasband, 2018) was used to perform densitometric measurements and the values obtained were expressed as optical density (OD) in arbitrary units; for each image, we converted RGB to 8-bit color depth, segmented the layers of interest and measured the relative intensity of pixels in each region. The analysis was made at 20× in series of 12–15 sections of whole dentate gyrus. Optical density of KCC2 was estimated in three regions: molecular and granule layers and hilus of the dentate gyrus. Each value of OD was normalized using background subtraction.

Statistical Analysis

Data were probed for normal probability distributions with the Levene test and for equality of variances with Shapiro Wilk test (SPSS software, v.25), if they come from one, Student's *t*-test was performed, otherwise, the non-parametric Mann-Whitney *U*-test was applied. Differences were considered significant at $p < 0.05$. Data were expressed as mean ± standard deviation (SD) if its statistical distribution was normal or as median with interquartile range (IQR) if they were non-parametric data.

RESULTS

Body Weight

The KD was well tolerated during the 3 months of study. The body weights of both ND and KD groups at the beginning of the treatment were not statistically different, showing equality of initial conditions. Body weights of ND and KD rats continuously increased along the treatment. After 3 months of treatment there were no significant differences in body weight between the ND and KD group despite observing a slight reduction of this parameter in the KD group. The values of the mean ± SD of body weight for groups ND and KD were: 47.23 ± 2.09 and 50.25 ± 8.33 g respectively at the beginning and 478.33 ± 51.49 and 467.45 ± 63.59 g respectively at the end.

Glucose

Assessment of blood glucose was performed at the beginning and end of the study. The results (mean ± SD) showed that peripheral blood glucose for the groups ND and KD at the time of weaning were: 132.25 ± 10.25 and 134.37 ± 11.46 (mg/dL) respectively, and at the end of the experiment, they were 92.12 ± 6.57 and 89.75 ± 11.98 (mg/dL) respectively. There were not statistically significant differences between groups at the beginning or at the end of the experiment (**Figure 1A**).

β-Hydroxybutyrate

In order to assess the effect of the ketogenic diet, ketone bodies were measured, particularly β-hydroxybutyrate. The median with IQR values of the β-hydroxybutyrate concentration in peripheral blood for the groups ND and KD at the time of weaning were 1.10 [IQR 0.9–1.3] and 1.10 [IQR 0.9–1.2] (mmol/L) respectively, and at the end of the experiment were 0.3 [IQR 0.2–0.3] and 1.25 [1.1–1.6] (mmol/L) respectively. At the beginning of the treatment, the values of this ketone body in both ND and KD groups were practically the same, showing thus equal initial conditions. However, it could be seen that at the end of the treatment, β-hydroxybutyrate concentration value for the KD group was higher than the value for ND group by 316%, which was statistically significant ($p < 0.01$, Mann-Whitney *U*-test) (**Figure 1B**).

NKCC1 Immunoreactivity

The cytoarchitecture of the dentate gyrus did not show variations, the lamination own of dentate gyrus was preserved properly in both hemispheres. The analysis of NKCC1 staining pattern was carried out in 12–15 sections of dentate gyrus of each rat and revealed the presence of clearly stained neuronal (scarce) and glial (abundant) cells as well as processes in all layers of dentate gyrus (**Figures 2A–E**). It was also easy to identify and demarcate the regions of analysis: molecular layer (**Figures 2A,B**), granule layer (**Figures 2A,C**) and hilus (**Figures 2A,D,E**) of the dentate gyrus. NKCC1-IR neuronal or glial cells were identified following criteria of size and morphologic appearance previously described

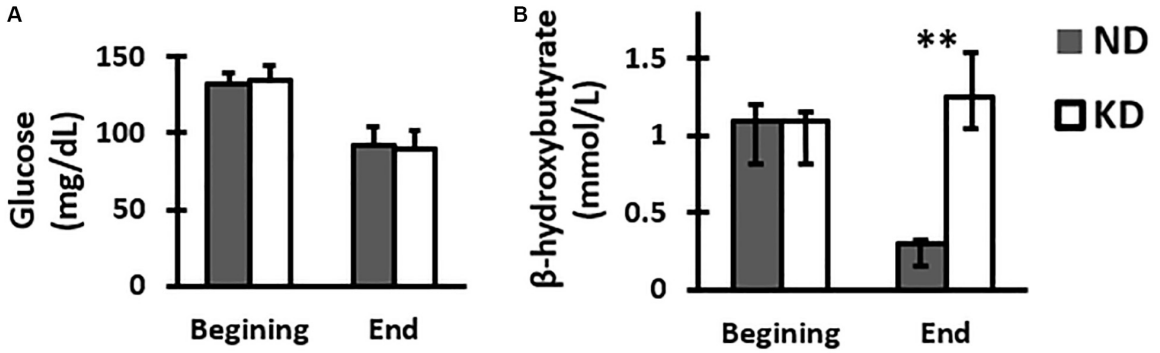

FIGURE 1 | Graph of the mean and SD of glucose **(A)** and median with IQR of β-hydroxybutyrate **(B)** concentrations in peripheral blood of group feed with normal diet (ND) or ketogenic diet (KD) at the beginning and end of treatment, $n = 8$ in each group. There were no significant differences between the two groups at beginning and end in the glucose levels. However, there was a significant increase in the β-hydroxybutyrate KD group when compared with ND group at the end of treatment (**$p < 0.01$, Mann-Whitney *U*-test).

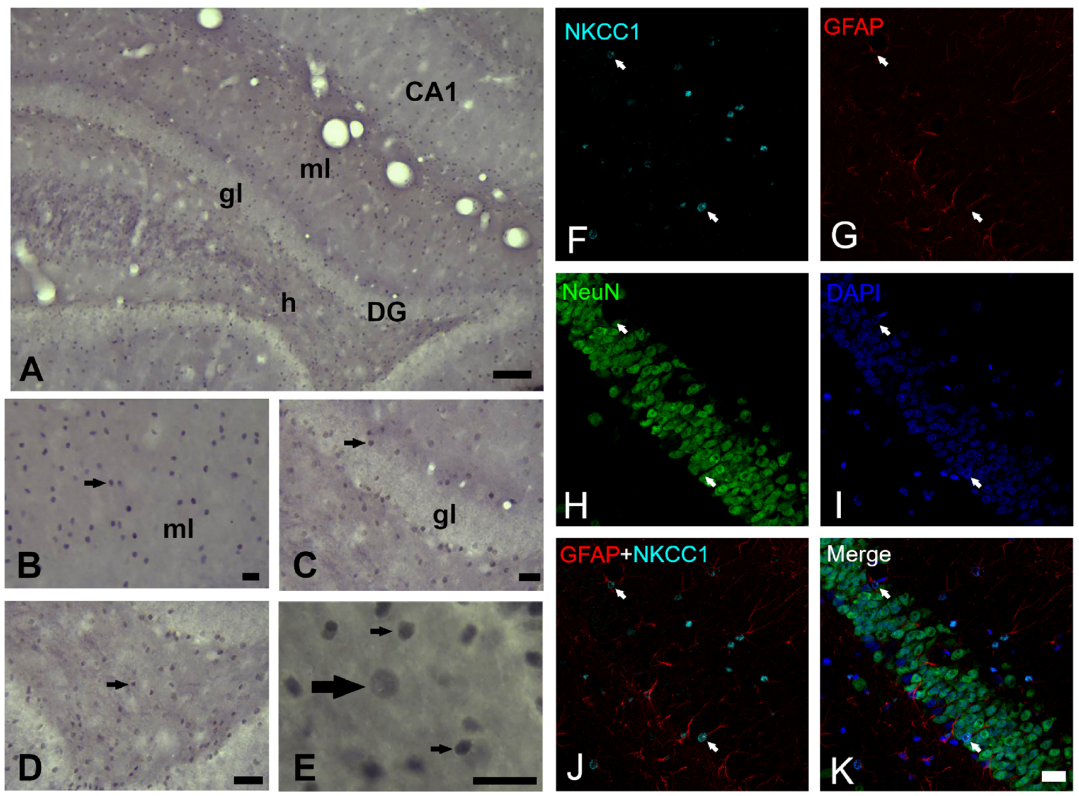

FIGURE 2 | NKCC1 expression in dentate gyrus of rats fed with a normal diet. **(A)** NKCC1-IR cells in the molecular (ml) and granule (gl) layer and hilus (h) of dentate gyrus (−3.3 mm posterior to Bregma). **(B–E)** NKCC1-IR cells (arrows) were found distributed through all dentate gyrus. **(E)** Higher magnification of hilus, NKCC1 immunostaining showed predominantly small, abundant and intensely stained cells, presumptive glial cells (thin arrows) and some scarce, large and weakly stained cells, presumptive neuronal cells (thick arrow). Quadruple immunofluorescence labeling of NKCC1 with NeuN (a neuron-specific marker), GFAP (a marker for astrocytes) and DAPI (a marker for nuclei) **(F–K)**. NKCC1-IR cells were mainly astrocyte-type glial cells, abundant, small and intensely stained cells (arrows indicate on all panels, NKCC1 and GFAP immunopositive astrocytes, except in NeuN panel where arrows indicate their absence). Scale bar: 100 μm **(A)**, 25 μm **(B–D)**, 10 μm **(E)** and 20 μm **(F–K)**.

(Brandt et al., 2010). Smaller cells with intense staining were considered glial cells, and those with large size and staining from slightly to considerably darker than background were considered as neuronal cells (**Figure 2E**). In the fluorescent co-staining of NKCC1 with GFAP, NeuN and DAPI (**Figures 2F–K**), it was observed that NKCC1-IR cells were predominantly astrocyte-type glial cells (abundant, small and intensely stained cells) (**Figures 2F,G,J,K**). A trend of NKCC1-IR neurons reduction was

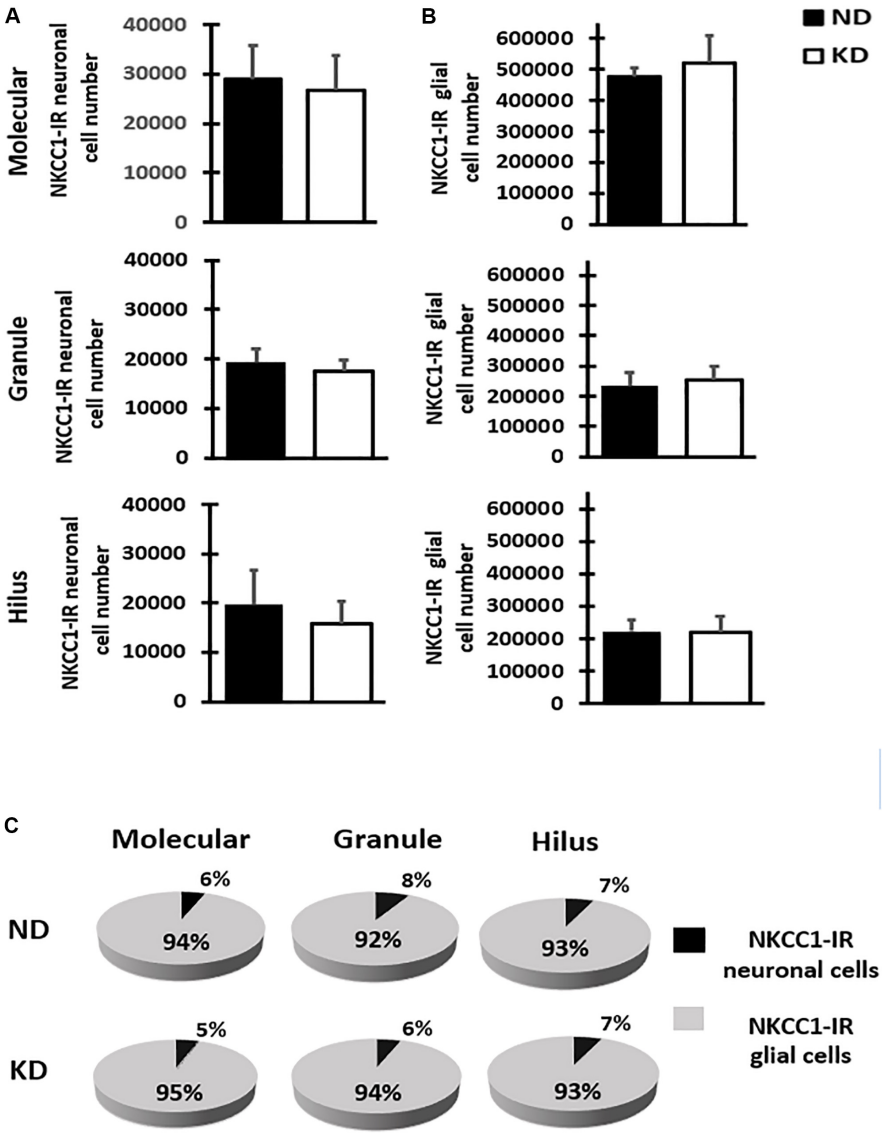

FIGURE 3 | Bar graphs show mean and SD of NKCC1-IR neuronal **(A)** and glial **(B)** cells number estimated by stereology in the three layers of dentate gyrus of rats fed with normal diet (ND) or ketogenic diet (KD), $n = 8$ in each group. The estimate of cell number showed that KD does not significantly change the NKCC1-IR neuronal or glial cell number when compared with ND rats in the molecular and granule layers, and hilus of the dentate gyrus. **(C)** Fractional composition of NKCC1-IR neuronal and glial cells in the three layers of dentate gyrus in control group (ND) and ketogenic group (KD). The data are presented as a percentage of total of NKCC1-IR neuronal and glial cells in each layer.

observed in the KD group, however, there were no significant differences in the molecular and granule layers and hilus of dentate gyrus after 3-months of diet (**Figure 3A**). A trend to increase NKCC1-IR glial cells was observed in the KD group, however, there were also no significant differences in the number of NKCC1-IR glial cells between ND and KD groups (**Figure 3B**). A fractional composition of NKCC1-IR neuronal and glial cells in the three layers of dentate gyrus of ND and KD groups is shown in **Figure 3C**. In the ND group, the percentage of NKCC1-IR neuronal and glial cells was 6 and 94% respectively in the molecular layer, 8 and 92% in the granular layer, and 7 and 93% for hilus (**Figure 3C**). In the KD group, the percentage of NKCC1-IR neuronal and glial cells was 5 and 95% respectively for the molecular layer, 6 and 94% for granular layer and 7 and 93% for hilus (**Figure 3C**).

KCC2 Immunoreactivity

Evaluation of the cation-chloride cotransporter KCC2 expression by optical density was carried out in 12–15 sections of dentate gyrus of each rat (**Figure 4A**). The cytoarchitecture of the dentate gyrus and hippocampus remained unchanged between the ND and KD groups, dentate gyrus lamination was similar in both conditions (**Figures 4B,C**). With respect to the staining pattern, it was observed that the three layers of the dentate

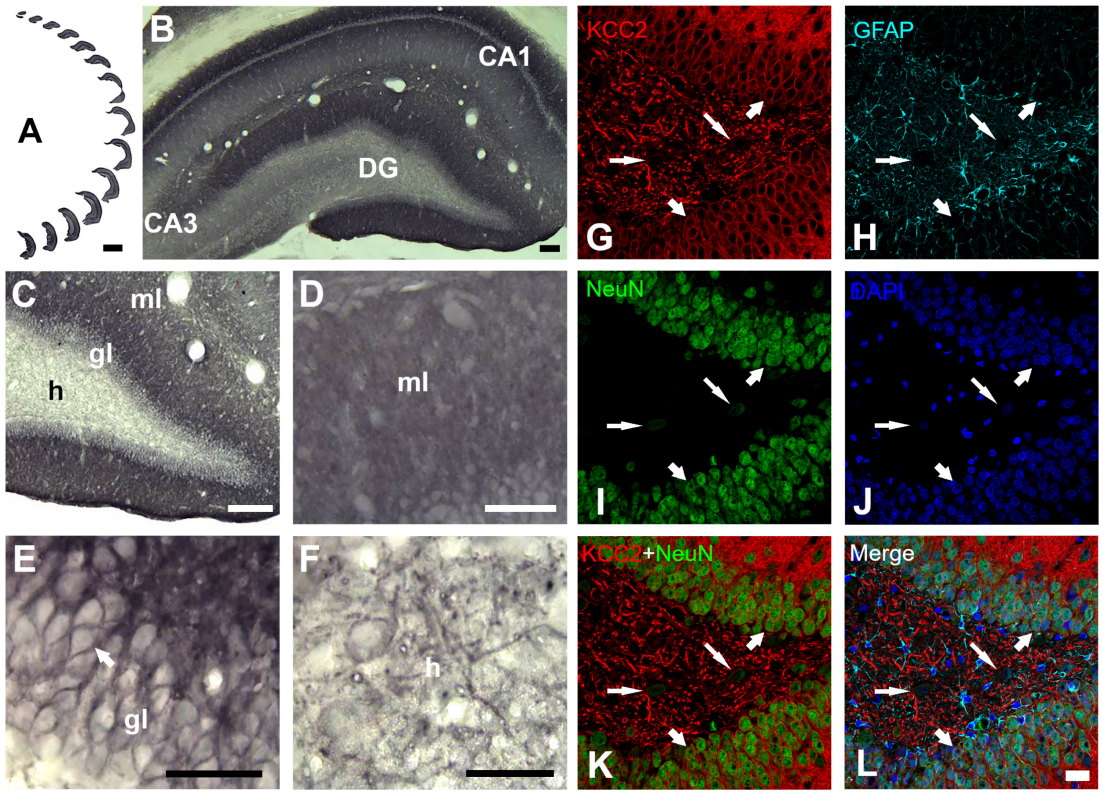

FIGURE 4 | KCC2 expression in dentate gyrus of a rat fed with normal diet. **(A)** Serial sections of the whole dentate gyrus of the right hemisphere. **(B)** Panoramic and **(C)**, higher magnification view of dentate gyrus, ml, molecular layer; gl, granule layer and h, hilus. Higher magnification of molecular layer **(D)**, granule layer **(E)** and hilus **(F)**. KCC2 immunoreactivity in the granule layer was observed in the plasmalemmal region of the granule cell body (perisomal) **(E)**. In the hilus **(F)**, KCC2 expression was observed around the polymorphic cells and in neural processes. **(G–L)**, quadruple fluorescent labeling of KCC2 with NeuN (a neuron-specific marker), GFAP (a marker for astrocytes) and DAPI (a marker for nuclei) of a rat feed with normal diet. KCC2 immunoreactivity (thick arrows) was mainly observed in both cytoplasmic projections and plasmalemmal region of the granule cell body (perisomal), indicating that KCC2 is present in granular neurons **(G–L)**. KCC2-IR was also observed in neural fibers and NeuN weakly – stained cells of the hilus, probably mossy cells or hilar interneurons (thin arrows). Scale bar: 1,000 μm **(A)**, 100 μm **(B)**, 50 μm **(C)**, 25 μm **(D–F)**, 20 μm **(G–L)**.

gyrus showed a diffuse immunoreaction, as in the rest of the hippocampus, allowing the identification and delimitation of each of the regions of analysis: molecular layer, granule layer and hilus of dentate gyrus. When the staining pattern was analyzed at a higher magnification, it was observed mainly in the molecular layer (**Figure 4D**). In the granule layer, the immunoreactivity of KCC2 cotransporter was around the neuronal somas i.e., in the plasma membrane, whereas the somas of the granule layer were not dyed (**Figure 4E**). In the hilus, KCC2 staining was observed around polymorphic cells and in neural processes (**Figure 4F**). The staining coloration oscillated between the black color and different shades of gray. Fluorescent co-staining of KCC2 (**Figures 4G–L**) with NeuN, GFAP and DAPI, showed that the staining pattern for KCC2 was similar to that obtained with immunohistochemistry with biotinylated secondary antibody, and it was abundantly located in the molecular layer. In the granule layer, KCC2 expression was around of the granular cells bodies, indicating that KCC2 was found in neurons (**Figures 4G,K,L**), but not in glial cells. In the hilus, KCC2-IR strongly stained neural processes and faintly stained the soma of scarce cells, probably interneurons or mossy cells. The analysis of KCC2-IR OD in the three layers of dentate gyrus showed that KD did not change KCC2 expression in the molecular layer of the dentate gyrus. However, there was a significant increase in KCC2 OD relative values in the granule layer ($p < 0.000$, Student's t-test) and hilus ($p < 0.000$, Student's t-test) after the treatment with KD (**Figure 5**).

DISCUSSION

The present study is the first to demonstrate, that long-lasting administration (3 months) of KD induces differential effects on the expression of cation-chloride NKCC1 and KCC2 cotransporters. Ketogenic diet modified the KCC2 cotransporter expression but not NKCC1 in the dentate gyrus of rats. Specifically, an increased KCC2 cotransporter expression was observed in the granule layer and hilus of dentate gyrus, which was not observed in the molecular layer. The ketogenic diet did not modify the NKCC1-IR neuronal and glial cells number in any of the layers analyzed. Interestingly, there were more NKCC1-IR glial cells than NKCC1-IR neural cells. The low neural expression

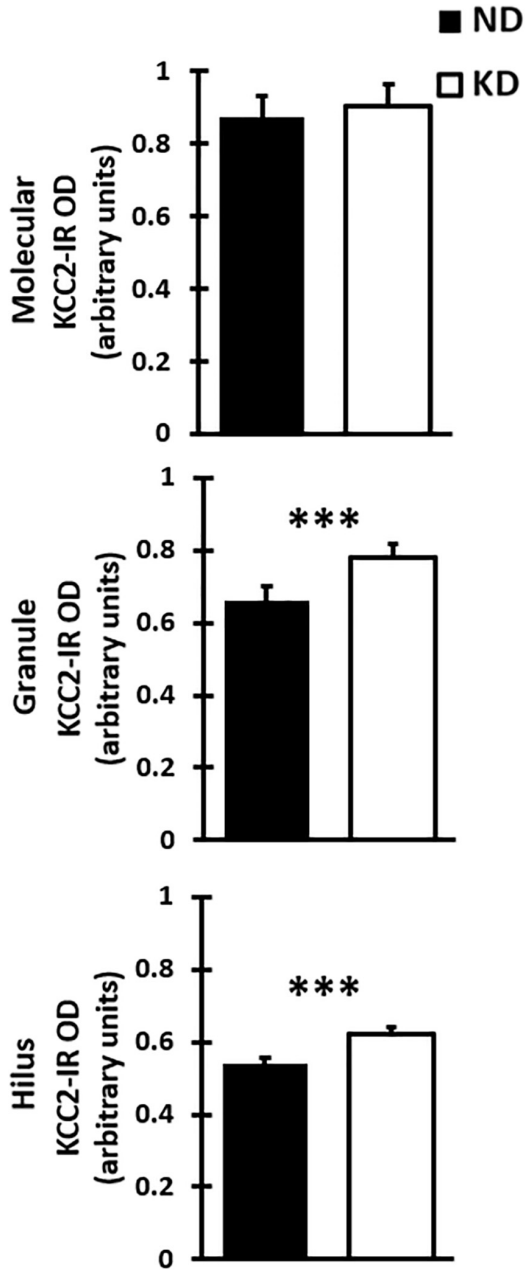

FIGURE 5 | Bar graph shows mean and SD of optical density (OD) of KCC2 expression in the three layer of the dentate gyrus of rats fed with normal diet (ND) or ketogenic diet (KD), $n = 8$ in each group. The KD does not significantly change the OD when compared with ND rats in the molecular layer. However, there was a significant increase in optical density of KD group when compared with DN group in the granule layer and hilus of dentate gyrus (***$p < 0.000$, Student's *t*-test).

of NKCC1 is in line with previous studies in adult rats (Brandt et al., 2010). These findings led to the conclusion that KD applied for 3 months increased the expression of KCC2 cation-chloride cotransporter in rat dentate gyrus.

These results complement our previous work, where it was shown that KD *per se* does not modify the expression of the cation-chloride cotransporters NKCC1 and KCC2 when the diet is provided for only a month (Gómez-Lira et al., 2011). Hence, the results of this work and those of our previous study indicate the necessity of long-term administration of ketogenic diet (3 months) to achieve effects in the KCC2 cotransporter.

The dentate gyrus is an important region of the hippocampal formation which has been the focus of synaptic plasticity, memory and learning process (Alkadhi, 2019), and epilepsy (Henderson et al., 2006) studies among others in rodents. So, this structure is an ideal model to elucidate the differential effects produced by KD. The dentate gyrus normally functions as a filter (Cohen et al., 2003) and GABAergic synaptic inhibition in the dentate gyrus is thought to endow this hippocampal subregion with the ability to function as a low pass filter, impeding excessive or aberrant activity from propagating into the circuit making the hippocampus to be more prone to seizure (Bonislawski et al., 2007).

Abnormalities in the dentate gyrus are likely to play a major role in the pathophysiology of various neurological diseases such as epilepsy and brain injury. Also, it is noted in experimental epilepsy and brain injury that the dentate gyrus is more excitable in these situations, probably due to alterations in GABAergic inhibition (Bonislawski et al., 2007), produced in turn by changes in the cation-chloride cotransporters.

The intracellular chloride concentration determines the strength and polarity of GABA-mediated neurotransmission, thus the efficacy of GABA-mediated inhibition depends on the low intracellular chloride concentration maintenance, regulated in turn by KCC2, which is the main chloride exporter. In this work, we brought into evidence increased KCC2 cotransporter in the dentate gyrus of rats after a treatment with KD. Further studies are needed to determine whether this increase in KCC2 expression underlies a hyperpolarizing effect of GABA-mediated neurotransmission.

The beneficial effect of KD in the diseases where GABAergic system and the dentate gyrus are damaged is probably due, at least in part, to the increase of KCC2. In addition, an increase of KCC2 in dentate gyrus may contribute in improving the function of the GABAergic system and modulate neuronal excitability, benefiting both cognitive deficits and epilepsy. This hypothesis needs to be evaluated in future studies.

In contrast, when chloride extrusion is disrupted due to decreased expression of KCC2, the intracellular chloride concentration inside the neuron increases, diminishing the driving force for GABA-mediated inhibitory currents which results in significant disinhibition (Bonislawski et al., 2007), especially in the dentate gyrus, as seen in neurological and neuropsychiatric diseases like epilepsy, traumatic brain injury, schizophrenia, autism, in addition to stress (Schulte et al., 2018). Interestingly in these diseases, the beneficial effect of ketogenic diet has been demonstrated. KD could also have positive effects on other diseases such as neuropathic pain, Rett syndrome associated with autism, tuberous sclerosis and stroke in which KCC2 reduction has been seen.

In this work, we demonstrated that KD produces an over expression of KCC2 cotransporter in neuronal cells in the rat dentate gyrus. It is possible that these cells may also exhibit

an upregulation of KCC2 activity that leads to a decrease in $[Cl^-]_i$ and consequently, an increase the magnitude of the inhibitory response to GABA. However, future studies should be conducted to explore the effect of a ketogenic diet on NKCC1 and KCC2 phosphorylation by WNK-SPAK/OSR1 pathway [WNK (kinase with no lysine (K)), SPAK (STE20-related proline-alanine-rich kinase) as well as OSRI (oxidative stress-responsive kinasa-1)], their upstream regulatory serine-threonine kinases. WNK kinases are not only effector kinases that work in conjunction with the SPAK/OSR1 kinases to regulate cation-chloride cotransporters by phosphorylation, but may also serve as intracelular Cl^- sensors (Moriguchi et al., 2005; Arroyo et al., 2013; Huang et al., 2019).

At the end of the experiment, β-hydroxybutyrate peripheral blood concentration increased, indicating the effectiveness of ketogenic diet used in this work. In addition, a state of ketosis endures after 3 months of treatment. A proposed mechanism for the ketogenic diet action is through the β-hydroxybutyrate.

Some studies have reported that KD reduces seizure-like activity (Bough et al., 2003; Wang et al., 2016), and normalizes various aberrant aspects of synaptic transmission (Stafstrom et al., 1999), in addition, attenuates pathological sharp waves (Simeone et al., 2014). Bough et al. (2003), showed that ketogenic calorie-restricted diet enhances GABAergic inhibition *in vivo* in rat dentate gyrus.

In contrast, KD feeding does not affect baseline excitability in the normal hippocampus, Stafstrom et al. (1999), found that KD does not modify aspects of synaptic transmission. These differences could be due to the duration of treatment with the ketogenic diet and the structure analyzed in the brain. As reported, KD *per se* presents differential effects that affect KCC2 expression in different structures such as the cerebral cortex (Wang et al., 2016) or the dentate gyrus (results of this work). The effect also depends on the treatment time. For instance, KCC2 increases in cerebral cortex in 1 month of treatment with KD, but not in the dentate gyrus, while this effect is observed in the dentate gyrus with 3 months of treatment.

Previous studies have proposed the possibility that KD effects may reach optimum status in hyperexcitable states as in epileptic condition. Clearly, more studies of KD effects on dentate gyrus regional expression of NKCC1 and KCC2 in a model of epilepsy are required. Little is known about regional alterations in dentate gyrus after applying a KD diet for 3 months.

Wang showed that KD *per se*, as in the present work, increases the expression of KCC2 without altering NKCC1. This author evaluated the expression of NKCC1 and KCC2 in an animal model of epilepsy and found that the PTZ (pentylenetetrazol) group treated with KD presented an overexpression of KCC2, and a reduction in the expression of NKCC1. These previous evidences and the results of the present work indicate that a mechanism of KD, as a non-pharmacological treatment for the control of the epilepsy, is probably by increasing KCC2 expression in the motor cortex (Wang et al., 2016), or in our case, in the dentate gyrus. However, further research is needed to confirm this last hypothesis.

In conclusion, KD provided during 3 months increases KCC2 expression but not NKCC1 in the dentate gyrus of rat. The significant increase of KCC2 expression could explain, at least in part, the beneficial effect of KD in the diseases where the GABAergic system is altered by increasing the inhibitory efficiency thus, causing the abolition of dysfunction of the dentate gyrus.

DATA AVAILABILITY STATEMENT

The data sets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Committee of Care and Use of Laboratory Animals (CICUAL) of National Institute of Pediatrics (México City).

AUTHOR CONTRIBUTIONS

LG-R conceived and designed the study. LG-R, KJ-C, TJ-Z, MT-R, RR-J, AT, EC-U, and PD conducted research, experiments, and data collection. LG-R, AT, NC-R, MR-G, and LC-A participated in drafting the manuscript. All authors critically revised the manuscript and gave final approval for the submitted version.

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