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BUILDING THE GATEWAY TO CONSCIOUSNESS – ABOUT THE DEVELOPMENT OF THE THALAMUS

Topic Editors
Tomomi Shimogori and Steffen Scholpp

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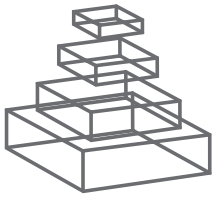

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BUILDING THE GATEWAY TO CONSCIOUSNESS – ABOUT THE DEVELOPMENT OF THE THALAMUS

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Since years, patterning and function of some brain parts such as the cortex in the forebrain and the optical tectum or cerebellum in the midbrain/hindbrain region are under strong investigation. Interestingly the diencephalon located in the caudal forebrain has been ignored for decades. Consequently, the existing knowledge from the development of this region to function in the mature brain is very fragmented. The central part of the diencephalon is the thalamus. This central relay station plays a crucial role in distributing incoming sensory information to appropriate regions of the cortex. The thalamus develops in the posterior part of the embryonic forebrain, where early cell fate decisions are controlled by local signaling centers.

In this Research Topic we discuss recent achievements elucidating thalamic neurogenesis - from neural progenitor cells to highly specialized neurons with cortical target cells in great distance. In parallel, we highlight developmental aspects leading from the early thalamic anlage to the late the organization of the complex relay station of the brain.

First we will address the very early events in neural plate patterning which leads to the subdivision in forebrain, midbrain and hindbrain primordia. This is followed by the specification of the diencephalon. One main aspect of the issue will be the induction and specification of the thalamic anlage. Patterning within elaborate brain regions, such as the neocortex or the cerebellum, is known to require instructive cell populations – 'local organizers'. The work of several labs has identified a similar organizing structure within the thalamus - the mid-diencephalic organizer (MDO). Organizers are located at prominent morphological discontinuities or boundaries in the neural primordium. Indeed, the MDO is localized at the zona limitans intrathalamica – the border between the prethalamus (formerly known as ventral thalamus) and the thalamus (formerly known as dorsal thalamus). Organizers are needed to establish concentration gradients of morphogenetic signal molecules in adjacent responsive tissues. The most prominent of the organizer's signals, Sonic hedgehog, is necessary for conferring regional identity on the prethalamus and thalamus and for patterning their differentiation. Several articles will focus on different aspects of the induction

and function of the MDO in zebrafish, chicken and mouse. Recent advances have been made to understand the function of other major signaling pathways here the Fgf pathway and the canonical Wnt / ß-catenin pathway. Similarly, the MDO is also a potent source for Fgf ligands and canonical Wnt ligands. We will elucidate the function of these signaling pathways and show that these pathways are required to establish integrity of the tissue. A further aspect will be the influence of the embryonic roof plate on thalamus development. This aspect has been completely overlooked in the last years. After patterning and specification, the thalamus becomes parcellated into several nuclei – independent functional units, which are specialized on transmitting information from a specific sensory organ to areas in the cortex. How these cells cluster form these entities will be discussed in several articles. Then, we will address the question how do neurons from a thalamic nucleus find their correct target area in the cortex? The area of the formation of the major nerve bundles the thalamo-cortical connection is under investigation from several labs. We will elucidate this in detail with the focus on intrinsic cues in thalamic neurons, but also on extrinsic cues released from tissues through which the axons have to navigate. In the last part of the issue we will add two articles, which will discuss similarities and differences within thalamic development across species. We feel that a comparative summary of the issue will have a great benefit as it will bundle common genetic and morphological aspects. In conclusion this ebook will help to elucidate many aspects of the development of the thalamus and thus be helpful on our way towards the understanding of the building of the gateway to consciousness.

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EDITORIAL
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Building the gateway to consciousness—about the development of the thalamus

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The thalamus is a twinned bulb-shaped structures that form at the top of the brainstem on either side of the third ventricle. The thalamic complex is located in the posterior forebrain and includes the prethalamus and thalamus (formerly known as ventral thalamus and dorsal thalamus, respectively). This complex is the major sensory relay station of the brain, receiving all inputs (except olfaction) and connecting reciprocally with the overlying cortex therefore, Crick and Koch (2003) has described the thalamus as "the gateway to consciousness." Although, the thalamus has been anatomically characterized in vertebrates the underlying genetic mechanism leading to the formation of this complex brain area is largely unknown.

In this special issue we tried to cover many aspects describing the development of the posterior forebrain i.e., the thalamus. The posterior forebrain can be subdivided in the epithalamus, the prethalamus (**Figure 1**, indicated in yellow), the thalamus (**Figure 1**, indicated in green), and the pretectum. Mechanisms regulating the formation and setting the boundaries between them are described in the article of Chatterjee and Li (2012). The crucial structure for the development of the thalamus is a small group of cells secreting different signaling molecules and is localized at the intrathalamic boundary, the zona limitans intrathalamica (ZLI). This cell population has been termed the mid-diencephalic organizer (MDO) or alternatively as ZLI organizer. The MDO releases three different families of signaling factors, Shh, Wnt, and Fgf. This network has been described in the articles of Hagemann and Scholpp (2012) focusing mainly on events in zebrafish and in Martinez-Ferre and Martinez (2012), who describe the situation in chick. Both group described that the principal signal of the MDO is Shh, which is conserved in different animals (**Figure 1**, expression domain of Shh indicated in red). Therefore, we elucidate the function of Shh in mice more in detail in two articles. Epstein (2012) focuses in his article on the description of the function of this signaling factor, whereas Haddad-Tóvolli et al. (2012) focuses on the regulators downstream of Shh signaling, the GLI transcription factors. Pre-patterning of the thalamic tissue is essential for the axonal projections of the thalamus and the most important axonal output of this structure is the thalamo-cortical projection. Price et al. (2012) and Grant et al. (2012) review recent progress of the development of these projections and axonal guidance. The zebrafish serves as a novel vertebrate model organism in thalamic development, however, the comparability to the mammalian systems is difficult. In a comparative review (Mueller, 2012) summarizes anatomical similarities and differences of the thalamus in different model organisms. Dorsally adjacent to the thalamus is the location of the habenula. The development of the connection to the thalamic tissue has been elucidated by two articles from Beretta et al. (2012) and Aizawa et al. (2011).

After our opinion, the special issue "Building the gateway to consciousness—about the development of the thalamus" summarizes recent efforts in understanding the formation of thalamus in vertebrates and we carefully selected carefully articles covering the entire field. However, it is impossible to elucidate any aspect in sufficient detail and we would like to apologize to our colleagues for potential gaps. We wish all scientist as much as fun in reading the articles as we had in assembling it and sincerely hope that this special issue helps the field of thalamus development to find the scientific recognition it deserves.

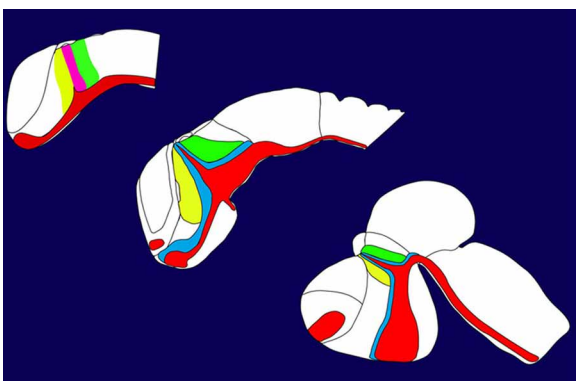

FIGURE 1 | Development of the thalamus in vertebrates.

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REVIEW ARTICLE
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What is the thalamus in zebrafish?

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Current research on the thalamus and related structures in the zebrafish diencephalon identifies an increasing number of both neurological structures and ontogenetic processes as evolutionary conserved between teleosts and mammals. The patterning processes, for example, which during the embryonic development of zebrafish form the thalamus proper appear largely conserved. Yet also striking differences between zebrafish and other vertebrates have been observed, particularly when we look at mature and histologically differentiated brains. A case in point is the migrated preglomerular complex of zebrafish which evolved only within the lineage of ray-finned fish and has no counterpart in mammals or tetrapod vertebrates. Based on its function as a sensory relay station with projections to pallial zones, the preglomerular complex has been compared to specific thalamic nuclei in mammals. However, no thalamic projections to the zebrafish dorsal pallium, which corresponds topologically to the mammalian isocortex, have been identified. Merely one teleostean thalamic nucleus proper, the auditory nucleus, projects to a part of the dorsal telencephalon, the pallial amygdala. Studies on patterning mechanisms identify a rostral and caudal domain in the embryonic thalamus proper. In both, teleosts and mammals, the rostral domain gives rise to GABAergic neurons, whereas glutamatergic neurons originate in the caudal domain of the zebrafish thalamus. The distribution of GABAergic derivatives in the adult zebrafish brain, furthermore, revealed previously overlooked thalamic nuclei and redefined already established ones. These findings require some reconsideration regarding the topological origin of these adult structures. In what follows, I discuss how evolutionary conserved and newly acquired features of the developing and adult zebrafish thalamus can be compared to the mammalian situation.

Keywords: isocortex, forebrain, ray-finned fish, reticular thalamic nucleus, teleost, thalamic eminence, thalamocortical

INTRODUCTION

The thalamus of mammals and other vertebrates is a prominent, multinucleated structure in the diencephalon (Jones, 2007; Nieuwenhuys et al., 2007). Often called the "gateway to consciousness," the thalamus regulates attention and alertness. As an interface between isocortex and deeper brain structures, the thalamus distributes, modifies, and filters ascending and descending information from and to various parts of the brain. Due to its relevance in human brain pathology, the thalamus is best studied in mammalian systems, particularly in rodents (hamster, mouse, rat, cats, and primates; Jones, 2007). According to these studies, the mammalian thalamus is functionally subdivided into four types of nuclei: sensory relay, motor, associative, and limbic ones. All

Abbreviations: A, anterior thalamic nucleus; AO, anterior octaval nucleus; aP1, alar plate prosomere 1; aP2, alar plate prosomere 2; aP3, alar plate prosomere 3; BLA, basolateral amygdala; BNSM, bed nucleus of the stria medullaris; bP1, basal plate prosomere 1; bP2, basal plate prosomere 2; bP3, basal plate prosomere 3; chor, commissura horizontalis; CN, cochlear nucleus; CP, caudate putamen; CP^{o}, centroposterior thalamic nucleus; cpost, commissura posterior; cTh, caudal thalamus proper; Ctx, isocortex; D, dorsal telencephalon (pallium); Dc, central zone of the dorsal telencephalon; Dl, lateral zone of the dorsal telencephalon; DON, descending octaval nucleus; dot, dorsomedial optic tract; Dm, medial zone of the dorsal telencephalon; DP, dorsal pallium; Dp, posterior zone of the dorsal telencephalon; DP^{o}, dorsoposterior thalamic nucleus; Th, thalamus (proper); E, epiphysis; EmT, eminentia thalami; EN, entopeduncular nucleus; fr, fasciculus retroflexus; GP, globus pallidus; H, hypothalamus; Ha, habenula; Had, dorsal habenular nucleus; Hav, ventral habenular nucleus; Hd, dorsal zone of the periventriuclar hypothalamus; Hip, hippocampus; Hv, ventral zone of periventricular nucleus; I, intermediate thalamic nucleus; IC, intercalated thalamic nucleus; InCo, inferior colliculus; lfb, lateral forebrain bundle; LH, lateral hypothalamic nucleus; lot, lateral olfactory tract; LP, lateral pallium; LP^{o}, lateroposterior thalamic complex; MGN, medial geniculate nucleus; MO, medullar oblongata; MP, medial pallium; OB, olfactory bulb; oc, optic commissure; P, pallium; P^{o}, posterior thalamic (preglomerular) nucleus; P1, prosomere 1; P2, prosomere 2; P2, prosomere 3; PG, preglomerular complex; PGa, anterior preglomerular nucleus; PGc, caudal preglomerular nucleus; PGl, lateral preglomerular nucleus; PGm, medial preglomerular nucleus; pirCtx, piriform cortex; Po, preoptic region; PPa, parvocellular preopticnucleus, anterior part; PPv, periventricular pretectal nucleus, ventral part; PPd, periventricular pretectal nucleus, dorsal part; PPp, parvocellular preoptic nucleus, posterior part; Pr, pretectum; PSm, magnocellular superficial pretectal nucleus; PSp, parvocellular superficial pretectal nucleus; PT, posterior tuberculum; PTd, dorsal posterior tubercular region; PVO, paraventricular organ; rTh, rostral thalamus proper; RTN, reticular thalamic nucleus; S, subpallium; SC, suprachiasmatic nucleus; SD, saccus dorsalis; SG, subglomerular nucleus; SO, superior olive; SOP, secondary octaval population; SuCo, superior colliculus; Teg, tegmentum; TeO, tectum opticum; TGN, tertiary gustatory nucleus; TLa, torus lateralis; TPB, thalamo-pallial border; TPp, periventricular nucleus of posterior tuberculum; TPm, migrated nucleus of posterior tuberculum; TS, torus semicircularis; TSc, central nucleus of torus semicircularis; V, ventral telencephalon (subpallium); VAO, ventral accessory optic nucleus; Vd, dorsal nucleus of the area ventralis; VL, ventrolateral thalamic nucleus; VM, ventromedial thalamic nucleus; VP, ventral pallium; VT, ventral thalamus; Y, sulcus ypsiloniformis; ZLI, zona limitans intrathalamica.

of these nuclei hold neurons that project to the isocortex. In fact, the thalamus and the isocortex in placental mammals are tightly correlated as two important findings highlight: firstly, lesions of particular cortical areas or removal of the whole isocortex lead to the degeneration of thalamic nuclei (Rose and Woolsey, 1943; Loopuijt et al., 1995; Kaas, 2009). Secondly, mammals with a less differentiated and smaller isocortex exhibit a less nucleated and prominent thalamus (Jones, 2007).

Hence, the thalamus and its connections to the isocortex are considered crucial to the evolution of human cognition and behavior. Comparative neurobiologists are trying to reconstruct the evolutionary history of the thalamus through cladistic outgroup comparisons and the identification of shared characters in diverse non-tetrapod anamniotes. Evolutionary developmental (evo-devo) studies, in contrast, focus on anamniote model organisms by comparing gene expression patterns and molecular compositions that define primordial brain structures. Among these model organisms, the teleost zebrafish has gained particular importance because of the availability of a large number of mutants and transgenic lines. In comparison to zebrafish, basally derived actinopterygians such as bichirs and sturgeons (**Figure 1**) are developmentally much less investigated. Frequently, the absence of detailed data about outgroups of teleosts undermines the determination of a character in question as being homologous or convergent to that of another group of species. However, differences in molecular composition and developmental history can be strong arguments against certain homologies (e.g., the teleostean anterior thalamic nucleus versus the mammalian dorsal lateral geniculate nucleus, (dLGN; see below).

This review compares the zebrafish thalamus (**Figure 2**) with the one of mammals. And while my focus will be on the thalamus proper, I also discuss surrounding thalamic regions and thalamus-like structures and their connections to the pallium. I do so because fundamental differences between the zebrafish and the mammalian forebrain make a direct comparison both impossible and inadequate. For example, defining features of the mammalian thalamus proper, such as the thalamocortical connections, are inexistent in teleosts. In fact, teleosts lack a sophisticated

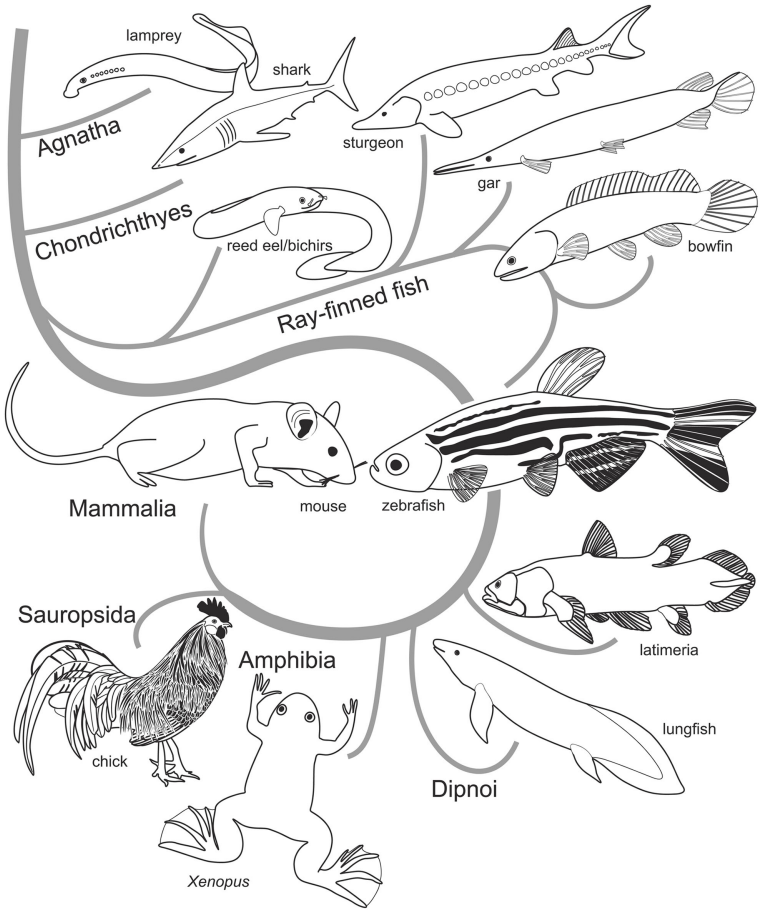

FIGURE 1 | Cladogram showing relationships of extant vertebrates. Determining homologies in brains of distantly related species, such as zebrafish (teleosts) and mouse (mammals), relies on comparative knowledge in regard to the ancestral situation provided by outgroup comparisons (Hennig, 1966). That is, to establish a neural character in zebrafish as homologous to a topologically corresponding structure in the mammalian brain, the neural character needs to be present in the last common (extinct) ancestor of these species. A high likelihood for a structure being homologous is given when the character in question exists in basally emerged ray-finned fish such as bichirs, sturgeons, and gars and in anamniote tetrapods (amphibians) and sauropsids (birds and reptiles).

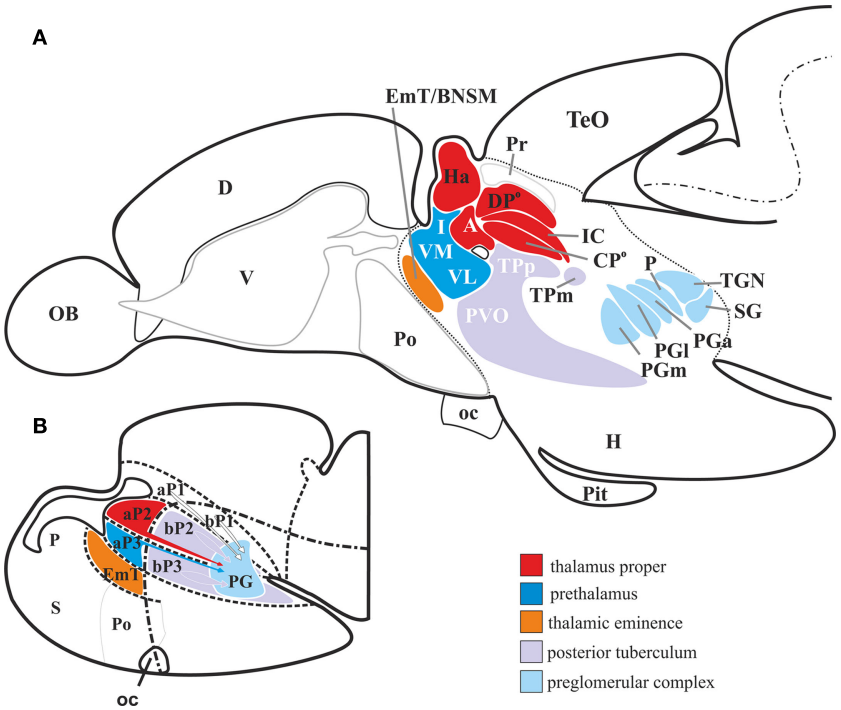

FIGURE 2 | Schematic drawing of the *wider thalamus* in the larval and adult zebrafish. (A,B) The alar plate prosomere 2 [aP2, red in **(B)**] derived dorsal thalamic nuclei [red in **(A)**] are located dorsally to the zona limitans intrathalamica. These are the habenular nuclei, the auditory dorsoposterior (DP°), the intercalated (IC), the visual centroposterior (CP°), and the anterior thalamic nuclei **(A)**. The alar plate prosomere 3 (aP3) derived ventral thalamic nuclei (blue) are the intermediate (I), the ventromedial (VM), and the ventrolateral (VL) thalamic nuclei. The posterior tubercular nuclei are derived from basal plate portions of prosomere 2 and 3 (bB2 + 3). The nuclei of the preglomerular complex [light blue in **(A)**], which together serve as the major thalamo-like sensory relay station, are likely of multiprosomeric origin [indicated with arrows in **(B)**]. Abbreviations see list.

six-layered isocortex, which in mammals provides chief instances of sensory processing. Teleosts, however, do possess a dorsal pallial division that topologically corresponds to the mammalian isocortex (**Figure 3**). Yet, the zebrafish dorsal pallium does not hold sensory areas that receive projections from relay stations comparable to the mammalian thalamus proper (Mueller et al., 2011). For example, the auditory thalamic nucleus (CP°) of teleosts projects to the amygdala (Dm) and to the hippocampal (Dl) division, regions that are, like their mammalian counterparts, involved in emotional response behaviors and spatial orientation respectively (Portavella et al., 2002; Northcutt, 2006). The thalamus proper is also less prominent in zebrafish than in mammals. This is the case because teleosts possess a preglomerular complex, an elaborated migrated agglomeration of nuclei related to the posterior tuberculum absent in mammals. In fact, the preglomerular complex serves as a predominant sensory relay station in the teleostean diencephalon (Wullimann and Northcutt, 1990; Northcutt, 2008; Yamamoto and Ito, 2008).

I discuss in what follows the thalamus proper and structures of the caudal diencephalon that form what I call the *wider thalamus*. My approach aims at relating similarities and differences in the zebrafish brain to defining features of the mammalian thalamus proper. First, I examine the prosomeric Bauplan of the zebrafish forebrain and recent findings that subdivide the thalamus proper into a rostral and a caudal part. Then I look at brain regions that qualify either as thalamus proper or as thalamus-like structures. My closing focus is placed on the functional organization of two ascending sensory systems in teleosts: the acoustic and the visual. While the auditory pathway gives a case of conservation, the visual pathway illustrates a case of non-conservation.

OVERVIEW

In this review, I consider the forebrain of carp-like (cyprinid) teleosts such as zebrafish and goldfish as representative for their fish clade. The cyprinid forebrain is relatively simple in structure, which facilitates the comparison with other vertebrates (Rupp et al., 1996). What is more, most of the developmental data on the teleostean forebrain comes from zebrafish research. In contrast, most of the connectional and functional data stem from studies of goldfish. I look at developmental and hodological data of zebrafish and goldfish to arrive at a generalized picture of the cyprinid forebrain. While a forebrain comparison across the teleostean clade exceeds the focus of my contribution, excellent reviews have already addressed such a comparison (Braford and Northcutt, 1983; Northcutt and Davis, 1983; Northcutt and Wullimann, 1988; Nieuwenhuys and Meek, 1990; Meek and Nieuwenhuys, 1998).

With the term *wider thalamus*, I refer to the thalamus proper (formerly "dorsal thalamus"), the prethalamus (formerly "ventral thalamus"), the thalamic eminence, the periventricular posterior tuberculum, and the migrated preglomerular complex. This broad definition corresponds to the one used by Bergquist (1932), who

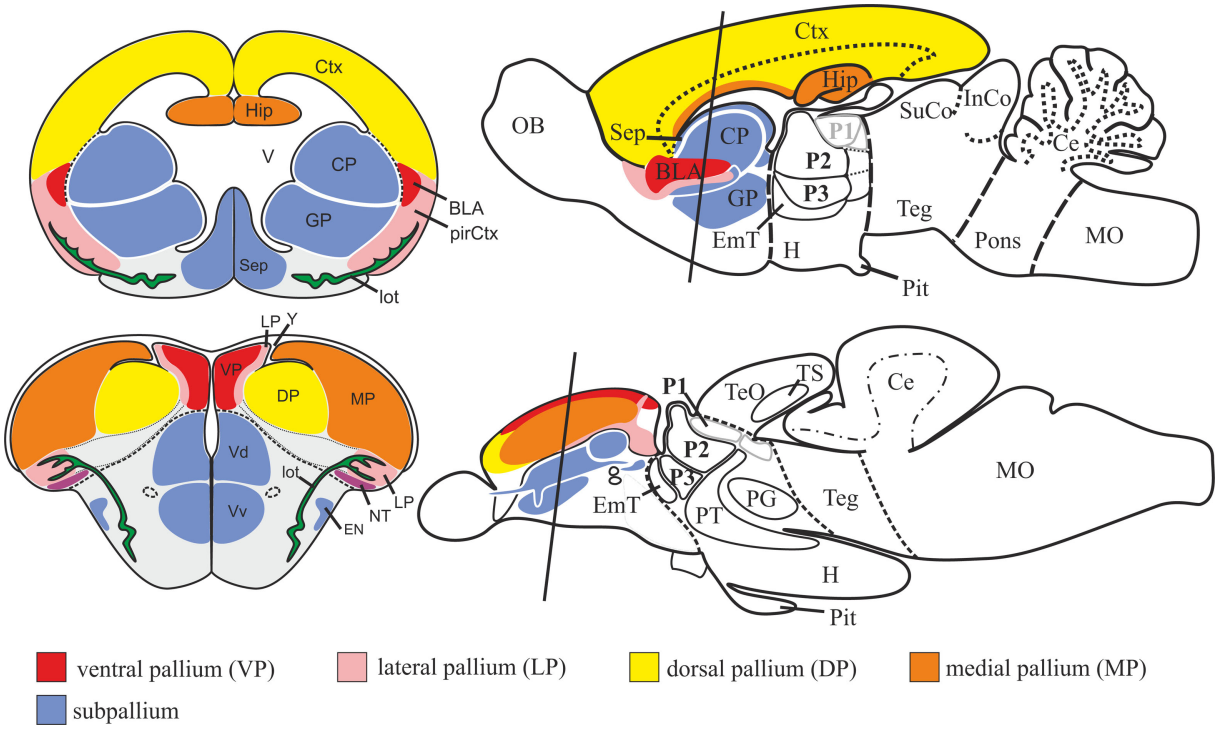

FIGURE 3 | Schematic drawing illustrating topological correspondences between cyprinid (zebrafish/goldfish; teleostean) and rodent (mouse/rat; mammalian) telencephala. The zebrafish telencephalon is characterized by two massive lobes covered by a dorsally located *T*-shaped ventricle. The mouse telencephalon, in contrast, consists of two bilateral hemispheres surrounding centrally located ventricles. The topology of the zebrafish pallium is similar to the one in mouse and can be explained through topographical shifts of its constituting pallial divisions during a complex outward folding process. The zebrafish pallium, like its mammalian counterpart, consists mainly of four pallial divisions: a medial pallium (MP) homologous to the mammalian hippocampus (Hip), a dorsal pallial division (DP) topologically corresponding to the mammalian isocortex (Ctx), and ventral (VP) and lateral (LP) pallial divisions homologous to the mammalian pallial (basolateral) amygdala (BLA) and piriform cortex (pirCtx) respectively. After Mueller et al. (2011). Abbreviations see list.

first described the segmental organization of the longitudinally bent vertebrate forebrain. Bergquist's work is still foundational to the prosomeric forebrain model we use today (Puelles and Rubenstein, 1993, 2003). In the nomenclature of the zebrafish diencephalon I follow Braford and Northcutt (1983) with some modifications by Wullimann and colleagues (Wullimann et al., 1996; Rink and Wullimann, 2004; Wullimann and Mueller, 2004a).

Teleosts clearly share with other vertebrates the thalamus proper, the habenula, and the prethalamus (**Figure 2A**). According to classical comparative works (Bergquist, 1932; Nieuwenhuys, 1963; Braford and Northcutt, 1983; Wullimann, 1998), the thalamus proper in zebrafish is subdivided into the anterior thalamic nucleus (A), the dorsal posterior thalamic nucleus (DP^o), the central posterior thalamic nucleus (CP^o), and the ventral (Hav) and dorsal (Had) habenular nuclei. The ventral and dorsal habenular nuclei are homologous to the mammalian medial and lateral habenular nuclei respectively (Amo et al., 2010). Some prethalamic structures are present in zebrafish but absent in the mammalian brain. These are the intermediate (I), the ventromedial (VM), and the ventrolateral (VL) thalamic nuclei. These nuclei can be found in amphibians as well but not in mammals (Braford and Northcutt, 1983; Neary and Northcutt, 1983; Butler and Northcutt, 1993; Rupp and Northcutt, 1998).

The posterior tuberculum and the preglomerular complex (**Figure 2A**) are two dominant structures in the basal diencephalon of zebrafish, which also have no apparent counterparts in amniote tetrapods like birds and mammals. Yet, a large posterior tuberculum is present in jawless vertebrates (lampreys), cartilaginous fishes (sharks and manta rays), lungfish, and amphibians (Nieuwenhuys et al., 1998). In mammals, the basal plate derivatives of prosomere two and three are homologous to the dorsal and ventral posterior tubercular fields of developing zebrafish (Wullimann and Puelles, 1999). In the mature zebrafish brain, the posterior tuberculum comprises the periventricular nucleus of the posterior tuberculum with its characteristic large pear-shaped dopaminergic cells, the paraventricular organ, and the posterior tuberal nucleus. All of them are intercalated between prethalamus and hypothalamus (Rupp et al., 1996; Wullimann et al., 1996). Some of these dopaminergic neurons found in the posterior tuberculum of zebrafish project to the subpallium and have been compared and homologized with an anteriormost diencephalic, not mesencephalic, division of the mammalian ascending mesodiencephalic dopaminergic groups A8–A10 in mammals (Wullimann and Rink, 2001; Rink and Wullimann, 2002). Other studies demonstrate that the majority of the dopaminergic neurons in the posterior tuberculum depend on the expression of Orthopedia (Otp)

and Nkx2.1 homeodomain proteins, similar to A11-dopaminergic groups present in the pretectum and thalamus proper of mammals (Ryu et al., 2006, 2007). These A11-dopaminergic neurons are the major far-projecting dopaminergic neurons that in zebrafish project to the subpallium, the diencephalon, the hindbrain, and the spinal cord similar to the situation in mammals (Tay et al., 2011).

The preglomerular complex (**Figure 2A**) consists of the anterior, the lateral, the medial, and the caudal preglomerular nuclei (PGa, PGl, PGm, PGc). It also comprises the tertiary gustatory nucleus (TGN), the so-called posterior thalamic (P), and the subglomerular (SG) nucleus. These nuclei have been interpreted as migrated derivatives of the embryonic posterior tuberculum (Braford and Northcutt, 1983; Northcutt, 2008). They serve as sensory relay stations projecting to different parts of the pallium, comparable to sensory thalamic nuclei in mammals. Based on their projection patterns to pallial parts of the adult zebrafish telencephalon and on expression patterns of *pax6* in larval zebrafish, these nuclei have been interpreted as homologous to thalamic nuclei of mammals (Yamamoto and Ito, 2008). The diencephalic organization of teleosts differs also gross anatomically from the one of mammals due to the dominance of the periventricular posterior tuberculum and the migrated preglomerular complex.

THE PROSOMERIC MODEL

During the last decades, a number of evolutionary and developmental (evo-devo) studies have addressed questions of forebrain homologies across vertebrates through the comparison of gene expression patterns. In the developing mouse brain, for example, patterning genes such as *Otx1/2*, *Pax2/6*, *Emx1/2*, *Dlx1/2*, *Nkx2.1/2.2*, and *shh*, define longitudinal and transverse forebrain domains across all vertebrate clades. Initially, the vertebrate forebrain has been interpreted as being divided into six transverse units called prosomeres (Puelles and Rubenstein, 1993). A modified version with only three prosomeres and non-prosomeric telencephalic and hypothalamic parts serves today as the standard for cross-species comparisons (Wullimann and Puelles, 1999; Puelles and Rubenstein, 2003). This new framework of the prosomeric model subdivides the forebrain into a posterior prosomeric diencephalon (P1–P3) and an anterior non-prosomeric telencephalon and hypothalamus. **Figure 2B** shows the schematic division of the prosomeric forebrain of larval zebrafish. The caudal diencephalon, furthermore, consists of alar and basal plate portions. The thalamus proper is a developmental derivative of the alar plate prosomere two (aP2, red in **Figure 2B**). In longitudinal perspective, the thalamus proper is placed anteriorly to both the pretectum, which originates from the alar plate prosomere one (aP1), and the prethalamus, which originates from the alar plate prosomere three (aP3; blue in **Figure 2B**). Thalamus proper and prethalamus ventrally border with posterior tubercular portions, i.e., basal plate of prosomeres two (bP2) and three (bP3), respectively. In mammals, these basal plate regions develop into the fields of Forel and the retromammillary area (Puelles and Rubenstein, 2003).

In zebrafish and other teleosts, an agglomeration of nuclei, the preglomerular complex, flanks the periventricular posterior tuberculum. In the past, the preglomerular complex (light blue in **Figure 2A**) has been treated as a basal plate derivative of the posterior tuberculum (Bergquist, 1932). It is, however, more likely that other alar and basal plate territories such as the pretectum (aP1), the nucleus of the medial longitudinal fascicle (bP1), the thalamus (aP2), and the prethalamus (aP3) contribute cells to the preglomerular complex through radial and tangential migration. Immunohistological stainings against proliferating cell nuclear antigen (PCNA) and bromodeoxyuridine (BrdU) longterm labels on brain sections from larvae between 2 and 5 days postfertilization have shown small chains of migratory cells between different alar and basal plate portions and the primordial preglomerular complex (M2; Wullimann and Puelles, 1999; Mueller and Wullimann, 2002). The expression of *pax6* in the developing brain of both zebrafish and medaka also suggest that alar plate 2 and 3 contribute neurons to the preglomerular complex (Wullimann and Rink, 2001; Ishikawa et al., 2007). Yet, the development of the preglomerular complex has not been studied in detail. In fact, the place of origin of none of its nuclei has been determined through fate mapping. This is why I provisionally treat the preglomerular complex as an entity with undetermined place of origin and designate it a *migrated* (=lateral) neighbor of the (periventricular) posterior tuberculum.

Recent findings regarding the zebrafish thalamus proper require a slight modification of the prosomeric model. In particular, studies on patterning mechanisms demonstrate that the development of the thalamus proper is more complex than previously assumed (Scholpp and Lumsden, 2010). In mammals, diffusible sonic hedgehog (*Shh*) molecules released from the mid-diencephalic organizer (MDO) in the zona limitans intrathalamica (ZLI) and FGF signaling released by a dorsal signaling source influence the development of the anterodorsal part of the thalamus (Kataoka and Shimogori, 2008; Scholpp et al., 2009; Vue et al., 2009; Scholpp and Lumsden, 2010). The combinatorial action of these morphogens leads to the production of GABAergic cell groups in the thalamus proper, which otherwise consists of glutamatergic cells. Based on these findings, the thalamus proper is now subdivided in a rostral (rTh) and a caudal (cTh) domain. The rostral part gives rise to the predominantly GABAergic intergeniculate leaflet (IGL) and contributes GABA cell populations to the ventral geniculate nucleus (Vue et al., 2007, 2009). The majority of the glutamatergic relay nuclei, such as the (dLGN; visual), the medial geniculate nucleus (MGN; auditory), and the ventrobasal complex (somatosensory) likely develop from the caudal portion of the thalamus proper (cTh; Jones and Rubenstein, 2004).

The thalamic eminence (EmT), an ambiguous thalamic structure, is located in the optic stalk area at the border of pallium and thalamus (zebrafish: orange in **Figures 2A,B**). This region is called the telencephalon impar (Nieuwenhuys et al., 2007). In the mammalian brain, the EmT is visible only during developmental stages. In the original prosomeric model, the EmT develops from the alar plate of prosomere four (aP4; Puelles and Rubenstein, 1993). Only in amphibian species like salamanders and frogs is the EmT prominent also in the adult brain. I treat the EmT as a developmental unit lying anterior to the prethalamus in the longitudinal view as proposed in the original prosomeric model (Puelles and Rubenstein, 1993; Wullimann and Puelles, 1999; Mueller and Wullimann, 2009). More recently, Puelles and Rubenstein (2003) have argued that the EmT is a dorsal part of the prethalamus. Since then, a number of studies have placed the EmT within the

prethalamus. However, studies have shown that prethalamus and EmT can be clearly distinguished based on their distinct molecular profiles during early phases of neurogenesis (Wullimann and Mueller, 2004a; Osório et al., 2010).

THE WIDER THALAMUS IN ADULT ZEBRAFISH

Molecular mechanisms patterning the thalamus proper appear highly conserved across vertebrates (Scholpp and Lumsden, 2010). Like in its mammalian counterpart, the zebrafish thalamus proper during early developmental stages divides into a rostral (rTh) and a caudal (cTh) domain (Scholpp et al., 2009; Peukert et al., 2011). The rostral zone is defined by expression of *ascl1*, a basic helix–loop–helix transcription factor (bHLH-TF) coding gene involved in the production and patterning of GABAergic phenotypes. The caudal zone (cTh), in contrast, is defined by expression of *neurog1*, which encodes for a bHLH-TF determining glutamatergic phenotypes. Both neuronal phenotypes depend on different levels of sonic hedgehog (shh) released by the MDO. High concentrations of shh and the presence of her6 promotes GABAergic cell fates whereas low levels and the absence of her6 induces glutamatergic thalamic neurons (Scholpp et al., 2009; Scholpp and Lumsden, 2010). How these early patterns of GABAergic versus glutamatergic territories relate to the adult distribution is still unclear. While only detailed and complex lineage studies could produce here reliable answers, the prosomeric distribution of GABAergic territories across vertebrates allows the proposition of some reasonable hypotheses in regard to the topology of the zebrafish thalamus (Brox et al., 2003; Mueller et al., 2006; Robertson et al., 2007).

In what follows, I describe the zebrafish thalamus proper as a largely glutamatergic derivative of the prosomere 2 (aP2) clearly distinguishable from the GABAergic prethalamus of the prosomere 3 (aP3) lying anteriorly to the ZLI (**Figure 4B**; **Figure 4E** for overview). There are, however, some GABAergic nuclei lying posteriorly to the ZLI that may be interpreted as derivatives of the embryonic rostral thalamus proper (rTh/aP2). In contrast, the majority of the nuclei classically treated as thalamus proper likely are predominantly glutamatergic derivatives of the caudal thalamus (cTh/aP2).

Two GABAergic nuclei in the thalamus proper lying close to the ZLI appear to originate in the *rostral thalamus proper* (rTh): the anterior thalamic nucleus (A) and the only recently recognized intercalated thalamic nucleus (IC; Mueller and Guo, 2009).

The *anterior thalamic nucleus* (A, **Figures 4B,C**) of cyprinids and other teleosts topologically corresponds to the anterior nucleus of amphibians. It likely processes emotional and, according to some authors also visual, information (Braford and Northcutt, 1983; Neary and Northcutt, 1983). It still remains to be determined how the anterior thalamic nucleus, a putative derivative of the rostral thalamus proper, relates to mammalian structures derived from this domain. In rodents, the rostral thalamus develops to the IGL and contributes to the ventral geniculate nucleus. The latter, though, is primarily a derivative of the prethalamus (Nakagawa and O'Leary, 2001; Brox et al., 2003; Jones and Rubenstein, 2004; Vue et al., 2007, 2009; Mueller and Guo, 2009; Inamura et al., 2011). In zebrafish neither the ventral lateral geniculate nucleus (vLGN) nor the IGL have been identified. Some authors suggest that the anterior thalamic nucleus of zebrafish is field homologous to the dLGN in mammals, because the anterior thalamic nucleus is located in a topological similar position (Butler's "lemnothalamus"; Butler, 1995; Butler and Hodos, 2005). The mammalian dorsal geniculate nucleus, however, is predominantly glutamatergic and thus probably belongs to the caudal thalamus (cTh). The teleostean GABAergic anterior thalamus is, furthermore, unlikely homologous to nuclei of the mammalian anterior thalamic complex, because the latter also most probably belongs to the cTh although detailed fate mapping studies are still missing (compare Vue et al., 2009).

The previously unrecognized GABAergic *intercalated thalamic nucleus* (IC; **Figure 4D**) is sandwiched between dorsoposterior (DP^o) and centroposterior (CP^o) nuclei. It is possible that the IC develops from the rostral thalamus (rTh) due to its position close to the ZLI. Fate mapping studies still need to confirm this hypothesis. The IC has not been found in any other anamniote vertebrate. Its delineation in zebrafish drastically reduces the extent of the dorsoposterior and centroposterior nuclei and redefines their borders.

Most of the thalamic nuclei are free of GABAergic neurons, as can be expected for glutamatergic derivatives of the cTh. To this group belong the habenular (Hav, Had; **Figure 4A**), the visual dorsoposterior (DP^o; **Figures 4C,D**), and the auditory centroposterior (CP^o; **Figure 4D**) thalamic nuclei.

In adult zebrafish brains derivatives of the prethalamus are GABAergic derivatives which lie anteriorly to the ZLI. They are the ventromedial (VM), the ventrolateral (VL), and the intermediate (I) thalamic nuclei (**Figure 4A**). These nuclei are present only in anamniotes but not in mammals (Braford and Northcutt, 1983).

Finally, the putative *reticular thalamic nucleus* (RTN; **Figures 4B,C**) in zebrafish is a lose *GAD67*-positive GABAergic aggregation of cells surrounding the anterior (A) and dorsoposterior (DP^o) thalamic nuclei (**Figures 4B,C**). It is unclear, if this nucleus is homologous to the one in mammals. The mammalian RTN, a derivative of the prethalamus (P3), lies rostroventrally adjacent to the sensory thalamus proper (P2) of adult rodents and other mammals. Topographically, the mature zebrafish RTN sits in a similar position and is closely associated with the sensory thalamus proper. It is possible that the zebrafish RTN exerts similar modulatory functions to the one in mammals. Both the topological origin and the function of the zebrafish RTN, however, are undefined (Mueller and Guo, 2009).

In larval zebrafish, the EmT (**Figures 5A–D,F**) has been located based on expression patterns of *neuroD* and *eomesA* (Wullimann and Mueller, 2004a). In zebrafish and mammals, the EmT appears to give rise to the glutamatergic bed nucleus of the stria medullaris (BNSM; Mueller and Guo, 2009). The BNSM in adult zebrafish sits in the stalk (peduncular) region, a transitional area at the border to the telencephalon impar (Mueller and Guo, 2009). In adult zebrafish the remainder of the EmT has not yet been identified. As **Figures 5E1+2** show, the EmT lies within the optic stalk area close to the BNSM. It is a periventricularly located proliferative cell mass located ventrally to the caudalmost telencephalon. Both EmT and BNSM are *GAD67*-negative (**Figures 5E1+2**; **Figure 5G** for overview). What I define as EmT in the mature zebrafish has previously been misinterpreted as a telencephalic proliferation zone. In the atlas of the adult zebrafish brain, for example, the EmT is considered the proliferative zone of the posterior pallial nucleus

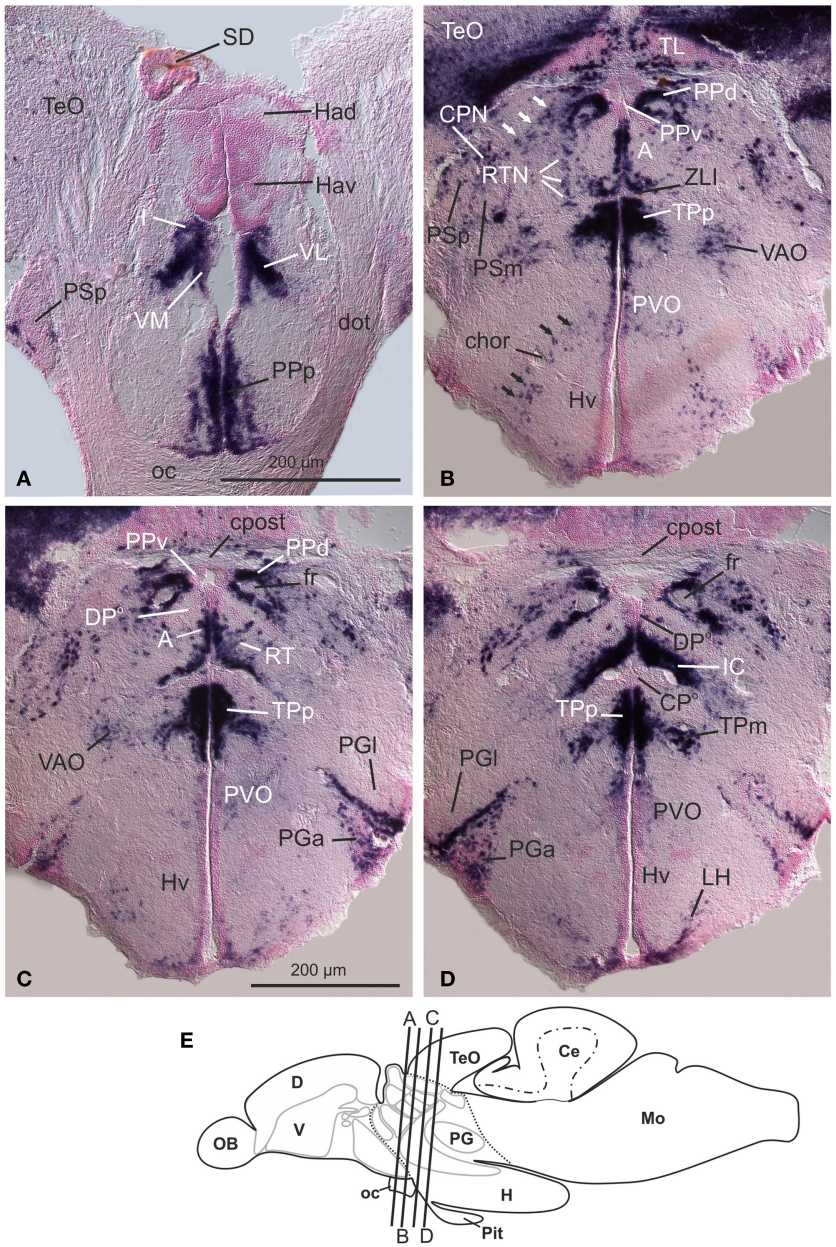

FIGURE 4 | Distribution of *GAD67*-mRNA reveals prosomerically organized thalamic derivatives in the brain of adult zebrafish (A–D). Ventral thalamic (prethalamic), GABAergic nuclei such as the intermediate, ventrolateral, and ventromedial nuclei (I, VL, and VM in **(A)** are defined by expression of *GAD67*-mRNA. In the longitudinal view, these nuclei are located anterior to the zona limitans intrathalamica (ZLI, nicely displayed in **(B)**. Dorsal thalamic nuclei, such as the habenular nuclei [Had, Hav in **(A)**], and dorsoposterior (DP°) and centroposterior (CP°) nuclei, are predominantly glutamatergic and *GAD67*-negative. However, some dorsal thalamic nuclei close to the ZLI are also GABAergic and likely derivatives of the rostral thalamus proper (rTh). These are the anterior **(A,B)** and the intercalated nucleus [IC; **(D)**]. **(E)** Schematic drawing of the zebrafish brain that indicates the approximate position of transverse sections. From Mueller and Guo (2009). Abbreviations see list.

(Dp; Wullimann et al., 1996). In other teleost fish, the EmT is sometimes erroneously considered the proliferative zone of the nucleus taeniae (Nieuwenhuys, 2011).

THE PREGLOMERULAR COMPLEX, A CONVERGENT THALAMUS-LIKE AREA IN TELEOSTS

After examining thalamic nuclei of the alar plate portions of prosomeres 2 and 3, I now move to those structures usually treated as basal plate derivatives of prosomeres 2 and 3: the periventricular posterior tuberculum and its migrated neighbor, the preglomerular complex. Both are important for the comparative interpretation of the teleostean wider thalamus. This is the case because in teleosts the nuclei of the preglomerular complex – in contrast to those of the thalamus proper – are reciprocally connected with pallial parts, mostly the mediodorsally located pallial amygdala (VP/Dm) and the laterodorsal hippocampal

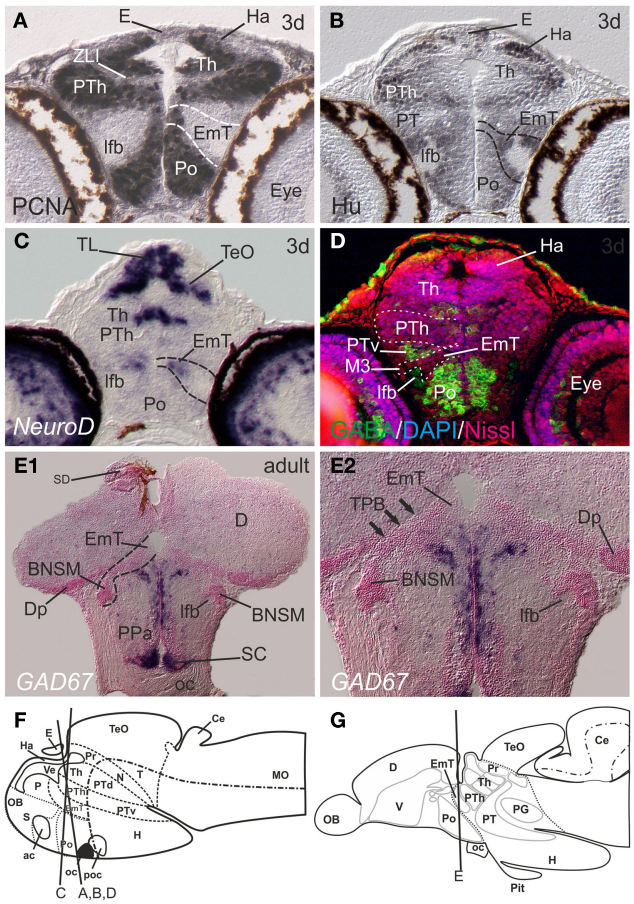

FIGURE 5 | The location of the eminentia thalami (EmT) in postembryonic and adult zebrafish. (A–E) In larval zebrafish, the periventricular proliferative zone [PCNA-positive in **(A)** and Hu-negative in **(B)**] of the EmT is located between ventral thalamus (VT) and preoptic region (Po). **(C)** Postmitotic EmT-cells expressing *neuroD*-mRNA migrate toward the lateral forebrain bundle (lfb). **(D)** Both, proliferative and postmitotic cells of the EmT in the larval brain are free of GABA cells, whereas surrounding regions, such as the ventral posterior tuberculum (PTv) and preoptic region (Po) show many GABA cells. Note, the ventral thalamus is usually defined by a heavy presence of GABA-expressing cells: here, the ventral thalamus appears GABA-negative, because its proliferative zone is displayed only. **(E1 + E2)** The EmT in the brain of adult zebrafish is located ventrally adjacent to the posterior zone (Dp) of the pallium, ventrally to the thalamo-pallial border. The derivative of the EmT, the peripheral bed nucleus of the stria medullaris (BNSM, former ventral nucleus of the entopeduncular complex) surrounds the lateral forebrain bundle. Schematic drawings of larval **(F)** and adult **(G)** zebrafish brain that indicates the approximate position of transverse sections. Abbreviations see list.

division (MP/Dl). Indeed, the majority of the ascending sensory system in teleosts reaches the pallium through the preglomerular complex. It has been suggested, that specific sensory nuclei in the preglomerular complex exist for gustatory, somatosensory, auditory, and visual systems (Wullimann, 1998; Wullimann and Mueller, 2004b). Recent connectivity studies in goldfish, however, show that the majority of these preglomerular nuclei process two or more sensory modalities and, are therefore multimodal (Northcutt, 2006, 2008; Yamamoto and Ito, 2008).

The preglomerular complex in adult cyprinids, like goldfish and zebrafish, consists of a group of satellite nuclei associated with the TGN, which in the past have been termed nucleus glomerulosus (Northcutt, 2008). The preglomerular complex is subdivided in a rostral and a caudal group. The rostral group is formed by three nuclei located at the rostral pole of the TGN: the anterior (PGa), the lateral (PGl), and the medial (PGm) preglomerular nuclei (**Figures 6A–D**; **Figure 6E** for overview). The caudal group is formed by the tertiary gustatory nucleus and the closely associated posterior thalamic (P) and subglomerular (SG) nuclei (**Figures 6A–D**).

In goldfish, the anterior nucleus (PGa) is a predominantly auditory relay nucleus projecting to the dorsomedially positioned pallial amygdala (VP/Dm). It receives projections from the auditory central torus semicircularis and from other auditory nuclei, such as the perilemniscular nucleus, the anterior tuberal nucleus, and the medial pretoral nucleus (Yamamoto and Ito, 2008). The lateral preglomerular nucleus (PGl) receives multiple inputs and is subdivided into different rostral and caudal parts. Different parts of the rostrolateral PGl convey visual, lateral line, and auditory ascending information (goldfish: Yamamoto and Ito, 2008; catfish: Striedter, 1990). The TGN and the closely associated posterior thalamic nuclei receive primarily gustatory projections (crucian carp: Morita et al., 1980, 1983).

Conflicting interpretations exist in regard to possible homologies of nuclei of the preglomerular complex with nuclei of the mammalian thalamus proper. It is assumed that the preglomerular complex evolved in the common ancestor of chondrostean sturgeons and teleosts as a derivative of the posterior tuberculum (Bergquist, 1932; Northcutt, 2008). Based on *Pax6* expression patterns in developing medaka, brains, parts if not all of the preglomerular complex are hypothesized as homologous to the thalamus proper (P2) of other vertebrates (Ishikawa et al., 2007). Yet, only sparse data supports this finding. Within the rostral group, for example, the predominantly glutamatergic lateral nucleus of the preglomerular complex (PGl) might indeed share its developmental origin with the centroposterior thalamus (CP^o), because in anterior sections the PGI is positioned in the ventrally bent, radial domain of the centroposterior thalamic nucleus (**Figure 6A**). Both of these nuclei (CP^o and PGl) are largely free of *GAD67*-expression in adult zebrafish. Yet detailed fate mapping studies that could clarify the situation are missing. Some PCNA, BrdU, and *neuroD*-gene expression studies in brains of zebrafish larvae between 2 and 5 days postfertilization indicate chains of migrating cells from the pretectum, the thalamus proper, and the prethalamus toward the primordial preglomerular complex (M2). All of these proliferation zones may contribute to the preglomerular complex in addition to the cells that derive from the posterior tubercular (bP2/3) proliferation zones (Wullimann and Puelles, 1999; Mueller and Wullimann, 2002).

Outgroup comparisons do also not support a homology between nuclei of the teleostean preglomerular complex and the thalamus proper of other vertebrates. Basal ray-finned fish like cladistian bichirs possess a well-developed posterior tuberculum but lack a preglomerular complex. The same holds true for basal sistergroups of ray-finned fish, such as cartilaginous sharks, manta rays, and agnathan lampreys. According to Northcutt (2006), the preglomerular complex evolved in the last common ancestor of

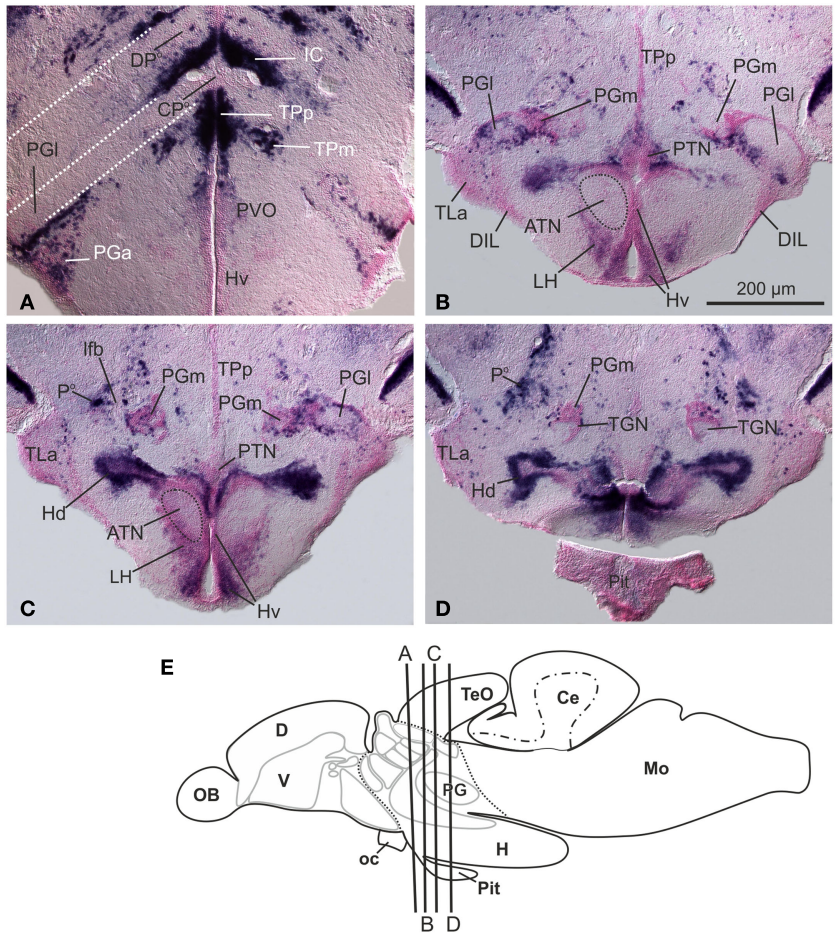

FIGURE 6 | Distribution of *GAD67*-mRNA at the level of the preglomerular complex and hypothalmus (A–D). Note that the anterior part of the lateral preglomerular nucleus [PGl in **(A)**] lies in the radial domain of the centroposterior thalamic nucleus [CP°; **(A)** is a magnification of **Figure 4D**]. The so-called posterior thalamic nucleus [P° in **(D)**] of the preglomerular complex consists of mostly *GAD67*-expressing, GABAergic cells. **(E)** Schematic drawing of the zebrafish brain that indicates the approximate position of transverse sections. From Mueller and Guo (2009). Abbreviations see list.

sturgeons and teleosts and is more complex in the latter group. We need then to assume that the teleostean preglomerular complex is a distinctive anatomical feature, an autapomorphy, and a convergent, thalamus-like sensory relay station. In addition, most of the preglomerular complex processes more than one modality which makes its functional organization distinctly different from the mammalian thalamus (Northcutt, 2008).

THE AUDITORY THALAMUS: A CASE OF CONSERVATISM

The topological organization of thalamic and prethalamic derivatives in teleosts resembles the situation in anamniote tetrapods (amphibians: Neary and Northcutt, 1983; Brox et al., 2003). Their functional significance and connectivity, however, is still poorly understood. Teleost fish do possess a well-described, effective auditory system, which is basically formed by the same central nervous system components found in tetrapod vertebrates (McCormick, 1982, 1992). This makes the auditory system a perfect candidate to trace how sensory information is relayed through the teleostean thalamus proper in comparison to mammals. In contrast, the visual system in teleosts appears to be very different from the mammalian one displaying widespread retinofugal projections to the preglomerular complex, prethalamic, pretectal nuclei, as well as to the tectum opticum.

Teleosts are capable of performing a range of auditory challenging tasks: they can discriminate distinct frequencies, locate sound sources, and they are capable of analytic and synthetic listening (Schuijf et al., 1977; Fay, 1992; Popper and Fay, 1993). The ears of teleost fish and land vertebrates necessarily differ from each other. Teleosts lack structures directly comparable to either the mammalian cochleae or the amphibian auditory papillae. The inner ear of teleosts processes sound via otolithic endorgans, such as the sacculus, lagena, and the utriculus (Furukawa and Ishii, 1967; Fay, 1978). Sensory fibers innervating the hair cells of the endorgans of the inner ear form the octaval, VIIIth nerve, which transmits both vestibular and acoustic information. The brain area receiving primary auditory information is located in the medulla oblongata and is named after its innervating nerve, the octaval area (**Figure 7**). This brain region represents the major source of afferents to the auditory torus semicircularis, an alar plate derived midbrain station en route to the teleostean thalamus proper. A column of five nuclei is arranged in a rostrocaudal sequence in the ventral octaval area. These nuclei are the anterior, magnocellular, descending,

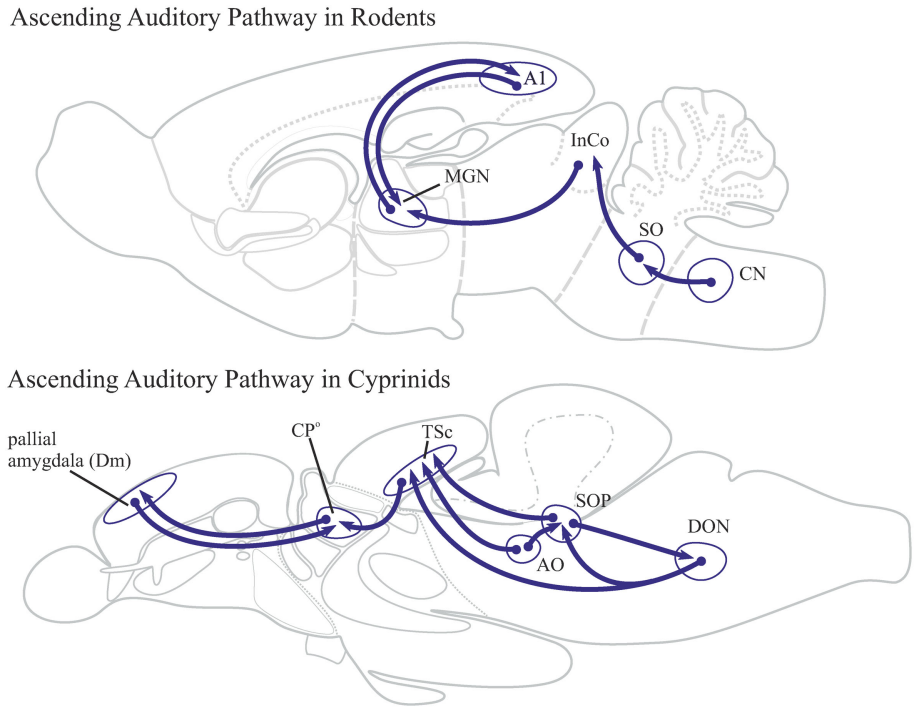

FIGURE 7 | Ascending auditory pathways in rodents and cyprinids. The ascending auditory pathway in cyprinids is similar to those found in mammals. The teleostean secondary octaval population (SOP) is homologous to the mammalian superior olive (SO). The supererior olive (SO) of rodents projects onto the inferior colliculus (IC), which conveys auditory information to the medial geniculate nucleus. The latter is a dorsal thalamic nucleus, which is reciprocally connected with the auditory cortex (A1). In cyprinids, however, the secondary octaval population conveys auditory information to the central nucleus of the torus semicircularis (TSc). This nucleus projects to the auditory centroposterior thalamic nucleus (CP°), which is reciprocally connected with the dorsomedial located pallial amygdala (Dm) of the telencephalon. The centroposterior nucleus does not project to the dorsal pallium (Dc = central zone) of teleosts.

tangential, and posterior nuclei (Northcutt, 1980; McCormick, 1982; Meredith and Butler, 1983).

Primary auditory centers in cyprinids are the dorsal part of the anterior octaval nucleus and the dorsomedial part of the descending octaval nucleus (Echteler, 1984, 1985). Secondary octaval projections from these two nuclei form part of the lateral longitudinal tract in cyprinids and other teleosts which is homologous to the mammalian lateral lemniscus. The name lateral lemniscus comes from the Greek word *lemniskos* signifying "filet" which refers to the lateral filet-like position of its lemniscal fibers. In teleosts, they project to a medially positioned central nucleus of the torus semicircularis, an auditory nucleus of the dorsal midbrain homologous to the mammalian inferior colliculus (InCo; **Figure 7**). Another part of the lateral lemniscus in cyprinids projects to a secondary octaval population of neurons, which may be homologous to the superior olive of mammals (Echteler, 1984; McCormick and Hernandez, 1996). The torus semicircularis is reciprocally connected to the auditory centroposterior (CP°) thalamic nucleus in cyprinids and other teleosts. In teleosts, this auditory relay nucleus projects to the dorsomedial lying pallial amygdala (VP/Dm). Thalamocortical projections are absent in teleost fish. The majority of the thalamotelencephalic projections are targeted to the subpallial basal ganglia, the dorsal part (Vd) of the ventral telencephalon (Northcutt, 2006). Currently, the auditory centroposterior (CP°) thalamic nucleus is the only known nucleus reciprocally connected to the pallial amygdala (VP/Dm) in the cyprinid goldfish and in two vocal batrachoid teleosts, the plainfin midshipman and the gulf toadfish (Goodson and Bass, 2002; Northcutt, 2006).

VISUAL THALAMIC AREAS: A LOST QUEST FOR THE GENICULATE PATHWAY

The visual system is highly developed in most teleosts. Goldfish and zebrafish are tetrachromats and their color vision is based on four cone types. Mammals, in contrast, are predominantly trichromats possessing three cone types (Neumeyer and Arnold, 1989; Robinson et al., 1993). The retinal ganglion cells of teleosts project to five primary target sites: the optic tectum, the thalamus, the pretectum, the accessory optic system, and the preoptic area (Northcutt and Wullimann, 1988). Two of them will be discussed here: the optic tectum and the thalamic projection sites. The optic tectum is the dominant visual center in the teleostean brain. It processes the vast majority of visual information that concern movement, shape and color of objects. The teleostean optic tectum is homologous to the mammalian superior colliculus, which is a relatively small portion in the dorsal midbrain.

Studies that describe visual thalamic circuits in teleosts (**Figure 8**) and other ray-finned fish in comparison to the mammalian situation focus on geniculate and extrageniculate visual pathways (Wullimann, 1998). In mammals around 90% of the retinal fibers ascend to the dLGN. This thalamic sensory nucleus

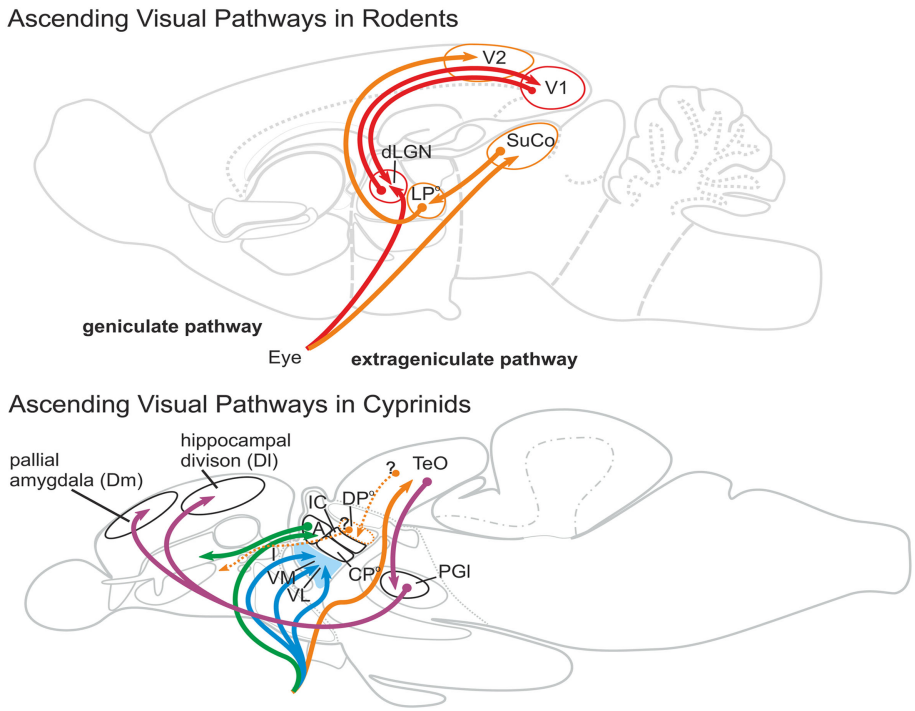

FIGURE 8 | Ascending visual pathways in rodents and cyprinids. In mammals, 90% of the primary visual projections target the dorsal lateral geniculate nucleus (dLGN), which is reciprocally connected with the primary visual cortex (V1). This pathway is called the geniculate pathway (red). The extrageniculate pathway (orange) comprises primary visual projections that target the superior colliculus (SuCo). The superior colliculus projects to the lateroposterior dorsal thalamic complex (LP°) of rodents. The lateroposterior complex is reciprocally connected with the secondary visual cortex (V2). Ascending visual pathways in goldfish could not establish geniculate and extrageniculate pathways. Primary visual projections target, for example, ventral thalamic nuclei (blue: intermediate (I), ventromedial (VM), ventralateral (VL) thalamic nuclei), as well as the anterior nucleus (green, A), and the tectum opticum (orange) which is homologous to the mammalian superior colliculus (SuCo). Based on studies in some teleosts, the dorsoposterior thalamic nucleus (DP°) of cyprinids does likely receive visual information from the tectum opticum (TeO) and, thus, likely represents the visual thalamic nucleus. However, this nucleus does project to the striatopallidum (Vd), but not to any pallial area. The only thalamus-like visual relay nucleus is the lateral preglomerular nucleus, which projects to the dorsomedial pallial amygdala (Dm) and to the laterodorsally located hippocampal divsion (Dl; projections in purple). Thus, geniculate and extrageniculate pathways as found in mammals are absent in teleosts.

relays primary visual information to V1, an important entrance part of the visual cortex. This visual pathway is called the geniculate pathway. In contrast, the mammalian extrageniculate pathway is characterized by retinal projections to the superior colliculus, which projects to a group of caudal thalamic nuclei, mainly the pulvinar or lateroposterior complex. These tectorecipient thalamic visual nuclei project to all visual cortical areas (V2–V4) except to V1 and to the lateral amygdala (Doron and Ledoux, 1999).

During the eighties when tracers such as horseradish peroxidase and DiI became available, retinothalamic projections were investigated in a variety of teleost fishes, particularly in goldfish. Northcutt and Wullimann (1988) reviewed these visual tracing studies for the revised nomenclature of the teleostean diencephalon provided by Braford and Northcutt (1983). Ascending visual pathways are schematically summarized in **Figure 8**. Thalamic projection sites found in all teleosts include primarily prethalamic derivatives: the ventromedial (VM), intermediate (I), and ventrolateral (VL) nuclei. Connectivity and comparability to amniote vertebrates regarding these prethalamic nuclei are unknown (Butler and Hodos, 2005). The anterior thalamic nucleus (A) is the only teleostean thalamic nucleus, which receives primary visual projections and projects to a part of the telencephalon, the region that is interpreted as the striatopallidum (Vd). As a retinorecipient thalamic nucleus, the anterior thalamic nucleus (A) has either been discussed as a field homolog of the dorsal geniculate nucleus (Butler and Hodos, 2005) or it has been considered part of the geniculate pathway (Wullimann, 1998). Yet like its amphibian homolog, the teleostean anterior thalamic nucleus is a GABAergic dorsal thalamic nucleus and likely a derivative of the newly defined rostral thalamus proper. In contrast, the mammalian dLGN is glutamatergic and probably a derivative of the cTh proper. A teleostean nucleus homologous to the mammalian dLGN might be absent in teleosts, similar to what has been found in amphibians (Roth et al., 2003).

Do teleosts possess a tectorecipient dorsal thalamic nucleus that serves as an extrageniculate pathway to the telencephalon? The mammalian pulvinar/lateroposterior complex is a dorsal thalamic structure that receives visual projections from the superior colliculus. Nuclei of this structure predominantly project to all primary visual cortical areas outside V1. Echteler and Saidel (1981) suggested that the dorsoposterior thalamic nucleus (DP°) in goldfish serves as a visual thalamic relay nucleus which projects to the

dorsomedial (Dm) part of the pallium. Recent DiI tracing studies in goldfish, though, demonstrate that the dorsal posterior nucleus in goldfish projects to the subpallium and not to pallial domains such as the putative homologs of the amygdala, the hippocampus, or the cortex (Northcutt, 2006). Tracing studies in the elephantnose fish (*Gnathonemus petersii*) show projections from the optic tectum to the dorsal posterior thalamic nucleus supporting a visual function. Also a homologous nucleus in the teleost catfish (*Ictalurus punctatus*) receives tectal projections (Striedter, 1990). Corresponding findings are still lacking for cyprinids like carp, goldfish, or zebrafish. Two reports in goldfish did not demonstrate projections from the tectum to the dorsoposterior nucleus (Sharma, 1975; Luiten, 1981).

Taken together, in cyprinids thalamic regions involved in visual processing clearly differ from mammalian visual systems (**Figure 8**). The teleostean ascending visual system can hardly be compared to the mammalian extrageniculate pathway. There is currently no evidence for a nucleus of the thalamus proper projecting to any other part of the pallium like the pallial amygdala, the dorsal pallium, or the hippocampal division. Thus, teleosts do not have homologous visual thalamoamygdalar projections. Future research needs to clarify if the dorsal posterior thalamic nucleus in cyprinids receives tectal input and if is functions are restricted to visual processing only.

CONCLUSION

The thalamus proper of placental mammals is a complex structure in the caudal diencephalon. It serves as a sensory relay station connecting the isocortex with the larger brain. What is the thalamus in zebrafish? Conserved patterning mechanisms and similar processes during neurogenesis allow for a comparison between zebrafish and mammals at early stages of development. The thalamus proper in adult zebrafish, however, strongly differs from the mammalian one. The question "What is the Zebrafish Thalamus?" then is truly a question after (1) the elements in the zebrafish brain that correspond topologically and (2) the elements that execute similar connectivity and functions. Certain nuclei of the thalamus proper, such as the anterior (A) and the dorsoposterior (DP^o) thalamic nuclei apparently correspond topologically to derivatives of the rostral and caudal Thalamus respectively. Unlike counterparts in mammals, their projections however are targeted to the ventral telencephalon and not to a region that corresponds to the mammalian isocortex. Defining features of the mammalian thalamus proper, such as thalamocortical connections and the dLGN, are absent in zebrafish. Most importantly, it is the preglomerular complex and not the thalamus proper that act in zebrafish as major sensory relay station. Zebrafish as a genetically traceable model organism has only begun to reveal the developmental processes and neural circuits defining the thalamus in teleosts. Genetic approaches that include molecular-anatomical analyses of late developing and mature zebrafish brains are needed to solve the evolutionary questions outlined here.

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The tale of the three brothers – Shh, Wnt, and Fgf during development of the thalamus

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The thalamic complex is an essential part of the brain that requires a combination of specialized activities to attain its final complexity. In the following review we will describe the induction process of the mid-diencephalic organizer (MDO) where three different signaling pathways merge: Wnt, Shh, and Fgf. Here, we dissect the function of each signaling pathway in the thalamus in chronological order of their appearance. First we describe the Wnt mediated induction of the MDO and compartition of the caudal forebrain, then the Shh mediated determination of proneural gene expression before discussing recent progress in characterizing Fgf function during thalamus development. Then, we focus on transcription factors, which are regulated by these pathways and which play a pivotal role in neurogenesis in the thalamus. The three signaling pathways act together in a strictly regulated chronology to orchestrate the development of the entire thalamus.

Keywords: diencephalon, forebrain, thalamus, development, zebrafish, Shh, Wnt, Fgf

INTRODUCTION

In sixteenth century France, people liked listening to the fairy tale of the "*Trois frères et le petite maison*." In this story, in order to maintain and add to the splendor of their father's small house, three brothers had to compete for its inheritance by showing their skills as a barber, a blacksmith, and a swordsman. It was a difficult task since although all of them were excellent in one particular field; none of them could match their father's all-round skills and wisdom. So, it quickly became obvious that only upon combining their skills would their father's home attain its full splendor. Thus, the three brothers worked together happily in the small house until they grew old.

In analogy to "*le petite maison*," the thalamic complex is also a small but essential part of the brain that requires a combination of specialized activities to attain its final complexity. It is located at the top of the brainstem and can be subdivided into the anterior prethalamus and the posterior thalamus. The thalamic complex serves as the major relay station for sensory information in the brain, receiving nearly all sensory inputs and connecting them reciprocally with the overlying cortex. In contrast, the prethalamus sends hardly any axons to the cortex, but is defined by projections into the underlying thalamus. However, our knowledge about the development of the thalamic complex is still fragmented and here we will summarize the recent advances in the field.

During development, the brain becomes segmented along the anterior-posterior axis to ensure the formation of complex and functionally independent brain parts. The development of the thalamic complex serves as an example for the importance of this segmentation process. The thalamic complex in vertebrates is subdivided by a compartment boundary separating the prethalamus anteriorly from the thalamus posteriorly (**Figures 1A–C**). This transverse boundary between prethalamus and thalamus is called the *Zona Limitans Intrathalamica* (ZLI; Bergquist, 1932). According to the prosomeric model of Puelles and Rubinstein, the ZLI is the border between prosomere 2 (P2) and prosomere 3 (P3; Puelles and Rubenstein, 1993, 2003).

Prominent morphological boundaries co-localize often with instructive cell populations – "local organizers" that are required to pattern adjacent brain regions. The *isthmus* with the *midbrain-hindbrain boundary organizer* serves as a well-studied example for a compartment boundary with an organizer orchestrating the development of the adjacent tectum and cerebellum. It was suggested that the thalamus might similarly require instructive information from an organizer to orchestrate its patterning and maturation (Shimamura et al., 1995). A key feature of organizers is therefore their ability to influence nearby cell behavior in a non-cell autonomous manner through long-range signaling by secreted morphogens. Indeed, heterotopic transplantation of mid-diencephalic donor cells induces ectopic thalamic and prethalamic genes in surrounding host tissue (Garcia-Lopez et al., 2004; Vieira et al., 2005; Guinazu et al., 2007). At the ZLI we find a cell population, which expresses signaling members of the three protein families Wnt, Shh, and Fgf. These secreted proteins have been implicated in local organizing functions elsewhere in the brain. Also, cells within an organizer generally do not intermingle with those of adjacent regions. Such cell lineage restriction helps to stabilize the size and position of the organizer and ensures the production of a stable morphogen gradient from its source, which is imposed by the morphological characteristic of the ZLI. Cell lineage restriction has been shown to operate at the border of the ZLI (Zeltser et al., 2001; Garcia-Lopez et al., 2004). Thus, the cell population at the ZLI has all characteristics of a local organizer and is therefore termed *mid-diencephalic organizer* (MDO; also known as ZLI organizer).

In the following review we will describe the induction process of the MDO where three different signaling pathways merge: Wnt, Shh and Fgf (Scholpp and Lumsden, 2010) and we will dissect the function of each signaling pathway in the thalamus

in chronological order of their appearance (**Figure 2**). We will therefore first describe the Wnt mediated induction of the MDO and compartition of the caudal forebrain, then the Shh mediated determination of proneural gene expression before discussing recent progress in characterizing Fgf function during thalamus development. We will then focus on transcription factors, which are regulated by these pathways and which play a pivotal role in neurogenesis in the thalamus. Like in the French tale, these three signaling factors act together in a strictly regulated chronology to orchestrate the development of the entire thalamus and none of them would be able to achieve such an elaborate structure on its own.

INDUCTION AND POSITIONING OF THE MDO

Prior to the formation of the anatomical ZLI boundary, the induction of the signaling center MDO in the diencephalon has been proposed to result from the interaction between the anterior (prechordal) and the posterior (epichordal) region of the neural plate. *In ovo* grafting and co-culture experiments in chick have shown that interaction between prechordal and epichordal neural tissues can induce *Shh* expression at their interface (Vieira et al., 2005; Guinazu et al., 2007). Molecular data obtained from experiments in different organisms may explain those co-culture results in a different way. Therefore, we have put a novel model forward incorporating findings from fish, chick, and mouse (Scholpp and Lumsden, 2010).

Firstly, by analyzing the function of transcription factors of the orthodenticle homeobox (Otx) family during regionalization of the neural plate in zebrafish, we found *Otx1* and *Otx2* expression in the primordium of the MDO and thalamus (Scholpp et al., 2007). Indeed, down-regulation of Otx1/Otx2 immediately before MDO formation resulted in failure to induce the *organizer*. These findings are consistent with the analysis of *Otx1–/–/Otx2±* double mutant mice, which also lack the MDO (Acampora et al., 1997). Since *Otx* genes are widely expressed in the forebrain and midbrain we asked the following questions. Firstly, what mechanism ensures the correct positioning of the MDO in the mid-diencephalon? Secondly, how is the tapering shape of the MDO established with a broad ventral domain and narrowing dorsal domain that coincides with *shh* expression?

Members of the *Fez* family of transcription factors are expressed in the prethalamic anlage from late gastrulation onward and abut the MDO territory (Hashimoto et al., 2000; Matsuo-Takasaki et al., 2000; Staudt and Houart, 2007). Ectopic expression of FezF2 at the MDO in mice as well as its ubiquitous expression in fish leads to a block in organizer formation (Hirata et al., 2006;

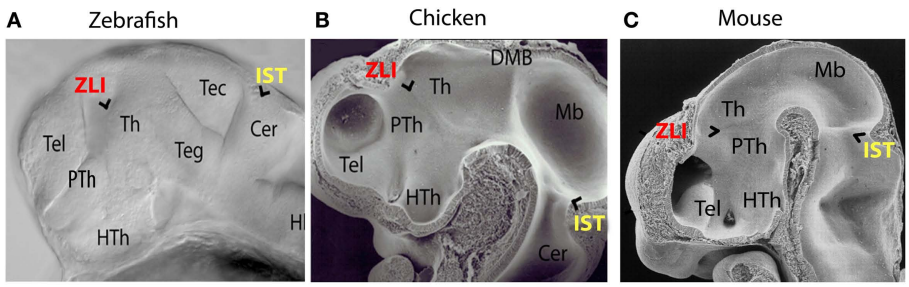

FIGURE 1 | Morphological comparison of embryonic brain regions in different vertebrate embryos. (A) Zebrafish 36 hpf. **(B)** Chicken HH20. **(C)** Mouse 11.5 dpf (with courtesy of Salvador Martinez). In vertebrates the *zona limitans intrathalamica* (ZLI) forms as a morphological ridge between the prethalamus and the thalamus, whereas the *isthmus rhombencephali* (IST) marks the constriction between midbrain and hindbrain. Th, thalamus; PTh, prethalamus; HTh, Hypothalamus; Tel, Telencephalon; Teg, tegmentum; Tec, tectum; Cer, cerebellum; Mb, midbrain; DMB, diencephalic-mesencephalic boundary; Hi, hindbrain.

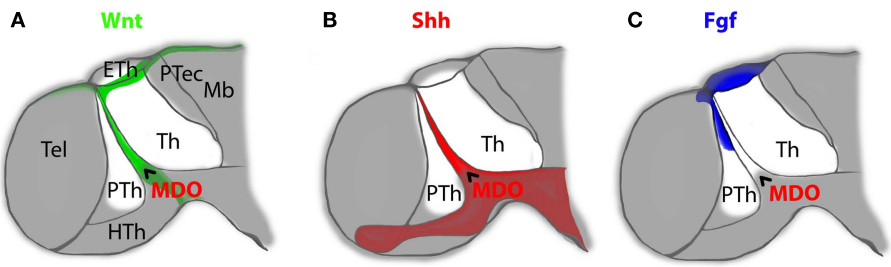

FIGURE 2 | The mid-diencephalic organizer (MDO) is the source for members of three morphogen signaling families in the ZLI of the embryonic vertebrate brain. Expression of Wnt signaling molecules [**(A)**, green] overlaps with Shh producing cells of the MDO [**(B)**, red], whereas expression of members of the Fgf family [**(C)**, blue] is located in the prethalamus and overlaps partially with the Wnt signaling source in the dorsal roof plate. Taken together, these three families of signaling molecules encircle the developing thalamus from dorsal (Wnt and Fgf), ventral (Shh) and rostral (Wnt, Shh, and Fgf). Th, thalamus; PTh, prethalamus; HTh, Hypothalamus; Eth, epithalamus; Tel, Telencephalon; Ptec, pretectum; Mb, midbrain; MDO, mid-diencephalic organizer.

Jeong et al., 2007). This suggests that *Fez* is required for setting the anterior border of the organizer by repressing the expression of organizer marker genes such as *shh*. Similarly, Irx1, a member of the transcription factor family of Iroquois genes, positions the posterior border of the MDO (Hirata et al., 2006; Scholpp et al., 2007). As shown in zebrafish and mouse, *Irx1b* morphant embryos exhibit a posterior expansion of the MDO territory, as the transcriptional repressor is absent. Therefore, we conclude that the organizer of the thalamus is induced within an Otx-positive area that is subsequently refined to a small stripe between prethalamus and thalamus by the repressive function of Fez and Irx. Which mechanism subsequently ensures that the MDO primordium persists between Fez and Irx expression? During early somitogenesis in zebrafish, Wnt3 and Wnt3a are co-expressed in the MDO anlage and have a pivotal role in the induction of the MDO (Mattes et al., 2012). Wnt3/Wnt3a deficient embryos lack the MDO primordium and as a result the prethalamic territory abuts the thalamus. However, the size of the thalamus and prethalamus are unchanged arguing against a fate change of the MDO. Indeed, lack of canonical Wnt signaling leads to increased cell death of the MDO, suggesting Wnt3 and Wnt3a act as survival factors for organizer cells in fish (Mattes et al., 2012). Furthermore, in embryos deficient for Wnt3/Wnt3a/FezF2 or Wnt3/Wnt3a/Irx1b the MDO is restored. This suggests that the prethalamus as well as the thalamus are competent to form the organizer (Mattes et al., 2012). However, expression of Fez or Irx suppresses MDO development in these areas, hence shaping the MDO to a small strip of cells between prethalamus and thalamus. In the next paragraph, we will discuss the function of the MDO during thalamus development.

Wnt/β-CATENIN–SIGNALING, A NEW PLAYER IN THALAMIC DEVELOPMENT

Wnt signaling is important to set up the initial anterio-posterior pattern of the neuraxis and it also has a more local function later during diencephalic regionalization (Wilson and Houart, 2004). Our knowledge of the latter is still fragmented while expression patterns of Wnt ligands, their receptors (Frizzled) and signaling mediators (Lef1, Tcf) in the mid-diencephalon do speak for a function in this location (Quinlan et al., 2009). Wnt3a and Wnt8b for example are expressed in the dorsal region of the thalamus and the MDO in mouse, chick, and fish from the precursor stage onward (Salinas and Nusse, 1992; Garcia-Lopez et al., 2004; Scholpp et al., 2007; **Figure 2A**). Furthermore, inhibition of canonical Wnt signaling by Dkk-1 in chick explants transforms the thalamus into prethalamus during the early regionalization phase (Braun et al., 2003). It has also been shown that the prethalamus marker Lhx5 can activate the expression of the extracellular Wnt inhibitors Sfrp1a and Sfrp5 in zebrafish embryos (Peng and Westerfield, 2006). A significant demonstration of the importance of Wnt signaling in thalamus development is seen in zebrafish lacking Wnt3 and Wnt3a recently (Mattes et al., 2012). These embryos fail to develop an MDO. Wnt3/Wnt3a are required to maintain the MDO anlage in early somitogenesis by suppressing apoptosis of the organizer cells. Interestingly, this analysis has further shown that a narrow time window of 4 h of Wnt signaling is sufficient to trigger survival of the MDO cells. Similarly, mice lacking the essential Wnt co-receptor low-density lipoprotein receptor LRP6 fail to develop a normal MDO and thalamus (Zhou et al., 2004). These data suggest that canonical Wnt signaling is required for the induction of the MDO and for thalamus development, whereas the development of the prethalamus requires inhibition of canonical Wnt signaling. This hypothesis is supported by the observation that the expression of components of the Wnt signaling machinery as well as Wnt target gene expression are enriched in the thalamus, but absent in the prethalamus of all vertebrates analyzed so far – from zebrafish to rhesus monkey (Jones and Rubenstein, 2004; Shimogori et al., 2004; Murray et al., 2007; Bluske et al., 2009; Quinlan et al., 2009; Peukert et al., 2011). The question remains, which aspects of canonical Wnt signaling are directly required for the development of the thalamus?

CELL–CELL ADHESION IN THE CAUDAL THALAMUS

Recently, Wnt signaling has been implicated in compartition of the caudal diencephalon. β-Catenin, the key effector of the Wnt pathway was initially discovered for its role in cell adhesion (Huber et al., 1996; Bienz, 2005). ß-Catenin promotes adhesiveness by binding to the transmembrane, Ca2+-dependent homotypic adhesion molecule cadherin, and links cadherin to the intracellular actin cytoskeleton. Although several classes of molecules are involved in morphogenetic events, cadherins appear to be the major group of adhesion molecules mediating the formation of morphological and functional boundaries in the developing brain (Takeichi, 1977). Therefore it seemed reasonable to assume that Wnt signaling in combination with cadherin function regulates cell adhesion in the thalamus. Indeed, in the late developing diencephalon classical cadherins, such as Chd2, Chd6b, Chd7, mark areas of gray matter, which correspond to presumptive brain nuclei (Redies et al., 2000). However, these studies are not able to explain segmentation in the caudal forebrain. Recently, we could show that the function of other members of the cadherin superfamily, the non-clustered protocadherins, links compartition in the developing diencephalon to Wnt signaling.

During somitogenesis in zebrafish, a member of this group, *pcdh10b* (formerly known as OL-protocadherin) has been shown to modulate cell adhesion in the paraxial mesoderm and somite segmentation (Murakami et al., 2006). However, the expression of *pcdh10b* also marks the alar part of P2, the thalamus, and we could show that canonical Wnt signaling regulates the expression of *pcdh10b* in the developing thalamus of the zebrafish (**Figure 3A**). Stabilization of ß-catenin leads to a broadening of the expression domain of *pcdh10b* whereas inhibition of Wnt signaling blocks *pcdh10b* expression (Peukert et al., 2011). Hence, alteration of *pcdh10b* expression in the thalamus leads to an intermingling of thalamic cells with the neighboring brain areas, predominantly with the pretectum in P1. Our data were supported by the fact that *pcdh10b* knock-down or overexpression also lead to a similar intermingling of cells in developing somites (Murakami et al., 2006). Interestingly, *pcdh10b* expression is strongest in thalamic progenitors, and non-detectable in mature post-mitotic neurons. Indeed, if progenitors are forced into differentiation by overexpression of Lhx2 we find a down-regulation of *pcdh10b* expression (Peukert et al., 2011). Consistently, Lhx2/Lhx9 deficient embryos display a strong expression of *pcdh10b* within the thalamus and in the adjacent brain areas as neuronal differentiation is blocked in

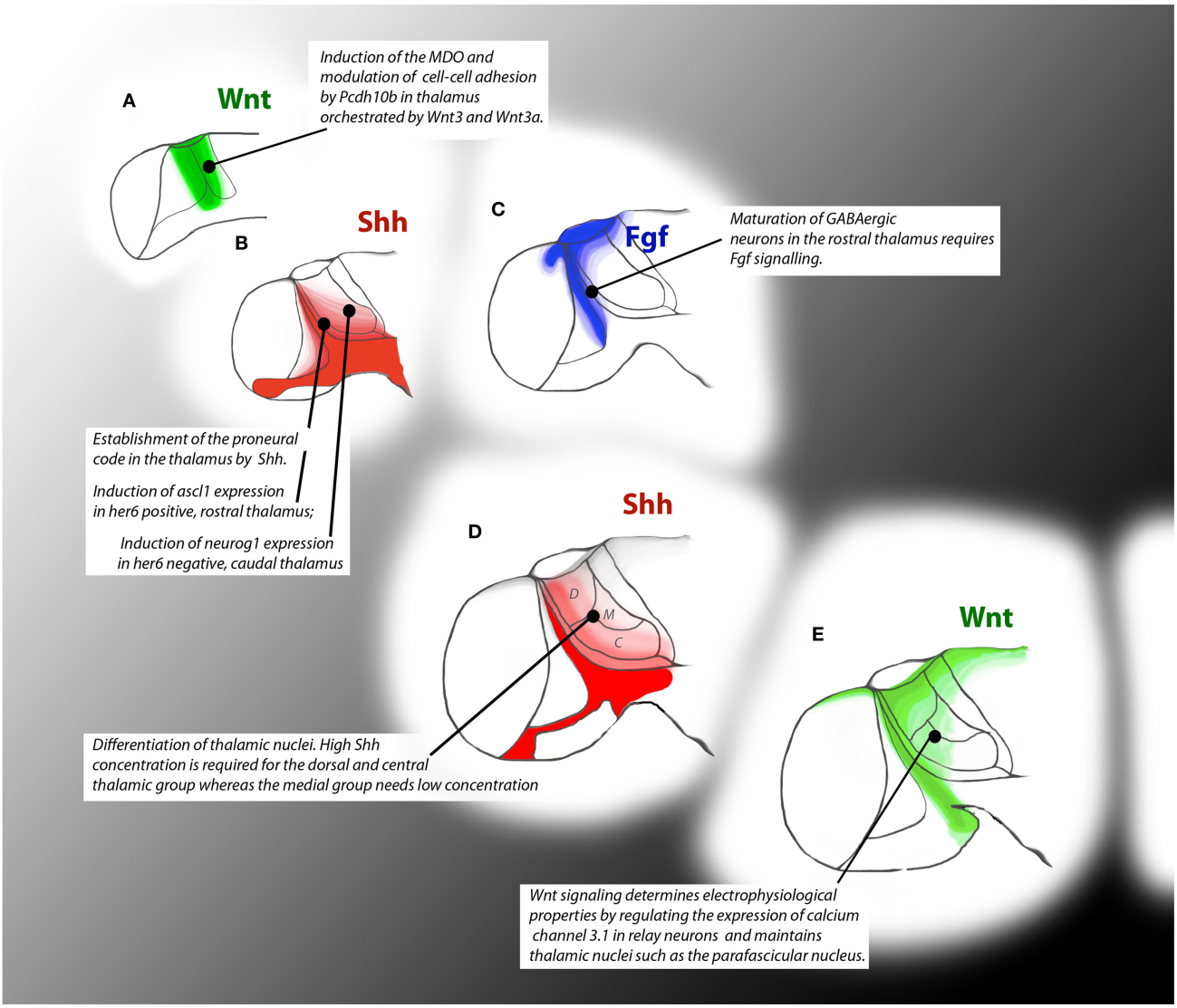

FIGURE 3 | Timeline of signaling events during thalamus formation in zebrafish embryos. Colored patterns show areas of influence of the depicted signaling pathways. At 15 hpf Wnt signals from the caudal forebrain are required for the induction of the MDO and for compartition of the thalamus **(A)**. At 24 hpf Shh signaling is required for the onset of neurogenesis by induction of proneural genes **(B)** whereas Fgf signaling influences maturation of GABAergic neurons **(C)**. At 36 hpf, graded Shh signal specifies the territory of the thalamic nuclei **(D)**. At 48hpf, Wnt signaling guides neurogenesis by influencing post-mitotic characteristics of thalamic neurons **(E)**.

these embryos. Thus, Wnt induced Pcdh10b helps to establish the thalamus as a compartment during forebrain development.

Similarly, in Gbx2 knock-out mice, thalamus cells start to intermingle with pretectum cells (Chen et al., 2009). Interestingly, these authors observe a non-cell autonomous function for this transcription factor and propose a restriction mechanism mediated by an unknown cell adhesion factor. Based on our recent data, we suggest that Gbx2 and Lhx2/Lhx9 are acting in a common pathway. Lack of Gbx2 in mice may lead therefore to a similar phenotype observed for the Lhx2/Lhx9 deficient morphant zebrafish embryos: an alteration of Wnt signaling in the caudal diencephalon which influences the expression of non-clustered Pcdhs and thus compartition.

In other vertebrate models, *Pcdh10* expression has been reported only at later stages in development, in chicken HH28 and in mouse E15 (Hirano et al., 1999; Nakao et al., 2005), arguing against a comparable role in these model organisms. However, Pcdh10 together with Pcdh8, 12, 17, 18, and 19 belong to the group of structurally related non-clustered δ2 protocadherins and several members show expression during somitogenesis in mice (Kim et al., 2011). In contrast to the function of classical cadherins only a few Pcdhs show adhesive activity *in vivo*. This includes *Xenopus* Papc, which is regulated by non-canonical Wnt signaling (Schambony and Wedlich, 2007). Other Pcdhs appear to have more varied functions such as homophilic or heterophilic interactions. One possibility is that they may function as regulators of

cell–cell adhesion by modulating cadherin adhesiveness. However, there is recent evidence that the intracellular domain of Pcdhs may contribute also to signaling (Kim et al., 2011). This role of Pcdh in signaling as well as the role of ß-catenin in cell–cell adhesion within the thalamus should be explored further.

REGIONALIZATION OF THE THALAMIC COMPLEX REQUIRES Shh SIGNALING

Besides canonical Wnt ligands, the best-characterized signals the MDO releases into the thalamic complex are members of the Hedgehog family. Expression of hedgehog proteins has been found in the MDO of all vertebrate model organisms examined, including lamprey (Osorio et al., 2005), zebrafish (Barth and Wilson, 1995), frog (Ruiz and Altaba, 1998), chick (Puelles and Martinez-de-la-Torre, 1987), and mouse (Shimamura et al., 1995). Furthermore, recent studies in chick (Kiecker and Lumsden, 2004; Vieira et al., 2005), fish (Scholpp et al., 2006), and in mice (Jeong et al., 2011) have shown that Shh released from the MDO regulates regionalization and patterning of the thalamus. Loss of Shh signaling results in the loss of genetic fate determinants and of thalamic identity in chick and fish (Hashimoto-Torii et al., 2003; Kiecker and Lumsden, 2004; Vieira et al., 2005; Scholpp et al., 2006). In addition, elevated Shh signaling expands thalamic identity in the forebrain: misexpression of Shh induces ectopic expression of thalamic markers such as Ascl1, Neurogenin, Abx2, Olig2, and Olig3 in the pretectum in chick and mouse (Echevarria et al., 2003; Vue et al., 2009). Thus, Shh is an important external cue that controls the expression of transcription factors within the thalamic complex.

Recent studies in mouse suggested that Shh is also required during regionalization of the thalamus. The thalamus can be subdivided into two different neuronal populations, the rostral thalamus (rTh) which is located close to the MDO and that contains mainly GABAergic inhibitory neurons and the caudal thalamus (cTh) which is located further away from the MDO and that represents the glutamatergic sensory thalamus (Vue et al., 2007). The activity of Shh as a morphogen has been linked directly to this differentiation process (**Figure 3B**). Reduction of Shh signaling from the MDO leads to a misspecification of GABAergic interneurons of the rTh and consequently to the loss of its derivative structures such as the ventral geniculate body and intergeniculate leaflet (Vue et al., 2009; Jeong et al., 2011). Thus, high concentrations of Shh are required for the formation of the rTh and low concentrations are required for the development of the cTh. These findings can be translated into a Gli transcription factor dependent activation of target genes: Activation of Gli1 leads to the induction of target genes of the rTh such as Sox14 and electroporation of Gli2 has been shown to activate cTh-markers such as Gbx2 in chick embryos (Hashimoto-Torii et al., 2003).

One pitfall of the thalamic morphogen gradient hypothesis is that concentration differences of only one morphogen within a field of cells fails to establish sharp borders between cell populations (Meinhardt, 2009). Which mechanism then is sharpening the border between the GABAergic rTh and the glutamatergic cTh? The hairy/enhancer of split genes E(spl) may play an important role in neurogenesis and glia formation (Chapouton et al., 2011). We recently found that one of these bHLH proteins, the hairy-like factor 6 (Her6) in fish – the ortholog of Hes1 in mouse – regulates neuronal identities by determining expression of different proneural genes in the thalamus (Scholpp et al., 2009). Its expression is specifically maintained in the rTh, whereas in the cTh it is down regulated prior to induction of proneural genes. Therefore, Her6 is a good candidate for the establishment of a sharp border between these thalamic territories. Indeed, Ascl1 is induced by Shh signaling from the MDO in the Her6 positive domains of the rTh, whereas in the Her6 negative cTh, Shh induces the expression of the proneural gene, neurogenin1. This expression code of proneural genes translates subsequently into the formation of GABAergic inhibitory neurons from the Ascl1 positive precursors in the rTh. The Neurogenin1 positive cells will differentiate into glutamatergic relay neurons of the cTh. Interestingly, in the mouse retina it has been shown that Shh signaling stabilizes ßHLH factors (Wall et al., 2009), suggesting a positive maintenance mechanism for the formation of Her6 positive, GABAergic cells close to the Shh source.

In the following chapter, we will discuss the important role of Fgf signaling in formation of the rTh parallel to the Shh gradient and the ßHLH Her6 dependent refinement mechanism.

Fgf SIGNALING IN THALAMIC DEVELOPMENT

Fgfs signaling is well known for its important roles during development of the central nervous system (Hebert, 2011) and, in addition to Shh and Wnt, has been implicated in the regulation of diencephalic development. Among the over 20 Fgf ligands, Fgf3, Fgf8 and the paralog genes Fgf15 and Fgf19 may play pivotal roles in this process. Early blockage of Fgf3 and Fgf8 mediated signaling for example leads to an alteration in prosencephalon patterning, including the absence of the prethalamic territory (Walshe and Mason, 2003). Later in development, Fgf15 and Fgf19 are expressed in the thalamus and prethalamus of mouse, chick, and fish (Ishibashi and McMahon, 2002; Miyake et al., 2005; Gimeno and Martinez, 2007). Their expression in the thalamus depends on long-range Shh signaling from the organizer. In fish, blockage of Fgf19 leads to a down-regulation of GABAergic inhibitory neurons in the prethalamus (Miyake et al., 2005). Likewise, electroporation of a truncated version of the Fgf-receptor-3 in the mouse thalamus leads to a lack of *Ascl1* positive GABAergic neurons of the rTh (**Figure 3C**; Kataoka and Shimogori, 2008). Consistently, increased Fgf activity leads to a broadening of the rTh area and a caudal shift of sensory nuclei in the cTh (Kataoka and Shimogori, 2008). This suggests that Fgf signaling is required for the development of GABAergic neurons in the prethalamus and in the rTh, most likely downstream of Shh. In parallel to its function in inducing GABAergic neurons in the thalamic complex, Fgf signaling seems to have also an influence on the development of glutamatergic neurons in the cTh. Analysis of Fgf8 hypomorphic mice revealed a reduction of *Gbx2* expression in the thalamic neuroepithelium of the cTh (Martinez-Ferre and Martinez, 2009). The question remains about which Fgf ligand is required during the development of the thalamus? Fgf8 expression is found predominantly in the dorsal part of the diencephalon, in the most dorsal tip of the MDO and in the epithalamus, whereas Fgf15 and Fgf19 are expressed only in the ventral prethalamus. Therefore, Fgf8 supposedly exerts its function in the dorsal part whereas

Fgf15 and Fgf19 influence rather the ventral area of the thalamus. However, the individual molecular mechanism of their local activity during diencephalic patterning still needs to be analyzed in detail. One may consider two experimental caveats for future analysis. Overexpression-based analyses may provide only insufficient answers as Fgf ligands tend to bind unspecifically to a number of Fgf receptors if the tissue is overloaded with the signaling molecule. Furthermore, timing of the experimental intervention as well as of the read-out is of great importance as Fgf signaling plays influential roles also during early phases of neural development. Another question for the future would be how Fgf signaling interacts with the other signaling pathways in order to pattern the nascent thalamus correctly? A first line of experiments in mice suggests that Fgf signaling may induce the expression of Wnt3a, whereas Shh expression in the MDO was not modified upon alteration in Fgf signaling (Kataoka and Shimogori, 2008; Martinez-Ferre and Martinez, 2009).

LATE DEVELOPMENTAL PHASE – Lhx2/Lhx9 MEDIATED NEUROGENESIS

As described above one consequence of the three external cues is the induction of certain transcription factors in the thalamus. For example, lack of Shh expression at the organizer leads to the absence of Neurog and Gbx2 in the thalamus in chick as well as zebrafish (Hashimoto-Torii et al., 2003; Kiecker and Lumsden, 2004; Vieira et al., 2005; Scholpp et al., 2006) and there is evidence that Fgf signaling may act upstream of Lhx2 in fish (Seth et al., 2006). Although there are many more transcription factors specifically expressed in the thalamus (Suzuki-Hirano et al., 2011), only a few have been functionally characterized, such as Gbx2, Neurog, Lhx2, Lhx9, and Lef1. Gbx2 knock-out mice lack the thalamus-specific post-mitotic neuronal markers *Id4* and *Lef1*, and subsequently lack cortical innervations by thalamic axons (Miyashita-Lin et al., 1999). Furthermore, Gbx2 mediated differentiation is required for compartition of the caudal forebrain in mouse (Chen et al., 2009). Together with Shh, Gbx2 is important for the establishment of the thalamic nuclei in mouse (**Figure 3D**; Szabo et al., 2009; Vue et al., 2009). Although Neurog2 knock-out mice show a similarly severe failure in neuronal connectivity to the cortex, the expression of *Lhx2*, *Id2*, and *Gbx2* is unchanged in these animals, suggesting that in the absence of *Neurog2* thalamic neurons are not re-specified at the molecular level (Seibt et al., 2003). Recently, the highly conserved apterous group proteins, Lhx2 and Lhx9, have been implicated in forebrain development. Lhx2 is required in mouse for the maintenance of cortical identity and to confine the cortical hem, allowing proper hippocampus formation in the adjacent pallium (Potter et al., 1997; Mangale et al., 2008). The Apterous transcripts are present in the nervous system of the cephalochordate *Amphioxus*, i.e., AmphiLhx2/9 (Takatori et al., 2008), and co-expression of *Lhx2* and *Lhx9* has been documented in the diencephalon of vertebrates, such as zebrafish (Ando et al., 2005), *Xenopus* (Bachy et al., 2001; Moreno et al., 2004), and mouse (Nakagawa and O'Leary, 2001). Thus, although recent studies of Lhx2-mutant mice showed no alteration during thalamic neuronal regionalization (Lakhina et al., 2007), co-expression of Lhx9 may compensate. Furthermore, although the function of Lhx9 has not been described, its expression pattern suggests a role during forebrain development (Nakagawa and O'Leary, 2001). We could indeed show now that in fish Lhx2 is functionally redundant to Lhx9 and that they regulate thalamic development. Single knock-down of Lhx2 or Lhx9 has no diencephalic phenotype but their simultaneous knock-down leads to stalling of thalamic neurogenesis at the late progenitor stage (Peukert et al., 2011). In the thalamus, Lhx2/Lhx9 may regulate genes that are essential to complete neuronal development, such that *Lhx2/Lhx9* deficient neurons do not reach the terminal neuronal stage. Similar to the *Gbx2*-/- knock-out mouse (Miyashita-Lin et al., 1999), we find that the expression of *deltaA, neurog1*, and *pcdh10b* is increased in lhx2/lhx9 morphant zebrafish embryos (Peukert et al., 2011). During neuronal development in fish, neurog1 has been shown to activate *delta* genes directly by binding several E-box elements in the *delta* promoter region (Hans et al., 2004). This suggests that in lhx2/lhx9 morphant embryos, neuronal progenitor development is arrested at the level of *deltaA*/*neurog1* expression. Consequently, terminal thalamic neuronal markers such as Id2a and Lef1 are absent in Lhx2/Lhx9 morphant embryos. Thus, we propose that Lhx2/Lhx9 are essential determinants for neurons to reach the late stage of thalamic development. Interestingly, in the spinal cord Lim HD factors together with ßHLH factors have been shown to be required for cell cycle exit in mouse (Lee and Pfaff, 2003). The Lim containing factor Isl-1 and Lhx3 together with the ßHLH factors Neurog2 and NeuroM act in a combinatorial manner to direct motor neuron differentiation. In the thalamus, we find a similar process: Lhx2/Lhx9 activate the expression of post-mitotic differentiation markers such as *id2a*, *lef1*, and *elavl3*.

Wnt SIGNALING AND NEUROGENESIS

Evidence is accumulating that Wnt signaling plays an important role in the regulation of thalamic neurogenesis also at later stages. Analysis of a Wnt-reporter and also mosaic loss-of-function studies for β-catenin indicate strong activity of Wnt signaling in the ventricular zone of the neural tube (Freese et al., 2010). Similarly, *pcdh10b,* a novel Wnt target gene, is expressed in the ventricular zone of the thalamus (Peukert et al., 2011). Furthermore, differentiating neurons are characterized by the expression of members of the DNA-binding protein inhibitor genes (Id) as well as Lef1 (Jones and Rubenstein, 2004) and these markers are activated by Wnt signaling in the thalamus (**Figure 3E**; Peukert et al., 2011). However, the expression of these markers is most prominent in the subventricular zone as well as in the mantle zone. This suggests that Wnt signaling together with Lhx2/Lhx9 has a role in turning mitotically active neuronal precursors into mature post-mitotic neurons in the thalamus. Consistently, mice mutant for the Wnt co-receptor Lrp6 have a defective expression of thalamic neuronal markers such as Lef1 (Zhou et al., 2004). Interestingly, we observed an upregulation of Wnt activity in the *lhx2/lhx9* morphant embryos and consistently an upregulated expression of the Wnt target gene *axin2* as well as the novel target *pcdh10b* in the ventricular zone of the thalamic neuroepithelium. However, further Wnt target genes, i.e., Lef1 are down regulated in the morphant embryos. This suggests that thalamic neuronal differentiation is coupled to a second competence phase for Wnt signaling in the mantle zone (Peukert et al., 2011). Also, the late and restricted onset of *lhx2/lhx9* expression in the thalamus and their requirement for *id2a* and *lef1*

Table 1 | Summary of functions of the three signaling pathways during thalamus development with related animal models in chronological order of publication.

	Function	Model organism	Reference
Wnt	Wnt signaling is important for patterning in the thalamic complex	Chicken	Braun et al. (2003)
	Fate map of the Wnt8b positive MDO	Chicken	Garcia-Lopez et al. (2004)
	Mutation in the Wnt co-receptor Lrp6 leads to loss of the thalamus	Mouse	Zhou et al. (2004)
	Collection of expression patterns of members of the Wnt signaling pathway	Chicken	Quinlan et al. (2009)
	Compartition in the caudal forebrain requires the Wnt-dependent induction of the adhesion modulator Pcdhl0b	Zebrafish	Peukert et al. (2011)
	Wnt3 and Wnt3a are required for the induction and survival of the MDO	Zebrafish	Mattes et al. (2012)
Shh	Shh marks the MDO at the prechordal/epichordal neural plate boundary	Mouse	Shimamura et al. (1995)
	The Shh positive ZLI is a compartment in the caudal forebrain	Chicken	Zeltser et al. (2001)
	Shh acts as a morphogen in thalamus regionalization	Chicken	Hashimoto-Torii et al. (2003)
	Shh is the principal organizing signal of the MDO	Chicken	Kiecker and Lumsden (2004), Vieira et al. (2005)
	Shh-a and Shh-b orchestrate thalamus regionalization	Zebrafish	Scholpp et al. (2006)
	Shh influences the neural progenitor domains in the thalamus	Mouse	Vue et al. (2007)
	Shh is important for the differentiation of the medial and intralaminar thalamic nuclei	Mouse	Szabo et al. (2009)
	Her6 specifies the mode of Shh action in the rostral and caudal thalamus	Zebrafish	Scholpp et al. (2009)
	Shh signaling from the MDO and basal plate is important for the formation of the rostral thalamus	Mouse	Jeong et al. (2011)
Fgf	Maintenance of the rostral inhibitory thalamus is Fgf dependent	Mouse	Kataoka and Shimogori (2008)
	Fgf regulates Wnt3a expression in the MDO and influences thalamus maturation	Chicken	Martinez-Ferre and Martinez (2009)

expression may explain the thalamic neuronal specificity of the Wnt target *lef1*. A consequence of Lef1 activity is the regulation of the expression of the Ca2+-channel Cav3.1 in mouse (Wisniewska et al., 2011). Therefore, we suggested that the second phase of Wnt signaling is required – via the activation of Lef1 – to reach the late stage of thalamic neuronal development and to determine the electrophysiological properties of thalamic neurons.

SUMMARY

Light has now been shed on the formation and function of the newly characterized MDO. Here, we summarized how the action of three different signaling pathways coordinates thalamic development through the MDO. Members of the three protein families, Wnt, Shh, and Fgf, are actively signaling in the mid-diencephalon and have different functions during development of the thalamic complex (**Table 1**). They regulate the compartition of the thalamus, drive neurogenesis, influence the neurotransmitter fate of thalamic neurons, and determine the electrophysiological characteristics of thalamic neurons. Considering the multifunctionality of these signaling pathways themselves and the expression of a cohort of further ligands of these pathways at the MDO, further research is needed into their possible function during thalamic development. In parallel, we still need to investigate how these signaling pathways influence each other. However, it has become clear that all three brothers together are needed to build the flourishing house of the thalamus, "*le petite maison*" of the brain.

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Patterning and compartment formation in the diencephalon

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The diencephalon gives rise to structures that play an important role in connecting the anterior forebrain with the rest of the central nervous system. The thalamus is the major diencephalic derivative that functions as a relay station between the cortex and other lower order sensory systems. Almost two decades ago, neuromeric/prosomeric models were proposed describing the subdivision and potential segmentation of the diencephalon. Unlike the laminar structure of the cortex, the diencephalon is progressively divided into distinct functional compartments consisting principally of thalamus, epithalamus, pretectum, and hypothalamus. Neurons generated within these domains further aggregate to form clusters called nuclei, which form specific structural and functional units. We review the recent advances in understanding the genetic mechanisms that are involved in the patterning and compartment formation of the diencephalon.

Keywords: thalamus, compartment boundary, organizer, fate mapping, ZLI, Gbx2, Shh, prosomere

INTRODUCTION

Multiple bulges called brain vesicles become visible in the anterior neural tube soon after closure of the neural tube, due to tissue expansion and constriction between the vesicles. The anteriormost vesicles are called the telencephalon and diencephalon, and together they form the future forebrain. The telencephalon gives rise to the neocortex and basal ganglia, while the diencephalon generates structures that connect the neocortex and the forebrain limbic systems with the rest of the central nervous system. The diencephalon is therefore dubbed as the "interbrain" as it functions as a crucial relay and integration center, and modulates sensory, motor, and cognitive functions. Based on histological landmarks and gene expression patterns, the diencephalon is divided into segments called prosomeres, from which major anatomical structures including the pretectum, the habenula, thalamus, prethalamus, and hypothalamus arise. Compartmental development is an evolutionarily conserved mechanism that controls generation of diversity and specificity among different progenitor domains. Furthermore, the compartment boundaries often serve as signaling centers that regulate development of cells in the neighboring compartments. Here, we will review recent studies addressing the patterning and compartment formation of the diencephalon. These studies have identified the signaling molecules that pattern the diencephalon and the intrinsic determinants of different prosomeres and subdivisions of these prosomeres. Recent studies have also demonstrated that there are several lineage restriction boundaries, some of which correspond to the prosomeric borders or subdivisions. These new findings combined with other exciting advances in this field have added to our expanding knowledge of diencephalic development.

PROSOMERE MODEL: DIVISION OF THE DIENCEPHALON

Morphological studies in chick embryos have suggested that the forebrain and in particular the diencephalon can be divided into multiple transverse segments called neuromeres that are orthogonal to the long axes of the neural tube (Bergquist and Kallen, 1954, 1955; Kaellen, 1965; Vaage, 1969; Puelles et al., 1987). Based on morphology, position of axonal tracts and expression of cell-adhesion molecules, Figdor and Stern divided the diencephalon into four transverse neuromeres (D1–D4), with the rostral-most D1 adjacent to the telencephalon and D4 abutting the mesencephalon (Figdor and Stern, 1993). In the same year, Bulfone et al. (1993) showed three distinct segments called prosomeres (p1–p3) in the diencephalon by analyzing four developmental regulator genes, *Dlx1*, *Dlx2*, *Gbx2*, and *Wnt3*. This and subsequent studies have led to the "prosomere model," which divides the developing forebrain into six prosomeres (Puelles and Rubenstein, 1993, 2003; Rubenstein et al., 1994). The p1–p3 segments give rise to the pretectum (p1), the epithalamus and thalamus (previously called the dorsal thalamus) (p2), and prethalamus (p3, previously called ventral thalamus) and eminentia thalami (**Figure 1**). P1 and p2 correspond to D1 and D2, while p3 may be subdivided into D3 and D4. Although the number and their nature as lineage-restricted compartments have been controversial, the prosomere model provides a conceptual framework for understanding forebrain development by imparting morphological meaning to gene expression patterns, and thus is essential for genetic and comparative studies of the developing brain across species.

ZLI, A COMPARTMENT BOUNDARY OR A SELF-CONTAINED COMPARTMENT?

Compartmental development is a fundamental mechanism for coordinating growth and patterning of the embryonic field in both invertebrates and vertebrates (Lumsden and Krumlauf, 1996; Kiecker and Lumsden, 2005). Rhombomeres in the vertebrate hindbrain are lineage-restricted compartments, in which cells freely intermingle within the same rhombomere but not with cells

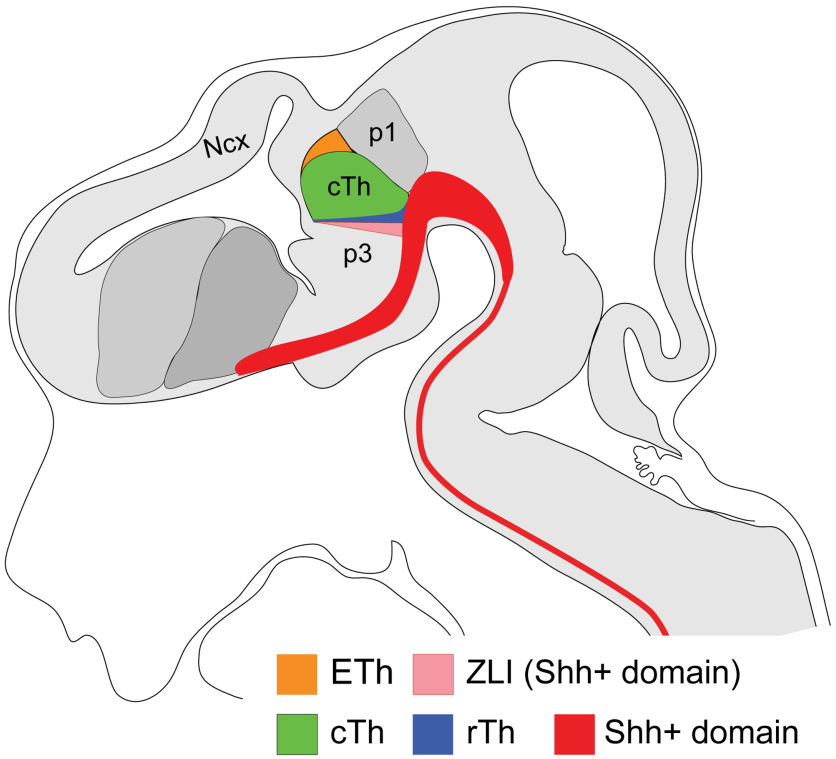

FIGURE 1 | Schematic representation of a sagittal view of the various domains of the diencephalon in mouse embryos at E12.5. The prosomere model predicts that the diencephalon consists of three principal prosomeres/segments. Prosomere1 (p1) corresponds to the pretectum and p3 corresponds to the prethalamus. The p2 domain consists of the progenitor domains of the rostral thalamus (rTh), the caudal thalamus (cTh), and the epithalamus. The progeny of the latter give rise to the Gbx2-positive thalamus proper. The ZLI wedge arises from the Shh positive basal plate and acts as an organizer. The entire area shaded in gray indicates the neural tube. Ncx: Neocortex.

of the neighboring rhombomeres (Fraser et al., 1990). Each of these rhombomeres displays unique molecular identity and functional organization (Lumsden and Krumlauf, 1996). It is thus interesting to determine whether segmental development occurs in the developing forebrain in a similar fashion. By labeling single or small groups of cells in the diencephalon of chick embryos, Figdor and Stern showed that cells of each neuromere could intermingle freely within the neuromere but were restricted from crossing the border after the formation of morphological ridges at the border (Figdor and Stern, 1993). Compartmental development of the diencephalon is further supported by fate-mapping studies in chick-quail chimera (Garcia-Lopez et al., 2004). However, clonal analyses using retrovirus in chick embryos found broad dispersal of labeled clones without respecting prosomeric boundaries, casting doubts on the compartmental development within the diencephalon (Arnold-Aldea and Cepko, 1996; Golden and Cepko, 1996). Furthermore, Larsen et al. (2001) showed that there was no obvious cell segregation at the border between p1 and p2 in chick embryos. However, they observed lineage segregation between p1 and the mesencephalon, as well as a lineage boundary between p2 and p3 (Larsen et al., 2001). Wedged in between p2 and p3 is a transverse domain called the zona limitans intrathalamica (ZLI), which is defined by the expression of *Sonic hedgehog* (*Shh*; Zeltser et al., 2001; **Figure 1**). Fate-mapping studies showed that ZLI cells were segregated from those of p2 or p3 cells (Zeltser et al., 2001). The author thus suggested that the ZLI is a self-contained compartment (Zeltser et al., 2001), rather than a compartment boundary (Garcia-Lopez et al., 2004). During embryogenesis, the prospective ZLI is first defined by the absence of expression of *Lfng* (Zeltser et al., 2001), which encodes a glycosyltransferase that modulates Notch signaling. Its homolog plays an important role in compartment boundary formation in *Drosophila* (Cho and Choi, 1998; Dominguez and de Celis, 1998; Papayannopoulos et al., 1998; Rauskolb et al., 1999). Perturbation of Lfng function disrupted formation of the compartment boundaries flanking the ZLI in chick embryos (Zeltser et al., 2001), suggesting that Lfng-mediated cell sorting contributes to the establishment of the ZLI compartment. Similar to the chick embryo, the prospective ZLI is also defined as a negative expression domain of *Lfng* and its paralog *Mfng* in mice (Zeltser et al., 2001; Baek et al., 2006). However, no developmental defect in the neural tube lacking *Lfng* has been reported so far in mice (Zhang and Gridley, 1998). Further studies are needed to determine whether Lfng and/or Mfng play a similar role in regulating ZLI formation in mice.

Notch signaling is known to play an important role in the formation of compartment boundaries in various systems (Cheng et al., 2004; Tossell et al., 2011a,b). Interestingly, a Notch effector gene, *Hes1*, is expressed in the boundary regions of the neural tube,

such as the isthmus, ZLI, and rhombomeric borders (Baek et al., 2006). Hes proteins inhibit neurogenesis and promote gliogenesis (Kageyama et al., 2008). Generation of neuron-free zones is associated with the formation of specialized cells, called boundary cells, at the border of the compartments (Baek et al., 2006; Jukkola et al., 2006). Importantly, various compartment boundaries, including the ZLI, were missing in mouse embryos lacking both *Hes1* and its related gene *Hes5* (Baek et al., 2006). Similarly, knock-down of *her6* (equivalent to mammalian *Hes1*) leads to loss of the organizer (Scholpp et al., 2009). These observations demonstrate the essential role of *Hes* genes in the formation of these compartment boundaries. As other signaling pathways such as FGF and Shh have been shown to directly regulate *Hes1* expression (Ingram et al., 2008; Wall et al., 2009; Sato et al., 2010), it remains to be determined how Notch interacts with other signaling pathway to regulate Hes genes in the formation of the ZLI compartment. Therefore, studying regulation and function of *Hes* genes may provide insights into the establishment of the ZLI compartment.

THE FORMATION AND FUNCTION OF THE ZLI ORGANIZER

Compartment boundaries often serve as a signaling center, also called an organizer, to regulate cell fate specification of progenitors in the neighboring compartments (Irvine and Rauskolb, 2001; Kiecker and Lumsden, 2005). For example, the isthmic organizer at the midbrain-hindbrain junction patterns the developing midbrain and cerebellum through Fgf8 signaling (Wurst and Bally-Cuif, 2001; Sato et al., 2004). Genetic fate-mapping studies have demonstrated that the midbrain-hindbrain border is a lineage restriction boundary (Zervas et al., 2004; Langenberg and Brand, 2005; Sunmonu et al., 2011). The ZLI expresses multiple signaling molecules including Shh, and members of the Wnt and FGF families (Echevarria et al., 2003). Transplantation and genetic manipulation experiments have demonstrated that the ZLI acts as an organizing center and Shh is the main component of the ZLI organizer activity (Hashimoto-Torii et al., 2003; Kiecker and Lumsden, 2004; Vieira et al., 2005; Scholpp et al., 2006). In thalamic explants, different concentrations of Shh proteins induced differential expression of *Sox14* and *Gbx2*, supporting the notion that Shh proteins secreted from the ZLI act as a morphogen to control the pattern formation of the p2 domain (Hashimoto-Torii et al., 2003).

Based on development of the organizer in different systems, Meinhardt has proposed that the formation of an organizing center involves initial specification of two populations of cells in adjacent territories and subsequent induction of cells at the common border to express signaling molecules (Meinhardt, 1983). Grafting and co-culture experiments indeed demonstrated that juxtaposition of prethalamic and thalamic tissues was sufficient to induce Shh expression at the interface (Vieira et al., 2005; Guinazu et al., 2007). Several studies have revealed the molecular basis for positioning the prospective ZLI and specification of the prethalamus and the thalamus in chicken (Kobayashi et al., 2002; Braun et al., 2003; Echevarria et al., 2003; Vieira et al., 2005), zebrafish (Scholpp et al., 2006), *Xenopus* (Rodriguez-Seguel et al., 2009), and mice (Hirata et al., 2006). It was proposed that mutual repression between *Six3* and *Irx3* positioned the prospective ZLI in chick embryos (Kobayashi et al., 2002; Braun et al., 2003). However, the ZLI was present in *Six3*-deficient mice, indicating that *Six3* is not essential for ZLI formation (Lavado et al., 2008). Zinc-finger genes *Fezf1* and *Fezf2* are expressed in the rostral forebrain juxtaposed with the rostral limit of *Irx1* expression in mouse embryos (Hirata et al., 2004, 2006). Deletion of *Fezf1* and *Fezf2* in mice or only *fezf2* in fish disrupts formation of ZLI and abnormal expression of genes characteristic for the thalamus or pretectum in the prethalamus (Hirata et al., 2006; Jeong et al., 2007). These results demonstrate that *Fezf1* and *Fezf2* are important for the formation of the prethalamus and the ZLI. However, deleting *Fezf1* and *Fezf2* did not completely abolish the induction of the prethalamic territory. Furthermore, a fate-mapping study in zebrafish revealed that the fate of the prethalamus was established during gastrulation (Staudt and Houart, 2007). These results suggest that other factors in addition to Fezf1 and Fezf2 may be involved in the induction of the prethalamus.

In mouse embryos, the expression domain of *Fezf1* and *Fezf2* abuts that of *Irx1* (Hirata et al., 2006). Furthermore, genetic studies showed that mutual repression between *Fezf* and *Irx* genes positioned the prospective ZLI in *Xenopus* (Rodriguez-Seguel et al., 2009) and mouse embryos (Hirata et al., 2006). In mice, there are six *Irx* genes in two genomic clusters, and genes of the same cluster have similar expression pattern in the diencephalon (Peters et al., 2000; Houweling et al., 2001; Gomez-Skarmeta and Modolell, 2002). The potential redundancy and close linkage of different *Irx* genes in the mouse genome create difficulties to uncover their function by gene targeting knock-out experiments (Peters et al., 2002; Anselme et al., 2007). The best available tool to analyze *Irx* function in mouse so far is the naturally occurring *Fused toes* mutant, which shows a fuzzier and reduced expression of *Shh* in the ZLI and the basal plate at E9.5, suggesting that the ZLI formation is probably affected due to loss of the *IrxB* cluster (Anselme et al., 2007). However, the interpretation is complicated by the simultaneous loss of three other additional genes (*Fts*, *Ftm*, and *Fto*) of unknown function in the nervous system (Peters et al., 2002).

In zebrafish, the expression domains of *fezf* and *irx1b* (*irx7* as well) do not juxtapose with each other (Scholpp et al., 2007). Furthermore, knock-down of *irx1b* resulted in a caudal expansion of the ZLI, rather than a loss of the ZLI (Scholpp et al., 2007). During gastrulation, members of *Otx* family, *Otx1* and *Otx2*, are broadly expressed in the prospective forebrain and midbrain (Simeone et al., 1992). In zebrafish embryos, *otx2* is down-regulated in the anterior neural tube and only maintained in a region posterior to the prethalamus, including the ZLI and thalamus, at the 10-somite stage (Scholpp et al., 2007). Knock-down of otx1 and otx2 proteins immediately before ZLI formation prevented expression of ZLI markers, including *shh*, and conditional expression of *otx2* cell-autonomously rescued *shh* expression in the prospective ZLI (Scholpp et al., 2007). Based on these observations, Scholpp et al. have proposed that the positioning and induction of ZLI is determined by the *fezf–otx* interface, while *fezf* and *irx1b* define the anterior and posterior limits of ZLI domain, respectively (Scholpp et al., 2007; Scholpp and Lumsden, 2010). However, strong *Otx2* expression was maintained in the prethalamus and ZLI in mouse embryos at least until E12.5 after the formation of the ZLI at E10.5 (Chatterjee and Li, unpublished observations).

Furthermore, loss-of-function studies have shown that both *Otx1* and *Otx2* are required, in a dosage dependent manner, for the formation of both diencephalon and mesencephalon, including the prethalamus, ZLI, and thalamus, in mice (Acampora et al., 1997; Kurokawa et al., 2004; Puelles et al., 2006; Sakurai et al., 2010). Therefore, future studies are necessary to determine the mechanism underlying the positioning and induction of ZLI in mouse embryos.

Shh expression expands from the basal plate dorsally into the wedge-shaped ZLI. Cell fate mapping have shown that the characteristic progression of *Shh* expression is not a result of ventral-to-dorsal cell movement (Zeltser, 2005; Scholpp et al., 2006; Staudt and Houart, 2007). Therefore, *Shh* expression in the ZLI is probably induced by a polarized signaling in the ventral–dorsal direction (Zeltser, 2005). Using chick forebrain explant culture, Zeltser showed that Shh signaling from the basal plate was required for ZLI Shh expression in the alar plate and the dorsal progression of the ZLI organizer is regulated by inhibitory signals from the dorsal diencephalon (Zeltser, 2005). However, using a similar approach, Guinazu et al. (2007) demonstrated that *Shh* expression in the basal plate was dispensable for the induction of the ZLI. The latter result is supported by genetic studies in zebrafish and mice (Scholpp et al., 2006; Jeong et al., 2011). In zebrafish, the ZLI *shh* expression was present in *one-eyed pinhead* mutants, which lacked the axial mesoderm and basal plate *shh* expression (Scholpp et al., 2006). By deleting a 525-bp intronic sequence upstream of *Shh* coding region called *Shh* Brain Enhancer-1 (SBE-1), Jeong et al. (2011) specifically abolished *Shh* expression and activity in the basal plate of the mouse forebrain. Normal expression of *Shh* and other ZLI markers like *Sim1* and *Sim2* was found in the ZLI of these SBE-1 deletion mice (Jeong et al., 2011). Altogether, these observations indicate that *Shh* expression from the basal plate is not crucial for ZLI development in vertebrates. A recent report have shown that the requirement of ß-catenin/Wnt signaling in the formation of the ZLI organizer in the zebrafish (Mattes et al., 2012). Blocking ß-catenin signaling or knocking down of both *wnt3* and *wnt3a* mostly prevented ZLI organizer formation within a narrow time window of 10–14 h post-fertilization in zebrafish. Interestingly, the loss of ZLI *shh* expression was associated with enhanced apoptosis of the organizer cells and inhibition of apoptosis by simultaneous knock-down of p53 rescued the ZLI organizer in the absence of *wnt3/wnt3a*. These observations suggest that ß-catenin/Wnt signaling is mainly required for the survival of ZLI organizer cells. The molecular nature of signals that directly induce formation of the ZLI organizer remains to be determined.

In the aforementioned study, Guinazu et al. (2007) also showed that signals from the dorsal diencephalon antagonized ZLI formation, and they suggested that retinoic acid (RA) is a dorsal ZLI inhibitor. Indeed, *Cyp1b1*, which encode cytochrome p450 family of mono-oxygenases to promote RA synthesis, is expressed in the chick epithalamus (Chambers et al., 2007; Guinazu et al., 2007). Furthermore, *in ovo* electroporation experiments showed that Cyp1b1 acted cell-non-autonomously to inhibit *Shh* expression in the prospective ZLI, in agreement with the involvement of a diffusible molecule like RA (Guinazu et al., 2007). However, no brain defect has been reported in *Cyp1b1*-null mice (Buters et al., 1999; Libby et al., 2003).

SUBDIVISION AND COMPARTMENTAL DEVELOPMENT WITHIN THE P2 DOMAIN

The major p2-derived structure is the thalamus, which functions as a relay station and integration center for almost all sensory and motor information to and from the cortex (Jones, 2007). However, in addition to the thalamus, the p2 domain is further subdivided to give rise to the epithalamus, located dorsal to the thalamus (Rubenstein et al., 1994). Gene expression and short-term lineage tracing experiments have recently revealed that the thalamic progenitor domain can be further divided into rostral and caudal areas (**Figure 1**). The caudal thalamus (cTh) gives rise to glutamatergic neurons, which project to the cortex, and cell bodies of these neurons constitute the nuclear complex that is traditionally viewed as the thalamus (Jones, 2007; Vue et al., 2007; Chen et al., 2009). In contrast, the rostral thalamus (rTh) produces GABAergic neurons, which do not project to the cortex (Vue et al., 2007). Significant progress has been made in the study of the development and compartmentalization of the p2 domain. The rest of the review will focus on patterning and compartment formation within the p2 domain.

ZLI ORGANIZER SIGNALS DETERMINE THE POSITION OF THE BORDER BETWEEN THE rTh AND cTh

By analyzing an array of transcription factors that are known for their important roles in regulating cell fate decision, Vue et al. (2007) identified the rostral thalamic progenitor domain (rTh), which is located immediately caudal to the ZLI. The rTh expresses *Nkx2.2*, *Ascl1*, and *Olig3*, while the cTh expresses *Neurog1*, *Neurog2*, and *Olig3* (Vue et al., 2007). Moreover, Kataoka and Shimogori showed that the rTh, also called the Rim, is a heterogeneous structure containing distinct populations of cells that express *Tal2*, *Six3/Gad67*, *Nkx2.2/Sox14*, and *Arx*, respectively (Kataoka and Shimogori, 2008). Vue et al. (2009) demonstrated that enhancing or attenuating Shh signaling led to the enlargement or reduction of the rTh domain, demonstrating that *Shh* is important for positioning the border between the rTh and cTh. Significantly, analyzing mouse mutants lacking SBE-1 showed that, although the *Shh* expression in the ZLI was normal, rTh cells were mis-specified, indicating that the specification of the rTh is dependent on Shh signaling from both the ventral midline and the ZLI (Jeong et al., 2011). Fgf8, a secreted morphogen of the Fgf superfamily, is expressed in the dorsal diencephalon (Crossley et al., 2001; Kataoka and Shimogori, 2008; Martinez-Ferre and Martinez, 2009). Manipulations of Fgf8 signaling by *in utero* electroporation of *Fgf8* or *sFGFR3*, encoding a soluble form of FGFR3 that blocks most FGF, including Fgf8, activity, resulted in respective enlargement or reduction of the rTh and its derived nuclei (Kataoka and Shimogori, 2008). Changing Fgf8 activity did not affect Shh and Wnt signaling (Kataoka and Shimogori, 2008). Neither did changing Shh signaling affect the expression of *Fgf8* and its downstream effectors (Vue et al., 2009). These results suggest that the Shh and Fgf8 signaling pathways converge in patterning the p2 domain via independent mechanisms. Experiments in zebrafish have recently shown that *her6*, a homolog of *Hes1*, is important for defining the rTh (Scholpp et al., 2009). *her6* is initially expressed throughout p2, and gradually restricted to the rTh as neurogenesis progresses and *neurog1* and *neurog2* expression is induced in cTh

cells. Furthermore, *her6* is required and sufficient to suppress *neurog1/2* and to induce *ascl1*. Given their known function in directly regulating *Hes1* (Ingram et al., 2008; Wall et al., 2009; Sato et al., 2010), Shh and Fgf8 may independently regulate the formation of the rTh via *Hes*1.

Short-term or long-term lineage tracing using *Ascl1-EGFP* or *Tal1-creER* transgenic mouse lines have demonstrated that the rTh cells give rise to GABAergic neurons of the ventral lateral geniculate nucleus (vLG) and intergeniculate leaflet (IGL; Vue et al., 2007; Jeong et al., 2011). Traditionally, the vLG and IGL are considered structures derived from the prethalamus, because they display clear differences from thalamic nuclei in neurochemistry and connectivity (Jones, 2007). Indeed, fate-mapping studies showed that in addition to other prethalamic nuclei, *Dlx5/6*-expressing cells in the prethalamus contributed to the vLG, demonstrating that the vLG is composed of heterogeneous neurons originating from the rTh and the prethalamus (Jeong et al., 2011). These findings imply that the compartment boundaries between p2 and the ZLI, as well as between p3 and ZLI mainly restrict the movement of progenitor cells, but not postmitotic cells. Similarly, different restrictions on progenitor cells versus postmitotic cells were previously found at the rhombomeric or pallial–subpallial boundaries (Fishell et al., 1993; Wingate and Lumsden, 1996). Therefore, compartmental boundaries are mainly required for a proliferating cell population with labile cell fates. As the fate of postmitotic cells become specified, boundary restriction becomes dispensable (Kiecker and Lumsden, 2005).

THE cTh IS A SELF-CONTAINED COMPARTMENT

The cTh is also defined by the expression domain of homeobox gene *Gbx2* (Bulfone et al., 1993). The onset of *Gbx2* expression appears to be associated with cell cycle exit of thalamic neurons (Bulfone et al., 1993; Nakagawa and O'Leary, 2001; Chen et al., 2009). The importance of *Gbx2* in thalamic development is demonstrated by the fact that deleting *Gbx2* disrupts the histogenesis of the thalamus and abolishes almost the entire thalamocortical projections in mice (Miyashita-Lin et al., 1999; Hevner et al., 2002). Furthermore, *Gbx2* is required for the survival of thalamic neurons (Szabo et al., 2009). Using a *Gbx2-creER(T2)* knock-in mouse line, we performed inducible genetic fate-mapping studies where we showed that the entire thalamic complex was derived from the *Gbx2*-lineage, and the fate-mapped *Gbx2*-lineage formed sharp boundaries surrounding the thalamus (Chen et al., 2009). Interestingly, *Gbx2*-lineage did not contribute to the vLG, while fate-mapping studies using *Tal1-creER* or *Nkx2.2-cre* showed that rTh-derived cells mostly contributed to the vLG but not cTh-derived nuclei, demonstrating that cells derived from the rTh and cTh do not intermingle during development (Chen et al., 2009; Jeong et al., 2011). In the absence of *Gbx2*, the *Gbx2*-lineage abnormally contributed to the habenula and pretectum (Chen et al., 2009). Interestingly, although the dorsal and caudal borders of the thalamus were disrupted in *Gbx2*-deficient mice, the anterior and ventral borders appeared unaffected by the loss of *Gbx2*, demonstrating that different mechanisms may be employed in establishing these thalamic boundaries. We suggest that the disruption of the boundaries is not caused by mis-specification of the thalamic neurons. In fact, in chimeric or genetic mosaic embryos that were composed of wildtype and *Gbx2*-deficient cells, the thalamic boundaries were mostly restored, demonstrating that *Gbx2* has a cell-non-autonomous role in regulating the formation of thalamic boundaries (Chen et al., 2009).

The disruption of the thalamic boundaries caused by loss of *Gbx2* suggests that the establishment of the lineage boundaries surrounding the thalamus is likely achieved through active cell sorting at the border rather than by a general lack of movement of the fate-mapped cells (Chen et al., 2009). It is conceivable that *Gbx2* regulates expression of cell-adhesion molecules that in turn control cell segregation between the thalamus and the pretectum. In agreement with the fate-mapping result in mouse embryos, lineage restriction was discovered at the border between thalamus and pretectum in zebrafish embryos (Peukert et al., 2011). *pcdh10b*, which encodes a cell-adhesion molecule protocadherin, is expressed in thalamic progenitors with its caudal expression border in register with the thalamic-pretectal border (Peukert et al., 2011). Morpholino mediated knock-down of *pcdh10b* disrupted the lineage restriction boundary between the thalamus and pretectum (Peukert et al., 2011). Significantly, expression of *pcdh10b* is regulated by LIM-homeodomain (HD) transcription factors lhx2 and lhx9 (Peukert et al., 2011). We have recently demonstrated that *Gbx2* is essential for the normal function of *Lhx2* and *Lhx9* in mice (Chatterjee and Li, submitted). These findings suggest that *Gbx2*, *Lhx2*, and *Lhx9* may work in the same pathway to regulate the expression of *Pcdh10b*, which in turn controls the compartment boundary between the thalamus and pretectum. As suggested by the authors, it remains to be tested whether Pcdh10b is the cell-adhesion molecule whose deregulation causes the loss of thalamic boundaries observed in *Gbx2*-deficient embryos.

DEVELOPMENT OF THE HABENULA

The p2 domain can be further subdivided into the epithalamus and the thalamus. The epithalamus gives rise to the habenula and pineal gland, which are evolutionarily conserved structures and are present in virtually all vertebrates. In recent years, there is a resurging interest in the habenula because of its regulatory roles in emotive decision-making and its implications in psychiatric disorders (Hikosaka, 2010). The habenula is further divided into the medial and the lateral habenula. The habenula receives input from the cortex, limbic system and basal ganglia through the stria medullaris (Sutherland, 1982; Hikosaka et al., 2008). The fasciculus retroflexus, also known as the habenular–interpeduncular tract, forms the output tract of the habenula and connects with the dopaminergic and serotonergic nuclei in the midbrain and hindbrain. The connectivity of the habenula suggests that it acts as an important node linking the forebrain to the midbrain and hindbrain monoamine systems that are involved in modulating emotional behaviors (Hikosaka, 2010). Indeed, functional imaging studies revealed that the habenula was hyperactive in patients with major depression and in healthy people when receiving negative feedback regarding a failed performance (Morris et al., 1999; Ullsperger and von Cramon, 2003; Hikosaka, 2010).

The habenula has been extensively studied in lower vertebrates like fish, amphibians, and reptile for its remarkable asymmetry in morphology, connectivity, and gene expression (Concha and Wilson, 2001; Halpern et al., 2003; Aizawa et al., 2005; Gamse et al.,

2005). However, relatively little is known about the specification and differentiation of the habenula in mammals. The habenula was once considered to be anatomically continuous with the pretectum (Rose, 1949), and this structure is often overlooked or mis-labeled in literature. There are currently no specific markers for the progenitor domain of the habenula, and the epithalamus shares expression of many marker genes with the pretectum or the thalamus. By contrast, postmitotic neurons of the habenula display distinct molecular markers (Quina et al., 2009). POU-domain homeobox gene *Pou4f1* (also known as *Brn3a*) is expressed in the developing habenula and is essential for habenular development (Xiang et al., 1996; Quina et al., 2009). By comparing the expression profile of the E16.5 habenula with other brain regions or between wildtype and *Pou4f1*-deficient habenula, Quina et al. (2009) have identified genes that are specifically expressed in postmitotic habenular neurons, including *Nurr1*, a downstream target of *Pou4f1*. The authors showed that despite of being highly heterogeneous in its composition, the habenula by itself has a molecular identity that is distinct from other brain regions including the neighboring thalamus. Genetic studies have identified molecules that are important for the development of the habenular neuronal traits, such as axon fasciculation and targeting (Giger et al., 2000; Kantor et al., 2004; Quina et al., 2009). However, how the habenular identity is specified remains unresolved.

Genetic fate-mapping studies have shown that the *Gbx2*-lineage is mostly restricted from crossing the border between the epithalamus and thalamus (Chen et al., 2009). By contrast, transplantation studies showed that cells derived from epithalamic grafts contributed to the mantle zone of the thalamus in chick embryos (Garcia-Lopez et al., 2004), or in organotypic explants of mouse diencephalon (Martinez-Ferre and Martinez, 2009). There results suggest that by some unknown mechanism the border between the epithalamus and thalamus mainly restrict cell movements in the unilateral direction. Long-term fate-mapping studies are necessary to determine the contribution and function of epithalamus-derived cells in the thalamus.

SIGNALING PATHWAYS THAT CONTROL DIFFERENTIATION OF THE cTh AND THE EPITHALAMUS

It has been shown that when the entire p2 domain is forced to express constitutively active Smoothened after E10.5 in mouse embryos, the cTh and the epithalamus persist despite the caudal expansion of the rTh (Vue et al., 2009). This suggests that additional signaling mechanisms are involved in controlling the identity of the cTh and epithalamus (Bluske et al., 2009). Recent studies have shown that Wnt and Fgf signaling play a role in development of the thalamus and the epithalamus.

Wnt signaling is important for partitioning of the anterior neural tube into the telencephalon and diencephalon with high Wnt activity inducing diencephalon while inhibition of Wnt promoting telencephalon (Heisenberg et al., 2001; Houart et al., 2002). Using explant culture and *in ovo* electroporation, Braun et al. (2003) demonstrated that Wnt activity sets up the difference in competence of rostral and caudal forebrain tissues by inducing differential expression *Irx3* and *Six3* in these two domains. Furthermore, manipulation of Wnt activity demonstrated that Wnt signaling played a key role in determining the position and angle of the ZLI relative to the longitudinal axis of the neural tube, probably by regulating expression of *Irx* genes (Sylvester et al., 2010). In addition to its patterning role in progenitors, Wnt signaling may be also important for maintaining distinct characteristics in different postmitotic neurons derived from different prosomeres or subdivision of a prosomere. For example, it was shown that Wnt activity is required for maintaining *Gbx2* and *Dlx2* expression in tissues that express *Irx3* and *Six3* respectively (Braun et al., 2003). Inactivation of Wnt receptor Lrp6 results in caudalization of p1and p2, along with ectopic expression of prethalamic markers in the thalamus and failure of ZLI formation (Zhou et al., 2004). At later stages, Wnt signaling continues to play a role in the differentiation of thalamic nuclei (Liu et al., 2008), and the development of thalamocortical projections (Wang et al., 2002; Zhou et al., 2008, 2009). Multiple Wnt ligands, as well as components of the Wnt signaling pathway, including receptors (Fz1/2/4/7/8/9/10), inhibitors (Sfrp1/2/3 and Axin1/2), and effectors (Tcf1 and Tcf4), are expressed in discrete domains of the diencephalon (Zhou et al., 2004; Bluske et al., 2009; Quinlan et al., 2009). One remaining challenge is to determine the identity and the mechanism of Wnt ligands in regulating development of the diencephalon.

Recent studies have revealed that FGF signaling is also important for the development of the diencephalon, although its role is less understood. *Fgf15* (or *Fgf19* in chick or fish embryos) is expressed in the thalamus, and mediates Shh function in cell proliferation within the diencephalon at the early stages in mouse embryos (Ishibashi and McMahon, 2002; Miyake et al., 2005; Gimeno and Martinez, 2007). Inactivation of *fgf3* and *fgf8* disrupted formation of the prethalamus in zebrafish (Walshe and Mason, 2003). *Fgf8* is expressed in the dorsal midline of the diencephalon. Forced expression of *Fgf8* by *in utero* electroporation promotes rTh identity in a Shh-independent pathway (Kataoka and Shimogori, 2008). By analyzing an *Fgf8* hypomorphic allele, Martinez-Ferre et al. showed that attenuating Fgf8 caused reduction of the habenula and thalamus (Martinez-Ferre and Martinez, 2009). The authors also showed an expansion of *Wnt1* expression in the thalamic midline and loss of *Wnt3a* expression in *Fgf8* mutants. It is possible that the phenotype observed in the habenula in this instance is mediated by Wnt signaling and needs to be verified by additional studies.

CONCLUSIONS AND PERSPECTIVES

Genetic studies have clearly demonstrated that the ZLI organizer, or specifically Shh signaling, plays an important role of patterning the diencephalon. However, the molecular mechanisms that underlie the establishment of the ZLI or regulate *Shh* expression is not completely clear. Mutual repression between region-specific genes has emerged as a common mechanism in establishing boundaries, including the ZLI, within the vertebrate neural tube. Previous studies suggest that the ZLI is induced at different interfaces, such as *Six3/Irx3* in chicken (Kobayashi et al., 2002; Braun et al., 2003), *fezf/otx* in zebrafish (Scholpp et al., 2007), and *Fezf/Irx* in mice (Hirata et al., 2006). Additional studies are required to resolve whether these apparent differences are truly specific to different species, or they reflect differences in the temporal dynamics of gene expression in different species. The timing of the establishment of the ZLI organizer appears different in various species. For

example, *wnt8b* and *shh*, two ZLI organizer markers, are induced in a transverse band of cells corresponding to the prospective organizer during early and late somitogenesis in zebrafish (Scholpp et al., 2009), whereas the ZLI *Shh* expression is not formed until much later at E10.5 in mice. It will be interesting to determine how such differences contribute to the evolution of the forebrain. It has been suggested that the relative size of the telencephalon versus diencephalon among ecological variants of the same species of cichlid fishes can be determined by creating different angles of the ZLI structure (Sylvester et al., 2010). This suggests that even though the same genetic mechanism might initially determine the position of the ZLI, further variation can be achieved to allow better adaptation to the external world.

It has been previously shown the compartment boundary mainly restricts cell movements of proliferating progenitors but not postmitotic cells (Fishell et al., 1993; Wingate and Lumsden, 1996; Kiecker and Lumsden, 2005). Lineage-restricted boundaries that were recently revealed by fate-mapping studies in mice seem to also apply to postmitotic cells (Chen et al., 2009; Jeong et al., 2011). Unlike the cortex, where neurons are arranged in laminar structures, diencephalic neurons aggregate to form distinct nuclei. The molecular mechanism that regulates specific grouping of neurons during differentiation of nuclei remains largely unknown. In the developing central neural tube, the expression of several cadherin molecules is restricted to developmental compartments as well as nuclei (Redies and Takeichi, 1996; Redies et al., 2000; Yoon et al., 2000). Therefore, establishment of compartments in the diencephalon and nuclei may share common mechanisms. Interestingly, genetic inducible fate mapping of *Gbx2* expressing cells at different stages have revealed that different thalamic nuclei display a distinct onset and duration of *Gbx2* expression. These observations raise an interesting possibility that the dynamic and differential expression of *Gbx2* may lead to segregation of *Gbx2*-positive neurons from *Gbx2*-negative neurons, which have not yet started to express *Gbx2* or have lost *Gbx2* expression.

As we progress in terms of our knowledge in this field, many of the questions raised will be answered and many more interesting will come up. We have not yet reached the threshold of knowing about the diencephalon. So we hope that this excitement will continue.

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Regulation of thalamic development by Sonic hedgehog

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The thalamus is strategically positioned within the caudal diencephalic area of the forebrain, between the mesencephalon and telencephalon. This location is important for unique aspects of thalamic function, to process and relay sensory and motor information to and from the cerebral cortex. How the thalamus comes to reside within this region of the central nervous system has been the subject of much investigation. Extracellular signals secreted from key locations both extrinsic and intrinsic to the thalamic primordium have recently been identified and shown to play important roles in the growth, regionalization, and specification of thalamic progenitors. One factor in particular, the secreted morphogen Sonic hedgehog (Shh), has been implicated in spatiotemporal and threshold models of thalamic development that differ from other areas of the CNS due, in large part, to its expression within two signaling centers, the basal plate and the zona limitans intrathalamica, a dorsally projecting spike that separates the thalamus from the subthalamic region. Shh signaling from these dual sources exhibit unique and overlapping functions in the control of thalamic progenitor identity and nuclei specification. This review will highlight recent advances in our understanding of Shh function during thalamic development, revealing similarities, and differences that exist between species.

Keywords: thalamus, diencephalon, forebrain, zli, Shh, morphogen

THE PROSOMERE MODEL OF FOREBRAIN DEVELOPMENT

Almost 20 years ago, Puelles and Rubenstein (1993) described a model to help explain how the complex architecture of the mouse forebrain is generated from discrete developmental territories termed prosomeres. The purpose of the prosomere model was to relate the bent longitudinal axis of the forebrain to that of more posterior regions of the neural tube and to define its primary subdivisions along the anteroposterior (a/p) and dorsoventral axes. Initially, the spatial patterns of 45 genes were mapped onto the prosomeric model with many respecting the hypothesized transverse and longitudinal boundaries of the forebrain (Puelles and Rubenstein, 1993). Over the years, hundreds of new genes have been added to the list and further testing of the model has led to its reinterpretation (Puelles and Rubenstein, 2003). As it stands, the prosomere model stipulates that the caudal forebrain is organized into three prosomeres (p1–p3) corresponding to the pretectum, thalamus, and prethalamus, respectively, whereas the rostral forebrain (telencephalon and hypothalamus) represents a complex protosegment not divided into prosomeres (**Figure 1**).

Functional genetic experiments performed over the past decade in several model organisms have further validated the prosomere model and have greatly enhanced our understanding of the molecular mechanisms underlying forebrain formation and evolution (Hébert and Fishell, 2008; Scholpp and Lumsden, 2010). A common theme that has emerged from these and other studies of nervous system development is that compartmentalization of the neuroepithelium into functional units is facilitated by its exposure to extrinsic factors secreted from localized signaling centers (Jessell, 2000; Hébert and Fishell, 2008; Scholpp and Lumsden, 2010). In the case of the caudal forebrain, the secreted morphogen Sonic hedgehog (Shh), has been shown to play a multifaceted role in regulating the growth and identity of distinct neuronal progenitor subtypes within the thalamic complex, as well as the formation of the zona limitans intrathalamica (zli), a dorsally projecting boundary between p2 and p3 that also serves as a critical signaling center for thalamic and prethalamic development (**Figure 1**). This review will highlight the diverse functions of Shh at different stages of thalamic development, including a feature unique to the caudal forebrain whereby Shh secreted from two orthogonal sources (basal plate and zli) contributes to a morphogenic signaling gradient that patterns an alar structure, the thalamic primordium.

Shh SIGNALING IN THE SPINAL CORD: LESSONS LEARNED FROM 20 YEARS OF STUDY

Much of what we know about Shh signaling has come from studies of its role in spinal cord development. A summary of the principal concepts learned from this work is described below and will serve as a framework for comparison with the roles of Shh signaling during thalamic development. For more comprehensive reviews on this subject the reader is encouraged to consult the following references (Dessaud et al., 2008; Matise and Wang, 2011).

Sonic hedgehog is a secreted protein that provides positional information to a wide variety of developing tissues, including the CNS (Dessaud et al., 2008; Ingham et al., 2011; Matise and Wang, 2011). *Shh* is expressed in the axial mesoderm (prechordal plate and notochord) and ventral midline (floor plate) of the overlying neural tube throughout most of the a/p neuraxis (Echelard et al., 1993; Roelink et al., 1994). It is from these sources that a ventral to dorsal concentration gradient of Shh is established in the ventral neural tube. Over the past several years a compelling body of evidence has been generated to explain how the Shh signaling gradient

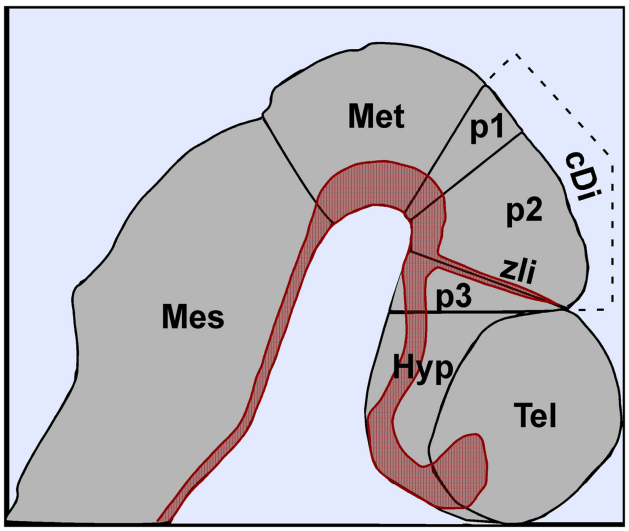

FIGURE 1 | Regionalization of the central nervous system along the anteroposterior axis of the mouse at embryonic day 10.5. The caudal diencephalon is divided into three prosomeres (p1, p2, p3) corresponding to the pretectum, thalamaus, and prethalamus, respectively. The zona limitans intrathalamica (zli) is located at the p2–p3 boundary. The expression of *Shh* in the ventral midline and zli is depicted in red. Abbreviations: cDi, caudal diencephalon; Hyp, hypothalamus; Mes, mesencephalon; Met, metencephalon; Tel, telencephalon.

is interpreted by neuronal and glial progenitors to account for the diverse array of cell types present in the ventral spinal cord. The prevailing model stipulates that the fate of a given progenitor is determined by the level and duration of Shh signaling to which it is exposed (Ericson et al., 1996, 1997; Dessaud et al., 2007, 2010). For instance, the identity of the ventral-most neuronal progenitors in the spinal cord (p3 domain) is determined by the highest concentration of Shh for the longest period of time, whereas, the identities of progenitors occupying progressively more dorsal positions in the spinal cord (pMN, p2–p0) are dependent on correspondingly lower levels of Shh signaling for shorter periods of time (Dessaud et al., 2007, 2010).

To fully appreciate the intricacies of the molecular mechanism by which ventral neuronal progenitors interpret the level and duration of Shh signaling, a brief overview of the Shh signal transduction cascade is necessary. In the absence of Shh ligand, the pathway is kept in an off state by Patched (Ptch1), a 12-pass transmembrane protein that also functions as an integral component of the Shh receptor complex (Marigo et al., 1996; Stone et al., 1996; Allen et al., 2011). Ptch1 suppresses Shh signaling by antagonizing the function of Smoothened (Smo), a 7-pass transmembrane protein with an essential role in Hedgehog signal transduction (Chen and Struhl, 1996; Zhang et al., 2001; Taipale et al., 2002). Blockage of Smo activity results in the phosphorylation and proteolytic processing of the zinc finger containing transcriptional regulators, Gli3, and to a lesser extent Gli2, into transcriptional repressors (Wilson and Chuang, 2010). When Shh binds to the Ptch1 receptor complex, the repression on Smo is relieved, thus permitting the production and nuclear entry of full-length Gli proteins and their transcriptional activation of target genes, including Gli1 and Ptch1.

In response to its position along the Shh morphogen gradient, a progenitor cell elicits distinct temporal profiles of Gli activity (Stamataki et al., 2005; Dessaud et al., 2007, 2010). This is a dynamic process given that progenitors become desensitized to Shh over time, as a result of the negative feedback loop with Ptch1. Therefore, to keep its position along the dorsoventral axis, the progenitor cell must maintain a certain threshold of Shh signaling over time (Dessaud et al., 2010).

Each progenitor domain can be identified by the expression of a distinct set of homeodomain and bHLH transcription factors (Briscoe and Ericson, 2001; Lupo et al., 2006). Boundaries between progenitor domains are generated over time by the mutual repression of complementary pairs of transcription factors (Muhr et al., 2001). Once the boundaries are fixed, the unique combination of transcription factors assigned to a given progenitor domain further directs the fate of differentiating neurons.

THE MULTIPLE ROLES OF Shh DURING THALAMIC DEVELOPMENT

While significant advances have been made in elucidating the requirements of Shh signaling in posterior regions of the CNS, it is only recently that similar progress has been described for the diencephalon (Scholpp and Lumsden, 2010). The thalamic primordium develops from the alar plate of p2. *Shh* expression is localized to the basal plate of p1–p3 by the 12-somite stage of development and over a day later (25-somites) is initiated in the zli, where it becomes fully activated by E10.5 (**Figure 1**).

The exposure of the thalamus to two *Shh* signaling centers has made it somewhat of a challenge to reconcile the specific roles of either one in regulating the growth, patterning and neuronal identity of thalamic progenitors. However, recent studies using a combination of genetic and tissue perturbation approaches in mouse, chicken, and zebrafish embryos have developed a clearer picture of the multifaceted roles of Shh signaling during thalamic development (Hashimoto-Torii et al., 2003; Kiecker and Lumsden, 2004; Vieira et al., 2005; Scholpp et al., 2006; Szabó et al., 2009; Vue et al., 2009; Jeong et al., 2011).

EARLY ROLES FOR Shh AS A MITOGEN

The dependency of thalamic development on Shh is temporally regulated. As early as the 15-somite stage of mouse development, $Shh^{-/-}$ embryos show reduced proliferation and survival of diencephalic precursors (Ishibashi and McMahon, 2002). This mitogenic role for Shh occurs well before zli formation and is therefore attributed to Shh signaling from the prechordal plate and/or ventral midline of the diencephalon. Since the cell proliferation defects in $Shh^{-/-}$ embryos were also observed in alar regions of the diencephalon, well out of range of Shh secreted from ventral sources, a Shh-dependent relay signal was proposed to regulate the growth of thalamic progenitors. Fgf15 appeared to be an ideal candidate to fulfill this function as its dorsal growth promoting properties were dependent on Shh, at least when overexpressed in cultured mouse brain explants (Ishibashi and McMahon, 2002). However, recent loss of function studies do not support this conclusion, as $Fgf15^{-/-}$ mutants show increased proliferation of dorsal neural progenitors

and decreased neurogenesis in the developing midbrain and neocortex, consistent with Fgf15 functioning as a growth suppressor (Borello et al., 2008; Fischer et al., 2011). The identity of the Shh-dependent regulator of thalamic growth and survival remains to be identified. Wnt ligands are good candidates based on the finding that several are expressed in the thalamic primordium, as well as the fact that the expression of *Tcf4*, a transcriptional mediator of Wnt signaling, is downregulated in the diencephalon of $Shh^{-/-}$ embryos (Ishibashi and McMahon, 2002). While Wnt signaling may, in some instances, act antagonistically to Shh in the specification of some neural cell fates (Robertson et al., 2004), this is likely to be context dependent, as Wnt signaling is also dependent on Shh for the proliferation of neural progenitors in the spinal cord (Alvarez-Medina et al., 2009).

Zli FORMATION

The epichordal/prechordal interface marks the territory from where the zli will emerge (Vieira et al., 2005). The expression of the homeodomain transcription factor Otx2 on the posterior (epichordal) side of the zli and the zinc finger proteins Fezf1 and Fezf2 on the anterior (prechordal) side of the p2/p3 border are required for zli formation (Hirata et al., 2006; Jeong et al., 2007; Scholpp et al., 2007). Whether these transcription factors play a direct role in regulating *Shh* expression in the zli, or provide a permissive environment for *Shh* to be transcribed, remains unresolved. Interestingly, mouse mutants lacking the bHLH trancription factors Hes1 and Hes5 also show a loss of *Shh* expression in the zli (Baek et al., 2006). It is intriguing to speculate that Hes1/5 might be functioning downstream of a cross repressive interaction between Otx2 and Fezf2 to regulate Shh expression in the zli. Of course, other regulatory relationships are equally possible.

The zli is a key partition between the thalamic and prethalamic territories and also serves as an important signaling center for the regionalization of the a/p axis of the caudal diencephalon (Scholpp and Lumsden, 2010). The zli extends dorsally from the ventral midline at the p2/p3 boundary, coinciding with the anterior limit of the notochord (**Figure 1**; Vieira et al., 2005). *Shh* expression is first detected in the zli at the 25 somite stage of chick and mouse embryos and expands dorsally at a rate of $\sim$20 μm/h until it reaches a length of 600 μm (Zeltser, 2005). The dorsal progression of *Shh* transcription was inhibited in chick embryos upon insertion of a microbarrier between the basal plate and the zli, or when Shh signaling was blocked with a constitutively active form of Ptch1 (Kiecker and Lumsden, 2004; Zeltser, 2005; Vieira and Martinez, 2006). These results suggested that *Shh* expression in the zli is regulated by a ligand-dependent feed-forward signaling mechanism.

A Shh-dependent vertical signaling model to explain the spread of Shh transcription along the zli is not entirely consistent with the finding that *Shh* continues to be expressed in the zli of mutant mouse embryos lacking principle components of the Shh transduction cascade, including Gli2, Gli3, and Smo (Hashimoto-Torii et al., 2003; Vue et al., 2009). Since it is likely that the inactivation of Smo function in *Nestin-cre*; $Smo^{loxp/loxp}$ embryos occurred after *Shh* expression was already initiated in the zli, these results might suggest that the maintenance, but not the initiation, of *Shh* expression in the zli is independent of Shh signaling. A more substantial challenge to the vertical signaling model comes from the observation that *Shh* expression was initiated in the zli of $Gli2^{-/-}$; $Gli3^{-/-}$ mouse mutants, as well as zebrafish oep mutants (which lack the nodal co-receptor tdgf1/cripto), despite the lack of *Shh* expression in the basal plate of the caudal diencephalon, which was thought to be the initiating source of the vertical signal (Hashimoto-Torii et al., 2003; Scholpp et al., 2006). Clearly, more work will be needed to sort out the molecular details of zli formation. A more thorough analysis of the critical cis and trans determinants of *Shh* expression in the zli may help explain the direct regulatory mechanisms underlying the formation of this structure (Epstein et al., 1999; Jeong et al., 2006).

The thalamus and prethalamus express different sets of genes in response to Shh signaling from the zli (Kiecker and Lumsden, 2004; Scholpp et al., 2006; Vieira and Martinez, 2006). To explain how this differential response to Shh is orchestrated, Kiecker and Lumsden (2004) proposed that the thalamus and prethalamus are prepatterned. In support of their hypothesis, they showed that the homeobox gene Irx3 acts as a thalamic competence factor. When misexpressed in the prethalamus of chick embryos, Irx3 ectopically activated genes typically expressed posterior to the zli in a Shh-dependent manner. While loss of function studies with Irx3 are likely confounded by functional redundancy with other family members, it is nonetheless intriguing that in zebrafish Irx1b morphants, the zli is posteriorly expanded at the expense of the thalamus, suggesting that Irx1b is necessary to restrict zli formation on the epichordal side of the zli (Scholpp et al., 2007).

Shh PATTERNS THE THALAMUS ALONG A MORPHOGENIC GRADIENT

The spatial arrangement of thalamic nuclei is important for generating the precise topographical relationship needed to fulfill its role as a relay center. Despite the many advances in our knowledge of the early events regulating thalamic growth, and regionalization, we still know relatively little concerning the mechanisms by which heterogeneous clusters of thalamic neurons become specified and aggregate into discrete thalamic nuclei. One particular challenge has been to correlate the patterns of gene expression initiated by Shh and other signaling pathways at early stages of thalamic development with discrete nuclei and/or neuronal subtypes that form at later postnatal stages (Nakagawa and O'Leary, 2001; Jones and Rubenstein, 2004; Vue et al., 2007, 2009; Szabó et al., 2009; Suzuki-Hirano et al., 2011; Yuge et al., 2011).

The neurons contributing to thalamic nuclei are derived from at least two distinct progenitor domains. The caudal population of thalamic progenitors, pTH-C, is the larger of the two groups, and gives rise to all thalamic nuclei that relay sensory information from the periphery to primary sensory regions of the neocortex via thalamocortical axons (**Figure 2**; Vue et al., 2007). The rostral population of thalamic progenitors, pTH-R, comprises a narrow band of cells sandwiched between pTH-C and the zli (**Figure 2**). Thalamic neurons derived from pTH-R progenitors are thought to contribute to two dorsolaterally positioned thalamic nuclei, the ventrolateral geniculate nucleus (vLG), and the

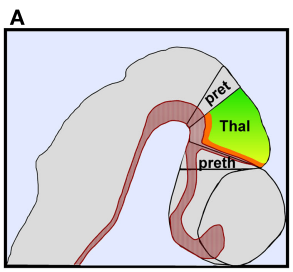

FIGURE 2 | A morphogenetic gradient of Shh signaling establishes distinct thalamic progenitor domains. (A) Schematic representation of a lateral view of the caudal diencephalon color-coded as follows: *Shh* expressing cells (red); pTH-C_2 domain (yellow); pTH-C_1 domain (green); pTH-R domain (orange). **(B)** Comparison of a representative set of genes expressed in thalamic and ventral spinal cord progenitor domains.

intergeniculate leaflet (IGL), neither of which project axons to the cortex (Horowitz et al., 2004; Morin and Blanchard, 2005; Jones, 2007; Vue et al., 2007, 2009).

In addition to its role in early patterning events, Shh signaling is also required to specify neuronal subtypes that contribute to a broad array of thalamic nuclei. The current model proposes that graded Shh signaling is necessary and sufficient to promote distinct classes of thalamic progenitors (Hashimoto-Torii et al., 2003; Scholpp et al., 2009; Szabó et al., 2009; Vue et al., 2009). The pTH-R domain, which develops closest to the zli, is dependent on the highest concentration of Shh, whereas, the rostroventral (pTH-C_2) and caudodorsal (pTH-C_1) populations of pTH-C progenitors, developing several cell diameters away from the zli, are dependent on progressively lower concentrations of Shh (**Figure 2**; Hashimoto-Torii et al., 2003; Szabó et al., 2009; Vue et al., 2009).

The conditional inactivation of either Shh or Smo in the diencephalon results in the loss of pTH-R progenitors and their post-mitotic derivatives in the vLG and IGL, as well as a sizeable reduction in the population of pTH-C progenitors and the Gbx2 expressing, cortex-projecting, thalamic neurons that differentiate from these cells (Szabó et al., 2009; Vue et al., 2009; Jeong et al., 2011). Although Szabó et al. (2009) and Vue et al. (2009) both described varying degrees of thalamic deficits in the absence of Shh, the phenotypes described by Szabó et al. (2009) were more severe and correlated with a greater loss of Gbx2 expression, which was likely attributed to the use of an earlier acting and more robust Cre line (*Foxb1-Cre* versus *Netrin-Cre*) to delete *Shh* from the thalamic primordium.

How does Shh signaling determine the different classes of thalamic progenitors? At first glance, a model similar to that described for neuronal subtype identity in the ventral spinal cord could be envisioned, whereby distinct thalamic progenitors are specified by their exposure to different thresholds of Shh signaling activity. However, another possibility is that the two classes of Shh-dependent thalamic progenitors, pTH-R and pTH-C, are specified by two spatially distinct sources of Shh, the basal plate, and zli, respectively. For the latter model to be valid, different phenotypes should arise from the inactivation of Shh from discrete signaling territories.

To help resolve this question, Jeong et al. (2011) examined mutant mice containing a targeted deletion of a *Shh* regulatory element required for *Shh* expression in the basal plate of the caudal diencephalon, but not the zli. This analysis showed that the expression of high threshold target genes in pTH-R (*Nkx2.2*, *Ascl1*, *Tal1*) was reduced, concomitant with an expanded expression domain of lower threshold, pTH-C target genes (*Ngn2*). While this result may, in part, reflect temporal differences in the dependency of pTH-R on Shh, it might also be the case that prolonged Shh signaling activity from both diencephalic sources is required to promote pTH-R identity. Given that the blockade of Shh signaling from the zli in chick embryos also results in a loss of *Nkx2.2* expression in the pTH-R domain, the most parsimonious explanation of the data is that both sources of Shh surrounding the thalamus are necessary for pTH-R identity (Kiecker and Lumsden, 2004; Jeong et al., 2011). Therefore, Shh secreted from two signaling sources, the basal plate and zli, supplies the Shh signaling gradient that shapes thalamic progenitor identity over time.

The similarity in signaling mechanisms by which Shh regulates neuronal progenitor subtype identity in the thalamus and ventral spinal cord also extends to the use of some of the same transcriptional regulators mediating these cell fate decisions (**Figure 2**). For instance, pTH-R and p3 neuronal progenitors form closest to their respective sources of Shh in the thalamus and spinal cord, respectively, and both populations express Nkx2.2. The rostroventral pTH-C (classified as pTH-C_2) and pMN domains reside at slightly greater distances from their respective sources of Shh and both express the bHLH transcription factors Olig2, Ngn1, and Ngn2. Finally, the caudodorsal region of pTH-C in the thalamus (classified as pTH-C_1) and p0 domain of the spinal cord both express Dbx1 and are at the tail end of the Shh responsive territories.

FUTURE DIRECTIONS

The specificity of thalamic progenitors are not solely determined by Shh. Clearly, additional signaling pathways (Fgf, Wnt, and others) must play significant roles in generating the diversity of progenitor subtypes that have been, and remain to be, discovered in the thalamus (Braun et al., 2003; Zhou et al., 2004; Miyake et al., 2005; Vieira and Martinez, 2005; Kataoka and Shimogori, 2008; Bluske et al., 2009; Martinez-Ferre and Martinez, 2009; Peukert et al., 2011). Future research will undoubtedly uncover how these signaling pathways integrate to generate the transcriptional network that programs each of the thalamic progenitor domains and gives rise to the full complement of thalamic nuclei. Hopefully, in the not too distant future, our understanding of thalamic development will match that of the intricately detailed patterning events that occur in the spinal cord (Alaynick et al., 2011).

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Development of the corticothalamic projections

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In this review we discuss recent advances in the understanding of corticothalamic axon guidance; patterning of the early telencephalon, the sequence and choreography of the development of projections from subplate, layers 5 and 6. These cortical subpopulations display different axonal outgrowth kinetics and innervate distinct thalamic nuclei in a temporal pattern determined by cortical layer identity and subclass specificity. Guidance by molecular cues, structural cues, and activity-dependent mechanisms contribute to this development. There is a substantial rearrangement of the corticofugal connectivity outside the thalamus at the border of and within the reticular thalamic nucleus, a region that shares some of the characteristics of the cortical subplate during development. The early transient circuits are not well understood, nor the extent to which this developmental pattern may be driven by peripheral sensory activity. We hypothesize that transient circuits during embryonic and early postnatal development are critical in the matching of the cortical and thalamic representations and forming the cortical circuits in the mature brain.

Keywords: subplate, layer 6, layer 5, cerebral cortex, VB, LGN, reticular thalamic nucleus

INTRODUCTION

The elaborations and changes in cortical representation during evolution have been accompanied by equally impressive changes in the structure of the thalamus (Kaas, 2007). The thalamus is not merely a relay station passing on verbatim information to the cortex, rather the thalamus and cortex represent a highly integrated processing unit that dynamically regulates thalamic transmission of peripherally derived data for cortical processing (Sherman and Guillery, 1998). Layer 6 corticothalamic connectivity largely outnumbers the sensory input to the thalamus (Mitrofanis and Guillery, 1993) providing the feedforward and feedback mechanisms essential in this processing unit. Furthermore the thalamus relays layer 5 cortical output to other distal cortical areas (Guillery and Sherman, 2002), thus distributing cortico-cortical information and integrating disparate cortical areas into a global network. This network provides a substrate for the widespread synchronization of cortical and thalamic cell populations. The high frequency oscillations associated with this synchrony are suggested to underlie discrete conscious events (Steriade, 2000), highlighting the importance of layer 5-derived cortical innervation of the thalamus. As such cortical innervation of the thalamus is highly important yet its development has received little attention.

THE ADULT CORTICOTHALAMIC RELATIONSHIP

All cortical areas receive thalamic input and send projections to the thalamus (Caviness and Frost, 1980). The circuit involves three cortical cell populations and two orders of thalamic nuclei (**Figure 1A**). The cortical component consists of glutamatergic projection neurons residing in layers 4, 5, and 6. First order thalamic nuclei include dorsal lateral geniculate nucleus (dLGN), ventrobasal nucleus (VB), medial geniculate nucleus (MGN), the ventrolateral nucleus (VL), and the anterior thalamic group. These nuclei contain thalamic relay cells with specific projections that process peripheral sensory information and relay it to the cortex. Higher order nuclei include the pulvinar group, mediodorsal thalamic group, and lateral posterior nucleus. These nuclei contain thalamic matrix cells with diffuse projections that relate cortico-cortical information between different cortical areas (Jones, 2002; Sherman and Guillery, 2002).

NUCLEAR AND LAMINAR SPECIFICITY OF THE THALAMOCORTICAL AND CORTICOTHALAMIC CIRCUIT

Thalamic relay cells in first order thalamic nuclei receive modality specific sensory information from peripheral nerves. All peripheral sensory information is represented in the thalamus with the exception of olfaction (which is represented indirectly via piriform cortex projection to mediodorsal thalamic nucleus; Jones, 1985). Ascending projections from thalamic nuclei are primarily directed to modality matched cortical areas, i.e., dLGN projects to primary visual cortex (V1). The target cells of first order nuclei are situated largely in layers 4 and 6 (Frost and Caviness, 1980). Collaterals from these thalamocortical axons synapse onto the GABAergic neurons residing in the reticular thalamic nucleus (RTN). These RTN neurons project to the thalamus, connecting with thalamic relay cells thus closing an inhibitory feedback loop which is involved in modulating the activity of thalamic relay cells (Jones, 2002; Cruikshank et al., 2010).

Cortical innervation of thalamic nuclei depends on the laminar identity of the cortical neurons. Layer 6 projects to the first order thalamic nuclei from which it receives input, continuing modality specificity (**Figure 1B**); from V1 they project to dLGN (Guillery, 1967), from primary somatosensory cortex (S1) to VB (Jones and Powell, 1968; Hoogland et al., 1987), and from auditory cortex (A1) to MGN (Diamond et al., 1969). The layer 6 axons terminate in small but numerous glutamatergic synapses on the distal dendrites of the relay cells (Guillery, 1995; Rouiller and Welker, 2000; Jones, 2002). These axons provide modulator input, modifying

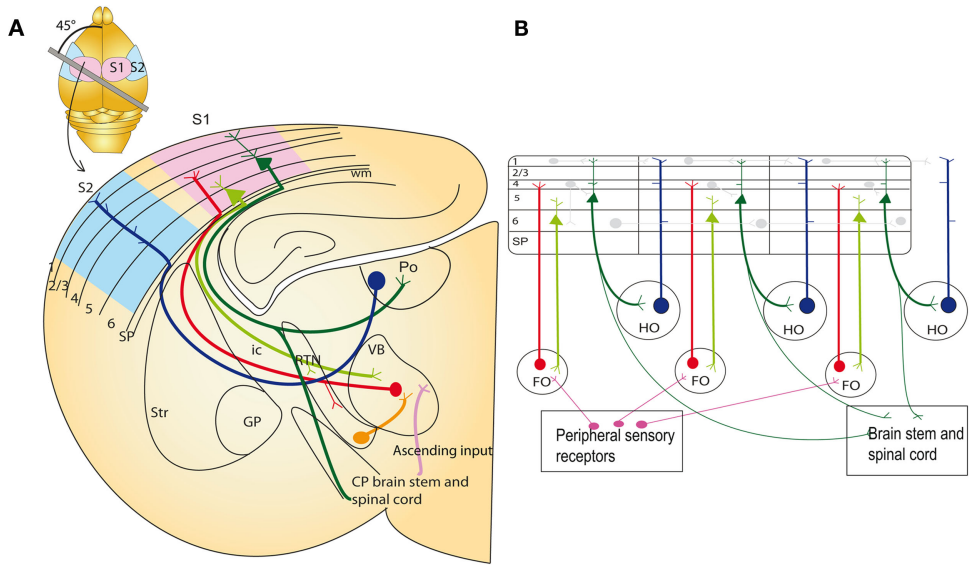

FIGURE 1 | Thalamocortical circuits in the adult on an idealized section containing somatosensory cortical connections (A) and schematic representation of the two major sets of thalamic projection neurons (B). **(A)** *Inset*: outline of the mouse brain with the line indicating the plane of section to obtain thalamocortical slice containing S1 with intact thalamocortical projections. For clarity S2 cortex connectivity is also indicated in the idealized section, although a different plane of section would be required to maintain connections. *Main image*: coronal schematic demonstrating the specificity of the connections between the cortex and thalamus using the somatosensory system as an example. The first order VB thalamic nucleus receives somatosensory peripheral input (pink). The VB then projects axons (red) to layer 4 of the primary somatosensory cortex (S1; light blue). Layer 6 "modulator" neurons (light green) in S1 project back to the VB. Layer 5 neurons (dark green) in S1 project to subcerebral structures and make a collateral branch to a higher order thalamic nucleus, e.g., posterior thalamic nucleus (Po). The higher order nuclei then project (dark blue) to an area of cortex that is different from the one they received input from (for example S2; light pink). This projection pattern generates an open loop. **(B)** Schematic illustration of the possible functional circuits generated by this reoccurring open loop connectivity. Sensory information is relayed through the first order thalamic nucleus to the cortex (red). This cortical area then projects from layer 6 reciprocally back to the first order nucleus (light green). Each area is also non-reciprocally connected to a higher order thalamic nucleus. The layer 5 input to the thalamus (dark green) is an "efference copy" of the layer 5 output to the motor system in the brainstem and spinal cord. This copy is forwarded to a higher cortical area (blue). Direct cortico-cortical connections are also depicted between cortical layers and cortical areas (pale gray lines). These circuits enable cortical areas to act with other cortical areas and motor apparatus in a coordinated manner. Modified from Sherman and Guillery (2002). CP, cerebral peduncle; FO, first order thalamic nuclei; GP, globus pallidus; HO, higher order thalamic nuclei; ic, internal capsule; RTN, reticular thalamic nuclei; SP, subplate; Str, striatum; S1, primary somatosensory cortex; S2, secondary association somatosensory cortex; Po, posterior thalamic nuclei; VB, ventrobasal thalamic nuclei; wm, white matter.

thalamic relay cell activity and thus gating pathways which transmit peripheral information (Sherman and Guillery, 1998). Layer 6 axons also provide collateral projections to the RTN, generating an inhibitory feedforward circuit thus modifying thalamic relay cell activity by at least two mechanisms (Guillery, 1995; Jones, 2002).

Higher order thalamic nuclei receive the majority of their driver inputs from collaterals of layer 5 corticobulbar and corticospinal neurons (Sherman and Guillery, 2002). These layer 5 "driver" neurons synapse in large glutamatergic terminals on the matrix cells (Sherman and Guillery, 1998). The higher order thalamic nuclei then project excitatory fibers to a different cortical area than the one they received input from. These projections do not aim for layer 4, they mainly target the upper and lower layers of the cortex (**Figure 1B**).

CONNECTIVITY ANALYSIS REVEALS COMPLEX THALAMOCORTICAL TRAJECTORY ARRANGEMENTS IN THE ADULT

The overall relationships between thalamus and cortex follow relatively simple principles (Caviness and Frost, 1980; Behrens et al., 2003), but the fine topography is complex and not fully understood. Retinal information is represented with different polarity in the primary and secondary visual areas (Hubel and Wiesel, 1977; Rosa et al., 1997). Recording visual representations in the dLGN and primary visual cortex, Connolly and Van Essen (1984) argued that the two-dimensional visual representation has to undergo a transformation between the thalamus and the cortex in a fashion that requires the crossing of the projections in one, but not the other dimension (Connolly and Van Essen, 1984). Indeed, tracing experiments by Nelson and LeVay (1985) demonstrated exactly this arrangement (**Figure 2**). Paired injections of tracers revealed that thalamocortical afferent trajectories rotate in the medio-lateral, but not the antero-posterior dimension in the cat V1 in the white matter, close to their target cortex (Nelson and LeVay, 1985). Adams et al. (1997) and Molnár (1998) argued that such thalamocortical transformations are common in several cortical areas.

Corticothalamic axons also undergo temporary trajectory changes, de- and re-fasciculating and rotating around one another (Bernardo and Woolsey, 1987; Lozsádi et al., 1996). These changes are visible at the RTN and the perireticular thalamic nucleus (PRN – a population of cells lateral to the RTN), close to their thalamic target (**Figure 3**).

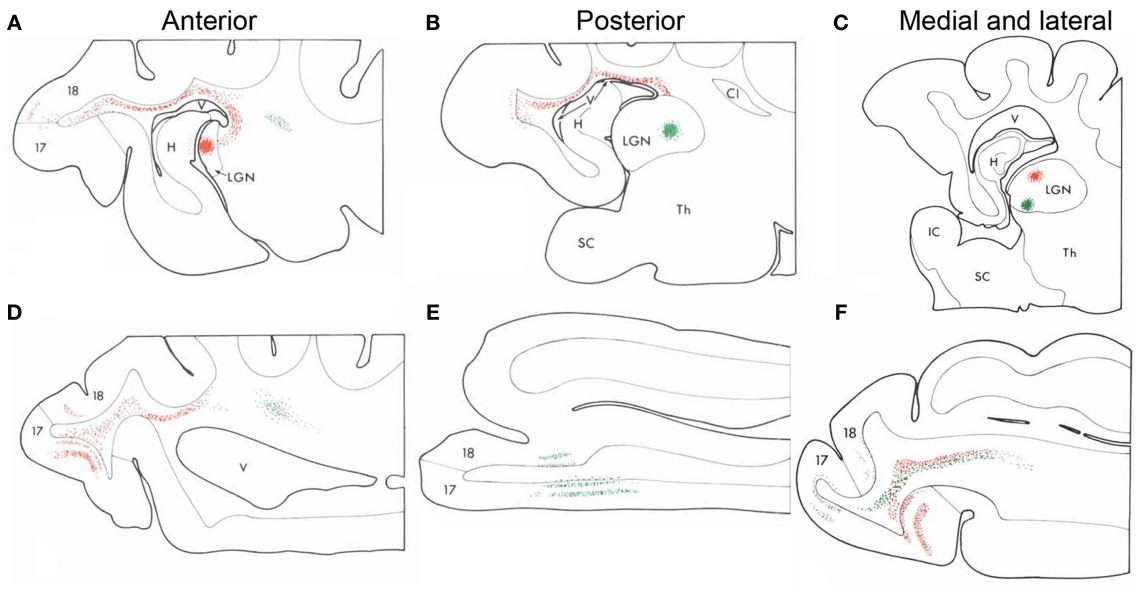

FIGURE 2 | Complex thalamocortical fiber trajectory changes in adult animals. Nelson and LeVay delivered paired injections of different tracers into the cat thalamus in an anterior and posterior **(A,B)** or medio-lateral arrangement **(C)**. The tracers revealed thalamocortical projections as they leave the thalamus, traverse the optic radiation and white matter before they enter the corresponding cortical regions. The antero-posterior pairs of injections revealed no crossing of the fibers at any sector of the trajectory **(D,E)**, whereas the medio-lateral pairs of thalamic injections revealed fibers that crossed each other close to the primary visual cortex **(F)**. These experiments demonstrated that lateral geniculate nucleus (LGN) afferents perform transformation in the medio-lateral, but not the antero-posterior fashion in the cat V1 close to the cortex in the white matter (Nelson and LeVay, 1985). Adapted from Nelson and LeVay (1985). H, hippocampus; IC, internal capsule; Th, thalamus; V, ventricle.

DEVELOPMENTAL ESTABLISHMENT OF COMPLEX THALAMOCORTICAL TRAJECTORY ARRANGEMENTS

Guillery and colleagues suggested that the fiber crossings observed in the region of the thalamus in the adult brain arise by rearrangements of the corticofugal projections during development (Lozsádi et al., 1996; Adams et al., 1997). Mitrofanis and Guillery (1993) suggested that during development the subcortical subplate and PRN and RTN serve as compartments where such rearrangements can occur. There are numerous similarities between these structures. Each compartment is more extensive during development than adulthood and contains largely transient cells that form part of the early circuits. Furthermore they share gene-expression patterns as demonstrated by correlation data from Allan Brain Atlas (**Figures 3C,D**) and comparative expression research (Montiel et al., 2011; Wang et al., 2011). Importantly during development they may act as accumulation compartments for growing fibers; thalamocortical axons accumulate in the subplate, while corticothalamic axons accumulate at the PRN and RTN. According to this hypothesis, coarse reciprocal connections are established during early development while distances are minimal. Fine-tuning of representations occurs subsequently using the two stable platforms provided by the subplate and RTN. There is anatomical and electrophysiological evidence for connections from thalamic projections to subplate neurons before the former invade the cortex. We later discuss the role of transient circuits that assist the formation and maturation of the earliest cortical circuits (Kostovic and Rakic, 1990; Allendoerfer and Shatz, 1994; Kanold and Luhmann, 2010). However, research into the corticofugal rearrangements and transient circuits at the thalamus is less established.

DEVELOPMENT OF CORTICOTHALAMIC PROJECTIONS

Thanks to improved labeling methods, time-lapse video-microscopy and new transgenic lines that express reporter genes there has been some progress in the understanding of the earliest corticofugal outgrowth in mice. After the preplate, the earliest post-mitotic cortical neurons migrate along radial glia to the nascent preplate around embryonic day (E) 10. Before they have even left the intermediate zone (between the germinal zone and cortical plate) the cells begin extending neurites (Noctor et al., 2004; Lickiss et al., 2012). This extension continues and becomes directed, laterally, medially, rostrally, or caudally, depending on transcription factor expression. Ctip2 is highly expressed in laterally projecting corticofugals with complementary high Satb2 expression in callosal projections (Molyneaux et al., 2007; Fishell and Hanashima, 2008). These corticofugal projections extend through the intermediate zone, deep to the cortex, until they reach the lateral internal capsule between E13 and E15.5 (Auladell et al., 2000; Jacobs et al., 2007). The lateral fibers arrive first and briefly pause until dorsally derived fibers have grown the extra distance (De Carlos and O'Leary, 1992; Molnár and Cordery, 1999). At E15.5 these projections resume extension, crossing the pallial–subpallial boundary (PSPB) and entering the internal capsule. After traversing the internal capsule the axons arrive at the diencephalon–telencephalon boundary (DTB). Here the axons enter the prethalamus where they encounter the cells of the PRN and RTN at E16. Here there is a second pause in corticofugal fiber front progression until E17.5 (Molnár and Cordery, 1999; Jacobs et al., 2007). Furthermore the heterogeneous corticofugal projections are "sorted" and separated here, with some continuing to the cerebral peduncle (layer 5), and others entering the thalamus

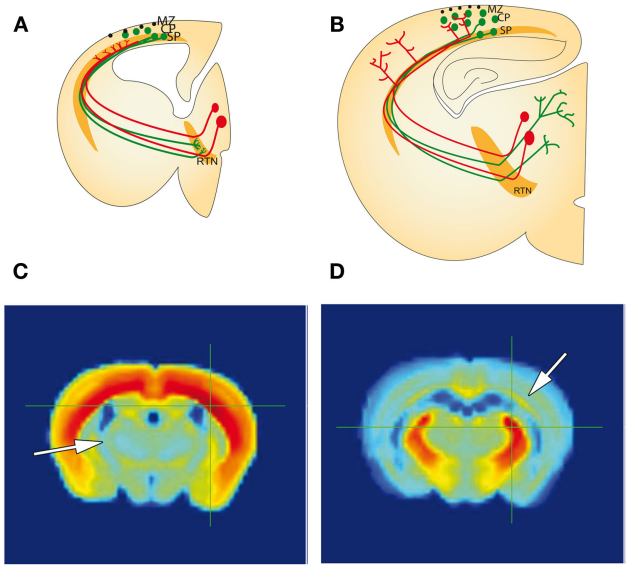

FIGURE 3 | Similarities between the cortical SP zone, and reticular thalamic nucleus during development (A,B), correlated gene expression (C,D). (A) Schematic representation of a forebrain section with the developing corticothalamic connectivity. Corticofugal (green) and thalamocortical (red) axons extend toward each other at early stages during embryonic development and arrive near their targets, but both pause short of their ultimate targets. Corticofugal projections from subplate and layer 6 accumulate in the reticular thalamic nucleus (RTN) and thalamocortical projections accumulate in subplate, respectively. **(B)** Toward the middle of the first postnatal week corticofugal and corticopetal axons enter the thalamus and cortical plate (CP), respectively, where they arborize and establish their contacts with their ultimate targets in thalamus and neocortex. There are signs of fiber crossings in the RTN and in the subplate indicating some rearrangements during development. (**A** and **B** modified from Montiel et al., 2011). **(C,D)** Gene correlation heat map from Allan Brain Atlas showing areal gene expression that correlates with expression in the SP **(C)** and RTN **(D)**. The crosshairs in each figure show the region sampled for gene expression. These gene correlations show that the SP (arrow in **D**) has a higher gene correlation with the RTN than other subpallial structures, and the RTN (arrow in **C**) has a higher gene correlation with the SP than other pallial structures. This comparison is in adult, however, and during development the similarities may be even more pronounced. CP, cortical plate; MZ, marginal zone; RTN, reticular thalamic nucleus; SP, subplate.

(layer 6 and layer 5 collaterals). The site of this sorting lies within the PRN and RTN (Mitrofanis and Baker, 1993).

CORTICOTHALAMIC WAITING PERIODS

The development and behavior of corticofugal projections has been studied with numerous methodologies, but is still not fully understood. Shatz and Rakic (1981) demonstrated with orthogradly transported tritiated proline injected into the occipital cortex of fetal rhesus monkeys that the development of corticofugal projections is synchronous with development of thalamocortical pathways, and that the corticofugal projections from V1 accumulate outside the lateral geniculate nucleus (LGN) for a protracted period (Shatz and Rakic, 1981). This suggests a similar "waiting period" for the corticothalamic projections outside the thalamus as the "waiting period" for the thalamocortical projections as they arrive at the cortex (Rakic, 1976). The exact timing and pattern of the early subplate, layer 6 and layer 5 fibers projecting subcortically and entering the thalamus is still not established. Waiting periods (in temporal order of fiber tract progression) have been demonstrated in the white matter or lateral internal capsule in ferrets (Clascá et al., 1995), in the RTN (Molnár and Cordery, 1999) and outside the LGN in monkey (Shatz and Rakic, 1981). A recent transgenic mouse line expressing tau-eGFP in subplate and layer 6 projections demonstrates two waiting periods, the first as the fiber front reaches the lateral internal capsule and a second as it arrives at the DTB. These mixed origin tau-eGFP fibers enter the thalamus in a clear temporal sequence depending upon the thalamic nucleus to be innervated (Jacobs et al., 2007). However, it has been debated whether subplate projections ever enter the dorsal thalamus (Allendoerfer and Shatz, 1994).

LAYER-SPECIFIC INGROWTH INTO THE THALAMUS

After the second waiting period at the RTN corticothalamic axons shift orientation once more and invade the thalamus, a process that takes several days with most thalamic nuclei being innervated postnatally in rats, mice, and hamsters (Miller et al., 1993; Molnár and Cordery, 1999; Jacobs et al., 2007). The innervation of the thalamus in mammalian species occurs in a temporal pattern which correlates with the functional establishment of behaviors associated with relevant sensory systems. Somatosensory and motor functions mature before visual and auditory functions; the somatosensory VB, and motor ventrolateral nucleus, are innervated earliest between E18.5 and P0.5, auditory MGN and visual dLGN are not fully innervated until P8 (Molnár et al., 1998a; Jacobs et al., 2007).

The three corticothalamic projection populations grow toward the thalamus with distinct temporal patterns; however which cortical layer reaches the thalamus first is contested within the literature. Subplate neurons are well placed to pioneer the course and could provide structural guidance to layer 5 and 6 axons. In support of this hypothesis, there are several species in which the first axons to reach the lateral internal capsule do so before the cortical plate cells become post-mitotic, therefore indicating that the projections arise from the earlier-born subplate cells in mice, rats and cats (McConnell et al., 1989, 1994; De Carlos and O'Leary, 1992; Molnár and Blakemore, 1995; Molnár et al., 1998a,b; Jacobs et al., 2007). Furthermore, in cats, ablation of subplate cells with timed kainic acid administration leads corticothalamic axons to fail to connect with appropriate thalamic nuclei (McConnell et al., 1994).

This, however, does not identify the temporal order in which subplate, layer 6 and layer 5 collaterals invade the thalamus. In hamsters, Miller et al. (1993) used retrograde carbocyanine dye tracing to assess timed invasion of the thalamus. At birth (postnatal day – P0) a limited number of layer 6 and subplate axons are back-labeled by thalamic DiI. This is quickly followed at P3 by the large ingrowth of layer 5 axons. The prevalence of layer 5 axons in the thalamus remains only until P7 at which point layer distribution reverses again (Miller et al., 1993). However similar tracing experiments in ferrets produced slightly different temporal patterns, demonstrating instead that layer 5 axons arrive in the thalamus several days before deeper layers (Clascá et al., 1995).

Thus the exact timing of each cortical layer's arrival is currently unresolved and may differ in different species. Combining these

results suggest that whilst subplate projections leave the cortex first, layer 5 projections may be the first to innervate thalamus, followed by layer 6. It is tempting to speculate that the extra time taken by subplate axons is a result of some rearrangement of representation during this period. How this rearrangement is controlled during the waiting period, or whether it is modulated by the input from the sensory periphery is not yet understood. The layer-specific timing of cortical innervation to the thalamus suggests future work should address questions of waiting period differences; do both layer 5 and layer 6 undergo the same waiting periods or does one population wait whilst the other forges ahead?

RECENT ADVANCES USING REPORTER GENE EXPRESSING LINES

The inability to label subplate, layer 6 and layer 5 neurons and their neurites selectively, hinders our understanding of the developmental integration of these neurons into the intra- and extra-cortical circuitry. This is now rapidly changing. Due to the advances in molecular taxonomy of cortical neurons, we have more tools to analyze circuits (Molnár and Cheung, 2006; Molyneaux et al., 2007; Hoerder-Suabedissen et al., 2009). These tools include subplate specific transgenic GFP animals, the Lpar1-eGFP (formerly Edg2-eGFP) mouse, the Golli-tau-eGFP mouse, and the CTGF-eGFP mouse (Jacobs et al., 2007; Hoerder-Suabedissen and Molnár, 2012b) (**Figure 4**). These mice express GFP primarily in the subplate. Below we present our work characterizing the subplate cells labeled in these animals.

Co-localizing GFP with neuronal and subplate markers revealed distinct, although overlapping, subpopulations within the subplate (Hoerder-Suabedissen and Molnár, 2012b). These subpopulations display different patterns of growth into the thalamic nuclei (**Figure 5**). Lpar1-eGFP fibers have not reached the RTN by P2. By P6 GFP+ fibers have entered VB in a pattern which suggests they innervate the hollows of the barreloids. No fibers have entered the LGN. By P14 the VB and LGN have been innervated.

In contrast Golli-tau-eGFP cortical fibers have different growth kinetics. At P2 the GFP+ fiber front is at the RTN and many fibers can be seen clearly entering VB. By P6 the fibers have fully entered VB and are patterned in the septa between barreloids. At this age the dLGN is not innervated, however, the first fibers are accumulating between VB and the ventral edge of the dLGN. At P14 the GFP+ fibers have innervated VB in the hollows of barreloids. The dLGN is now completely innervated although the vLGN is not (**Figure 5**).

Retrograde carbocyanine dye tracing at P8 demonstrates only 7% of the cells back-labeled from the thalamus are GFP+ in the Lpar1-eGFP mouse whereas 50% of back-labeled cells are GFP+ in the Golli-tau-eGFP mouse (**Figure 6**).

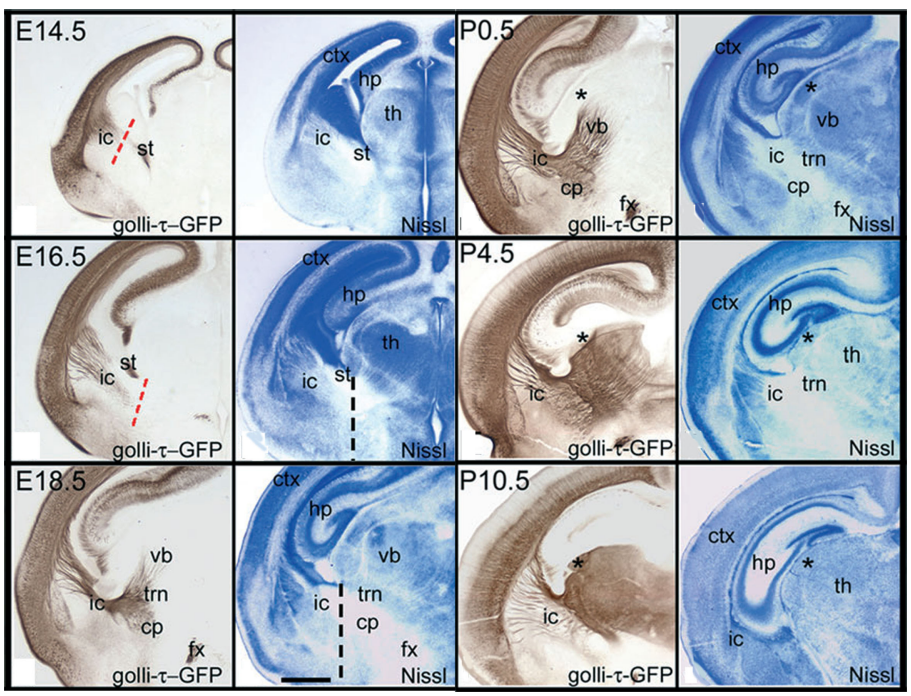

FIGURE 4 | Temporal pattern of subplate (layer 6b) cortical axons ingrowth to the thalamus of the GTE mouse. Adapted from Jacobs et al. (2007). The Golli-tau-eGFP mouse has GFP labeling in the axons of deep cortical layer axons, mostly in layer 6 and subplate. Visualization of these fibers demonstrates the growth of cortical axons toward the thalamus during development. At E14.5 cortical axons have reached the lateral cortex, but only a few have crossed the PSPB. By E16.5 the cortical fibers have reached the internal capsule and advanced toward the RTN (labeled TRN in panels), which they reach by E18.5. Between P0 and P4 cortical axons innervate the midline nuclei, but they advance relatively slowly and some thalamic nuclei (e.g., dLGN – asterisk) do not get a substantial innervation until the end of the first postnatal week. dLGN, dorsal lateral geniculate nucleus; GP, globus pallidus; LGE, lateral ganglionic eminence; MD, mediodorsal nucleus; MGE, medial ganglionic eminence; PSPB, pallial–subpallial boundary; RTN, reticular thalamic nucleus; Stri, striatum; VB, ventrobasal nucleus.

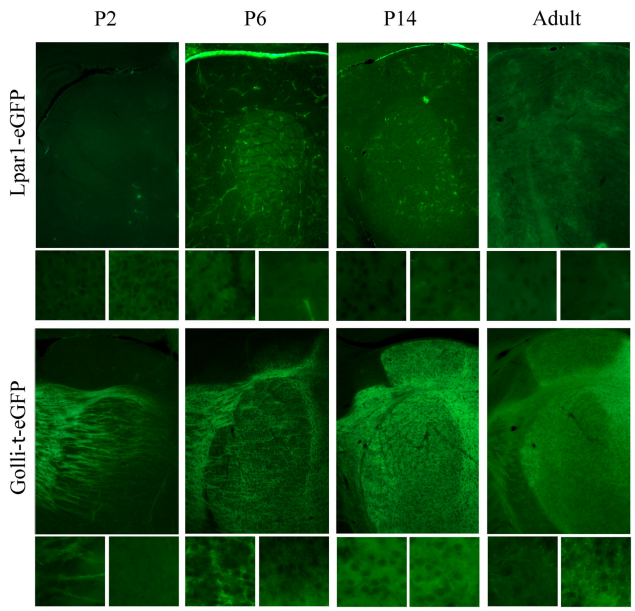

FIGURE 5 | The developing innervation of the thalamus by subpopulations of subplate neurons. Cortical sections, dorsal at top of image, lateral at left of image. Boxes beneath each image show higher magnification of the VB (left) and dLGN (right). Characterization of transgenic mouse lines Lpar1-eGFP and Golli-tau-eGFP identify clearly distinct, although overlapping, subpopulations of subplate neurons on coronal section of thalamus. These distinct populations innervate the thalamus with a different temporal pattern. At P2 Lpar1-eGFP fibers have not reached the RTN. By P6 GFP+ fibers have entered VB in a barreloid pattern. No fibers have entered the LGN. By P14 VB and LGN have been innervated. In the Golli-tau-eGFP the GFP+ fiber front is at the RTN and many fibers can be seen clearly entering VB at P2. By P6 the fibers have fully entered VB and are patterned in the septa between barreloids. The dLGN is not yet innervated however GFP+ fibers accumulate between VB and the ventral edge of the dLGN. At P14 the Golli-tau fibers have innervated VB in the hollows of barreloids. The dLGN is now completely innervated although the vLGN is not. dLGN, dorsal lateral geniculate nucleus; RTN, reticular thalamic nucleus; VB, ventrobasal nucleus; vLGN, ventral lateral geniculate nucleus.

Thus GFP+ fibers in each transgenic line exhibit distinct temporal patterns for entering the thalamus, project to the thalamus in different numbers, and upon entering establish a different pattern of innervation.

Our results demonstrate that not only does layer identity determine distinct ingrowth kinetics – but subpopulations of cells within layers also display specific properties of thalamic ingrowth.

MOLECULAR AND CELLULAR MECHANISMS OF CORTICOFUGAL DEVELOPMENT

DEVELOPMENTAL GENE-EXPRESSION BOUNDARIES

The early telencephalon is subdivided by the differential expression of genes including *Pax6* and *Gsh2* (Schuurmans and Guillemot, 2002). These gene-expression patterns generate distinct neural subpopulations and give rise to the regional patterns encountered by the corticofugal axons. These regions generate a complex genetic and structural landscape which provides cues for corticothalamic axons to navigate with.

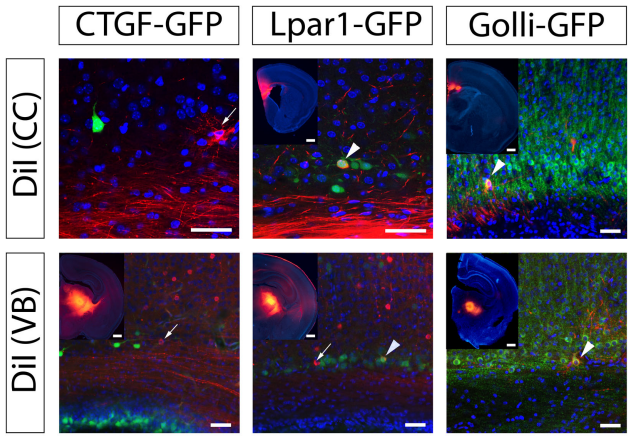

FIGURE 6 | Three subplate subpopulations have distinct projection profiles. Cortical sections, dorsal top of image, lateral is left of image. DiI back-labeling from the VB or the CC demonstrate that CTGF-eGFP labeled subplate cells are rarely-to-never back-labeled from distant structures suggesting they project locally. Insert shows DiI crystal position. The Lpar1-eGFP subpopulation is back-labeled from corpus callosum and VB; 7% of DiI+ cells from VB are Edg2-GFP+ ($n = 745$ cells) and 26% of DiI+ cells from CC are Edg2-GFP+ ($n = 114$ cells). The Golli-tau-eGFP subpopulation is also back-labeled from corpus callosum and VB; 50% of DiI+ cells from VB are Golli-tau-eGFP+ ($n = 65$ cells), and 33% of DiI+ cells from CC are Golli-tau-eGFP+ ($n = 45$ cells). VB, ventrobasal thalamic nucleus; CC, corpus callosum.

THE PALLIAL–SUBPALLIAL BOUNDARY

The PSPB is a key region during corticothalamic and thalamocortical development; there are numerous mutants which present axon guidance defects at the boundary (López-Bendito and Molnar, 2003). The PSPB (see **Figure 7**) extend along a graduated overlapping gene-expression domain separating the developing cortex from the striatum (hence its alternative name – the corticostriatal boundary). It is primarily generated and maintained by opposing gradients of Pax6 and Gsh2 expression. Pax6 is highly expressed in the dorsal pallium and conversely, Gsh2 is highly expressed in the subpallium (Carney et al., 2009). The dorsal pallium, which gives rise to excitatory cortical neurons, additionally expresses Tbr1, Emx1, and Ngn2. The subpallium expresses additional ventral organizers such as Mash1, Dlx2, and Nkx2.1 and generates inhibitory cortical interneurons and ventral telencephalic cells (Simeone et al., 1992; Stoykova and Gruss, 1994; Puelles et al., 2000). Within these subdivisions there are further discrete gene expression gradients differentiating the dorsal, lateral, and ventral pallium, and, within the subpallium, also differentiating the dorsal and ventral lateral ganglionic eminence, and the medial ganglionic eminence (Schuurmans and Guillemot, 2002; **Figure 7**).

The PSPB is an important decision point where early corticofugal projections turn sharply from their original ventrolateral trajectory to a medial one to enter the subpallium (Agmon et al., 1995; Molnár and Cordery, 1999). Early patterning of the pallium and subpallium by organizer genes including *Pax6* and *Gsh2* determines expression patterns of later guidance molecules. Pax6 is especially important for the generation of the PSPB. In *Pax6*

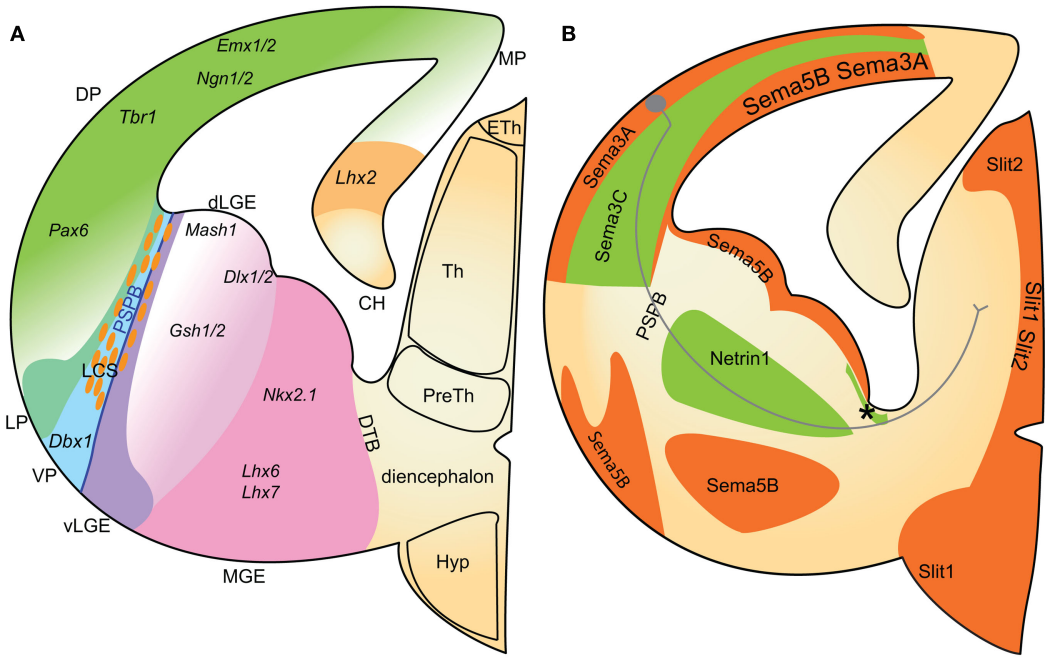

FIGURE 7 | Schematic representation of gene-expression boundaries in the developing telencephalon (A) and the expression patterns of identified axon guidance cues in relation to corticothalamic axons (B). **(A)** Early gene-expression boundaries subdivide the developing telencephalon into the pallium, in dorsal telencephalon, and the subpallium in the ventral telencephalon. The pallial–subpallial boundary lies between opposing gradients of Pax6 and Gsh2 expression in the pallium and subpallium respectively. Further subdivisions are generated with other discrete gene gradients. Dorsal pallium (DP) progenitors express Pax1, Emx1/2**,** and Ngn1/2 (green gradient). Proximal to the cortical hem, the medial DP progenitors express Lhx2 (yellow). Ventral pallium (VP) progenitors express Dbx1 (aqua/turquoise). Within the subpallium, dorsal lateral ganglionic eminence (dLGE; pink gradient) progenitors express Mash1 and Dlx1/2 and have higher expression of Gsh1/2 than ventral LGE cells (light purple). The MGE can be distinguished from LGE by expression of Nkx2.1, Lhx6, and Lhx7 in the MGE (pink). The lateral cortical stream cells (LCS; orange cells) migrate along radial glia at the PSPB. **(B)** Corticothalamic axons encounter combinations of molecular guidance cues as they navigate toward the thalamus. Chemoattractant Sema3C (labeled green) is expressed in a lateral to medial gradient in the intermediate zone. Netrin-1 is expressed in the internal capsule (labeled and asterisk). Within the thalamus, chemoattractive guidance cues for corticothalamic axons have not yet been identified. Corticothalamic chemorepulsive cues include Sema3A, Sema5B, Slit1, and Slit2 (orange). Combinations of chemorepellents are expressed in a pattern that flanks the route of corticothalamic axons. The positions of the pallial–subpallial and diencephalon–telencephalon boundaries are also represented. CH, cortical hem; dLGE, dorsal lateral ganglionic eminence; DP, dorsal pallium; DTB, diencephalon–telencephalon boundary; LCS, lateral cortical stream; LP, lateral pallium; MGE, medial ganglionic eminence; MP, medial pallium; PSPB, pallial–subpallial boundary; VP, ventral pallium; vLGE, ventral lateral ganglionic eminence.

LacZ knock-out mice the expression of corticofugal guidance cues Netrin-1, Sema3C, and Sema5A is disrupted (Jones et al., 2002). Furthermore the PSPB may be the site of a temporary physical barrier. The lateral cortical stream (LCS) is formed at the PSPB by ventrolateral migration of a heterogeneous population including Pax6+, pallium-derived and Dlx2+, subpallium-derived cells (Carney et al., 2006; Carney et al., 2009). This migratory stream, along a palisade of radial glia, may generate a physical boundary preventing axons from crossing into the subpallium until there are appropriate cues or a physical bridge becomes available. Evidence for this hypothesis is provided by the *Pax6*$^{-/-}$ mouse strain, where corticothalamic axons fail to cross the PSPB, most misrouting ventrally and following the current of the LCS. The few fibers that succeed in crossing do so in abnormally large fascicles (Hevner et al., 2002; Jones et al., 2002). *Pax6*$^{-/-}$ mice have a higher cellular density at the PSPB suggesting the mutation may increase the LCS thus enhancing a normal anatomical barrier and preventing crossing (Jones et al., 2002; Piñon et al., 2008). This physical barrier may be overcome by time dependent mechanisms such as axon–axon fasciculation, thus enabling precise temporal control of corticofugal guidance. Indeed this is supported by recent work showing thalamic axons require cortical axons in order to cross the PSPB, discussed in more detail later (Chen et al., 2012; Molnár et al., 2012).

THE DIENCEPHALON–TELENCEPHALON BOUNDARY

The DTB lies antero-laterally to the thalamus and prethalamus. The genetic identity of this boundary is much less clearly established than the PSPB and it is identified solely by nearby anatomical landmarks including the prethalamus dorso-posterior to it (López-Bendito and Molnar, 2003; Garel and Rubenstein, 2004; Hanashima et al., 2006). Close to DTB, within the internal capsule, there are turning points where corticothalamic axons reroute dorsally to invade thalamus and other corticofugal axons start their descent through the cerebral peduncle to the brainstem and spinal cord (Mitrofanis and Baker, 1993; Agmon et al., 1995; Lozsádi et al., 1996). This region appears important for sorting subpopulations of cortical projection neurons according to their target destination.

The DTB may also have similar barrier functions to the PSPB. Disruption of pioneer axons or fasciculation with thalamic axons prevents corticothalamic axons from entering the diencephalon or invading the thalamus correctly (McConnell et al., 1994; Hevner et al., 2002).

These developmental gene-expression boundaries contribute to corticothalamic guidance in three ways. (1) Generating physical boundaries that can be appropriately permissive or restrictive depending on developmental stage. (2) Acting as decision points for major trajectory changes. (3) Setting up important patterns of guidance molecules. The full extent of the contribution to guidance that these developmental gene-expression boundaries make is still under investigation. As is the importance of the temporal choreography of the development of functional corticothalamic circuits (Molnár et al., 2012).

STRUCTURAL GUIDANCE CUES

Since the first description of the early corticofugal axon front extending toward the thalamus (McConnell et al., 1989) a possible role of structural guidance cues in thalamocortical axon guidance has been postulated. These ideas were formulated as the "Handshake Hypothesis" (Blakemore and Molnar, 1990; Molnár and Blakemore, 1995; Molnár et al., 1998a,b), which suggests that early corticofugal and thalamocortical projections meet at the PSPB and the early corticofugal projections aid the thalamic fibers to cross through this region toward the cortex. The relationship between early corticofugal and thalamic projections has been debated. It has been suggested that they fasciculate with each other in internal capsule and intermediate zone (Molnár and Blakemore, 1995; Molnár et al., 1998a,b); but other studies suggest that they run in separate compartments (Miller et al., 1993) or interdigitate only in a restricted portion of their path (Bicknese et al., 1994). Some of these issues are related to the difficulties of delivering paired tracers into the equivalent regions of the cortex and the thalamus. The Golli-tau-eGFP mouse model (see above) demonstrates the intimate association of early corticofugal projections with thalamic afferents (Piñon et al., 2005) from the intermediate zone, PSPB and also throughout the lateral sector of the internal capsule.

Several mutants with thalamocortical guidance defects also display aberrant development of corticofugal projections (Hevner et al., 2002; López-Bendito and Molnar, 2003). Mutation of the thalamic gene *Gbx2* causes a reduced projection of thalamocortical axons into the internal capsule. Subsequently, corticothalamic axons fail to enter the diencephalon from the subpallium, thus suggesting that thalamic axons provide some cue to corticothalamic axons that may include physical fasciculation to help them cross the DTB (Hevner et al., 2002). Recent research has demonstrated that structural support from populations of other axons can be crucial to crossing early developmental gene boundaries (Chen et al., 2012). When cortical fibers are absent and thus do not provide structural support at the PSPB, thalamic axons in $Emx1^{cre}$; $Apc^{loxp/loxp}$ mouse fail to reach the cortex although the mutant cortex remains attractive to the thalamic fibers (Chen et al., 2012). The authors demonstrate that the phenotype can be rescued by the replacement of cortical fibers across the PSPB. As such the use of structural support from other fiber populations to cross gene boundaries is important and may be contributing to cortical crossing at the DTB.

Due to a better understanding of forebrain patterning and the availability of conditional knock-outs, there is a recent revival of focus on structural guidance cues to overcome physical barriers in the telencephalon. Zhou et al. (2008, 2009) have demonstrated that region specific *Celsr3* inactivation affects development of the internal capsule in different ways. In *Celsr3|Foxg1* mice the internal capsule is defective and thalamic axons either cross to the contralateral diencephalon or descend to the ventral surface of the telencephalon. In *Celsr3|Dlx5/6* mice, the internal capsule is also abnormal and thalamic fibers are misrouted to the amygdala. Furthermore, the early corticofugal axons fail to advance toward the thalamus, and instead stop at the PSPB, forming a mass resembling an amputational neuroma. In *Celsr3|Emx1* mice, the internal capsule is intact and there are normal thalamocortical connections (Zhou et al., 2008).

The molecular and cellular nature of these interactions is not understood. This limits the interpretation even in these conditional knockouts. The cell surface proteins involved in fasciculation have not been identified and it appears bidirectional signaling as well as just fasciculation may be important for axonal guidance (Hevner et al., 2002).

CORRIDOR CELLS

Early work on corticothalamic development identified a population of cells in the internal capsule, the perireticular cells of the PRN. Their position coincides with the point where corticothalamic axons deflect dorsally into the prethalamus and where layer 5 axon branches are sorted to project to thalamus or to subcerebral targets (Mitrofanis and Baker, 1993). These cells were previously suggested have a role in guiding the corticothalamic axons given the major rearrangement behavior upon reaching them (Mitrofanis and Guillery, 1993).

Since then a second population of cells named corridor cells, derived from the lateral ganglionic eminence, have been demonstrated to generate a critical permissive corridor, within the restrictive medial ganglionic eminence (MGE) (López-Bendito et al., 2006). Without these cells thalamocortical axons are repelled by MGE and fail to properly navigate to the cortex. These cells may also be required to generate a permissive substrate for cortical axons to grow across similar to their support of thalamic axons. Furthermore back-labeling has suggested these cells may overlap with the perireticular neurons and so may contribute to guiding corticothalamic axons and sorting layer 5 thalamic and subcerebral projections. This will become apparent with further research.

MOLECULAR GUIDANCE CUES

Recent advances have identified major families of well-known guidance molecules in the guidance of corticothalamic axons (**Figure 7B**).

Members of the semaphorin family provide several early, context dependent cues, and mutations of these genes generate subtle phenotypes suggesting multiple cues collaborate at each stage of guidance. Corticofugal axons are repelled from Sema3A expressing cortical plate and attracted toward the Sema3C expressing intermediate zone (Bagnard et al., 1998, 2001; Skaliora et al., 1998).

The intermediate zone forms a permissive lane between the cortical plate and the ventricular zone as Sema3A, along with Sema5B, prevents cortical axons overshooting into the cortical germinal zone (Bagnard et al., 1998; Lett et al., 2009). Sema3A also attracts cortical dendrites. The asymmetric distribution of cellular guanylate cyclase enables different responses of cortical axons and dendrites to Sema3A (Polleux et al., 2000).

The lateral-to-medial gradient of Sema3C attracts corticothalamic axons extending within the intermediate zone toward the lateral cortex (Bagnard et al., 1998, 2000). The complementary medial-to-lateral gradient of expression of Sema3A in the ventricular zone may also repel corticofugal axons coming from the medial cortex.

Chemoattractant Netrin-1 is expressed in the internal capsule and ventral telencephalon. This is complementary to Dcc expression (Netrin-1 attraction receptor) in corticothalamic axons (Oeschger et al., 2011). *In vitro*, Netrin-1 mediates long range attraction to E12.5 and E13.5 corticothalamic axons (Métin et al., 1997; Richards et al., 1997). This attraction can induce turning and therefore appears responsible for corticofugal growth cone reorientation toward the ventral telencephalon (Métin et al., 1997). Chemorepulsion may also guide turning due to Sema5B expression in the lateral cortex flanking the route of axons that cross the PSPB (Skaliora et al., 1998; Lett et al., 2009).

Ensuring that axons remain within the internal capsule involves several chemorepulsive interactions. During development Sema5B expression in the germinal zones of the ganglionic eminences and the globus pallidus borders the corticothalamic path through the internal capsule (Skaliora et al., 1998; Lett et al., 2009). Corticothalamic explants are repelled by Sema5B expressing cells and ectopic Sema5B in the internal capsule causes cortical axons to stall at the new Sema5B boundary. Furthermore RNA interference against *Sema5B* causes aberrant entry of cortical axons into the germinal zones (Lett et al., 2009). Thus Sema5B restricts the growth of cortical axons to their appropriate trajectory.

Inhibitory cell surface molecules Slit1 and 2, and receptors Robo1 and 2, also mediate the guidance of the corticothalamic axons within the ventral telencephalon and diencephalon. Slit1 and 2 are expressed in overlapping domains including the ganglionic eminences, prethalamus, hypothalamus, and the germinal zone of the dorsal thalamus (Braisted et al., 2000). Robo1 and 2 are expressed in complementary patterns in the cortical plate, intermediate zone, and dorsal thalamus (López-Bendito et al., 2007). In *Slit2* mutants, *Slit1* and *2* double mutants, and *Robo1* and *2* double mutants corticothalamic guidance is disrupted with the majority of corticofugal fibers continuing ventrally instead of turning at the PSPB, some reaching the basal telencephalic surface. Those which do correctly enter the ventral telencephalon then aberrantly cross the ventral midline (Bagri et al., 2002; López-Bendito et al., 2007).

Slit pathway components therefore ensure containment of corticothalamic axons within the internal capsule and direct corticothalamic axons dorsally upon reaching the DTB in order to enter the thalamus rather than crossing the midline (Bagri et al., 2002; López-Bendito et al., 2007; Braisted et al., 2009).

Guidance cues, which direct axons from specific cortical regions to the thalamic nuclei that they connect to in adulthood, are yet to be elucidated; however candidate cues are beginning to be identified. Using microarrays, Sur and colleagues identify gene-expression differences between the LGN and MGN. Axon guidance molecules including Ephs and ephrins, semaphorins, slits and netrin pathways were differentially expressed between the two nuclei (Horng et al., 2009). Furthermore cues *EphA7* and *Ntrk2* expression is up-regulated in both the LGN and the rewired MGN, in which after peripheral ablation of auditory nerves, the ingrowing retinal axons invade the MGN and the LGN. Thus distinct guidance cue expression may contribute to the specific neural connectivity between thalamic nuclei. Indeed a review in this research topic proposes the hypothesis that overlapping molecular expression in the thalamus may be responsible for the determination of areal axon guidance from thalamus to the cortex (Price et al., 2012). These overlapping and combinatorial gene-expression patterns may also be responsible for organizing cortical axon guidance into specific thalamic nuclei.

Molecular control of temporal dynamics such as the waiting periods has proved harder to elucidate although recent discoveries are beginning to suggest answers. Robo1 is expressed by corticothalamic neurons. It appears to act as a molecular slowing signal as *Robo1*$^{-/-}$ corticothalamic axons reach their targets a day early (Andrews et al., 2006). It appears this slowing signal is not mediated by the canonical Slit–Robo interaction as *Slit2* and *Slit 1* and *2* double mutants have a different phenotype in which most corticothalamic axons fail to reach the thalamus rather than being delayed and *Slit1* mutants do not have corticothalamic phenotypes (Bagri et al., 2002).

Signals that might cause different waiting periods for distinct populations of corticothalamic axons are currently unclear. Guidance pathway molecules, including Unc5c, differ between the subplate and lower cortical plate (Oeschger et al., 2011). Unc5c is a receptor which mediates repulsion to soluble Netrin-1. It is transiently up-regulated in subplate cells compared to layer 5/layer 6. This up-regulation coincides temporally with the first waiting period that corticofugal fibers undergo (E14.5 in mouse). Unc5c has been demonstrated to produce a waiting period during the guidance of primary sensory axons to the spinal cord (Watanabe et al., 2006).

Switching responses to Netrin 1 requires cortical axons to alter their molecular expression after crossing the PSPB. Fluctuating expression of guidance cue receptors over time allows a population of axons to grow through different compartments and only respond to relevant cues. We suggest a temporal pattern of receptor expression as seen with Unc5c may generate the waiting periods and specific temporal growth patterns. Furthermore differential expression of guidance cue receptors between populations of corticothalamic axons generates specific responses of each population to each cue. We propose such molecular differences may generate different waiting behavior in corticothalamic axon populations. Once layer-specific waiting periods are recognized this may provide insight into how temporal control helps cortical neurons path-find to the correct thalamic nucleus.

Many major molecular cues guiding corticothalamic axons have been identified. Complex combinations of various cues generate the specific and detailed connectivity patterns that characterize the connections between cortex and thalamus.

There are likely to be many more, and subtler, cues involved in the precise details of the developmental events. For example cortical layer-specific competency to respond to guidance

cues has not been addressed. Future work should determine cortical layer-specific or even neuronal subtype specific ingrowth to understand the relevance of the stage and subtype specific innervation of different thalamic nuclei by axons from particular cortical areas. Selective subplate, layer 6 and layer 5 gene expression profiling at the time of major decisions in axon growth would help to resolve some of these issues. Furthermore greater understanding of the sequence of circuit formation might give insight into self-organizing mechanisms during development. In addition to molecular cues there may be an activity related component although so far no phenotype has been described in various SNARE complex knock-out mice mutant (Washbourne et al., 2002; Molnár et al., 2002) or cocultures (Blakey et al., 2012). Furthermore evidence so far has shown no phenotype of early corticothalamic innervation in the Golli-taueGFP mouse after peripheral manipulations (Grant and Molnár, 2012).

UNDERSTANDING TRANSIENT CIRCUITS IN CORTEX

The role of transient circuits involving subplate neurons has been demonstrated in the developing visual cortex during ocular dominance formation (Ghosh et al., 1990) and orientation column formation (Kanold et al., 2003) in carnivores. Similar mechanisms might operate in the barrel field of the mouse primary somatosensory cortex (Piñon et al., 2009; Tolner et al., 2012). Thalamic axons reach the intermediate zone and subplate several days prior to innervating the cortex. During this accumulation of thalamic axons, layer 4 exhibits di-synaptic activation in response to thalamic fiber excitation suggesting that thalamic axons synapse with the subplate neurons that synapse onto layer 4 neurons. After 2 days thalamic activation of cortical layer 4 neurons is monosynaptic reflecting direct thalamic innervation (Zhao et al., 2009). Furthermore subplate ablation in cats stops normal up-regulation of glutamate receptor subunit, GluR1, in cortical dendrites thus leading to reduced strength at the thalamocortical synapse (Kanold et al., 2003). Selective ablation of subplate beneath limb or barrel cortex in rat confirms these results; ablation abolishes spontaneous and evoked spindle burst activity in limb cortex *in vivo* and thalamocortical connections to layer 4 are weaker than controls *in vitro* (Tolner et al., 2012). Therefore this early developmental feedforward innervation via the subplate is proposed to strengthen and stabilize the developing thalamic to layer 4 synapses.

This transient circuit also regulates maturation of cortical inhibition and ocular dominance columns. Subplate ablation disrupts neuronal receptor profile maturation. Cortical neurons fail to up-regulate KCC2 channel expression. Without KCC2 neurons maintain high internal chloride concentrations and GABA receptor activation continues to depolarize, rather than hyperpolarize, the membrane (Kanold et al., 2003). The loss of proper cortical inhibition causes paradoxical effects on ocular dominance columns; subplate ablation causes monocular deprivation to favor the deprived eye (Kanold et al., 2003; Kanold and Shatz, 2006). By adulthood the subplate circuit is transformed or dismantled, a large proportion of the early born subplate neurons have died (Price et al., 1997; Hoerder-Suabedissen and Molnár, 2012b), but some survive into adulthood as layer 6b.

We have little understanding of the mode of integration of subplate neurites into the cortical plate prior, during, and after thalamic innervation. Axonal and/or dendritic remodeling associated with thalamocortical ingrowth and periphery related patterning has recently been demonstrated by studies of single cell morphology (Hoerder-Suabedissen and Molnár, 2012a,b). The establishment of area-specific thalamocortical connections is also considered to be dependent on early circuits involving subplate neurons (Molnár and Blakemore, 1995; Catalano and Shatz, 1998; Shimogori and Grove, 2005). Shimogori and Grove (2005) demonstrated that thalamocortical projections could be shifted to different cortical areas by manipulating cortical gene-expression patterns. The site of these shifts was identified in subplate and white matter.

UNDERSTANDING TRANSIENT CIRCUITS IN THALAMUS

The interactions between the early corticofugal projections and the PRN and RTN are even less understood. A critical question which remains to be addressed is how corticothalamic axons integrate into functional circuits with thalamic and reticular (RTN) neurons.

The potential role of transient circuits in cortical connections to the thalamus has not been probed. As discussed previously the subplate shares many similarities with the PRN and RTN. These largely transient populations of cells, subplate and PRN, contain early born neurons, which display early mature synaptic connections (Mitrofanis and Guillery, 1993; Molnár and Cordery, 1999; Cruikshank et al., 2010). Given the importance of transient circuits involving the subplate, we propose transient circuits comprising corticothalamic axons, PRN and RTN neurons and the thalamus may shape mature corticothalamic connections.

Anatomical tracing confirms that corticofugal axons project to the RTN and reticular neurons project to the thalamus prior to corticothalamic axons invading thalamic nuclei (Mitrofanis and Baker, 1993; Molnár et al., 1998a; Molnár and Cordery, 1999). Optical recording using voltage sensitive dyes demonstrate functional excitatory synapses between the cortex and RTN in early postnatal rat (**Figure 8**). This circuit may also contribute to the depolarization seen in the ventroposterior lateral thalamic nucleus (VPL) at this age, although with this method it is difficult to distinguish between direct cortical and indirect RTN activation of the VPL. In the adult the RTN to thalamus circuit, unlike the subplate circuit, is a substrate for feedforward and feedback inhibition to the relay cells of the thalamus (Guillery and Sherman, 2002; Jones, 2002). Immediately after birth GABAergic IPSCs are recorded in mouse thalamic relay cells in response to reticular neuron activity. This inhibitory innervation increases over early postnatal weeks although adult properties are not fully established until P9 (Warren and Jones, 1997; Warren et al., 1997; Evrard and Ropert, 2009). Thus at birth the circuit is already exhibiting adult features.

However the embryonic connectivity has not been studied and may mimic the subplate's feedforward excitation to strengthen corticothalamic connections. Indeed the RTN axons are the first to innervate the thalamus – at E14 in rats. This is days before peripheral and cortical inputs (cortical innervation arrives 3 days later at E17) thus suggesting an important developmental role of the RTN-thalamic connectivity (Mitrofanis and Guillery, 1993).

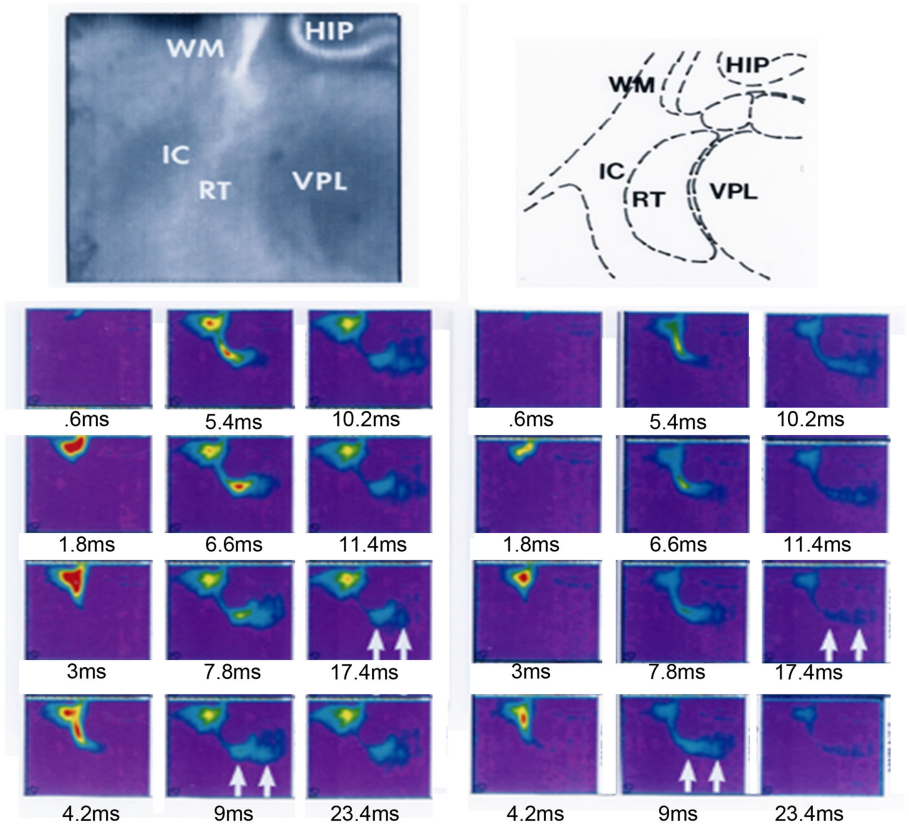

FIGURE 8 | Functional synapses revealed in the reticular thalamic nucleus after cortical stimulation in a thalamocortical slice from a P2 rat. A thalamocortical slice was prepared and stained with voltage sensitive dye RH482 according to the protocols previously described in Higashi et al. (2002, 2005). The slice was positioned to be able to observe the internal capsule, reticular thalamic nucleus (labeled RT in panels), part of the thalamus (VPL) and hippocampus (HIP). A stimulating electrode was placed into the white matter below the primary somatosensory cortex. Stimulus-induced changes in the intensity of transmitted light (700 ± 30 nm) were collected with a 128 × 128 pixel array of photosensors (70 μm^2/pixel) every 0.6 ms (Fujifilm HR Deltaron 1700, Japan) for up to 300 ms (each pixel measured the change in transmitted light intensity relative to a prestimulus reference image acquired just before the recording trial). The selected frames on the left record the response to stimulation after 0.6, 1.8, 3, 4.2, 5.4, 6.6, 7.8, 9, 10.2, 11.4, 17.4, and 23.4 ms; on the right the same frames are presented after the application of 40 μM 6,7-dinitroquinoxaline-2,3-dione (DNQX, Tocris, UK) and 50 μM 2-amino-5-phosphonopentanoic acid (APV, Sigma, USA) for 20–30 min. Sustained depolarization was observed in RTN and VPL in controls which was reduced after the DNQX and APV application, activity in RTN and VPL indicated by arrows in frames recorded at 9 and 17.4 ms in left and right frames. HIP, hippocampus; RTN, reticular thalamic nucleus; VPL, ventro-posterior lateral thalamic nucleus.

Such questions concerning how cortical axons initially integrate into thalamic circuits and what role the RTN contributes will be important in the coming years. The answers may be critical in our understanding of how the developmental process may go awry in pathologies of connectivity.

CONCLUSION

Classic anatomical research has elucidated the tightly scheduled timing and specificity of the development of corticothalamic axons. This research demonstrates how the complexity of the corticothalamic connection requires highly specific and combinatorial use of guidance mechanisms during development. From the start of their journey corticothalamic axons are encountering cell type specific molecular cues which guide them out of the cortex, along the intermediate zone, across the internal capsule and into the thalamus. They must respond to structural cues as they traverse developmental compartments at the PSPB and DTB which may direct gene-expression changes. Then as they reach the prethalamus the axons interact with intermediate cellular populations including the perireticular/corridor cells and the RTN cells perhaps gaining both guidance instructions and integrating into transient developmental circuits.

However there are still unresolved questions which we propose lie in three key areas. Firstly cortical cell subpopulations must be regarded separately, distinguishing layers 5, 6 and subplate and subpopulations within layers, rather than gathering fibers into heterogeneous groups which will likely have different cues. This will enable the next level of detail in understanding the development of highly complex circuits. Secondly research must probe the cellular and molecular identities of the telencephalic and diencephalic regions that corticothalamic axons encounter and how this compartmental environment is important for corticothalamic guidance. Thirdly work must look more closely at developmental circuitry including the details of transient circuitry and the balance between intrinsic guidance factors and external inputs. Modern techniques are now being harnessed and

are starting to yield results, including reporter gene expressing transgenic mouse lines and conditional knock-out mice, *in utero* electroporation and the recent availability of population specific markers. These models could be further exploited after sensory alterations and during cross-modal plasticity in order to probe the role of external input in generating highly specific corticothalamic circuits. Understanding the logic of development of the cortical input to thalamus is integral to the comprehension of the function of the thalamus and corticothalamic circuits.

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The importance of combinatorial gene expression in early mammalian thalamic patterning and thalamocortical axonal guidance

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The thalamus is essential for sensory perception. In mammals, work on the mouse has taught us most of what we know about how it develops and connects to the cortex. The mature thalamus of all mammalian species comprises numerous anatomically distinct collections of neurons called nuclei that differ in function, connectivity, and molecular constitution. At the time of its initial appearance as a distinct structure following neural tube closure, the thalamus is already patterned by the regional expression of numerous regulatory genes. This patterning, which lays down the blueprint for later development of thalamic nuclei, predates the development of thalamocortical projections. In this review we apply novel analytical methods to gene expression data available in the Allen Developing Mouse Brain Atlas to highlight the complex organized molecular heterogeneity already present among cells in the thalamus from the earliest stages at which it contains differentiating neurons. This early patterning is likely to invest in axons growing from different parts of the thalamus the ability to navigate in an ordered way to their appropriate area in the cerebral cortex. We review the mechanisms and cues that thalamic axons use, encounter, and interpret to attain the cortex. Mechanisms include guidance by previously generated guidepost cells, such as those in the subpallium that maintain thalamic axonal order and direction, and axons such as those of reciprocal projections from intermediate structures or from the cortex itself back toward the thalamus. We show how thalamocortical pathfinding involves numerous guidance cues operating at a series of steps along their route. We stress the importance of the combinatorial actions of multiple genes for the development of the numerous specific identities and functions of cells in this exquisitely complex system and their orderly innervation of the cortex.

Keywords: thalamus, cortex, transcription factor, axon guidance, guidance molecule, transgenic mouse, thalamocortical axon, pioneer axon

INTRODUCTION

AN OVERVIEW OF THALAMIC STRUCTURE AND FUNCTION

The thalamus is a bilaterally symmetrical structure located roughly centrally in the forebrains of mammals (**Figure 1**). In adults, its neurons are clustered into discrete groups called nuclei that are easily recognized in histological sections. Some nuclei connect a single functional pathway: for example, the dorsal lateral geniculate nucleus (LGN) receives axons from the retina and is connected to the visual cortex. The best-studied nuclei are those through which sensory information flows from the periphery to the cortex, namely the dorsal LGN (visual), the ventral complex (somatosensory), and medial geniculate complex (auditory; **Figure 1**). These sensory nuclei are found in a region of the thalamus traditionally called the dorsal thalamus to distinguish it from its neighbor, the ventral thalamus, which contains nuclei whose functions are less well understood and do not connect to the cortex. In fact, as one moves along the curved anteroposterior axis of the neural tube, the ventral thalamus is found *anterior* to the dorsal thalamus rather than ventral to it and the preference now is to refer to the dorsal thalamus as simply "the thalamus" and the ventral thalamus as "the prethalamus" (indicating its location *before* the thalamus). This nomenclature will be adopted here.

Each thalamic nucleus connects either to a specific cortical region or over a broader area comprising several related regions. Electrophysiological and axonal tracing methods have shown that the connections of the principal sensory nuclei project in an ordered topographical manner to the cortical area they innervate, generating maps of the sensory surface. It is estimated that between 70 and 99% of thalamic neurons project to the cortex and, across mammalian species, the size of the thalamus is proportional to the size of the cortex (Sherman and Guillery, 2005; Jones, 2007). Nuclei whose main role is to relay and process sensory information often have their neurons organized into discrete subregions, in some cases into layers, that relay specific aspects of the sensory modality they receive. For example, in the rodent whisker system, distinct types of sensory information such as that regarding touch versus movement of the whiskers is transmitted by separate components of the somatosensory nuclei. The thalamus is

more than merely a relay of information to the cortex. It has a much more active role, with information coming to the cortex being altered through cortical feedback via corticothalamic axons and the involvement of the reticular nucleus of the prethalamus (**Figure 1**; Sherman, 2007). Nuclei such as those near the midline (**Figure 1**) have been linked to general properties of the normal functioning brain, such as attention, arousal, and awareness (Van der Werf et al., 2002).

Thus, the thalamus and prethalamus are divisible and subdivisible into a highly complex set of anatomically and functionally distinct regions. As described in later sections, this complexity starts to be generated from the earliest embryonic stages at which the thalamus and prethalamus are first recognizable as a distinct structure, soon after closure of the neural tube.

AN OVERVIEW OF THE ANATOMICAL DEVELOPMENT OF THALAMUS AND ITS CONNECTIONS

In the mammalian embryo, the folding of the neural plate and its closure along the dorsal midline to form the neural tube generates the spinal cord posteriorly and the brain anteriorly. The early brain comprises three main vesicles, the forebrain (or prosencephalic) vesicle, the midbrain (or mesencephalic) vesicle, and the hindbrain (or rhombencephalic) vesicle. The forebrain vesicle expands to form two bilateral telencephalic vesicles and a central diencephalic vesicle that becomes engulfed by the more rapidly expanding telencephalic vesicles that rise up on either side of it. The telencephalon generates the cerebral cortex in its dorsal component (known as the pallium) and the basal ganglia in its ventral component (known as the subpallium). The major derivatives of the diencephalon are the thalamus, hypothalamus, prethalamus, and epithalamus.

The thalamocortical tract, which comprises the axons of thalamic neurons that innervate the cortex, starts to form soon after the thalamus and prethalamus develop as distinct anatomical entities and continues to grow and expand at the same time as thalamic nuclei emerge and mature. This makes it likely that the mechanisms of thalamic nuclear formation and thalamocortical axonal development are intimately intertwined. The formation of the thalamocortical tract is achieved by the guidance of growing axons from their cell bodies within the thalamus through a complex three-dimensional route to the cortex (**Figure 2**). The fact that thalamic axons take such a complex route over a relatively

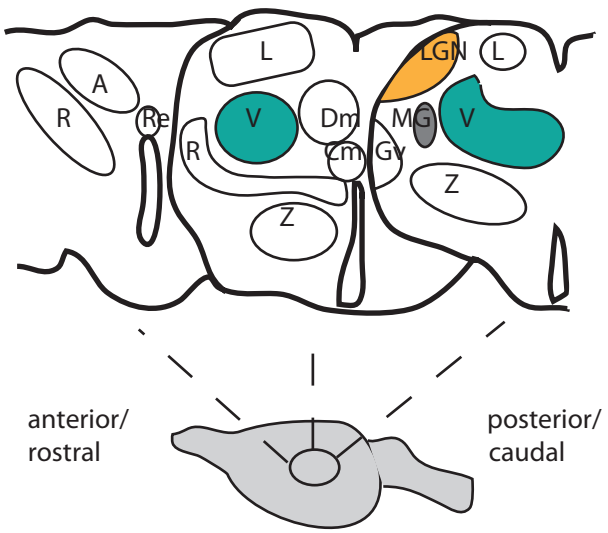

FIGURE 1 | The layout of some of the main nuclei in the adult rodent thalamus. At the top are drawings of three coronal sections cut through the thalamus from anterior (or rostral) on the left to posterior (or caudal) on the right. The diagram below indicates their approximate positions in the brain. Three principal sensory nuclei are colored: dorsal lateral geniculate nucleus (dorsal LGN, orange); ventral nuclei (V, green); medial geniculate (MG, gray). Examples of other major nuclei are: A, anterior nuclei; Cm, centromedial nucleus; Dm, dorsomedial nucleus; Gv, ventral lateral geniculate nucleus; L, lateral nuclei; R, reticular nucleus; Re, reunions nucleus; Z, zona incerta.

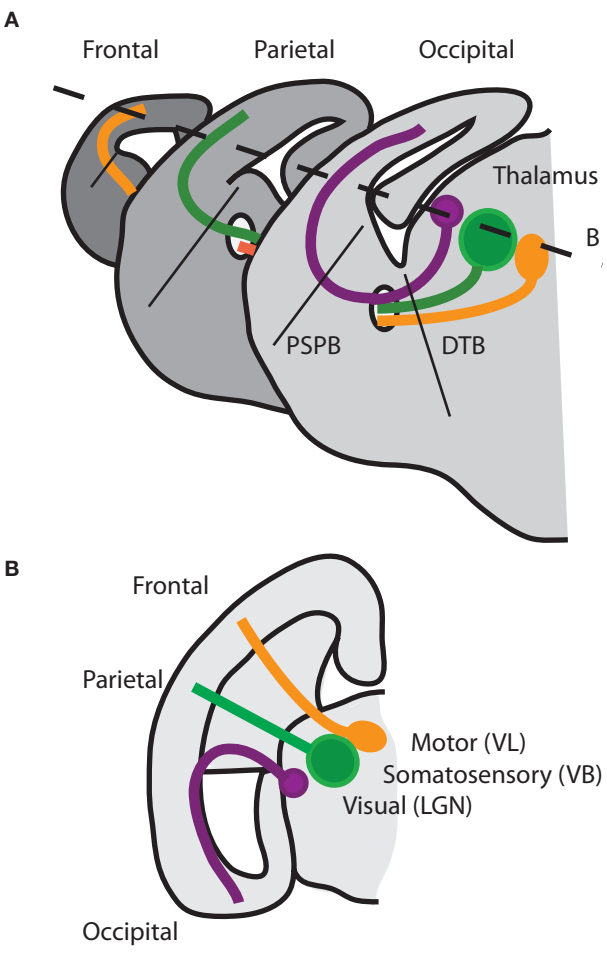

FIGURE 2 | The three-dimensional topography of thalamocortical connections in the mouse brain. (A) Three coronal sections through the brain around the time of birth, from anterior on the left to posterior on the right. Axons from different thalamic nuclei project in topographic order to innervate their specific cortical regions: dorsal lateral geniculate nucleus (dorsal LGN) innervates occipital (visual) cortex (purple); ventrobasal nuclei (VB) innervate parietal (somatosensory) cortex (green); ventrolateral nuclei (VL) innervate frontal (motor) cortex (orange). Thalamocortical axons cross the diencephalic–telencephalic boundary (DTB) and the pallial–subpallial boundary (PSPB). **(B)** Horizontal section taken in the plane shown by broken line in **(A)**.

long distance makes this tract a particularly fascinating example in which to analyze the mechanisms of axon guidance and suggests that the guidance mechanisms are likely to be multiple.

Initially, thalamocortical axons extend anteroventrally through the prethalamus; they then turn laterally into the ventral part of the telencephalon, or subpallium, by crossing the diencephalic–telencephalic boundary (DTB); finally, they turn once again, this time dorsally, to reach the cortex in the pallium by crossing the pallial–subpallial boundary (PSPB; **Figure 2**). At the same time, axons extend from the cortex along the same path, forming reciprocal corticothalamic connections with the thalamus (reviewed by López-Bendito and Molnár, 2003; Price et al., 2006). The thalamus and cortex are interconnected in an ordered fashion such that adjacent cells in the presynaptic structure project, as a general rule, to adjacent structures in the postsynaptic structure. Thalamocortical axons are ordered as they grow through the subpallium toward the cortex, indicating that topography is present in the tract before axons arrive at their final target (reviewed in Garel and Rubenstein, 2004). A detailed understanding of the precision of this topography is still elusive. One issue that needs to be resolved is exactly how a collection of thalamic nuclei and sub-nuclear structures distributed in three-dimensional space in the thalamus and a set of points distributed across the two dimensions (rostrocaudal and mediolateral) of the cortical sheet map onto each other. To what extent are the neighbor-relationships of axons from adjacent neurons in one structure maintained as they grow to the other? At present, our insight into the topography of thalamocortical projections remains relatively crude.

MOLECULAR PATTERNING OF THE DEVELOPING THALAMUS

PREVIOUS STUDIES

For thalamocortical axons to navigate a complex route through the forebrain while maintaining some degree of topographic order they need to be equipped with the molecules necessary to allow appropriate responses to the guidance cues they will encounter from other cell bodies or axons, including those of other thalamic neurons. One important way in which topographic order might be maintained is that the neurons of cells in different parts of the developing thalamus might express different sets of such molecules, preventing them from becoming mixed up. In other words, the pattern of molecules expressed across the thalamus might be critical for ensuring appropriate navigation. There is now strong evidence that this is the case.

The establishment of inter-neuronal differences in the expression of molecules involved in axon guidance, such as receptors for extracellular guidance cues, is controlled by the thalamic expression of transcription factors that determine which of the genes that encode such cues are activated or repressed in any particular cell. A critical question, therefore, is whether, and if so to what extent, different regions of the thalamus express different sets of regulatory transcription factors and downstream guidance molecules, such as receptors, before they start to grow axons toward the cortex. The sets of genes expressed within each thalamic region are certain to overlap partially and the molecular identity of each region will depend on the combination of transcription factors that it expresses and their levels.

By about halfway through gestation in mice, the thalamus comprises the progenitor cells that will generate most of the postmitotic thalamic neurons over the following days. A study by Vue et al. (2007) defined two major progenitor domains in the thalamus, one large posterior domain named pTH-C that expresses transcription factors such as Ngn2 and Olig2 and a much smaller anterior domain named pTH-R that expresses transcription factors such as Nkx2.2 and Mash1 (**Figure 3**). At a finer level, several detailed and elegant expression analyses have shown that from an age before thalamocortical axons start to navigate to the cortex thalamic progenitors and their newly generated postmitotic neurons are subdivided molecularly by the expression of transcription factors and other molecules into regions that are likely to be the forerunners of the thalamic nuclei of the mature structure (Nakagawa and O'Leary, 2001; Vue et al., 2007, 2009; Kataoka and Shimogori, 2008; Suzuki-Hirano et al., 2011; Yuge et al., 2011). More work is required to determine the relationship between these early patterns of thalamic gene expression and specific mature thalamic nuclei. Most previous studies looked for possible correspondence between the early expression of individual genes and the later development of specific thalamic nuclei and further insights might be gained by examining the early patterning conferred by combinations of transcription factors, since cells that co-express one particular combination are likely to develop differently to cells that express a different combination even if some of the individual genes expressed are the same. In the following section we describe an analysis using existing data on the expression of individual genes to deduce patterns of co-expression of these genes. The results of a theoretical analysis such as this might guide us toward combinations of genes that might be most worth studying experimentally.

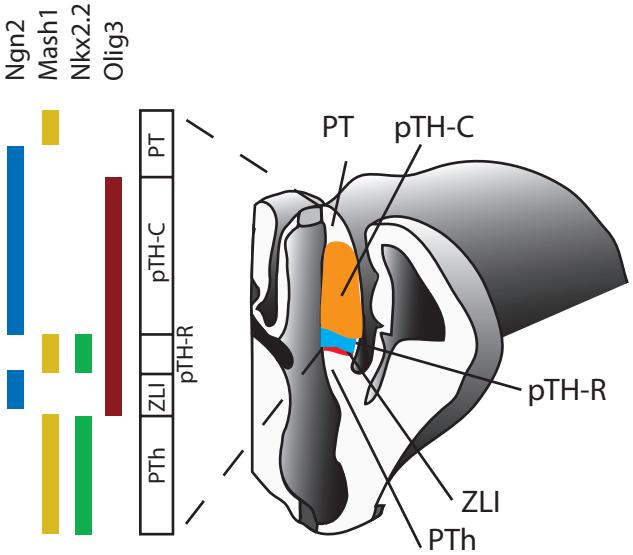

FIGURE 3 | Examples of transcription factor expression patterns in the progenitor domain of the embryonic mouse diencephalon. The thalamus is divided into the large pTH-C (orange) and the small anterior pTH-R (blue). Abbreviations: PT, pretectum; PTh, prethalamus; ZLI, zona limitans intrathalamica. Adapted from Vue et al. (2007).

AN ANALYSIS OF EXISTING DATA IN THE ALLEN DEVELOPING MOUSE BRAIN ATLAS

The Allen Developing Mouse Brain Atlas (ADMBA) contains images of both Feulgen Yellow DNA counterstained 20 μm-thick tissue coronal sections through mouse embryonic brains and *in situ* hybridizations providing data on the expression of a wide range of genes that pattern the early thalamus. Its detail and high-resolution images give it the potential to be a powerful resource for the analysis of gene expression and co-expression patterns. Such data has many possible uses. For example, previous work has developed methods for semi-automatic annotation of gene expression patterns as a step toward generating atlases of the expression of all genes in the brain (Carson et al., 2005). Here, we tested whether such data could be used to deduce the patterns of co-expression of key transcription factors in the embryonic thalamus at an age that predates the establishment of thalamocortical connections.

Serial sections were downloaded from E13.5 embryos. Within the thalamus, cell densities varied from low to high (**Figure 4**) and cells could be counted manually in such areas (**Figure 4B**), but this was very time-consuming and so an automatic method was devised. The method was based on the use of Hessian matrices to describe mathematically local morphological features across an image, such as the curvatures of edges (Sato et al., 1998). In the ADMBA, counterstained nuclei have edges that are clear enough to allow the application of this approach. The calculation of the eigenvalues of the Hessian matrices and their subsequent classification allows the identification of round or oval shapes that correspond to cells. Data from this automated approach were compared with data obtained manually. For each image, the distance was calculated between each point allocated manually to a cell nucleus and its nearest neighbor among points allocated automatically, and where the distance was less than the radius of a nucleus (5 μm) it was designated as a hit. This method gave hit rates of 90%, indicating excellent correlation between the automatic and manual methods, and so the former was used to measure density across all E13.5 coronal sections through the entire thalamus. The surface of each section was divided into tiles (20 μm × 20 μm) using a grid and cell density was measured in each tile.

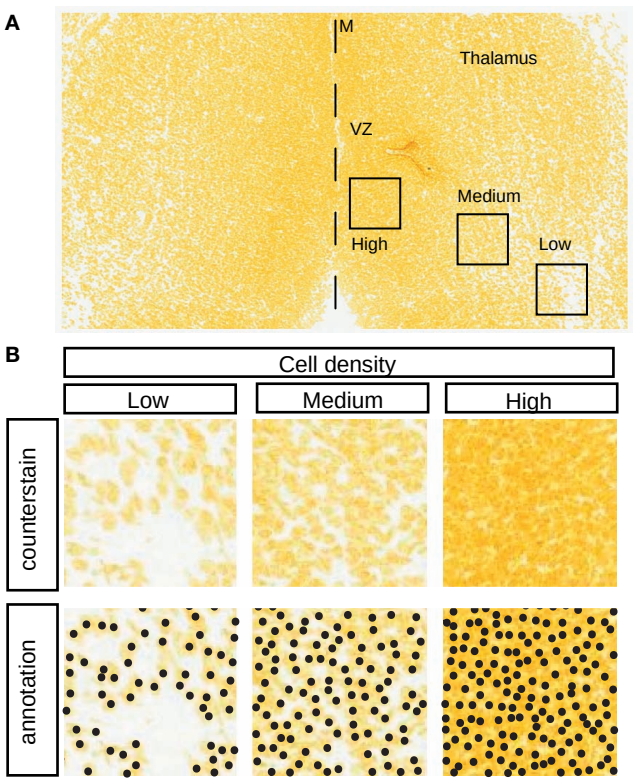

FIGURE 4 | Measuring cell density in the thalamus. (A) E13.5 coronal thalamic sections counterstained with the DNA stain Feulgen-HP yellow from ADMBA. There is substantial variation in cell density in different parts of the thalamus, for example cell density is very high in the proliferative ventricular zone (VZ) adjacent to the thalamic midline (M, dashed line). Boxed areas indicate areas of high, medium, and low density examined further in **(B)**. **(B)** Top panels show raw image examples of low, medium, and high cell densities taken from thalamic areas indicated in **(A)**. Bottom panels show manual annotation of these data where the operator identifies nuclei by eye and marks each one with a black dot.

Gene expression information was extracted from images of *in situ* hybridizations in the ADMBA based on color and brightness levels (an example of raw data is shown in **Figure 5A**). Each pixel across the image was categorized as: (i) no tissue (white; bright); (ii) non-expressing counterstained tissue (yellow; intermediate brightness); (iii) expressing tissue (gray/black; very dark). Each image was subdivided into 20 μm × 20 μm tiles (**Figure 5B**; each small square represents one tile) to generate data that could be readily matched to the density information obtained as described above. The level of expression for each tile was calculated with:

$$\text{tile expression level} = \frac{\text{number of expressing pixels}}{\text{number of expressing pixels} + \text{number of non-expressing pixels}}$$

This gave a number for each tile indicating of how much of the tissue in it showed *in situ* hybridization staining. This number did not take into account cell density.

Data were then combined from *in situ* hybridizations with each of a series of patterning genes. Data from images of *in situ* hybridizations for one gene were matched to those from immediately adjacent images of the expression of another gene. The range of possible double-labeling was then estimated.

In cases where the expression level (as defined by the equation above) of one gene and that of a second gene within a given tile add to more than 100%, we can conclude that there must be some double-labeling. The minimum proportion of double-labeling tells us whether two genes necessarily overlap in a given tile, and how big that overlap is. For example, if the two genes cover 60 and 70%, they must overlap by at least 30%, although they could overlap by as much as 60%. The maximum double-labeling gives a measure of how much of the tissue could be expressing both genes at the same time. Since we have values for the cell density in every tile (obtained as described above), we can combine data on proportions of double-labeled cells with cell densities in each tile to obtained values in terms of numbers of cells per tile (due to its

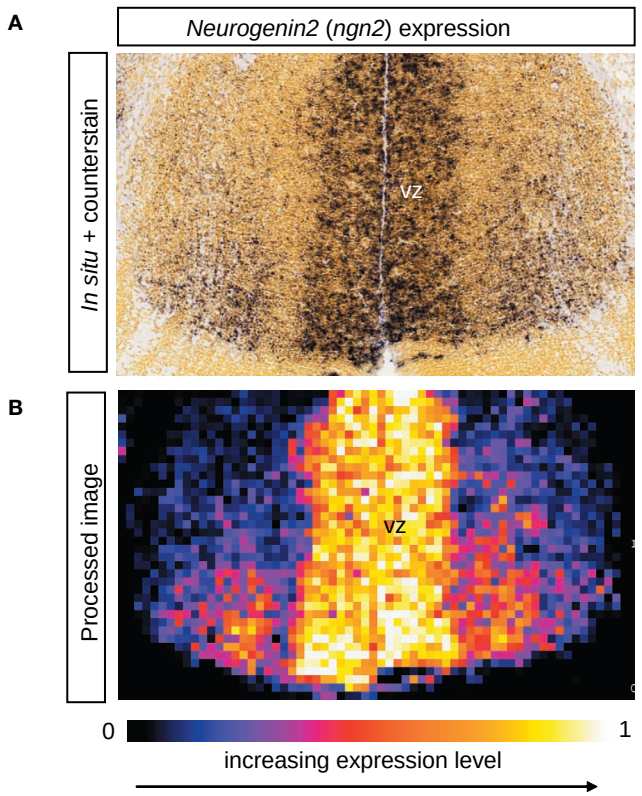

FIGURE 5 | Measuring gene expression from ADMDA images. (A) *In situ* hybridization for *neurogenin2* (*ngn2*) mRNA in E13.5 thalamus. The *in situ* signal is a brown color and the section is also counterstained with Feulgen yellow. **(B)** This processed image is obtained by dividing the image in **(A)** into 20 μm × 20 μm square tiles and calculating the *ngn2* expression level for each tile (see main text) with expression level ranging from 0 (minimum expression) to 1 (maximum expression) with tile-values color coded as illustrated. This analysis identifies highest *ngn2* expression in tiles at the ventricular zone (VZ) with expression diminishing laterally, in excellent agreement with visual inspection of the original *in situ* in **(A)**.

size, each tile contained a maximum of 11 cells). Thus, in each tile, the proportion of double-labeled cells was multiplied by the cell density.

The combination of minimum and maximum double-labeling values for each pair of genes provides information on the number of cells that *must* be co-expressing both genes and the potential number of cells that *could*. Examples of the outcomes of these analyses are shown in **Figures 6–8**. It is important to point out that these theoretical outcomes, which would need to be verified experimentally, necessarily involve assumptions. One of the assumptions, which might affect the estimates of minimum double-labeling, is that the expression pattern of each gene is similar through the depth of each 20 μm-thick section. This assumption might become less accurate in areas of high cell density in which, given an average cell diameter of about 10 μm, a maximum of two entire cells or one entire cell and two cell fragments might be located above each other at any point across the surface of the section. The more positions there are at which labeling is contributed by only one of the cells stacked above each other, the greater will be the overestimate of minimum double-labeling.

Figure 6 shows expression of the genes encoding the transcription factors Gbx2 and Ngn2, at two different rostrocaudal levels in the thalamus. A few cells in the strip of proliferating tissue adjacent to the third ventricle express *gbx2*, but most cells throughout the population of differentiating thalamic cells express it. Expression is particularly strong at the lateral edges of the thalamus (upper arrow in **Figure 6**). On the other hand, *ngn2* is expressed by proliferating cells adjacent to the ventricle as well as by differentiating cells positioned centrally within the thalamus. There is a gradient of *ngn2* expression, which increases with proximity to the zona limitans intrathalamica (ZLI), which marks the anterior boundary of the thalamus at this age. Examination of the possible range of *gbx2-ngn2* co-expression across the thalamus revealed that many cells must co-express in a medial strip adjacent to the proliferative zone. Moving laterally, at least a few cells in the anterior part of the thalamus, toward the ZLI, must co-express whereas none are likely to co-express in the most lateral regions where *gbx2* expression is strong. Thus, the thalamus can be subdivided at E13.5 into an anteromedial zone in which cells express *gbx2* and *ngn2* together and a lateral zone in which cells express *gbx2* alone. The relative location of the regions of anterior thalamus in which cells co-express *gbx2* and *ngn2* (lower arrow in **Figure 6**) correspond roughly to the relative location of the anterior nuclei of the adult thalamus. These nuclei are further subdivided and one can speculate that the most anteromedial zone of high double-labeling might develop into the mature anteromedial nuclei whereas the adjacent anterior zone with lower proportions of double-labeled cells might develop into another of the anterior group, e.g., the slightly more lateral anteroventral nucleus. Clearly, the early double-labeled cells need to be followed through development to confirm or refute such suggestions.

Figure 7 shows a further example, in this case of the expression and estimated co-expression of *olig2* and *otx2*. In this case, we can deduce that the most significant numbers of cells that must, or might, co-express these transcription factors form a small subset of cells (arrows in **Figure 7**) expressing one or other alone within the body of the thalamus in a medial location. This relative position suggests that these cells might conceivably generate the centromedial nucleus of the mature thalamus.

Figure 8 shows a third example, in this case of the expression and estimated co-expression of *gbx2* and *otx2*. In this case, co-expression levels are potentially greatest in anteromedial locations, suggesting a contribution of this combination of expression to the specification of the anteromedial nuclei.

Whereas the specifics of these suggested associations between gene co-expression patterns and mature nuclei are highly speculative, these analyses highlight an important overall conclusion, namely that the thalamus is extremely highly patterned molecularly from the earliest stages at which is contains significant number of postmitotic neurons and before it starts to send axons toward the cortex. This conclusion is reinforced by data on the expression of potential downstream effectors of the actions of these transcription factors, such as the Cadherins or the Eph/ephrins. **Figure 9** shows, in a rostral-to-caudal series of sections through the E13.5 thalamus,

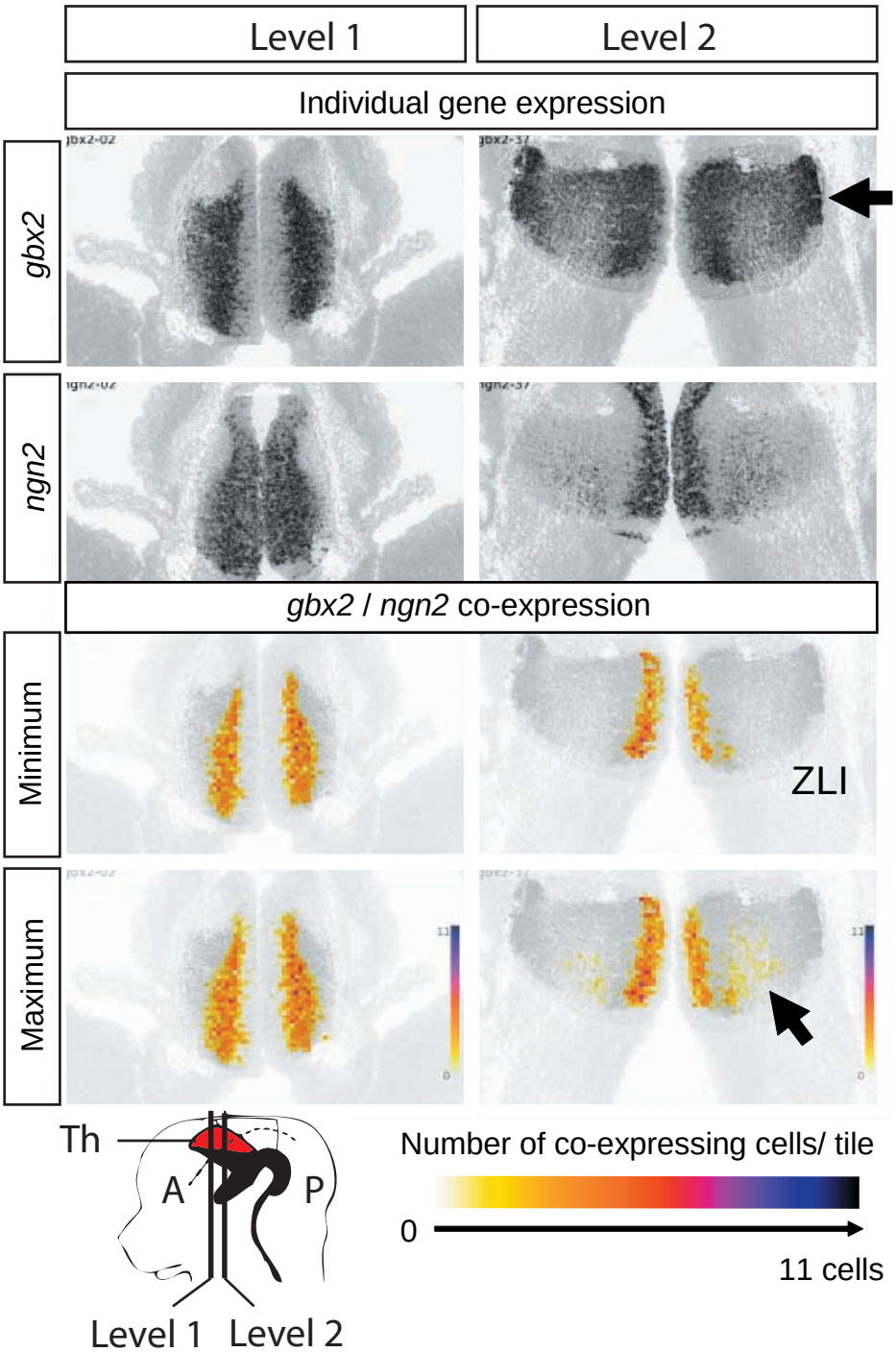

FIGURE 6 | *Gbx2* and *ngn2* co-expression in E13.5 thalamus. Top four panels show the raw data from ADMBA: *in situ* hybridizations for *gbx2* and *ngn2* at two coronal levels through the thalamus. The two levels are shown in the schematic at the bottom of the Figure. The lower four panels show representations of *gbx2* and *ngn2* co-expression obtained by combining gene expression and cell density information and calculating the (1) maximum and (2) minimum number of cells in each 20 μm × 20 μm tile co-expressing *gbx2* and *ngn2*. The values for each tile range from 0 to 11 (the maximum number of cells that can fit in a single tile) and are color coded as illustrated at the bottom. Arrows indicate high level of *gbx2* expression in the mantle zone (top) and double-label anteriorly close to the zona limitans intrathalamica (ZLI; bottom). In the schematic, the thalamus (Th) is in red.

the complex expression patterns of two example, *cadh8* and *epha4*, revealing the molecular diversity and complex patterning of the cell surface constitutions of even the earliest thalamic cells.

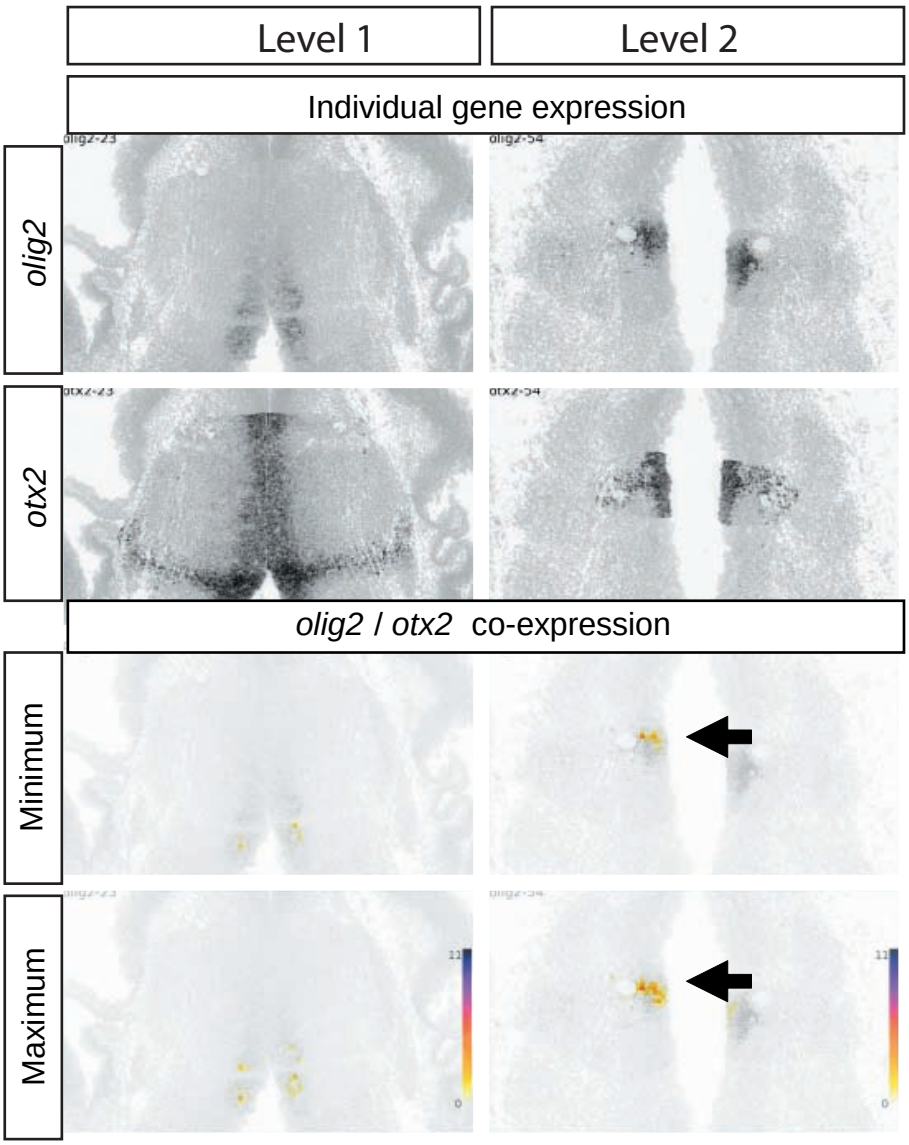

FIGURE 7 | *Olig2* and *otx2* co-expression in E13.5 thalamus. Top four panels show the raw data from ADMBA: *in situ* hybridization for *olig2* and *otx2* at two coronal levels through the thalamus (see **Figure 6**). The lower four panels show representations of *olig2* and *otx2* co-expression obtained by combining gene expression and cell density information and calculating the (1) maximum and (2) minimum number of cells in each 20 μm × 20 μm tile co-expressing *olig2* and *otx2* (as in **Figure 6**). A small thalamic area in the ventricular zone is identified where two to six cells in each tile must be co-expressing *olig2* and *otx2* (arrows).

EVIDENCE FOR THE IMPORTANCE IN THALAMIC AXONAL DEVELOPMENT OF REGIONALLY EXPRESSED THALAMIC TRANSCRIPTION FACTORS

One of the ideas expressed earlier in this review is that early thalamic patterning is likely to invest in axons growing from different parts of the thalamus the ability to navigate in an ordered way to their appropriate area in the cerebral cortex. If this is the case we would predict that disruption of the expression of pattering molecules would disrupt the ordered development of thalamocortical axons. A number of mouse knock-out studies have shown that a variety of transcription factors play an important role in thalamocortical tract development. Mice lacking transcription factors such as Pax6, Mash1, Tbr1, and Ebf1 have thalamocortical axon pathfinding defects (Kawano et al., 1999; Miyashita-Lin et al., 1999; Tuttle et al., 1999; Pratt et al., 2000, 2002; Garel et al., 2002; Hevner et al., 2002; Jones et al., 2002) but it is not always clear whether this is because of a direct effect within the thalamus rather than an indirect result of defects elsewhere. In the case of Pax6, *in vitro* co-culture experiments have provided evidence that Pax6 expression is required within the thalamus itself for correct axon guidance (Pratt et al., 2000). Explants from wild type ventral telencephalon were cultured in contact with wild type or *Pax6*$^{-/-}$

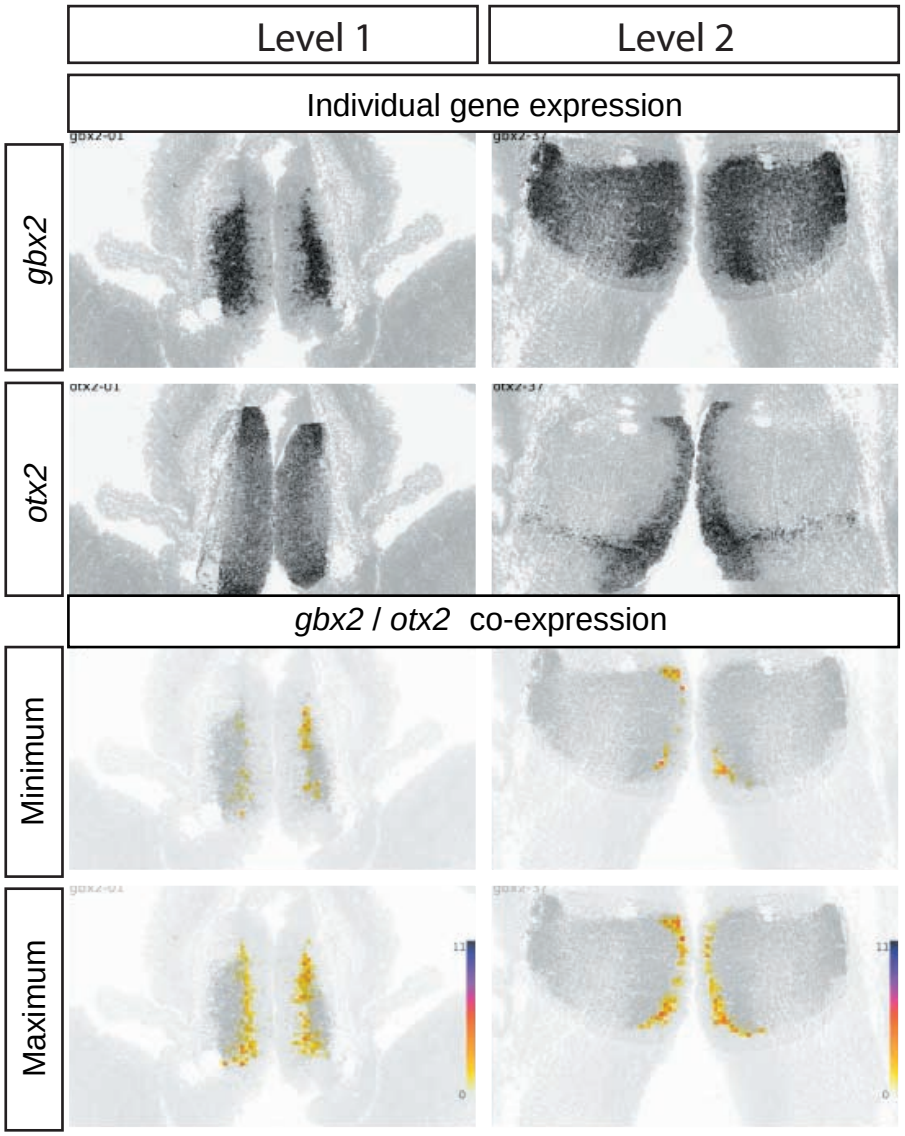

FIGURE 8 | *Gbx2* and *Otx2* co-expression in E13.5 thalamus. Top four panels show the raw data from ADMBA: *in situ* hybridizations for *gbx2* and *otx2* at two coronal levels through the thalamus (see **Figure 6**). The lower four panels show representations of *gbx2* and *otx2* co-expression obtained by combining gene expression and cell density information and calculating the (1) maximum and (2) minimum number of cells in each 20 μm × 20 μm tile co-expressing *olig2* and *otx2* (as in **Figure 6**). A strip of co-expressing cells, narrow in the mediolateral axis but extending along the dorsoventral axis of the thalamus, is identified where two to six cells in each tile must be co-expressing *gbx2* and *otx2*.

thalamus. When wild type thalamus was cultured with wild type ventral telencephalon, thalamic axons entered the ventral telencephalon and turned toward the cortex in a similar manner to that observed *in vivo*. When *Pax6*$^{-/-}$ thalamus was cultured with wild type ventral telencephalon a smaller number of thalamic axons entered the ventral telencephalon, they extended a shorter distance and failed to make a dorsal turn as in the previous experiment. This result showed that axons lacking Pax6 fail to grow in the correct manner even when confronted with an environment containing guidance cues that normal thalamic axons can respond to. This indicates that Pax6 expression is required within the thalamus for its axons to navigate.

One of the most interesting transcription factors in the present context is Ngn2, whose graded expression across the thalamus immediately prior to axonal outgrowth was discussed above and shown in **Figure 6**. By analyzing mice lacking Ngn2, Seibt et al. (2003) showed that Ngn2 specifies cell autonomously the projection of thalamic neurons to frontal cortical areas. The mechanism by which this is achieved is particularly interesting. Ngn2 determines the projection of thalamic neurons to specific cortical domains by specifying the responsiveness of their axons to cues encountered in tissues through which they navigate to attain the cortex. In other words, this transcription factor fits the role predicted by the hypothesis that early thalamic patterning is likely to

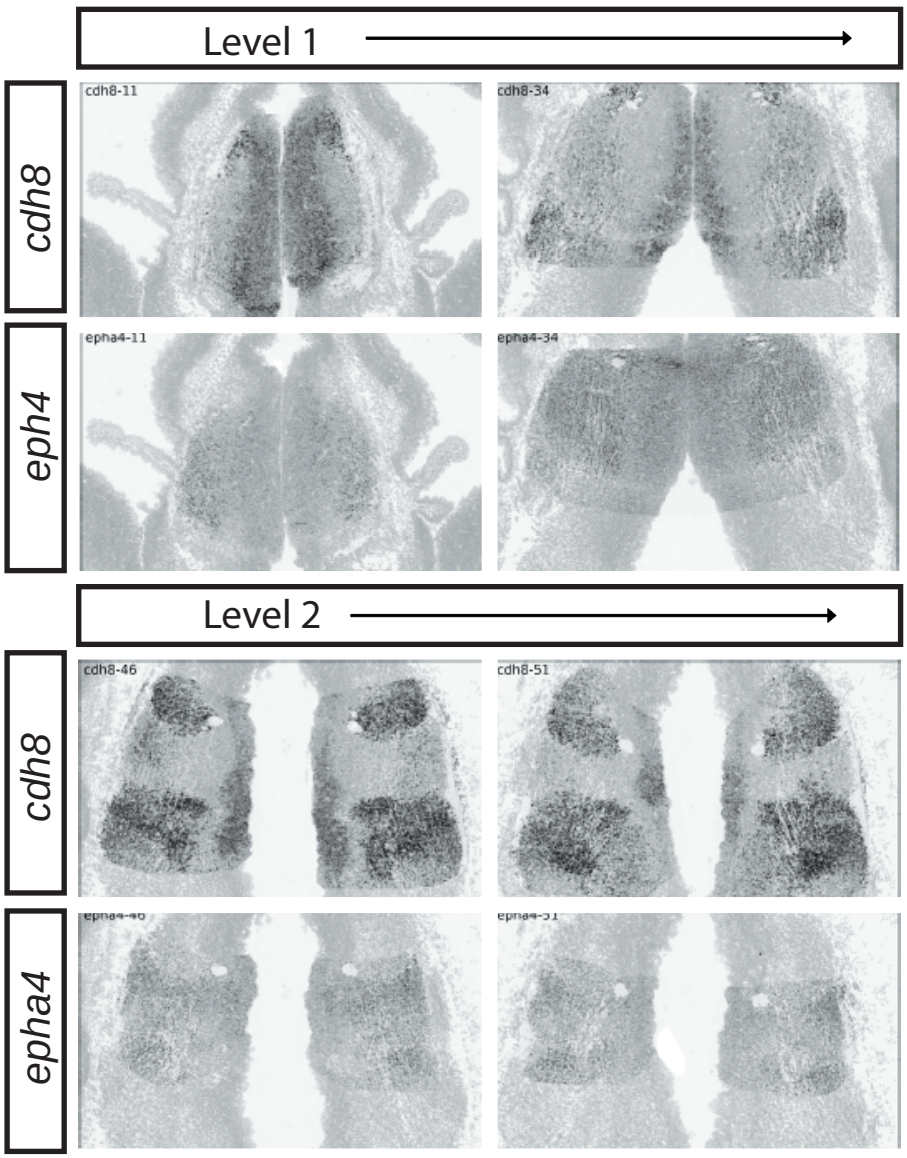

FIGURE 9 | Complex expression patterns of cell surface molecules in the developing thalamus at E13.5. *In situ* hybridizations for *cadh8* and *epha4* running from Level 1 past Level 2 (Levels shown in **Figure 6**). Images are taken from ADMBA.

invest in axons growing from different parts of the thalamus the ability to navigate in an ordered way to their appropriate area in the cerebral cortex.

The tissue through which thalamocortical axons navigate includes the ventral telencephalon, or subpallium, and much has been learned in recent years about the mechanisms that guide these axons through this and other intermediate regions. These mechanisms will be reviewed in the following sections.

THE GUIDANCE OF AXONS FROM THE THALAMUS

GUIDEPOST CELLS AND AXONS

Before considering further the mechanisms that might ensure that axons from each part of the developing thalamus target their correct cortical area, we shall consider what is known about the mechanisms that guide the earliest axons out of the thalamus and toward the cortex. It seems likely that these early axons grow in an orderly way but as yet direct evidence on how ordered they are is lacking. Much of our understanding of these early phases of thalamocortical growth center on the results of studies examining the role of other sets of *neurons* and *axons* that pioneer or guide the thalamocortical axons in the direction of the cortex. This work predates our knowledge of the *molecules* that provide guidance and so we shall discuss the importance of pioneer axons first.

The importance of early pioneer or guidepost cells and axons in the development of persistent axonal pathways was recognized decades ago in other systems in other species. Guidepost cells are found at intermediate positions along the route of a particular axonal tract where they provide guidance information

to navigating axons at important choice points. Initially described in the grasshopper limb bud, guidepost cells have since been proposed in other systems, including the developing mouse thalamocortical tract.

During grasshopper (*Locusta migratoria*) development the first two neurons that differentiate within the grasshopper limb bud designated as Ti1 extend their axons along a defined route through the limb bud toward the central nervous system (Bate, 1976). Axons from later born neurons follow the same path as the Ti1 pioneer axons to form a major nerve trunk within the adult limb (Keshishian, 1980). The route taken by these pioneers is not a straight one, however: the axons make two near 90˚ turns as they traverse the limb bud. At specific points along its route the Ti1 axon makes contact with three cells designated F1, F2, and CT1. It was proposed that these cells act as intermediate targets for the Ti1 axon. These cells were termed "guidepost cells." Their importance was later demonstrated by a study which selectively destroyed the CT1 guidepost cells during limb bud development. It was found that in the absence of these cells the Ti1 axon does not make the second 90˚ turn toward the CNS and instead growth of the axon is either arrested at this point or continues abnormally within the limb bud. This demonstrated that guidepost cells are necessary for the guidance of pioneer axons in the grasshopper limb (Bentley and Caudy, 1983).

In the developing mouse thalamocortical system, it has been proposed that populations of guidepost cells are present along the route taken by thalamocortical axons and that these cells act as intermediate targets for thalamocortical axons or corticothalamic axons. These cells act in a different manner to those found in the grasshopper limb bud in that they extend axons, and it is thought that these axons then act as a scaffold to guide the later thalamocortical and corticothalamic axons. Axon tracing studies have identified populations of cells in two main locations that are proposed to act as guideposts (**Figure 10**).

First, cells within the reticular nucleus of the prethalamus are known to project axons to the thalamus (Ohara and Lieberman, 1985). These axons form along the same route that later thalamocortical axons will follow and are present throughout the period during which thalamocortical axons navigate through the diencephalon (Mitrofanis and Baker, 1993; Mitrofanis and Guillery, 1993). A second population of cells project axons to the thalamus from the internal capsule at the same time as axons from the reticular cell group (Métin and Godement, 1996; Molnár et al., 1998; Braisted et al., 1999). It has been suggested that these two populations of axons guide thalamocortical axons through the diencephalon and into the ventral telencephalon (reviewed in Molnar et al., 2003; **Figure 10**).

This hypothesis is compatible with the results of several studies in mutant mice. In mice lacking the transcription factor Mash1, thalamocortical axons do not cross the diencephalic–telencephalic border but instead remain within the diencephalon. In these mice there are no early projections from cells within the primitive internal capsule to the thalamus (Tuttle et al., 1999). In mice lacking the transcription factor Pax6, the thalamocortical tract also fails to form properly and, again, this corresponds with a failure of axonal projections from the internal capsule to the thalamus (Jones et al., 2002; Pratt et al., 2002). In mice that lack Emx2 thalamocortical axons are misrouted in the ventral telencephalon and fail to reach the cortex. As in the previous examples this phenotype is associated with the loss of projections from the internal capsule and in addition a reduced number of axonal projections from cells within the prethalamic reticular nucleus (López-Bendito et al., 2002). Although these observations fit the proposal that the axons from guidepost cells located within the prethalamus and the primitive internal capsule are responsible for guiding thalamic axons into the ventral telencephalon, further work is needed since these mutant mice display wide-ranging morphological and molecular abnormalities and it is possible that the loss of guidepost cell axons may not be the primary cause of defects seen in the thalamocortical tract. More recent experiments by Zhou et al. (2008) demonstrated

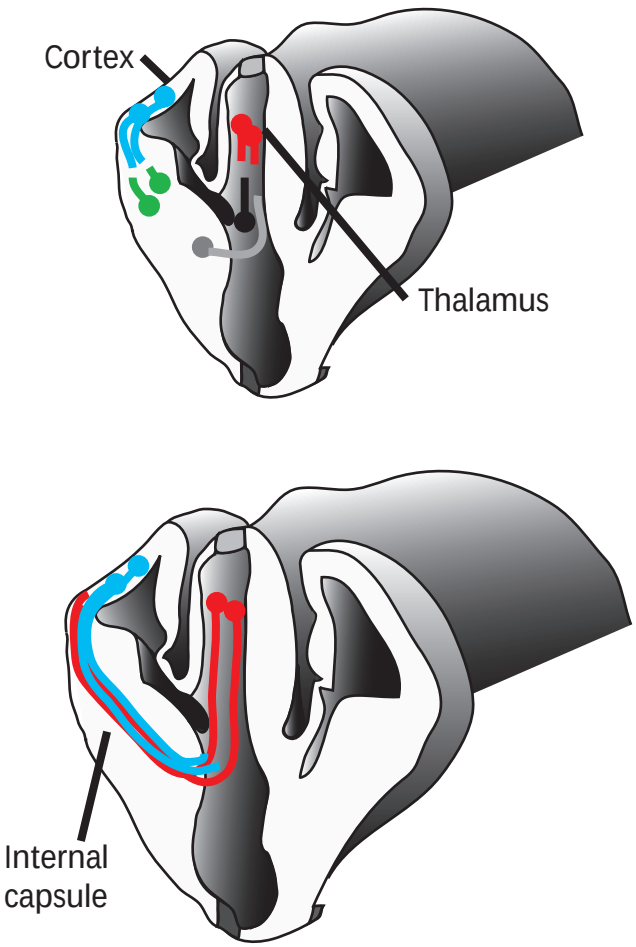

FIGURE 10 | Pioneer neurons and axons in the developing thalamocortical tract. As thalamic axons (red) grow they are thought to be guided by the axons of neurons in the reticular nucleus of the prethalamus (black) the site of the future internal capsule (gray). It has been suggested that these two populations of axons guide thalamocortical axons through the diencephalon and into the ventral telencephalon. Within the primitive internal capsule another population of cells project axons in the opposite direction, into the cortex (green). These axons are thought to guide descending corticothalamic axons (blue) into the ventral telencephalon across the pallial–subpallial boundary. Recent evidence suggests that for thalamic axons (red) to grow from subpallium to cortex they require guidance by cortical efferents (blue).

loss of both thalamocortical and corticothalamic axons in mice with conditional deletion of the *Celsr3* gene restricted to the *Dlx5/6*-expressing cells in the ventral telencephalon around the site of the internal capsule. They suggested that a likely cause of these defects was the need for *Celsr3* expression by guidepost cells, the probability of this explanation being increased by the restricted nature of the deletion to a region that contains such guidepost cells.

Within the primitive internal capsule another population of cells project axons in the opposite direction, into the cortex (Molnár et al., 1998; **Figure 10**). These axons are thought to guide descending corticothalamic axons into the ventral telencephalon across the PSPB (the important anatomical and gene expression boundary dividing the pallium from the subpallium). Recent experiments in our laboratory tested the hypothesis that descending corticofugal are, in their turn, important for guiding thalamocortical axons across the PSPB. The work used conditional mutagenesis to assess the effects of blocking corticofugal axonal development without disrupting thalamus, subpallium, or the PSPB. The results provided the most compelling evidence to date that for thalamic axons to grow across the PSPB from subpallium to cortex they require guidance by cortical efferents (Chen et al., 2012). The navigation of thalamic axons can be seen, therefore, as a series of steps with guidance being provided at each by different sets of reciprocal axons from intermediate or final targets.

THE GROWTH OF THALAMOCORTICAL AXONS THROUGH THE VENTRAL TELENCEPHALON; THE CORRIDOR

Guidance cues and receptors bound to the cell membranes can guide thalamic axons by providing permissive territory into which the axons are channeled. Recent studies have shown that thalamic axons will only navigate successfully through the medial ganglionic eminence (MGE) if cells from the lateral ganglionic eminence (LGE) expressing the guidance cue Neuregulin-1 migrate to form a permissive "corridor" through the otherwise non-permissive MGE (López-Bendito et al., 2006; Bielle et al., 2011a,b). This migration of cells from LGE to MGE is shown in **Figure 11**. The mechanisms that regulate it are not well understood but some experiments have implicated the transcription factor Pax6 and the repellant molecule Slit2 in this process (Simpson et al., 2009; Bielle et al., 2011a,b).

As thalamocortical axons navigate from the thalamus, Pax6 expression is found not only in the cortex, but also in the proliferative zone of the LGE (at lower levels than in the cortex) and in a stream of cells within the LGE descending on the ventral side of the internal capsule, as shown in **Figure 11**. The use of conditional mutagenesis to delete Pax6 from a specific region of Pax6 expression close to the internal capsule, through which thalamic axons navigate to cerebral cortex, caused many thalamic axons to take aberrant routes, either failing to turn normally into ventral telencephalon to form the internal capsule or exiting the developing internal capsule ventrally. In this case, early pioneer axons that project from the region of the future internal capsule to the thalamus were present, and the structure that was disrupted was the corridor, which was broader and less dense than normal (**Figure 11**). These findings indicate that ventral telencephalic Pax6 is important for formation of the corridor and the thalamic and cortical axons that grow through it (Simpson et al., 2009). *In vivo* and *ex vivo* experiments in mice demonstrated that the midline repellent Slit2 orients migration of corridor neurons (Bielle et al., 2011a,b). Whether Pax6 might regulate molecules such as Slit2 to generate the corridor remains to be tested.

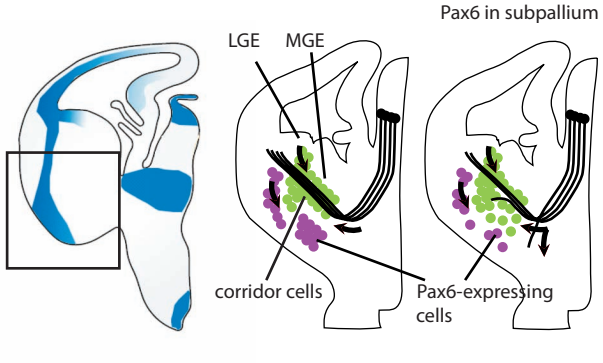

FIGURE 11 | Evidence that Pax6 plays a role in corridor formation. (A) Normal Pax6 expression (blue shading) in a section of mouse brain; cells in the boxed area are schematized in **(B,C)**. **(B)** Normally, Pax6-expressing cells (purple) are located ventral to the corridor/developing internal capsule in the medial ganglionic eminence (MGE); those located laterally comprise the lateral cortical stream migrating from the pallial–subpallial border (arrow). Islet1-expressing cells (green) migrate from the progenitor layer of the lateral ganglionic eminence (LGE; arrow) to form the corridor through which thalamocortical axons grow (arrow). **(C)** In conditional mutant embryos with selective reduction of Pax6 in specifically the ventral telencephalon but neither thalamus not cortex, there are fewer Pax6-expressing cells ventral to the corridor than normal (other populations of Pax6-expressing cells outside the region of *Pax6* deletion are not shown since they are not affected). Cells from the lateral ganglionic eminence migrate to form a corridor that is abnormally broad with a lower peak density of Islet1-expressing cells; many Islet1-expressing cells stray into the area depleted of Pax6 expression. Many thalamic axons fail to enter this abnormal corridor or exit it along its length. Data are taken from Simpson et al. (2009).

REGULATORY MOLECULES IMPLICATED IN THE GUIDANCE OF THALAMIC AXONS THROUGH THE SUBPALLIUM

A number of transcription factors other than Pax6 have been shown to be important for thalamocortical axons to navigate successfully through the subpallium. The most likely mechanism of action here is that these factors are required for the proper patterning of the subpallium and that, by regulating the cellular and molecular environment through which thalamocortical axons navigate, they create the necessary conditions and cues. For example, correct levels of the transcription factor Gli3, which is a well-known player in Sonic hedgehog signaling and whose mutation in humans causes brain and limb defects, are critical (Magnani et al., 2010). Mutant mice hypomorphic for *Gli3* ($Gli3^{Pdn/Pdn}$) show morphological abnormalities of the LGE associated with severe pathfinding defects of thalamocortical axons in the ventral telencephalon. Transplantation experiments demonstrated that the intrinsic ability of the $Gli3^{Pdn/Pdn}$ ventral telencephalon to guide thalamocortical axons is compromised, rather than the defects being caused primarily in the thalamus. The molecular networks that require

regulation by Gli3 to ensure correct thalamocortical development are not known.

Another example comes from the work of Lakhina et al. (2007). They showed that the transcription factor Lhx2 is essential for normal thalamocortical tract pathfinding because it has functions that are localized in the ventral telencephalon rather than in the thalamus. Interesting, the absence of Lhx2 in the ventral telencephalon selectively disrupts a subset of thalamic axons and their topography. Unlike the case with Gli3, this indicates that loss of Lhx2 causes a specific rather than a general perturbation of cues in this structure.

Crossing the knowledge-gap between high level transcription factors and the effectors of axons guidance that they ultimately regulate, we find quite a considerable body of literature on how the guidance cues themselves can influence the guidance of thalamocortical axons. It has been shown that certain guidance cues can have a chemoattractive or chemorepulsive effect on growing thalamic axons. One example of a well-known guidance molecule known to influence thalamocortical axon guidance in this way is Netrin-1. It is expressed in the ventral telencephalon and has been shown *in vitro* to act as an attractant for a subset of thalamic axons (Braisted et al., 1999, 2000; Bonnin et al., 2007). Netrin-1 may also act as a repulsive cue. Other studies have shown that Netrin-1 receptor DCC mediates an attractive response in the growth cone while receptor Unc5 mediates a repulsive response (reviewed by Moore et al., 2007). A close inspection of the dynamics of expression of different Netrin receptors by different sets of thalamic neurons is particularly important to understand the full activity of this molecule in thalamocortical axon guidance.

We now know that the topography of thalamocortical axon projections is initiated before they reach the cortex, in the ventral telencephalon. One of the interesting features of the expression of Netrin-1 in the ventral telencephalon is that it is graded from high levels anteriorly to low levels posteriorly. A question addressed by Powell et al. (2008) is whether this concentration gradient is required for the topographic sorting of thalamocortical axons to distinct cortical domains. Their work showed that Netrin-1 is a chemoattractant for anterior thalamic axons, in which DCC is highly expressed, but a chemorepellent for posterior thalamic axons, in which Unc5 receptors are highly expressed. DCC is required for the attraction of anterior thalamic axons to the Netrin-rich, anterior part of the ventral telencephalon whereas DCC and Unc5 receptors are required for the repulsion of posterior thalamocortical axons from this Netrin-rich region.

A study by Bonnin et al. (2007) has provided further information on how the activities of guidance molecules such as Netrin-1 can be modified by the actions of other molecules, thereby increasing the array of guidance cues offered to advancing thalamocortical axons. Altering serotonin levels in the embryonic mouse brain disrupts the precision of sensory maps formed by thalamocortical axons (e.g., Cases et al., 1996), suggesting that serotonin might influence thalamocortical axonal growth. Bonnin et al. (2007) found that posterior thalamocortical axons are repelled from Netrin-rich anterior ventral telencephalon in the presence of serotonin, suggesting that the distinct action brought about by combining serotonin and Netrin-1 is an important component in the subcortical sorting of thalamocortical axons en route to the cortex.

As well as Netrin-1 a number of other groups of proteins have been shown to act as guidance cues or receptors for thalamocortical axons. These include the Slits and their receptors, the Robos, which are thought to be particularly important for the repulsion of axons away from the hypothalamus (Bagri et al., 2002; Braisted et al., 2009). Mice deficient in Robo2 and, more dramatically, in both Robo1 and Robo2, display axon guidance errors in the development of thalamocortical connections (López-Bendito et al., 2007). *Slit2* and *Slit1/2* double mutants display malformations in thalamocortical targeting (Andrews et al., 2006).

A very recent and particularly striking example of how multiple guidance cues can combine to have often surprising effects on thalamocortical development is provided by the work of Bielle et al. (2011b). This work showed that the rostrocaudal topographic organization of the thalamocortical connections is initially established in the corridor in the ventral telencephalon (discussed above) and that the rostrocaudal gradients of Slit1 and Netrin-1 are critical for establishing this order in a rather surprising way. In these experiments, the authors found that for rostral thalamic axons Slit1 is a repellent and Netrin-1 had no chemotactic activity (in contradiction to previous findings discussed above: Powell et al., 2008). But when Slit1 and Netrin-1 were combined they generated attraction. The authors concluded that Slit1 has a dual context-dependent role in thalamocortical navigation and that a combination of cues produces an emergent activity that neither of them has alone.

Other molecules involved in thalamocortical development include the Semaphorins, a family of genes that encode both membrane bound and diffusible guidance cues. Examples include Sema6A which is expressed by thalamic axons and Sema3C which is expressed in the ventral telencephalon and the cortex (Jones et al., 2002; Little et al., 2009). In Sema6A mutants, axons from the LGN of the thalamus are misrouted in the ventral telencephalon although, remarkably, the misrouted axons eventually do find their way to the visual cortex via alternate routes at postnatal stages and reestablish a normal pattern of thalamocortical connectivity (Little et al., 2009). These findings emphasize the point that, although intermediate cues in the ventral telencephalon are important for guidance, specificity can be achieved through cortical cues, indicating the multiplicity of mechanisms that result in correct thalamic axonal targeting in the cortex.

Another family of molecules strongly implicated in the development of the topography of thalamocortical projections is the Ephs and Ephrins (Dufour et al., 2003, 2006; Bolz et al., 2004; Torii and Levitt, 2005). Ephrin A5 has a gradient of expression in the intermediate tissue through which thalamocortical axons grow and EphA3, EphA4, and EphA7 have gradients of expression in the thalamus. Analysis of mutant mice has shown that Eph/ephrin genes control the topography of thalamocortical axons by sorting them in the ventral telencephalon (Dufour et al., 2003). As in the case of the modulation of the response of thalamocortical axons to netrin by serotonin discussed above, in the case of the Eph/ephrins it has been found that the cell adhesion molecules L1 and Close homolog of L1 (CHL1) can interact with individual EphA receptors and cooperate with them to guide specific sets of

thalamocortical axons to their cortical targets (Demyanenko et al., 2011). These combinatorial actions are an important subject for future research.

FUTURE PROSPECTS

THE IMPORTANCE OF COMBINATIONS OF REGULATORY MOLECULES FOR THALAMIC AXONAL GUIDANCE

The development of precise, complex connectivity in the nervous system is regulated by a set of guidance molecules whose size is tiny compared to the number of connections that form. A question that requires answering is how so few guidance molecules specify the development of so many connections with such precision. In this review we have stressed the importance of levels and combinations of guidance molecules, with different levels and combinations having different effects on navigating thalamic growth cones. We can now appreciate that the range of instructions given to growing axons depends not only there being different guidance molecules with different properties but also on the fact that different levels, possibly other variables such as different isoforms, and different combinations of molecules modify the actions of these guidance cues. Many molecules that are not usually thought of primarily as guidance factors might, therefore, be involved in modulating the actions of the classical guidance molecules. We have already seen above that serotonin, thought of primarily as a neurotransmitter, can modify the actions of Netrin-1 (Bonnin et al., 2007). This means that the range of molecules that might be involved in specifying precise connectivity could be very much larger than the families of guidance molecules implicated to date.

THE POTENTIAL IMPORTANCE OF CO-FACTORS IN MODULATING GUIDANCE

As indicated above, additional specificity can be added to the function of relatively ubiquitous signaling pathways by molecules which act to modulate those pathways. For example, a potential mechanism for increasing specificity in this way is provided by enzymes that modify heparan sulfate (HS), the complex sulfated glycan component of cell surface and extracellular heparan sulfate proteoglycans. HS is modified by the addition and removal of sulfate moieties by specific heparan sulfotransferase and endosulfatase enzymes to generate a potentially enormous number of differentially sulfated structure (Turnbull et al., 2001). The high degree of structural diversity coupled with the fact that HS mediates the function of many morphogens and axon guidance molecules including Slit, Netrin, Wnt, FGF, VEGF, neuregulin, and Hh family members has prompted the "heparan sulfate code" hypothesis which postulates that differentially sulfated HS sugar structures act in combination with signaling proteins and their cell surface receptors to specify distinct cell fate and axon guidance responses (Bulow and Hobert, 2004; Lee and Chien, 2004; Holt and Dickson, 2005). Consistent with the hypothesis that this mechanism functions in the developing mammalian brain, distinct axon navigation phenotypes occur at the optic chiasm and

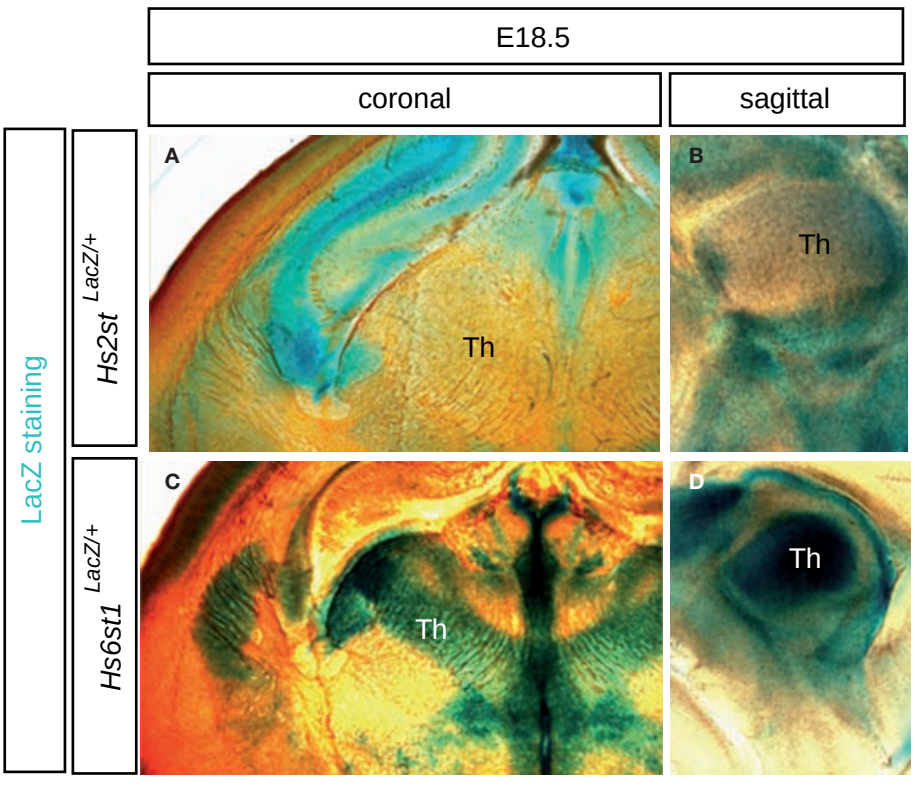

FIGURE 12 | Expression of heparan sulfate modifying enzymes *Hs2st* and *Hs6st1* in distinct patterns in the thalamus. Expression at E18.5 is revealed by LacZ staining (blue) of embryos heterozygous for a LacZ gene-trap integration in **(A,B)** *Hs2st* or **(C,D)** *Hs6st1*. Note that each enzyme is expressed in a highly distinct pattern. **(A,C)** are coronal sections and **(B,D)** are sagittal sections with anterior to the right. Th, thalamus.

in the corpus callosum in mouse knock-out models lacking the HS modifying enzymes Ndst1, Hs2st, or Hs6st1 (Grobe et al., 2005; Pratt et al., 2006; Conway et al., 2011a,b). While the role of HS modifying enzymes has not been studied in the thalamus and its connections in much detail to date, we have found that Hs2st and Hs6st1 are expressed in remarkably regionalized, and to some extent complementary, patterns in the embryonic thalamus (**Figure 12**). Thalamic development and axonal projection rely on many of the same molecular pathways as the optic chiasm and corpus callosum, for example Netrin and Slit mediated axon guidance, so it is tempting to speculate that regionally expressed HS modifying enzymes create regions of different HS bioactivity in the developing thalamus which are important for its development and connectivity, most likely in highly specific ways.

SCREENING FOR MORE GUIDANCE FACTORS

Recently, researchers have started to broaden the search for genes that might be involved in regulating thalamocortical development. One study used a forward genetic approach in which random mutagenesis was carried out in mice and mutations with effects on thalamocortical development were screened for by examining the morphology of the thalamocortical tract (the tract was specifically labeled in these mice since they carried a thalamus-expressed reporter transgene). Several lines carrying mutations in genes not previously known for roles in thalamocortical development were obtained (Dwyer et al., 2011; discussed in Lokmane and Garel, 2011). Such screens have the exciting potential to reveal additional genes regulating thalamocortical development without bias toward those whose proteins themselves have the ability to attract or repel growth cones. This approach is likely to identify new critically important co-factors whose analysis is probably going to be a major focus for future research in this area.

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Mouse thalamic differentiation: Gli-dependent pattern and Gli-independent prepattern

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Sonic hedgehog (Shh) signaling is essential for thalamic development. The Gli transcription factors act downstream of Shh – while Gli2 is the major activator (GliA), Gli3 acts primarily as a repressor (GliR). The thalamus is remarkable among dorsal structures because of its proximity to the mid-diencephalic organizer, a unique dorsal Shh source. This lends complexity to the interactions between Shh, Gli2, and Gli3, suggesting the presence of a dorsal Gli activator which elsewhere is found only ventrally, and making the dissection of thalamic Gli functions particularly interesting. A current model based on mutant phenotypes in telencephalon and midbrain postulates a degree of reciprocal antagonism of Shh and Gli3 in dorsal brain regions. To approach the role of Gli factors in thalamic specification we first analyzed mice deficient in *Gli2* or *Gli3*. In *Gli2* mutants, the thalamus is small and poorly differentiated with the exception of the medial and intralaminar nuclei which, in contrast, are specifically and severely affected by *Gli3* inactivation. *Gbx2* expression is very reduced in the *Gli3* mutant. Most thalamic nuclei are present in both mutants, although incompletely differentiated, as reflected by the loss of specific markers. The ventral posterior group, revealed by novel specific marker *Hes1*, is present in both mutants and extends axons to the telencephalon. To test the Gli3/Shh interaction we generated a novel mutant deficient in Gli3 and neuroepithelial Shh. The thalamus of the *n-Shh/Gli3* double mutants is very large and very poorly differentiated except for a broad domain of *Gbx2*, *Lhx2*, and *Calb2* expression. *In utero* electroporation experiments on wild type embryos suggest that a stage-specific factor acting early is responsible for this prepattern. We show that, in the thalamus, GliA acts downstream of Shh to specify pattern and size of the thalamic nuclei to the exception of the medial and intralaminar groups. Gli3A can partially substitute for Gli2A in the Gli2 mutant. GliR is essential for specification and growth of the medial and intralaminar nuclei, contributes to the specification of other thalamic nuclei and reduces thalamic size. GliA (from neuroepithelial Shh signaling) and GliR do not show reciprocal antagonism in the thalamus, and their joint abolition does not rescue the wild type phenotype.

Keywords: differentiation, double mutant, Gbx2, Gli2, Gli3, mouse, Sonic hedgehog, thalamus

INTRODUCTION

How different molecules and signaling pathways work together to build the intricate and precisely patterned structure of the brain is not yet clear. The thalamus is a complex of neuronal nuclei relaying sensory input to the cortex. It develops from the neuroepithelium of the caudal diencephalon also known as alar portion of p2 (Puelles et al., 1987; Puelles and Rubenstein, 1993), which generates five masses of tissue or pronuclei which further differentiate into thalamic nuclei gradually building a complex nested structure (Rose, 1942; Jones, 2007). Its development is a model of the formation of an intricate, three-dimensional brain region from a two-dimensional neuroepithelium.

The *Sonic hedgehog* (*Shh*) signaling pathway is essential for thalamic development (Kiecker and Lumsden, 2004; Vieira et al., 2005; Scholpp and Lumsden, 2010). Shh is a secreted morphogen conferring fate specification through a concentration gradient (Ericson et al., 1997; Jessell, 2000; Dessaud et al., 2008).

The Shh pathway is very complex, including several steps – posttranslational modifications, release of Shh to the extracellular space through a unique mechanism, regulation of Shh spread, and gradient formation through binding to several membrane-bound proteins and to a multipart receptor system as well as intricate intracellular signal transduction; each of these steps depends on the cooperation of a large number of ancillary proteins (Ingham

Abbreviations: ATN, anterior thalamic nuclei (central pronucleus); CL, centrolateral nucleus (medial pronucleus); CM, centromedial nucleus (medial pronucleus); LG/LGN, lateral geniculate nucleus; LGd/LGNd, lateral geniculate nucleus (dorsal); LP, lateral posterior nucleus (dorsal pronucleus); MD, mediodorsal nucleus (medial pronucleus); MG/MGN, medial geniculate nucleus; MV, medioventral nucleus (reuniens; medial pronucleus); PO, posterior nucleus (dorsal pronucleus); PT, paratenial nucleus (medial pronucleus); PTh, prethalamus; PV, thalamic paraventricular nucleus; STN, subthalamic nucleus; Th, thalamus; TL, telencephalon; VM, ventromedial nucleus (central pronucleus); VP, ventral posterior nucleus (central pronucleus); ZLI, zona limitans interthalamica.

and McMahon, 2001; Ingham and Placzek, 2006; Dessaud et al., 2008).

The Shh protein gradient is interpreted by the progenitor cells through regulation of the activity of zinc-finger transcription factor cubitus interruptus (Ci) in *Drosophila* (Methot and Basler, 2001) or its vertebrate homologs, the Gli transcription factors Gli1, Gli2, and Gli3 (Hui et al., 1994; Lee et al., 1997; Ruiz i Altaba, 1998). While Ci can act as a transcriptional activator or, after proteolytic cleavage, as a repressor (reviewed in Jacob and Briscoe, 2003), the functions of each of the three Gli as activator or repressor vary between species and between CNS regions inside the same species.

In particular, Gli1 and Gli2 exert some overlapping functions but also play some distinctive roles depending on animal species and organ. For instance, in the mouse, Gli2 is an Hh-dependent activator of the Hh response in the nervous system and in the limbs as well as in the lungs; it is also essential for early development (Mo et al., 1997; Ding et al., 1998; Matise et al., 1998; Motoyama et al., 1998). In contrast, although transcription of *Gli1* is indeed upregulated by Hh (and Gli1 can activate the Hh response), it is not required for neural patterning or early development (Park et al., 2000). Then again, Gli1 and Gli2 have overlapping activator roles as indicated by the fact that Gli1-mediated activation can rescue the Gli2-deficient phenotype in mouse (Bai and Joyner, 2001).

In the zebrafish, as opposed to the mouse, Gli1 is the major activator downstream of Hh while Gli2 shows activator and repressor roles in different regions of the embryo (Karlstrom et al., 2003). While zebrafish deficient in *Gli1* show an important neural phenotype, *Gli2* deficiency causes only small alterations in this species (Karlstrom et al., 2003). In agreement, experiments overexpressing Gli proteins in *Xenopus* embryos also suggest that Gli1 is a transcriptional activator while Gli2 can behave as both activator and repressor (Ruiz i Altaba, 1998; Mullor et al., 2001).

Whereas the roles of Gli1 and Gli2 are phylogenetically divergent, the dual role of Gli3 as repressor as well as weak activator, however, seems conserved in fish and mouse (Tyurina et al., 2005). Finally, although the functions carried out by individual Gli proteins differ between species, within a given species there is significant functional equivalency between Gli family members.

In the mouse, Gli2 and Gli3 are activated directly by Shh signaling, and they in turn activate transcription of *Gli1* (Dai et al., 1999; Sasaki et al., 1999; Park et al., 2000). Gli2 is the major activator downstream of Shh, Gli1 is also an activator, and Gli3 exists as activator (Gli3A) and, after proteasome-mediated truncation, as repressor (Gli3R); the formation of Gli3R is negatively regulated by Shh (Bai and Joyner, 2001; Bai et al., 2002, 2004; Persson et al., 2002; Nguyen et al., 2005; Stamataki et al., 2005; Tyurina et al., 2005). Since Shh attenuates the formation of Gli3R, the Shh gradient results in an opposite gradient of Gli3 repressor activity (Persson et al., 2002; Bai et al., 2004). In this way, the outcome of the activation of the Shh pathway is the induction in the developing neural tube of two gradients of transcriptional regulators with opposite polarities – activator (high to low from ventral or medial to dorsal or lateral) and repressor (high to low from dorsal or lateral to ventral or medial; Jacob and Briscoe, 2003; Ruiz i Altaba et al., 2007).

Shh acts on the thalamic primordium after being secreted by two neuroepithelial domains – the zona limitans and the basal midbrain (Shimamura et al., 1995), each with essential and specific effects (Scholpp et al., 2006; Vieira and Martinez, 2006; Jeong et al., 2011). In particular, the positioning of the zona limitans is under tight regulation (Scholpp et al., 2007). Activation of the Shh pathway in the thalamic progenitors leads to their appropriate specification and therefore to correct differentiation of thalamic nuclei (Szabo et al., 2009b; Vue et al., 2009).

The Gli factors cooperate to integrate Shh and possibly other essential thalamic pathways (Zhou et al., 2004; Kataoka and Shimogori, 2008) in a region- and stage-specific manner, resulting in the specification of cell number and identity and ultimately of the size and morphology of brain regions (reviewed in Stecca and Ruiz, 2010). Despite numerous original contributions and reviews investigating the role of Shh in the development of the thalamus in different species, the Gli code has not been explored yet in this structure. The quantitative relation between Gli2 and Gli3 and its effects on thalamic development, however, have been approached before (Hashimoto-Torii et al., 2003).

Double mutants lacking Shh signaling as well as *Gli3* partially rescue the Shh null mutant phenotype in the spinal cord and ventral telencephalon (Litingtung and Chiang, 2000; Aoto et al., 2002; Persson et al., 2002; Rallu et al., 2002; Wijgerde et al., 2002), and so it has been postulated that Shh and Gli3R cooperate in repressing and modifying a prepattern resulting from yet unknown factors (reviewed in Ruiz i Altaba et al., 2003). The effects of removing both *Shh* and *Gli3* in the thalamus have not been analyzed yet.

Here we begin to assess the role of Gli factors in thalamic development by analyzing thalamic nuclear differentiation in mouse embryos deficient either in *Gli2* (Mo et al., 1997) or in *Gli3* (the *Extra-toes* mutant; Hui and Joyner, 1993; Maynard et al., 2002; Genestine et al., 2007). Furthermore, we approach the question of the reciprocally antagonistic action of Shh and Gli3 by analyzing the thalamus of a novel double mutant deficient in neuroepithelial *Shh* and in *Gli3* (termed *n-Shh/Gli3* mutant) and by loss-of-function and gain-of-function experiments in mouse embryos.

Our results suggest a combinatorial specification of each thalamic nuclear group by *Gli2* and *Gli3*. An important exception is represented by the medial and intralaminar thalamic nuclei, which develop independently of Shh signaling but are Gli3R-dependent. Finally, simultaneous deficiency in neuroepithelial Shh and Gli3 reveals a prepattern defined by *Gbx2* and *Lhx2* expression and partial specification of the medial and intralaminar nuclei, as well as an increase in size. *In utero* electroporation experiments suggest that the effects of abolishing all Gli-mediated transcriptional regulation in the thalamus are nucleus- and stage-specific.

MATERIALS AND METHODS

MOUSE LINES

Animals were housed and handled in ways that minimize pain and discomfort, in accordance with German animal welfare regulations and in agreement with the European Communities Council Directive (2010/63/EU). The authorization for the experiments was granted by the Regierungspräsidium Karlsruhe (state authorities) and the experiments were performed under surveillance of the Animal Welfare Officer responsible for the Institute of Anatomy and Cell Biology. To obtain embryos, timed-pregnant females of

the appropriate crossings were sacrificed by cervical dislocation; the embryos were decapitated.

Gli2^{zfd} (Gli2 zinc finger-deleted) mice

This *Gli2* null mutant mouse line was generated (Mo et al., 1997) by replacing the exons encoding for zinc fingers 3 to 5. The deletion leads to an out-of-frame mutation causing disrupted transcription from the deletion site to the 3′ end of the *Gli2* gene. This results in translation of a truncated protein unable to bind to DNA, since the zinc fingers 4 and 5 are essential for DNA binding (Pavletich and Pabo, 1993).

Gli3$^{Xt/+}$ (Extra-toes) mouse line

Breeding pairs of heterozygotes were a kind gift from Dr. Thomas Theil (University of Edinburgh). This line carries a large deletion that inactivates the *Gli3* locus but does not affect flanking genes (Hui and Joyner, 1993; Maynard et al., 2002; Genestine et al., 2007).

n-Shh/Gli3 mutants

The double homozygous mutants for neuroepithelial *Shh* and *Gli3* were generated by crossings between *Foxb1$^{Cre/+}$*; *Shh$^{loxP/+}$* mice; and *Gli3$^{Xt/+}$* mice. The double mutants (*Foxb1$^{Cre/+}$*; *Shh$^{loxP/loxP}$*; *Gli3$^{Xt/Xt}$*, termed *n-Shh/Gli3* mutants) do not survive beyond birth. The *Foxb1$^{Cre/+}$* mice show a normal phenotype (Zhao et al., 2007, 2008), *Foxb1$^{Cre/Cre}$* homozygotes were never used in this study. The *Shh$^{loxP/+}$* conditional mutants were generated in the laboratory of Dr. Andrew McMahon (University of Harvard) and we obtained them through Jackson Labs (www.jax.org). The *n-Shh* mutants lack all *Shh* expression in the forebrain neuroepithelium (*Foxb1$^{Cre/+}$*; *Shh$^{loxP/loxP}$*; Szabo et al., 2009a,b).

IN SITU HYBRIDIZATION

Embryos or embryonic brains were dissected, fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned at 14 μm. RNA *in situ* hybridization was performed as described (Blaess et al., 2011).

IMMUNOHISTOCHEMISTRY

Immunofluorescent staining on sections was performed according to standard protocols. Primary antibodies were: mouse anti-Calretinin (Chemicon-Millipore, Billerica, MA, USA; MAB1568, 1:20, no unmasking, 2 days of incubation), mouse anti-neurofilament antibody 2H3 (DSHB, Iowa City; 1:100), rabbit anti-Hes1 (Chemicon AB5702; 1:100), mouse anti-NeuN (Chemicon MAB377; 1:100), rabbit anti-glial fibrillary acidic protein (GFAP) polyclonal (Chemicon AB5804; 1:100). Secondary antibodies: horse anti-mouse biotin (Vector BA2000; 1:200), Streptavidin-HRP (GE Healthcare RPN1231V; 1:100), anti-mouse IgG Alexa Fluor 488 (Invitrogen; 1:300), anti-rabbit IgG Alexa 594 (Invitrogen; 1:300).

DNA CONSTRUCTS FOR TRANSFECTION

We used an expression vector driven by pCAGGS (Niwa et al., 1991) and inserted, upstream of an internal ribosomal entry site (IRES) and either *EmGFP* (kind gift of Dr. Boris Fehse, University of Hamburg; Weber et al., 2010) or *tdTomato* (kind gift of Dr. Roger Y. Tsien, UCSD) as reporters, one of the following cDNAs:

(1) A mutated form of human *PTCH1* in which we deleted part (between *Mfe*I and *Nsi*I) of the second large extracellular loop (*PTCH1$^{\Delta\text{-}loop2}$*).
(2) A mutated form of mouse *Smoothened* (*mSmo*) in which we caused a G-to-T substitution by directed mutagenesis changing codon 539 from Trp to Leu (*SmoM2*; Xie et al., 1998).
(3) An ATG plus Kozak consensus sequence (Kozak, 1987) followed by the sequence encoding amino acids 471–645 of the mouse *Gli3* zinc-finger (*mGli-ZnF*).

Similar constructs have been published (Hynes et al., 2000; Briscoe et al., 2001; Cayuso et al., 2006).

IN UTERO ELECTROPORATION

This procedure was carried out as described (Saito and Nakatsuji, 2001; Saito, 2006) with the caveats for thalamus targeting (Kataoka and Shimogori, 2008; Vue et al., 2009; Matsui et al., 2011). In short, pregnant mice at E12.5 were anesthetized with a mixture of Halothane (Isoflurane, Baxter) and oxygen (0.5 l/min) administered with a Komesaroff Anaesthetic Machine. The uterus was exposed through laparotomy and the DNA solution (1 μg/μl) was injected with a glass micropipette in the ventricular system of the brain of each embryo through the uterine wall. Electric pulses were administered with a CUY21 electroporator (Nepagene, Japan; 5 square-wave pulses, 50 V, 50 ms on/950 ms off) and 5 mm tweezer electrodes. After the surgery, the embryos were allowed to develop *in utero* for 6 days and collected at E18.5 for analysis. The embryonic brains were dissected out, sectioned into 75 μm thick sections with a vibrating microtome (Compresstome VF-300, Precisionary Instruments Inc., Greenville, NC, USA) and observed and photographed with a Zeiss confocal microscope.

STATISTICS

Statistical assessment of the electroporation data was performed with Prism 5 software (GraphPad Software, San Diego, CA, USA).

RESULTS

REGIONALIZATION DEFECTS IN THE *Gli* MUTANTS

Whole mount detection of *Gli3* mRNA on wild type embryos at E9.5 shows robust expression in the thalamic primordium (arrowhead in **Figure 1A**). Expression of *Shh* on E12.5 embryos (**Figures 1B,C**) shows an elongated zona limitans in the *Gli3$^{Xt/Xt}$* mutant, suggesting an expansion of the thalamic primordium along the dorso-ventral axis. Expression of *Pax6*, a marker gene of the prethalamus (Stoykova and Gruss, 1994; Stoykova et al., 1996), confirms the presence of this region in the *Gli3$^{Xt/Xt}$* mutant, although it appears dorsally elongated (**Figures 1D,E**) in keeping with the expansion of the zona limitans (**Figures 1B,C**). *Ptch1*, a diagnostic marker gene for activation of the *Shh* pathway (reviewed in Lewis et al., 2001), was present (arrowheads in **Figures 1F,G**), consistent with the normal expression of *Shh* in the mutant zona limitans (**Figures 1B,C**). To further explore thalamic regionalization in the Gli3$^{Xt/Xt}$ mutant, we examined specific thalamic regional marker gene *Dbx1* (Shoji et al., 1996; Ishibashi and McMahon, 2002), which was appropriately expressed in the *Gli3$^{Xt/Xt}$* mutant thalamus (**Figures 1I,J**). A second thalamic marker gene, however, *Gbx2* (Miyashita-Lin et al.,

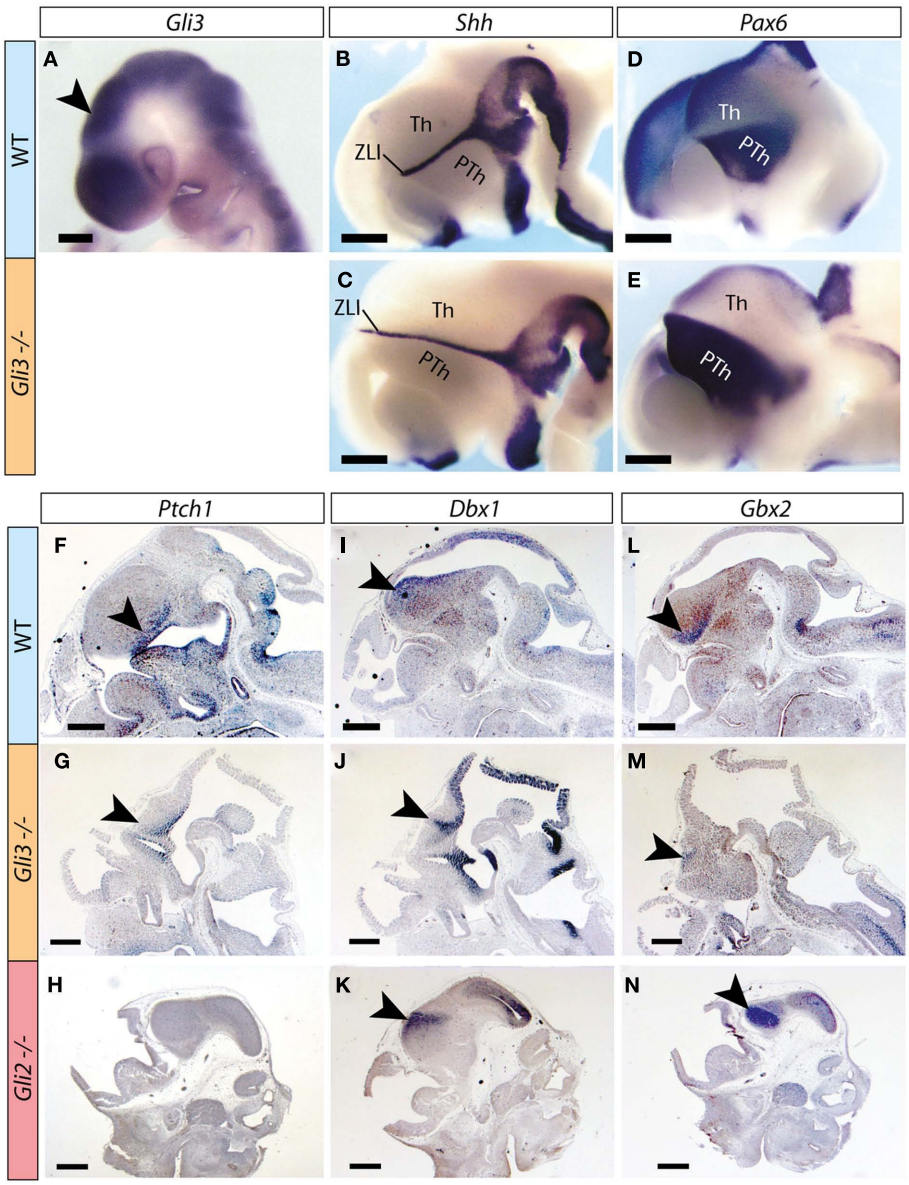

FIGURE 1 | Thalamic regionalization in the Gli3Xt/Xt mutant. **(A–E)** Whole mount *in situ* detection of markers in E9.5 **(A)** and E12.5 **(B–E)** mouse embryos, markers, and genotypes as indicated. Rostral to the left. Arrowhead in **(A)** points to the thalamic primordium. **(F–N)** *In situ* detection of marker expression on sections of E12.5 mouse embryos, markers, and genotypes as indicated. Arrowheads point to diencephalic expression. PTh, prethalamus; Th, thalamus; ZLI, zona limitans interthalamica. The brown precipitate in **(F,I,L)** corresponds to background staining. Scale bar in **(A)**, 200 μm; in **(B–N)**, 500 μm.

1999; Hevner et al., 2002), was indeed expressed in the mutants but the expression was very reduced in extension and intensity (**Figures 1L,M**).

Upon examination of the *Gli2* mutant embryo at the same age, however, we found severely downregulated or undetectable expression of *Ptch1* in the thalamus (**Figure 1H**), although the mRNAs of *Dbx1* and *Gbx2* were readily detected (**Figures 1K,N**).

We concluded that thalamic structures and expression of essential thalamic markers are induced in both $Gli3^{Xt/Xt}$ and $Gli2^{zfd/zfd}$ mutants

EVALUATION OF THALAMIC DIFFERENTIATION IN THE $Gli3^{XT/XT}$ AND THE $Gli2^{ZFD/ZFD}$ MUTANT THALAMI

To assess thalamic differentiation in the $Gli3^{Xt/Xt}$ and in the $Gli2^{zfd/zfd}$ mutants we performed an *in situ* analysis of specific marker expression (**Figures 2** and **3**; **Table 1**). Both mutants survive until E18.5, which was chosen as age of analysis. We used as markers several genes previously mapped to different thalamic nuclei and whose usefulness for phenotype assessment has been demonstrated (Szabo et al., 2009b): *Calb2* (*Calretinin*; Arai et al., 1992, 1994; Winsky et al., 1992; Frassoni et al., 1998), *Gbx2* (Jones

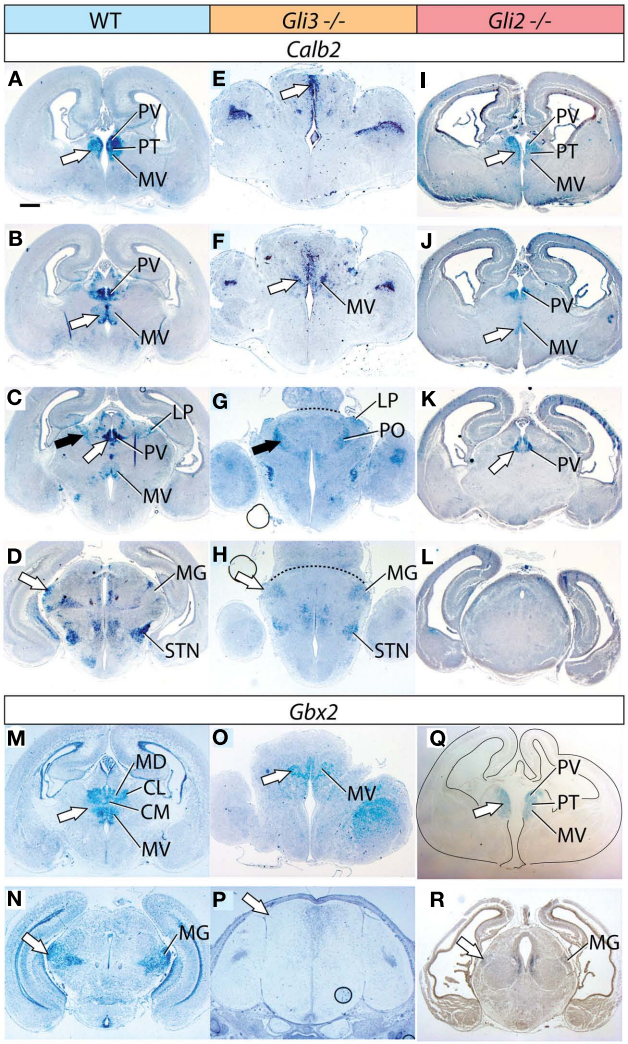

FIGURE 2 | Thalamic differentiation in the *Gli3*$^{Xt/Xt}$ and *Gli2* mutants (1). *In situ* detection of Calb2 **(A–L)** and Gbx2 **(M–R)** expression on transverse sections of wild type **(A–D,M,N)**, Gli3Xt/Xt **(E–H,O,P)**, and Gli2zfd/zfd **(I–L,Q,R)** E18.5 mouse brains. Comparable rostro-caudal thalamic levels are represented side-by-side. White and black arrowheads point at comparable structures across genotypes. The dotted line in **(G)** and **(H)** delimits the mutant thalamus from more caudal structures. The outline of the section has been delineated in **(Q)**. Scale bar, 500 μm.

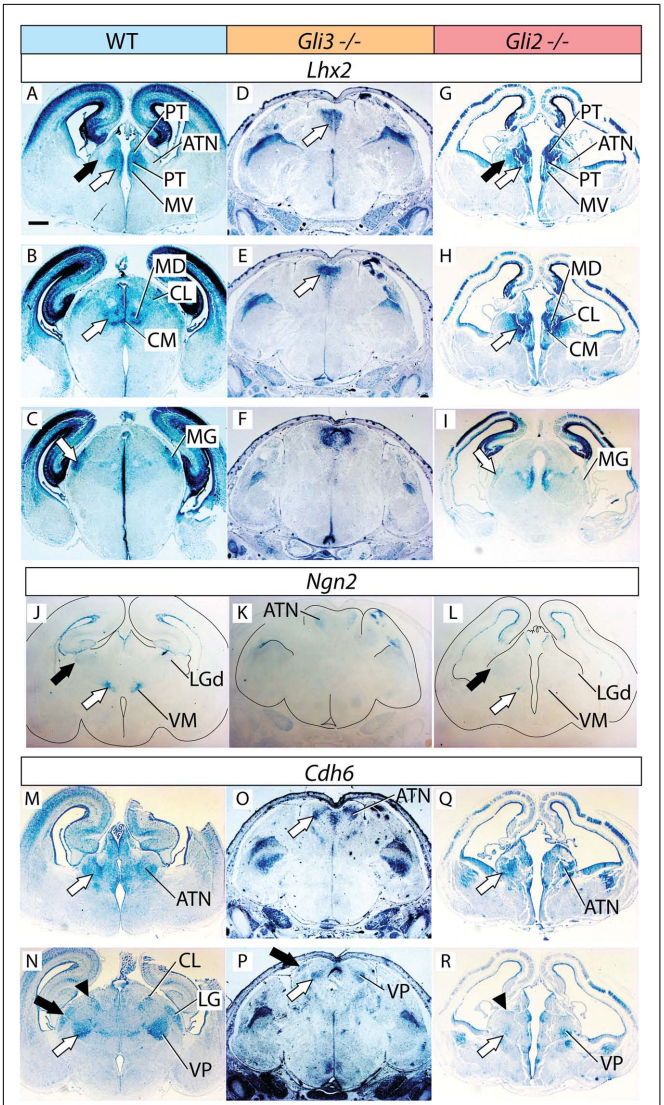

FIGURE 3 | Thalamic differentiation in the *Gli3*$^{Xt/Xt}$ and *Gli2* mutants (2). RNA *in situ* detection of Lhx2 **(A–I)**, Ngn2 **(J–L)**, and Cdh6 **(M–R)** expression on transverse sections of wild type **(A–C,J,M,N)**, Gli3Xt/Xt **(D–F,K,O,P)**, and Gli2zfd/zfd **(G–I,L,Q,R)** E18.5 mouse brains. Comparable rostro-caudal thalamic levels are represented side-by-side. White and black arrows point at comparable structures at comparable rostro-caudal levels across genotypes. The outline of the sections has been delineated in **(J–L)**. Scale bar, 500 μm.

and Rubenstein, 2004), *Lhx2* and *Ngn2* (Nakagawa and O'Leary, 2001), and *Cdh6* (Jones and Rubenstein, 2004). These results are summarized in **Table 1**. To these markers we add here expression of *Hes1* (*hairy and enhancer of split 1*), a transcription factor in the Notch pathway (Kageyama et al., 2000), as novel marker for the ventral posterior nuclear group of the thalamus at E18.5 (**Figures 4** and **6**; Lein et al., 2007). Our list is certainly not exhaustive, and the search for marker genes specific for different thalamic regions and nuclei is ongoing (Suzuki-Hirano et al., 2011; Yuge et al., 2011).

The developing thalamic mantle layer forms five neuronal masses or pronuclei out of which the adult thalamic nuclei gradually differentiate. The pronuclei and their derivatives are: medial pronucleus (medial and intralaminar nuclei), central pronucleus (anterior and ventral nuclear groups), dorsal pronucleus (lateral and posterior nuclear groups), lateral geniculate pronucleus, and medial geniculate pronucleus (Rose, 1942; Jones, 2007). We decided to use this classification for our analysis of the mutant thalamic phenotypes.

THE MIDLINE AND INTRALAMINAR NUCLEI ARE SEVERELY AFFECTED IN THE *Gli3*$^{XT/XT}$ BUT NOT IN THE *Gli2*$^{ZFD/ZFD}$ MUTANT THALAMUS

First, we sought to define the derivatives of the medial pronuclear group, including the medial nuclei [mediodorsal, medioventral

Table 1 | Markers and nuclei in the wild type and *Gli3*$^{Xt/Xt}$ and *Gli2*$^{zfd/zf}$*d* thalamus.

		Gli3$^{Xt/Xt}$	*Gli2*$^{zfd/zfd}$	*n-Shh/Gli3*
CENTRAL				
ATN	*Lhx2*	−−−	++	−−−
	Cdh6	++	++	−−−
VP	*Cdh6*	++	+	−−−
VM	*Ngn2*	−−−	+	−−−
Dorsal	*Calb2*	++	−−−	−−−
	Cdh6	−−−	+	−−−
LGN	*Ngn2*	−−−	+	−−−
	Cdh6	++	−−−	−−−
MGN	*Calb2*	++	−−−	−−−
	Gbx2	−−−	+	−−−
	Lhx2	−−−	+	−−−
Medial	*Calb2*	+	+++	++++
	Gbx2	+	+++	++++
	Lhx2	+	+++	++++

The symbol "+++" denotes normal expression; "++" and "+" denote reduced and very reduced intensity of expression, respectively; "++++" denotes expression more intense or over a larger area than in the wild type; and "−−−" denotes loss of expression.

(or reuniens), paraventricular, paratenial, and rhomboid] and the intralaminar nuclei (particularly the centromedial, paracentral, and centrolateral) by detecting the expression of marker genes *Calb2*, *Gbx2*, and *Lhx2*. *Calb2* shows a complex and characteristic expression pattern in this structure (Arai et al., 1992, 1994; Winsky et al., 1992; Frassoni et al., 1998), which makes it useful for a general assessment of differentiation (Szabo et al., 2009b; **Figures 2A–D**).

The *Gli3*$^{Xt/Xt}$ mutant shows a gross morphological alteration of the di-telencephalic junction (Theil et al., 1999; Fotaki et al., 2006; Rash and Grove, 2011) affecting all rostral and dorsal structures (**Figures 2E,F**). In contrast, *Calb2* expression was conserved in the midline (white arrows in **Figures 2E,F**) and recognizable on the basis of position and morphological traits in the medioventral nucleus (MV in **Figure 2F**), in the dorsal group (LP and PO, black arrows in **Figures 2C,G**) and in the medial geniculate nucleus (MG, white arrow in **Figure 2H**).

In the *Gli2* mutant, the thalamus seemed of normal morphology but of reduced size and the size reduction was most prominent in caudal sections (**Figures 2I–L**). *Calb2* was expressed in the medial group rostrally following a somewhat reduced normal pattern (**Figures 2I,J**). This marker however disappeared at more caudal levels and was not found in the dorsal group or in the medial geniculate (**Figures 2K,L**). We confirmed these results by antibody staining for calretinin (not shown).

Gbx2 labels the medial and intralaminar nuclei very specifically in the wild type (**Figure 2M**). Its expression is severely reduced but can still be detected in a midline domain in the *Gli3*$^{Xt/Xt}$ mutant (arrow in **Figure 2O**). In the *Gli2*$^{zfd/zfd}$ mutant (arrow in **Figure 2Q**), on the contrary, *Gbx2* expression is strong and specific in this nuclear group.

Lhx2 is also expressed characteristically by the derivatives of the medial pronucleus (white arrows in **Figures 3A,B**). Its expression is present only in a small, compact, medial and dorsal structure in the *Gli3*$^{Xt/Xt}$ mutant (arrows in **Figures 3D,E**). The *Gli2*$^{zfd/zfd}$ mutant shows robust and appropriate expression of *Lhx2* (white arrows in **Figures 3G,H**) indicating complete preservation of the medial and intralaminar nuclei.

We concluded that derivatives of the medial pronucleus (midline and intralaminar nuclei), are drastically altered in the *Gli3*$^{Xt/Xt}$ mutant but show essentially normal marker expression and size in the *Gli2*$^{zfd/zfd}$ mutant.

THE CENTRAL AND DORSAL PRONUCLEI ARE DIFFERENTIALLY AFFECTED IN THE *Gli3*$^{XT/XT}$ AND THE *Gli2*$^{ZFD/ZFD}$ MUTANTS

We used the markers *Lhx2*, *Ngn2*, and *Cdh6* in order to explore the anterior and ventral thalamic nuclear groups, derivatives of the central pronucleus. The anterior thalamic nuclei were labeled by *Lhx2* (black arrow in **Figure 3A**) and by *Cdh6* (white arrow in **Figure 3M**). In the *Gli3*$^{Xt/Xt}$ mutant, they lost *Lhx2* expression (**Figure 3D**) but are still labeled by *Cdh6* (white arrow in **Figure 3O**). The *Gli2*$^{zfd/zfd}$ mutant showed expression of the two markers in a rostral structure clearly identifiable as part of the anterior thalamic complex (*Lhx2*, black arrow in **Figure 3G**; *Cdh6*, white arrow in **Figure 3Q**).

Specific *Ngn2* expression in the ventromedial nucleus (white arrow in **Figure 3J**) was absent from the *Gli3*$^{Xt/Xt}$ mutant (**Figure 3K**) but present, very reduced in extension and intensity, in the *Gli2*$^{zfd/zfd}$ mutant (white arrow in **Figure 3L**). Finally, we detected *Cdh6*, a specific marker of the ventral posterior nuclei (white arrow in **Figure 3N**), in both mutants (white arrows in **Figures 3P,R**).

Derivatives of the dorsal pronucleus (lateral and posterior thalamic nuclei) can be labeled by *Calb2* (black arrow in **Figure 2C**) or *Cdh6* (arrowhead in **Figure 3N**) expression. The *Gli3*$^{Xt/Xt}$ mutant showed *Calb2* expression in structures reasonably identifiable as the lateral and posterior nuclei (LP, PO, black arrow in **Figure 2G**), but no *Cdh6* expression in the appropriate position (**Figure 3P**). The *Gli2*$^{zfd/zfd}$ mutant showed only expression of *Cdh6* in the possible lateral group (arrowhead in **Figure 3R**).

Both mutants express some, but not all of the specific markers for thalamic nuclei derived from the central and dorsal pronuclei. We concluded that differentiation of the central and dorsal pronuclei is altered in both mutants, albeit with differences in the effects and severity.

THE LATERAL AND MEDIAL GENICULATE NUCLEI

To investigate the differentiation of the lateral geniculate nucleus (dorsal) we used markers *Ngn2* (black arrow in **Figure 3J**) and *Cdh6* (black arrow in **Figure 3N**). We were able to detect only *Cdh6* in the lateral geniculate of the *Gli3*$^{Xt/Xt}$ mutant (black arrow in **Figure 3P**). The *Gli2*$^{zfd/zfd}$ mutant, on the contrary, expressed *Ngn2*, although at very low intensity, in the lateral geniculate (black arrow in **Figure 3L**) but no trace of *Cdh6* (**Figure 3R**).

The medial geniculate is labeled by expression of *Calb2* (MG, white arrow in **Figure 2D**), *Gbx2* (white arrow in **Figure 2N**), *Lhx2* (white arrow in **Figure 3C**), and *Cdh6* (Szabo et al., 2009b). We discarded *Cdh6* as a useful marker for this structure since it

labels also the lateral geniculate nucleus, which is contiguous to the medial along the antero-posterior axis and could cause confusion. Only *Calb2* was expressed in the appropriate caudal level of the $Gli3^{Xt/Xt}$ mutant thalamus (white arrow in **Figure 2H**). The $Gli2^{zfd/zfd}$ mutant showed no *Calb2* expression in the appropriate region. *Gbx2* (white arrow in **Figure 2R**) and *Lhx2* (white arrow in **Figure 3I**) were however present in the medial geniculate (although very reduced in extension and intensity).

In summary, the medial and intralaminar nuclei (medial pronucleus) showed a remarkable degree of preservation in the $Gli2^{zfd/zfd}$ mutant but were severely affected in the $Gli3^{Xt/Xt}$ mutant. Each of the other pronuclei (dorsal, central, lateral geniculate, medial geniculate) expressed at least one of its characteristic markers in both mutants. The loss or preservation of specific marker expression was often complementary between the $Gli2^{zfd/zfd}$ and the $Gli3^{Xt/Xt}$ mutant (**Table 1**). Finally, the $Gli2^{zfd/zfd}$ mutant thalamus was of reduced size and, the $Gli3^{Xt/Xt}$ mutant seemed to be larger than in the wild type, in agreement with previous morphometric work (Aoto et al., 2002).

THALAMOCORTICAL EFFERENTS IN THE *Gli* MUTANTS

In addition to our marker analysis with *in situ* hybridization we used antibodies against pan-neuronal marker protein NeuN (Wolf et al., 1996; green in **Figures 4A–C**) as well as against glial marker GFAP (arrow, red labeling in **Figures 4A–C**) in order to ascertain that the $Gli3^{Xt/Xt}$ as well as the $Gli2^{zfd/zfd}$ mutant thalamus are able to appropriately express general differentiation markers, as was the case (**Figures 4A–C**).

Essential to thalamic function are the thalamocortical efferents, whose development is a complex process depending on the appropriate fate specification of thalamic neurons by a cascade of key transcription factor genes (Lopez-Bendito and Molnar, 2003). Exploring the status of these efferents can provide important information about the degree of thalamic differentiation.

The thalamocortical axons of the somatosensory pathway originate from the ventral posterior complex (ventral postero-medial and ventral postero-lateral nuclei). At E18.5, transcription factor gene *Hes1* is expressed in a single, well-delimited thalamic region (Lein et al., 2007) which we surmised, because of its localization, could be the ventral posterior group. Antibody detection of Hes1 on wild type E18.5 thalamus showed staining of the ventral posterior complex (red in **Figure 4D**), and neurofilament co-detection demonstrated as expected thalamocortical axons streaming out of the complex (asterisk in **Figure 4D**). In the $Gli3^{Xt/Xt}$ mutant, *Hes1* was also expressed by a nuclear group (**Figure 4E**) extending very numerous axons into the direction of the telencephalon (asterisk in **Figure 4E**). The $Gli2^{zfd/zfd}$ mutant, however, showed weak and spatially reduced *Hes1* expression in the region of the ventral posterior complex. A reduced number of axons originated in this area (asterisk in **Figure 4F**).

We then used stitching of multiple confocal images to analyze the broad pattern of axons connecting thalamus and telencephalon in Gli2 and Gli3 null mutants (**Figures 4G–I**). Thalamocortical axons were revealed as much in the wild type brain (arrowhead in **Figure 4G**) as in the $Gli3^{Xt/Xt}$ (arrowhead in **Figure 4H**) and the $Gli2^{zfd/zfd}$ (arrowhead in **Figure 4I**) mutants. However there were clear differences between the mutants and between each mutant and the wild type. In the wild type, the thalamocortical axons form a well-developed layer in the cortex (double arrowhead in **Figure 4G**). The $Gli3^{Xt/Xt}$ mutant seems to send very large numbers of axons in the general direction of the telencephalon, but at E18.5 they had not been able to proceed further into this region (**Figure 4H**), consistent with the severe defects in the cortex of the $Gli3^{Xt/Xt}$ (Theil et al., 1999; Theil, 2005). The $Gli2^{zfd/zfd}$ mutant thalamus sends only very sparse thalamocortical axons, resulting in a thin labeled axonal layer in the cortex (double arrowhead in **Figure 4I**).

THE THALAMUS OF THE DOUBLE *Shh-Gli3*$^{XT/XT}$ MUTANT

Gli3R and Shh have reciprocally antagonistic functions (Litingtung and Chiang, 2000), and there is evidence that, in the telencephalon, deficit in both of them results in a phenotype closer to the wild type than that resulting from the mutation of each gene alone (Rallu et al., 2002). The implication is that other factors, acting earlier, are able to provide the telencephalon with a pattern (the so-called "prepattern"). During normal development, the combined action of *Shh* and *Gli3* would later eliminate this prepattern and impose a new pattern (i.e., the normal thalamic pattern). Only when both *Shh* and *Gli3* are experimentally inactivated is the prepattern uncovered (Ruiz i Altaba et al., 2003; Gutin et al., 2006; Hebert and Fishell, 2008).

Here we attempted for the first time to uncover such a possible "prepattern" in the thalamus by generating mice lacking *Shh* expression in the forebrain neuroepithelium as well as Gli3 expression. In previous work, we used a Cre-lox strategy to generate a mouse mutant lacking *Shh* expression in the forebrain neuroepithelium (the *n-Shh* mutant mouse; Szabo et al., 2009a,b). Here we crossed the $Foxb1^{Cre/+}$, the $Shh^{loxP/loxP}$, and the $Gli3^{Xt/+}$ mouse lines to obtain homozygous null mutant embryos for neural *Shh* as well as *Gli3* ($Foxb1^{Cre/+}$; $Shh^{loxP/loxP}$; $Gli3^{Xt/Xt}$, in what follows called "double mutants" or *n-Shh/Gli3* mutants; see Materials and Methods for details). These embryos are not born alive but can usually be collected at E18.5. At this age, the double mutant shows (**Figure 5**) a large thalamic primordium lacking characteristic dorsal structures (diencephalic roof and epithalamus-habenula). The lumen of the third ventricle, widened dorsally, correlates with the absence of the prethalamus (which is also absent in the *n-Shh* mutant; Szabo et al., 2009b).

We then explored thalamic differentiation in the wild type and double mutant by using as markers *Calb2* (**Figures 5A–F**), *Lhx2* (**Figures 5G–L**), *Gbx2* (**Figures 5M–P**), and *Cdh6* (**Figures 5Q–T**). As for *Ngn2*, we were not able to detect expression of this gene in the thalamus of the double mutant at all (not shown). A striking finding was a wide ventral band of *Calb2* (white arrows in **Figures 5D–F**), *Lhx2* (white arrows in **Figures 5J–L**), and *Gbx2* (white arrow in **Figure 5O**) expression extending along most of the rostro-caudal length of the double mutant thalamic primordium. Since these three markers are characteristic for the medial pronucleus (medial and intralaminar nuclei), and given the anatomical localization of the expression area, this result suggests that at least the ventral part of the medial pronucleus is present, and largely expanded, in the double mutant.

In addition, we noticed the presence of two small, paired, dorsal domains of weak marker expression that should

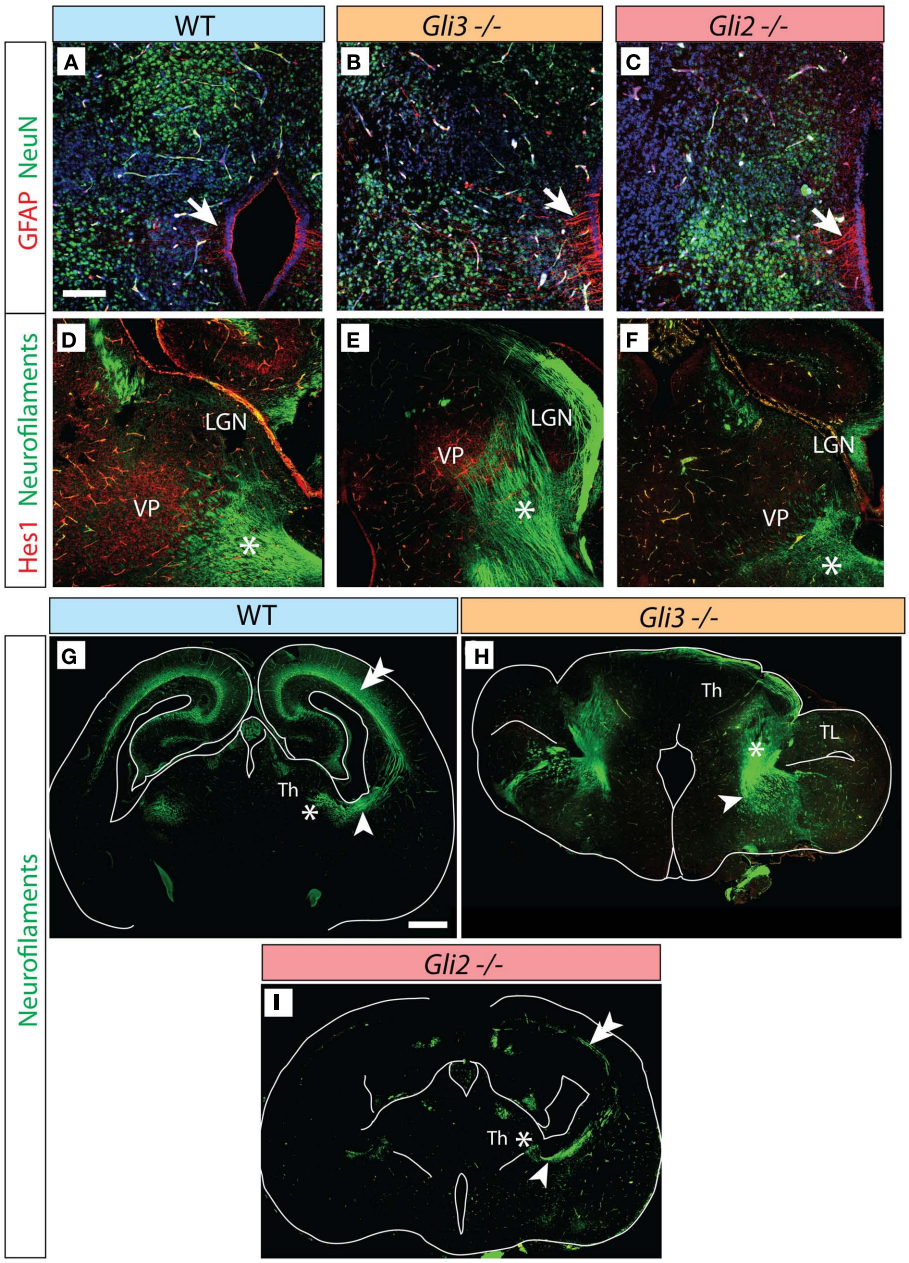

FIGURE 4 | Thalamocortical axons in the *Gli3*$^{Xt/Xt}$ and *Gli2* mutants. (A–C) Antibody detection of GFAP (red) and NeuN (green) on transverse sections of E18.5 wild type **(A)**, Gli3Xt/Xt **(B)**, and Gli2zfd/zfd **(C)** thalamus. **(D–F)** Antibody detection of Hes1 (red) and neurofilaments (green) on transverse sections of E18.5 wild type **(D)**, Gli3Xt/Xt **(E)**, and Gli2zfd/zfd **(F)** thalamus. **(G–I)** Confocal mosaic images of transverse sections through the E18.5 wild type **(E)**, Gli3Xt/Xt **(B)**, and Gli2zfd/zfd **(C)** brain showing antibody detection of neurofilaments, green. Scale bars, 100 μm **(A–F)** and 500 μm **(G–I)**.

correspond to two dorsal stripes visible with each of the four probes used, *Calb2* (arrowheads in **Figures 5D–F**), *Lhx2* (arrowheads in **Figure 5L**), *Gbx2* (**Figure 5P**), and *Cdh6* (arrowheads in **Figures 5S,T**). No other thalamic nucleus or structure could be recognized in the double mutant thalamus.

Furthermore, we determined that the double mutant thalamus expresses NeuN, a general marker of neurons, as well as GFAP, a marker of radial glia (**Figure 6A**)

THALAMOCORTICAL AXONS IN THE *N-Shh/Gli3* DOUBLE MUTANT

Next, we investigated the development of thalamocortical axons in the double-mutant through detection of neurofilaments and of Hes1 protein (see above, **Figures 2** and **3**). Hes1 was weakly expressed in the region giving rise to the presumed thalamocortical axons (**Figure 6B**). We were able to detect axons, although not very numerous, extending from the thalamic primordium in the direction of the telencephalon (arrowhead in **Figure 6C**). The telencephalon (including the cortex) of the double mutants

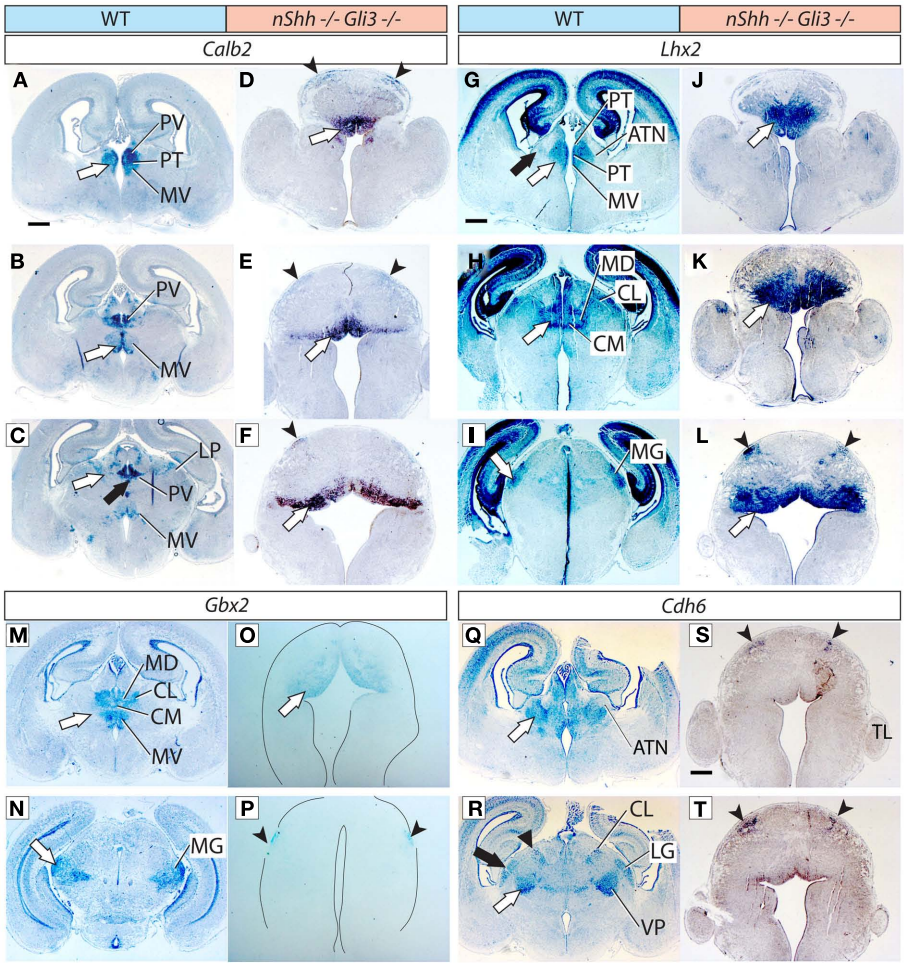

FIGURE 5 | Thalamic differentiation in the double *n-Shh/Gli3* mutant. *In situ* detection of Calb2 **(A–F)**, Lhx2 **(G–L)**, Gbx2 **(M–P)**, and Cdh6 **(Q–T)** expression on transverse sections of wild type **(A–C,G–I,M,N,Q,R)** and n-Shh/Gli3 **(D–F,J–L,O,P,S,T)** E18.5 mouse brains. Comparable rostro-caudal thalamic levels are represented side-by-side. White arrows and black arrows point at comparable structures across genotypes. The outline of the section has been delineated in **(O,P)**. Scale bar, 500 μm.

showed a very abnormal phenotype, and the axons seemed unable to enter the telencephalic primordium (**Figure 6C**).

These results provide evidence that, in the absence of *Gli3* expression and neuroepithelial *Shh* expression there is some appropriate thalamic differentiation.

AN APPROACH TO THE *Shh* VS *Gli3* RECIPROCAL ANTAGONISM THROUGH *IN UTERO* MANIPULATION OF THE *Shh* PATHWAY

The increase in size obvious in the double mutant thalamus indicated that an abnormal surge in proliferation, independent from neuroepithelial *Shh*, had been unleashed by the simultaneous deficiencies in neuroepithelial *Shh* and in *Gli3*. The most striking size increase corresponded to the most differentiated part of the double mutant thalamus, a wide ventral domain which could correspond to some of the nuclei of the medial group (**Figure 6**). Since we could not recognize any other thalamic nucleus, we reasoned that a surge in proliferation activity could specifically affect the progenitors of the medial group at an early stage, when progenitor pools are still expanding, rather than later, when the actual neurons are generated (E12.7–E14.4; Bayer and Altman, 1995; Clancy et al., 2007). Therefore, we wanted to know if a late abolition of neuroepithelial Shh and Gli3 activity would also cause an increase in proliferation.

We approached this question by experimentally altering the Shh pathway in the progenitors of the dorsal lateral geniculate nucleus (which develops early) on wild type embryos developing *in utero*. The lateral geniculate nucleus was chosen as a candidate since it belongs to a different pronuclear group developing comparatively early (E11.9–E12.7; Bayer and Altman, 1995; Clancy et al., 2007) but still late enough that it can be experimentally approached *in utero*.

In order to establish a basis for the interpretation of our results, we first performed experiments transfecting DNA constructs that either block or constitutively activate the Shh pathway activity cell autonomously (see below). Since we transfect a 2:1 mixture of green (control):red (experimental) constructs, the effect of any

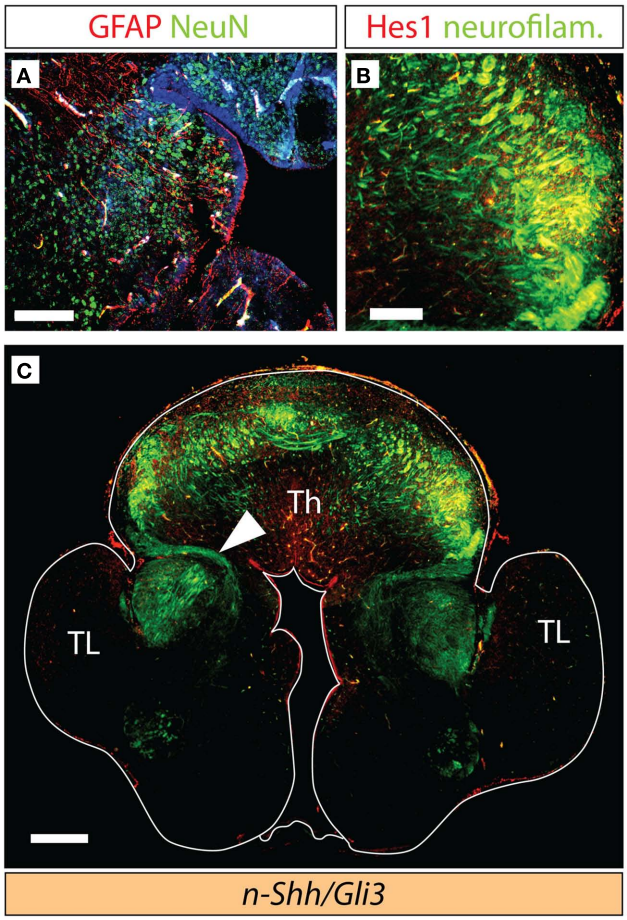

FIGURE 6 | Axonal tracts in the double *n-Shh/Gli3*$^{Xt/Xt}$ mutant. Antibody detection of markers on transverse sections through the diencephalon of the *n-Shh/Gli3*$^{Xt/Xt}$ double mutant brain at E18.5. (A) NeuN (green) and GFAP (red). **(B,C)** Neurofilaments [green in **(B,C)**] and Hes1 [red in **(B)**]. **(C)** Shows a confocal mosaic, **(B)** shows a detail of **(C)** under higher magnification. Scale bars 100 μm **(A)**, 200 μm **(B)**, and 500 μm **(C)**.

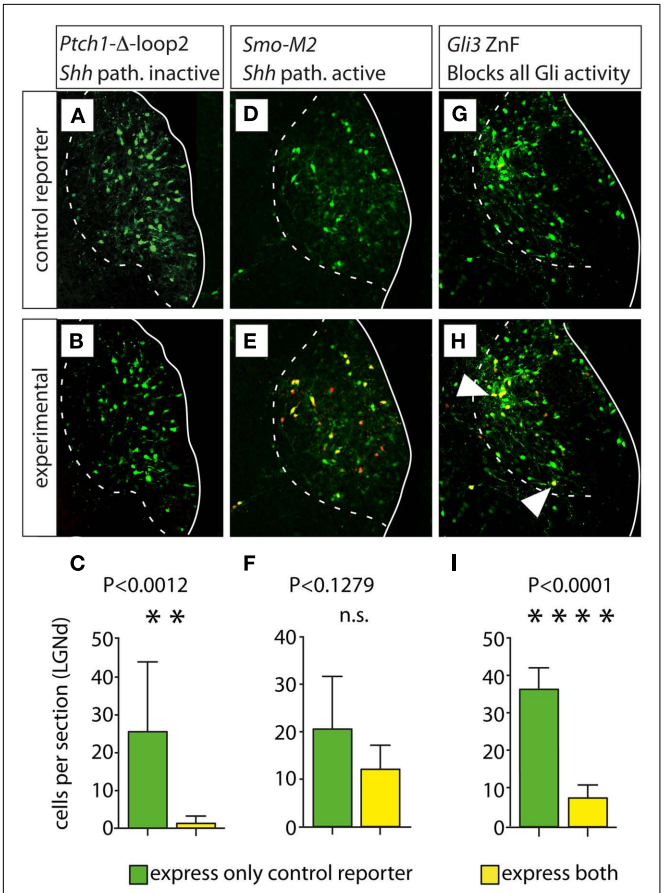

FIGURE 7 | Loss of all *Gli* activity reduces proliferation of lateral geniculate progenitors. (A,B,D,E,G,H) Confocal images of the E18.5 lateral geniculate nucleus in representative transverse Compresstome sections showing expression of green fluorescent reporter (top row) or both green and red fluorescent reporter (middle row) after in utero electroporation transfection at E12.5 of plasmid constructs as indicated. Cells expressing both the control and experimental construct are yellow in **(B,E,H)**. White arrowheads in **(H)** indicate double-labeled (yellow) cells. **(C,F,I)** Cell counting histograms (mean ± SD) after the corresponding transfections; n.s., non-significant.

experimental construct on proliferation can be measured by the ratio between green fluorescent cells and red fluorescent cells that result from the transfection. If the experimental construct has no effect at all, 100% of labeled cells will express the green reporter and 50% of them would express additionally the red reporter (since they are transfected with a 2:1 ratio); since green plus red fluorescence will appear yellow, we would expect 50% of all labeled cells to be green fluorescent (expressing only *EmGFP*) and 50% to be yellow fluorescent (expressing *EmGFP*- and *tdTomato*). Any increase or decrease in the proportion of red reporter-expressing cells (yellow) vs only green reporter-expressing cells will indicate increase or decrease in proliferation induced by the corresponding experimental construct.

BLOCKING *Shh* SIGNALING IN THE DEVELOPING LATERAL GENICULATE NUCLEUS

We first used *in utero* electroporation to transfect a 2:1 mixture of plasmids carrying either the reporter gene *EmGFP* (green) or a mutated form of the *Shh* receptor *Ptch1* (*Ptch1*$^{\Delta loop2}$) plus reporter gene *tdTomato* (red) into the thalamus of E12.5 embryos. Both constructs included a powerful constitutive promoter (see Materials and Methods for details). Transfection of this mutated form of *Ptch1* blocks *Shh* signaling cell-autonomously (Briscoe et al., 2001). The transfected embryonic brains were collected and sectioned after 6 days survival *in utero* (E18.5) and labeled cells were counted in the region of the lateral geniculate nucleus (five embryos, nine sections; **Figures 7A–C**).

This experiment yielded ca. 92.40% green cells (carrying the control construct expressing *EmGFP*) and only 5.60% yellow cells (carrying both the *EmGFP* control construct plus the *Ptch1*$^{\Delta loop2}$ construct with red reporter *tdTomato*) in the lateral geniculate nucleus (a small percent of the labeled cells could not be unequivocally attributed red or yellow color). This result confirms that cell-autonomous inactivation of the *Shh* pathway leads to decreased

proliferation (Ishibashi and McMahon, 2002; Szabo et al., 2009b). Additionally, an increase in Gli3R in absence of Shh signaling could increase cell death.

CONSTITUTIVE ACTIVATION OF *Shh* SIGNALING IN THE DEVELOPING LATERAL GENICULATE NUCLEUS

Next, we performed similar experiments using as experimental plasmid a DNA construct carrying *SmoM2*, a mutated form of the gene encoding Smoothened, a transmembrane protein essential for *Shh* signaling (Xie et al., 1998; Hynes et al., 2000). Expression of *SmoM2* activates the *Shh* pathway constitutively (Hynes et al., 2000). Transfection of *SmoM2* (four embryos, six sections) resulted in non-significant difference between the number of cells expressing only the control reporter (green cells) and the number of cells expressing both the control reporter and the red (experimental) reporter (yellow cells; **Figures 7D–F**). This is the result that we would expect if constitutive activation of the Shh pathway at E12.5 had no effect on progenitor proliferation (see above).

BLOCKING ALL TRANSCRIPTIONAL REGULATION BY Gli PROTEINS IN THE DEVELOPING LATERAL GENICULATE NUCLEUS

In order to mimic the effect of the double deficiency in neuroepithelial *Shh* and in *Gli3*, we generated DNA constructs carrying a mutated form of *Gli3* containing only the DNA-binding zinc-finger-domain (*Gli-ZnF*; Cayuso et al., 2006). Expression of *Gli-ZnF* results in the synthesis of a truncated Gli protein such that it specifically binds to DNA blocking all Gli-mediated transcriptional activation and repression; therefore, it can be considered the equivalent of a simultaneous loss of Shh and Gli3 (Cayuso et al., 2006).

This experiment (two embryos, five sections) resulted in 83.0% of green cells and 16.0% of yellow cells (**Figures 7G–I**). This indicates that blocking all GliA and GliR activity at E12.5 in the lateral geniculate (dorsal) does not increase proliferation at all (as would be expected on the basis of the *n-Shh/Gli3* mutant phenotype), but rather decreases it significantly.

DISCUSSION

We have attempted to clarify the Gli code as it applies to thalamic development in the mouse. Our conclusions are based on the analysis of mutant phenotypes, among them a novel double mutant, and on *in utero* experiments on wild type background. Although this approach is straightforward, several caveats apply to our interpretation of the data.

First of all, we use gene marker expression to determine if a phenotype is altered. One possible concern here is that we use only a tiny fraction of all existing specific marker genes. A full description of all specific nuclear markers for the thalamus is not yet available; it is also possible that the number of markers would be too large to be of practical interest. All things considered, though, the fact that the expression of some specific genes is altered is enough to state that the development of a specific nucleus is altered. If some nuclei are more altered than others, or if other markers would have shown a more complex phenotype, are valid questions that we cannot approach in this contribution.

Additionally, in our paper we do not address which genes or cellular processes act downstream of the Gli proteins in order to control thalamic development. While this is a very interesting field of inquiry for the future, it is obviously beyond the scope of this study. Finally, since the $Gli3^{Xt/Xt}$ mutants and the *n-Shh/Gli3* double mutants die before birth, here we do not analyze the adult thalamus – however, it is in principle possible that the defective phenotypes we describe could have been compensated somehow later in development.

FUNCTIONS OF Gli FACTORS DOWNSTREAM OF Shh IN THE THALAMUS

Expression of *Ptch1* is upregulated in cells that receive high levels of Shh and in which GliA is induced, and it is considered diagnostic of activation of the Shh pathway (reviewed in Lewis et al., 2001). We have not been able to detect *Ptch1* expression in the $Gli2^{zfd/zfd}$ mutant mice at E12.5, indicating that *Ptch1* expression is not induced or that expression levels are too low to be detected (**Figure 1**). We are not aware of other publications analyzing *Ptch1* expression in the $Gli2^{zfd/zfd}$ thalamus. In any case, the fact that *Ptch1* is downregulated only in the $Gli2^{zfd/zfd}$ but not in the $Gli3^{Xt/Xt}$ thalamus indicates that Gli2 is the main activator downstream of Shh in this structure. This suggests that the main functions of the elements of the Shh pathway are the same in the thalamus as the ones classically described in the spinal cord (Matise et al., 1998; Litingtung and Chiang, 2000).

Intriguingly, *Gli1* expression, another readout for Shh signaling, is very reduced but still detectable in the $Gli2^{zfd/zfd}$ mutants in the dorsal diencephalon at E10.5 (S. Blaess, unpublished results). This is remarkable, since *Ptch1* and *Gli1* are both readouts for high levels of *Shh* signaling. It is possible that the loss of Gli2 can be compensated by Gli3A only at early embryonic stages (before E12.5) but not late, or perhaps *Gli1* is expressed at slightly higher levels of in the mutants, that are easier to detect.

THE $Gli2^{ZFD/ZFD}$ THALAMIC PHENOTYPE

The $Gli2^{zfd/zfd}$ mutant thalamus is remarkably reduced in size and has a reduced marker expression. This agrees with the function of Gli2 as the major activator downstream of Shh. The fact that the thalamus is only reduced and not completely absent in the $Gli2^{zfd/zfd}$ mutants is most likely due to a compensatory role of Gli3A. In the absence of Gli2, the activator form of Gli3 can be induced by high levels of Shh signaling, and in combination with Gli1A, Gli3 can rescue at least partially the Gli2 deficiency (Bai et al., 2004). However, Gli3A is only a weak activator (Dai et al., 1999; Shin et al., 1999; Litingtung and Chiang, 2000; Motoyama et al., 2003; Bai et al., 2004) and cannot completely restore proliferation or differentiation. This is confirmed by the poor development of the thalamocortical projection in the $Gli2^{zfd/zfd}$ thalamus.

The thalamic primordium is exposed to Shh secreted by a large neuroepithelial domain comprised by a rostral, transverse portion (the zona limitans) and a ventral, longitudinal portion (the basal midbrain; Shimamura et al., 1995). Both portions are important to create a zone of very high Shh signaling ensuring proper specification of thalamic progenitors (Jeong et al., 2011). The curved shape of this rostral-plus-ventral domain is also observed in the immediately adjacent domain of *Ptch1* expression that is an indicator of high levels of Shh signaling and Gli2A activity (**Figures 1F,G**; see also Lein et al., 2007). Therefore, we would expect the most severe

Gli2$^{zfd/zfd}$ phenotype rostro-ventrally, i.e., in the area of highest Shh signaling. This is however masked by the good preservation of the derivatives of the medial pronucleus, i.e., the medial and intralaminar nuclei, whose development is not affected in mutants deficient in neuroepithelial *Shh* either (Szabo et al., 2009b; see below). These nuclei are of normal size in the *Gli2*$^{zfd/zfd}$ mutant and express their characteristic markers *Calb2*, *Gbx2*, and *Lhx2*. Since they occupy the central part and the rostral 2/3 of the thalamus, their good preservation in the mutant causes the misleading impression that the *Gli2*$^{zfd/zfd}$ thalamus is particularly small caudally.

THE *Gli3*$^{XT/XT}$ THALAMIC PHENOTYPE

The *Gli3*$^{Xt/Xt}$ mutant thalamus shows alterations in size and AP patterning. Specifically, the rostral–dorsal part of the mutant thalamus is severely altered by the loss of di-telencephalic junction (Theil et al., 1999; Fotaki et al., 2006; Quinn et al., 2009; Rash and Grove, 2011). The thalamus of this mutant is larger than the wild type thalamus (Aoto et al., 2002), suggesting that in this brain region Gli3 represses proliferation or survival. In the dorsal midbrain region, Gli3R is essential for restricting growth to a normal level and for correct AP patterning in the mid-hindbrain region (Blaess et al., 2008). The effect of Gli3 on growth is very much context dependent (Yu et al., 2009a) and, for example, the cerebellum is reduced in size in *Gli3*$^{Xt/Xt}$ mutants (Blaess et al., 2008).

Additionally, there are alterations of nuclear specification: the role of Gli3R in thalamic differentiation affects specific genes in specific nuclei (**Table 1**). This, together with the often complementary loss of specific markers in the *Gli3*$^{Xt/Xt}$ and the *Gli2*$^{zfd/zfd}$ thalamus is consistent with a "GliA/R code" in the thalamus which specifies every nucleus on the basis of GliA/R ratios determined by local Shh signaling levels (Hashimoto-Torii et al., 2003). That the thalamocortical axons do not enter the telencephalon can also be due to specific alteration in telencephalic differentiation in the absence of *Gli3* (Magnani et al., 2010).

The alterations in the *Gli3*$^{Xt/Xt}$ thalamus are likely due to loss of Gli3R rather than loss of Gli3A, since at the high levels of Shh signaling that result in Gli3A formation, Gli2A is also strongly upregulated and could compensate for the loss of Gli3A (Bai et al., 2004). This is also evident in the *Ptch1* expression in the *Gli3*$^{Xt/Xt}$ thalamus, which is not reduced in the mutants. Other studies emphasize the importance of the repressor function of Gli3 – in mouse mutants with reduced production of Gli3R (not Gli3A), cortical defects ensue similar to those found in the *Gli3*$^{Xt/Xt}$ mutant (Willaredt et al., 2008; Besse et al., 2011; Wilson et al., 2011). Furthermore, Gli3R is required to specify V0 interneurons in the ventral spinal cord (Persson et al., 2002) as well as for fate specification in cortical neurons (Wang et al., 2011) and for cerebellar foliation (Blaess et al., 2008; Kim et al., 2011). The Gli3 repressor function is also essential for limb (Hill et al., 2009), kidney (Cain et al., 2009), and thymus specification (Hager-Theodorides et al., 2009).

THE Gli3 REPRESSOR FUNCTION IS ESSENTIAL FOR THE DEVELOPMENT OF THE MEDIAL AND INTRALAMINAR NUCLEI

We have previously shown that the derivatives of the medial pronucleus (the medial and intralaminar nuclei) are specifically lost in *Gbx2* mutants (Szabo et al., 2009b). Early *Gbx2* expression defines the entire thalamic neuroepithelium (Martinez-de-la-Torre et al., 2002; Hashimoto-Torii et al., 2003), and the whole thalamus is derived from the *Gbx2* lineage (Chen et al., 2009). Around midgestation, however, *Gbx2* expression becomes restricted to a thalamic region including mostly the neurons of the medial and intralaminar group (Lein et al., 2007). We show that *Gbx2* expression is severely reduced in the *Gli3*$^{Xt/Xt}$ mutant at E12.5. In keeping with this reduction, the medial and intralaminar nuclei appear to be almost absent in the *Gli3*$^{Xt/Xt}$ mutant thalamus. It would also be possible that the development of the medial and intralaminar nuclei is rescued in the *Gli2*$^{zfd/zfd}$ mutant by Gli3A. However, since other rostral thalamic nuclei are affected in the *Gli2*$^{zfd/zfd}$ mutant, and the medial and intralaminar nuclei are not affected in mutants deficient in neuroepithelial Shh (Szabo et al., 2009b), we do not think that a Gli3 activator function is at work here.

These data indicate that thalamic specification depends not only on the Shh pathway but rather on the interplay between this and other signaling systems like the Wnt and Fgf8 pathways (Zhou et al., 2004; Kataoka and Shimogori, 2008; Bluske et al., 2009; Martinez-Ferre and Martinez, 2009; Quinlan et al., 2009), possibly even in cooperation (Rash and Grove, 2011). Since, as we show, expression of transcriptional repressor *Hes1*, a Notch effector, specifically labels a thalamic nuclear group at E18.5 (see also Lein et al., 2007), the Notch pathway is also a candidate to be involved in thalamic specification and differentiation. Additionally, all or some of these pathways could act through regulating the activity of proneural genes (Scholpp et al., 2009).

Shh VS Gli3 IN THE DORSAL DIENCEPHALON: A THALAMIC PREPATTERN?

Shh downregulates Gli3R by preventing the processing of full-length Gli3A by the proteasome into a Gli3R form (Marigo et al., 1996; Ruiz i Altaba, 1998; Wang et al., 2000; Kise et al., 2009). In mutants deficient in both Shh signaling and Gli3, ventral patterning defects observed in *Shh* null mutants are partially rescued in the spinal cord and in the telencephalon (Litingtung and Chiang, 2000; Rallu et al., 2002; Wijgerde et al., 2002), indicating that one important function of Shh is to antagonize the formation of Gli3R. The concept of a "prepattern" has emerged, i.e., a pattern of the neural tube independent of *Shh* and *Gli3* (Ruiz i Altaba et al., 2003).

Deleting *Shh* in a *Gli3*$^{Xt/Xt}$ background, however, does not fully rescue the *Gli3*$^{Xt/Xt}$ phenotype in the telencephalon (Aoto et al., 2002; Persson et al., 2002; Rash and Grove, 2007; Yu et al., 2009b). Moreover, in the midbrain or cerebellum, mutants with inactive Gli3 and inactive Shh signaling have a phenotype that is almost indistinguishable from Gli3 mutants, suggesting that the antagonistic function of Shh and Gli3 is not reciprocal (Blaess et al., 2008). Here we show that in the dorsal diencephalon the removal of Shh from the neuroepithelium in Gli3 mutants does not rescue the *Gli3*$^{Xt/Xt}$ phenotype, and even results in a much more severe phenotype. If we consider that the double mutant allows us to define for the first time the prepattern of the thalamus, that is, a pattern existing before any influence of Gli3 or neuroepithelial Shh, this prepattern would only include a rough rostro-caudal and dorso-ventral axis specification, overgrowth and partial specification of the medial pronuclear group as well as thalamocortical axonal extension.

The fact that the ventral component of the medial group (expressing *Gbx2*, *Lhx2*, and *Calb2*) seems to be not only present but is abnormally large in the double mutant, suggests that neuroepithelial Shh and Gli3 restrict the expansion of a specific progenitor pool in the medial region of the thalamic neuroepithelium (see below). In general, the increased size of the double mutant suggests that *Gli3* and neuroepithelial *Shh* cooperatively reduce the growth of the thalamic region. This is in agreement with the increase in proliferation in the spinal cord of the Gli2/Gli3 double mutants that has been linked to changes in the cell cycle (Bai et al., 2004).

The electroporation experiments blocking Shh and Gli3 at E12.5 on a wild type background show a reduction in cell number, i.e., the opposite effect as in the mutant. Therefore, blocking all Gli-mediated transcriptional regulation *per se* does not result in increased growth. This suggests that the factor or factors responsible for the prepattern act would act early, in a defined time-window. Their effect is therefore stage-specific.

Shh from the prechordal plate, which is intact in the *n-Shh/Gli3* double mutants (since it is intact in the *n-Shh* mutants;Szabo et al., 2009a) could be responsible for the patterning of the *n-Shh/Gli3* mutant thalamus, as well as the Fgf8, Wnt and maybe Notch pathways (see above).

CONCLUSION

We have analyzed for the first time the relation between Shh, GliA, and GliR in a dorsal portion of the mouse nervous system, the thalamus. We show that Shh acts upstream of GliA in the specification of pattern and size of most thalamic nuclei (except the medial and intralaminar groups). In a complementary manner, GliR is essential for specification and growth of the medial and intralaminar nuclei (although it also contributes to the specification of other thalamic nuclei and to restrict thalamic size). Additionally, we have found that if Gli2A is abolished, Gli3A can partially substitute for it in the thalamus. Finally, and against what has been established in ventral regions of the neural tube, GliA (from neuroepithelial Shh signaling) and GliR are not reciprocally antagonistic in the thalamus.

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Phylogeny and ontogeny of the habenular structure

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Habenula is an epithalamic nucleus connecting the forebrain with the ventral midbrain and hindbrain that plays a pivotal role in decision making by regulating dopaminergic and serotonergic activities. Intriguingly, habenula has also attracted interest as a model for brain asymmetry, since many vertebrates show left–right differences in habenula size and neural circuitry. Despite the functional significance of this nucleus, few studies have addressed the molecular mechanisms underlying habenular development. Mammalian habenula consists of the medial and lateral habenulae, which have distinct neural connectivity. The habenula shows phylogenetic conservation from fish to human, and studies using genetically accessible model animals have provided molecular insights into the developmental mechanisms of the habenula. The results suggest that development of the habenular asymmetry is mediated by differential regulation of the neurogenetic period for generating specific neuronal subtypes. Since the orientation and size ratio of the medial and lateral habenulae differ across species, the evolution of those subregions within the habenula may also reflect changes in neurogenesis duration for each habenular subdivision according to the evolutionary process.

Keywords: habenula, brain asymmetry, lateralization, monoamines, neurogenesis, interpeduncular nucleus, zebrafish, evolution

The habenula in the epithalamus connects the limbic forebrain with the midbrain and hindbrain where the monoaminergic neurons are concentrated. This nucleus has attracted growing interest because recent studies implicated it as a negative source of the reward signal in midbrain dopaminergic neurons (Hikosaka, 2010), and it plays critical roles in the pathophysiology of psychiatric disorders including depression (Lecourtier et al., 2004; Roiser et al., 2009; Sartorius et al., 2010; Li et al., 2011).

The habenular structure is well conserved across species, with all vertebrates examined possessing the efferent pathway of the habenula, which is called the fasciculus retroflexus or habenulo-interpeduncular tract which runs longitudinally from the epithalamus to the ventral midbrain (Concha and Wilson, 2001). This conservation allowed us to use model animals that are more amenable to genetic manipulation to analyze the development and function of the habenular circuitry (Okamoto et al., 2011). The habenula is also peculiar in that many vertebrates show conspicuous left–right asymmetry in its size and cytoarchitecture (Concha and Wilson, 2001), suggesting this brain region as a good model for the analysis of brain asymmetry.

Herein we discuss this expanding research field on the habenula and summarize recent findings from the genetic analysis of animal models like zebrafish.

HABENULA MODULATES ANIMAL BEHAVIORS VIA MONOAMINERGIC REGULATION

Habenula was previously implicated as a regulatory center for the dopaminergic and serotonergic systems in the central nervous system (CNS), based on studies showing that lesions in the habenula led to increased monoamine metabolism (Nishikawa and Scatton, 1985; Nishikawa et al., 1986). In addition, electric stimulation of the habenula inhibited the firing of dopaminergic and serotonergic neurons in anesthetized animals (Wang and Aghajanian, 1977; Christoph et al., 1986). However, it has remained unclear when the habenular neurons are activated in the behaving animals. Recent electrophysiological studies in monkeys also revealed activation of the lateral habenular neurons in response to the aversive stimuli and outcomes that seem inappropriate for the chosen behaviors, as opposed to the inactivation of midbrain dopaminergic neurons (Matsumoto and Hikosaka, 2007, 2009). These results substantiated the view that the habenula act as a negative source for the monoaminergic systems.

Based on these previous observations, lateral habenula was implicated in the pathophysiology of psychiatric disorders such as schizophrenia and depression in which dysregulation of the monoaminergic systems has long been postulated as the neural bases of these diseases (Sandyk, 1991; Ellison, 1994). Depressive patients show increased cerebral blood flow in the habenula (Morris et al., 1999; Roiser et al., 2009), and excitatory synapses onto the lateral habenular neurons are potentiated in learned helpless rats showing depression-like behaviors (Li et al., 2011). It was therefore hypothesized that increased excitability in the lateral habenula may underlie the pathophysiology of depression. Thus, reducing hyperexcitability in the habenula by surgical methods such as deep brain stimulation is a plausible future therapy strategy for drug-resistant depression (Sartorius et al., 2010).

However, only a limited number of studies have addressed the function of the medial habenula, probably because this structure is too small to be targeted by conventional experimental methods such as lesioning or stimulation. Genetic manipulation of the medial habenular neurons and their homologs confirmed that medial habenula is essential in controlling nicotine intake (Fowler et al., 2011) and fear expression (Agetsuma et al., 2010).

Despite this demonstrated functional significance of habenula, examining the function, and developmental mechanism for a subset of functional pathways remained difficult, mainly because there was no model animal with neural circuitry that is readily amenable to a genetic approach.

PHYLOGENETIC CONSERVATION OF THE MEDIAL AND LATERAL HABENULAR PATHWAYS

The mammalian habenula is subdivided into medial and lateral habenulae, each of which project to different targets. The medial habenula projects, almost exclusively, to the interpeduncular nucleus (IPN; Herkenham and Nauta, 1979), whereas the lateral habenula projects to a variety of nuclei such as rostromedial tegmental nucleus (Herkenham and Nauta, 1979; Jhou et al., 2009; Kaufling et al., 2009), raphe nuclei, substantia nigra, ventral tegmental area (Herkenham and Nauta, 1979), and the nucleus incertus (Goto et al., 2001; Olucha-Bordonau et al., 2003; **Figure 1A**). However, few studies have addressed whether subregions homologous to the medial and lateral habenulae in mammals are present in the other vertebrates, despite the fiber bundle called the fasciculus retroflexus connecting the habenula with the ventral midbrain in all vertebrates examined (**Figures 1A,B**).

Subdivision of the habenula into the medial and lateral habenulae occurs in reptiles, birds, and mammals (**Figures 2A–C**; Concha and Wilson, 2001). In lizard, the mediolateral organization of the habenula and its efferent connectivity is comparable to that found in mammals, in which the medial and lateral habenulae project to the IPN and raphe nuclei, respectively (Distel and Ebbesson, 1981; Engbretson et al., 1981). Although the medial habenula homolog that projects to the IPN is conserved from fish to mammals (red lines in **Figures 1A,B**), it remains unclear whether fish and amphibia have lateral habenular pathways. Fish and amphibian habenulae can be subdivided into dorsal and ventral habenulae based on the cytoarchitecture (**Figures 2D,E**; Braford and Northcutt, 1983; Kemali and Lazar, 1985), with some habenular neurons projecting to the serotonergic neuron-rich raphe nuclei, although studies showing this lacked detailed positional descriptions of such neurons with respect to the dorsal and ventral organization of the habenula (Kemali and Lazar, 1985; Yanez and Anadon, 1996; Guglielmotti and Fiorino, 1998; Tomizawa et al., 2001; Aizawa et al., 2005; Bianco et al., 2008).

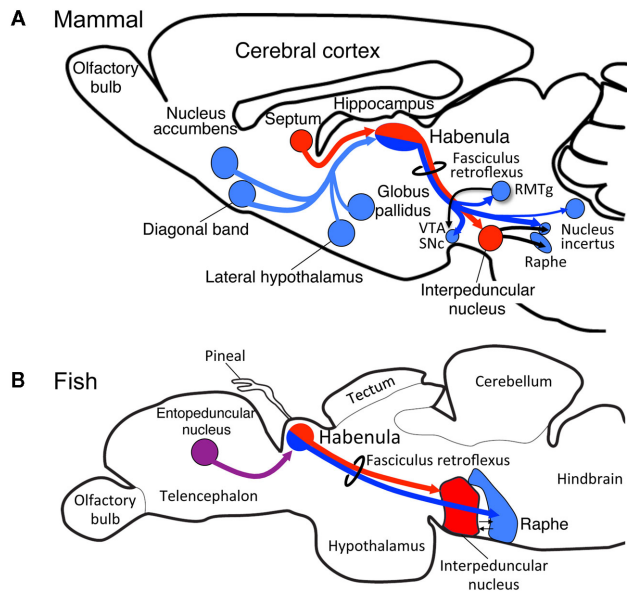

FIGURE 1 | Evolutionary conservation of the medial and lateral habenular pathways in vertebrates. Schematic diagram of sagittal sections from rat **(A)** and zebrafish **(B)** showing homologs for the medial (red) and lateral (blue) habenular circuitries. The entopeduncular nucleus [purple in **(B)**] sends axons to the habenula in zebrafish, although the target of those axons within the habenula remains unclear. RMTg, rostromedial tegmental nucleus; SNc, substantia nigra, pars compacta; VTA, ventral tegmental area.

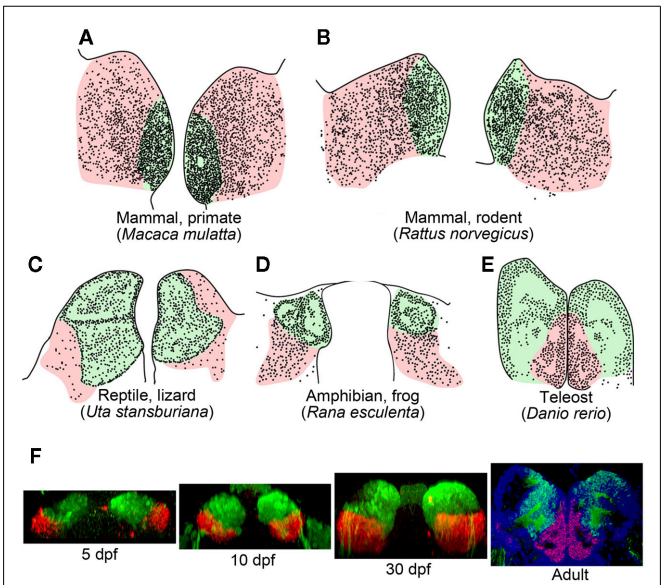

FIGURE 2 | Subnuclear organization of the habenula in vertebrates. **(A–E)** Schematic drawings of coronal sections taken at the level of the habenula, showing regions homologous to the mammalian medial (light green) and lateral habenulae (pink) of the macaque monkey **(A)**, rat **(B)**, side-blotched lizard **(C)**, European frog **(D)**, zebrafish **(E)**. The small dots indicate regions rich in cell bodies, and the remaining gaps are neuropils. **(A,B)** were adapted from Amo et al. (2010). **(C,D)** Schematics modified from data presented previously by Guglielmotti and Fiorino (1998) **(D)** and Engbretson et al. (1981) **(C)**. **(E)** Schematic drawing based on a coronal section of adult zebrafish stained with cresyl violet. **(F)** Development of the ventral habenula, based on expression patterns of the ventral habenular marker, *diamine oxidase*. Coronal views of Tg(*brn3a-hsp70:GFP*) zebrafish showing the distributions of *dao*-expressing cells in the ventral habenula (red) and GFP-expressing cells in the medial subnucleus of the dorsal habenula (green). Developmental stages are indicated beneath each panel. A section of adult zebrafish habenula (rightmost panel) was counter-stained by 4′,6′-diamidino-2-phenylindole (DAPI; blue). dpf, Days postfertilization.

Two possibilities may be considered to explain the evolution of the medial and lateral pathways in the habenula. One is that the lateral habenular pathway was newly added to the habenula, which originally projected only to IPN. Another possibility assumes the presence of both the medial and lateral habenula homologs in the lower vertebrates as in mammals, and expansion or shrinkage of the lateral habenula homolog later occurring in response to changing environmental demands on the brain. To address these possible explanations, we examined the level of medial and lateral habenulae conservation in zebrafish, a model that is genetically accessible and amenable for live imaging. Analysis of transgenic fish revealed that the dorsal habenula projects to the IPN and is thus homologous to the medial habenula of mammals (red lines in **Figures 1A,B**; Aizawa et al., 2005). In contrast, zebrafish ventral habenula sends axons to the median raphe (blue line in **Figure 1B**; Amo et al., 2010) and expresses *protocadherin 10a* (Hirano et al., 1999), a specific marker of the lateral habenula of mammals. Thus, the fish ventral habenula is homologous to the lateral habenula of mammals. These findings confirmed that the medial and lateral habenular pathways are well conserved between fish and mammals.

Since the ventral habenula is located ventromedially to the dorsal habenula in adult zebrafish brain (**Figure 2E**), the orientations of these structures appear opposite to that of the mammalian habenula in the mediolateral direction (**Figures 2A,B**; Amo et al., 2010). In contrast, the lateral habenula in macaque monkeys is expanded dorsally (pink in **Figure 2A**), whereas the medial habenula is pushed ventromedially and appears to be residual (light green in **Figure 2A**; Mikula et al., 2007). Gene expression analyses revealed that the ventromedially positioned ventral habenula (red in **Figure 2F**) in the adult zebrafish originated from the region of primordium lateral to the dorsal habenula (green in **Figure 2F**), and it then gradually migrated ventromedially as development progressed (Amo et al., 2010). This suggested that zebrafish habenulae emerge during development with mediolateral orientation similar to that of the mammalian medial and lateral habenulae.

The differential size ratio of the medial and lateral habenulae across species may reflect the extent of the cortical input through the basal ganglia to the lateral habenula (Yanez and Anadon, 1996). Since the number of neurons in the medial and lateral habenulae is determined by developmental processes such as proliferation, differentiation, and cell death, the evolutionary changes in subnuclear organization in the habenula may reflect species-specific modulation of the neurogenetic processes (Finlay and Darlington, 1995). Thus, enlargement of the lateral habenula by increasing the duration of neurogenesis might result in finer tuning of the monoaminergic systems to evaluate a larger amount of sensory information.

MOLECULAR MECHANISM FOR THE AXONAL GUIDANCE OF THE HABENULAR CIRCUITRY

Epithalamus including habenular primordium in mammals is in prosomere 2 domain (p2; **Figure 3A**). A recent study revealed that *Fgf8* expressed in the dorsal diencephalon including p2 (light blue in **Figure 3A**) was indispensable for development of the habenula,

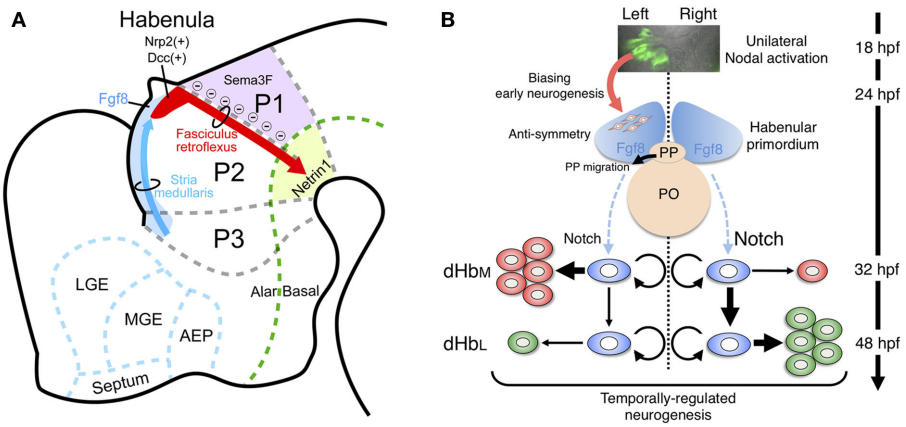

FIGURE 3 | Developmental mechanism of the habenula in genetically accessible model animals. (A) Schematic diagram of a sagittal section from E14 rat showing the habenular primordium (red), developing axons from the habenula (fasciculus retroflexus, red arrow) extending in between prosomere 1 (p1) and prosomere 2 (p2), and developing afferents to the habenula (stria medullaris) running along the dorsolateral surface of prosomere 3 (p3) and p2 (as blue arrow). The dorsal part of p2 and p3 expressed Fgf8 (light blue), which drives essential signaling for habenular development. Areas expressing guidance molecules for the habenular axons are designated by purple (Sema3F) and light green (Netrin1). AEP, anterior entopeduncular area; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence. The rostral and dorsal side is oriented at the left and top of the panel, respectively. **(B)** Neurogenetic model for the generation of left–right differences in the zebrafish dorsal habenula. At 18–24 h postfertilization (hpf), Nodal, and its downstream genes (green in the top panel showing dorsal view of Tg[*lefty1:GFP*]) are activated in the left dorsal diencephalon. Unilateral activation of Nodal signaling in the dorsal diencephalon directs the parapineal migration toward left (black arrow in the middle panel) and biases early neurogenesis in the habenular primordium with anti-symmetric nature in the presence of Fgf8 (red arrow between top and middle panels). Under those influences, common neural stem cells (light blue in the bottom panel) in the habenular primordium start to generate neural precursors for the lateral subnucleus (dHbl, red in the bottom panel) predominantly on the left side, under the influence of reduced Notch activity, and then neural precursors for the medial subnucleus (dHbM, green in the bottom panel) predominantly on the right side during late stages of neurogenesis peaked at 48 hpf. PO, pineal organ; PP, parapineal organ.

since mutants with reduced *Fgf8* showed a dose-dependent reduction in habenula size (Martinez-Ferre and Martinez, 2009). Neuronal precursors for the habenular neurons are associated with the neuroepithelial domain expressing transcription factor *Dbx1* in the dorsal part of p2 (Vue et al., 2007; Quina et al., 2009). That region first starts to generate lateral habenular precursors (E13 in rat), followed by the medial habenular precursors (E15 in rat) showing the lateromedial gradient in neurogenesis (Angevine, 1970; Altman and Bayer, 1979). Initial axonal growth from the habenular nucleus was observed as early as E13 in rats (Funato et al., 2000), and bundles of the efferent projection (fasciculus retroflexus) runs ventrocaudally between the p1 and p2 domains to form the segmental scaffold in the embryonic diencephalon (red arrow in **Figure 3A**; Figdor and Stern, 1993; Funato et al., 2000). Domain p1 expressing semaphorin 3F (Sema3F; purple in **Figure 3A**), which utilizes neuropilin-2 (Nrp2) as a receptor, showed repulsive effects on the Nrp2-expressing axons from the habenula *in vitro* (red in **Figure 3A**; Funato et al., 2000). Interaction between Sema3F and Nrp2 is needed in the formation of fasciculated habenular axons (fasciculus retroflexus), since mice lacking Sema3F or Nrp2 showed defasciculation or absence of this axonal bundle, respectively (Chen et al., 2000; Sahay et al., 2003). In addition to that repulsive guidance, the habenular axons expressing *Deleted in colorectal cancer* (Dcc; red in **Figure 3A**) are also guided by the attractive signal, Netrin1 (light green in **Figure 3A**; ligand for Dcc receptor), in the floor plate (Funato et al., 2000). Despite this accumulating evidence for the mechanisms guiding habenular axons, it remains unclear how and when the afferents to the habenula (stria medullaris; blue arrow in **Figure 3A**) develop. Molecular analysis of transgenic animals expressing fluorescent proteins in the habenular afferents or efferents is therefore an important way to decipher the mechanism driving habenular circuitry emergence.

HABENULA AS A MODEL SYSTEM FOR ANALYZING LEFT–RIGHT ASYMMETRY IN THE BRAIN

Brain asymmetry developed as a basic feature of the CNS across the vertebrates (Toga and Thompson, 2003). This feature is considered advantageous for efficient processing of neural information, such that each hemisphere is engaged in the differential regulation of behavior (Vallortigara and Rogers, 2005). However, few studies have addressed the molecular mechanism behind the generation of asymmetric brain, because there is no model animal in which neural development in both hemispheres is readily amenable to a genetic approach.

The region homologous to the medial habenula and the other epithalamic structures such as the parapineal/parietal organ show conspicuous left–right differences in many vertebrates including fish (Concha and Wilson, 2001). For instance, the left habenula has larger neuropil, and the parapineal organ innervating the left habenula is found only on the left side. Asymmetry in the habenula is observed not only in size and cytoarchitecture, but also in the axonal projection pattern to IPN (Aizawa et al., 2005; Gamse et al., 2005). Among the animals with asymmetry in the habenula, zebrafish are genetically accessible and amenable to imaging of morphogenetic processes because of their transparency during development. Furthermore, the availability of markers such as *kctd12* family genes, many of which are expressed asymmetrically in the zebrafish habenula (Gamse et al., 2003), enabled us to detect the consequent changes of the brain asymmetry in the mutant animals. Thus the habenular asymmetry in zebrafish provides an opportunity to examine the molecular mechanisms underlying the development of brain asymmetry.

DEVELOPMENTAL MECHANISM THAT DIRECTS THE EPITHALAMIC ASYMMETRY

Two possible developmental mechanisms have been suggested to drive epithalamic asymmetry; determination of the asymmetry direction, and generation of left–right difference. Prior to a series of key discoveries in this field, the first clue came from studies showing correlation between the direction of Nodal signal activation and asymmetry in the epithalamic region (Concha et al., 2000; Liang et al., 2000). Nodal genes are expressed in the left lateral plate mesoderm (LPM) and play a central role in asymmetric formation of the visceral organ in all vertebrates examined so far (Hamada et al., 2002). In fish, a Nodal-related gene (*cyclops*) is unilaterally activated in the left dorsal diencephalon at 18–20 h postfertilization (hpf; top panel in **Figure 3B**; Rebagliati et al., 1998; Sampath et al., 1998). Zebrafish mutant analyses also revealed that the direction of habenular asymmetry (laterality) and position of the parapineal organ were randomly determined when Nodal signaling in the diencephalon was absent or activated bilaterally (Concha et al., 2000). Indeed, live imaging of Nodal activation in the embryonic diencephalon confirmed that the direction of Nodal activation was concordant with that of the habenular laterality (Aizawa et al., 2005). This lateralized activation is reportedly dependent on the activity of Nodal-related gene *southpaw*, which emanates from the left LPM (Long et al., 2003). However, there could also be a brain-specific mechanism underlying the asymmetric Nodal activation, based on studies showing symmetric Nodal activation in the embryonic diencephalon despite intact Nodal expression only in the left LPM in mutants defective in either Six3 or Wnt/Axin1/β-catenin (Carl et al., 2007; Inbal et al., 2007). Following the transient activation of Nodal signaling on the left side, the primordial parapineal organ migrates from the midline toward the left side at 28–36 hpf (black arrow in the middle panel of **Figure 3B**; Concha et al., 2003). Laser ablation of the premigratory parapineal organ and mutants defective in parapineal migration both show symmetric habenula (Concha et al., 2003; Gamse et al., 2003; Snelson et al., 2008), suggesting that interaction between the parapineal and habenular anlage ensures elaboration of the lateralized epithalamus.

NEUROGENETIC ASYMMETRY DETERMINING CELL NUMBER IN THE LEFT AND RIGHT HABENULAE

Despite the pivotal role that Nodal signaling plays in determining the direction of habenular asymmetry during the early stages of development, a size difference is still apparent between hemispheres, even in mutants that lacked Nodal activation in the developing dorsal diencephalon (Concha et al., 2000). This suggested that Nodal signaling might be dispensable for the emergence of left–right differences in habenula size.

The developing epithalamus in zebrafish seems to display *fgf8*-dependent anti-symmetry (asymmetry without a directional bias)

in nature that is independent of Nodal signaling (middle panel of **Figure 3B**). This was shown in *acerebellar* zebrafish mutant embryos, which showed reduced *fgf8* function and symmetric habenula on both sides and a parapineal organ at the midline (Regan et al., 2009). A recent study also revealed that pharmacological inhibition of Nodal signaling disrupts the early asymmetric neurogenesis in habenula and parapineal migration, indicating that unilateral activation of Nodal signaling could act on the *fgf8*-expressing anti-symmetric domain in the embryonic epithalamus to bias both the habenular neurogenesis and parapineal migration toward the left side during early development (middle panel of **Figure 3B**; Roussigne et al., 2009).

Left–right difference in the number of neurons that belong to each subnucleus underlies the development of habenular asymmetry. The issue of habenular asymmetry is based on the mechanism by which these subnuclei are asymmetrically generated during development, since the dorsal habenula in zebrafish consists of medial (dHbM, green in the right most panel of **Figure 2F**) and lateral subnuclei (dHbL, blue region dorsolateral to dHbM in the right most panel of **Figure 2F**), each of which has a distinct projection pattern to the IPN (Aizawa et al., 2005; Gamse et al., 2005). The birthdate analyses by bromodeoxyuridine-pulse labeling experiments indicated that neural precursors for the dHbL (red in the bottom panel of **Figure 3B**) emerge at an earlier stage than those for the dHbM (green in the bottom panel of **Figure 3B**). In addition, more neural precursors are born on the left side than on the right side, most likely due to the right-dominant asymmetry in the activity of Notch signaling, which suppresses neurogenetic activity in neural stem cells (Louvi and Artavanis-Tsakonas, 2006). Since both the neural precursors for the dHbL and the dHbM are derived from common stem cells (blue in the bottom panel of **Figure 3B**; Aizawa et al., 2007), more neurogenesis in the left habenula induces quicker depletion of the stem cells in the left habenula than in the right habenula. This causes more neurogenesis from the remaining stem cells on the right side to give rise to more dHbM neurons on the right side than on the left side at later stages of embryonic development, which ultimately leads to the left–right asymmetric subnuclear organization of the dorsal habenula (Aizawa et al., 2007).

These studies together suggest that lateralization may work by regulating the reciprocal expansion or contraction of subregions within a particular brain region rather than by specifying unique structures on either left or right sides. This allows for variation of the brain structure between species, dependent upon the extent of lateralization, without the need for a complete re-definition of the structure in lateralized and non-lateralized species.

PERSPECTIVES

Recent evidence supports the view that modification of the duration of neurogenesis, during which the particular neuronal subtype is generated, can cause expansion or shrinkage of the particular habenula subdivision. This may in turn lead to a species-specific subnuclear organization of brain asymmetry and enable animals to adjust the subset predominance of the habenular circuitry according to the prevailing evolutionary adaptation. Further analysis of the molecular mechanisms that determine the number of neurons in each subregion of the habenula by using genetically accessible animals will be indispensable to understand how the habenula evolved in vertebrates.

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Habenula circuit development: past, present, and future

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The habenular neural circuit is attracting increasing attention from researchers in fields as diverse as neuroscience, medicine, behavior, development, and evolution. Recent studies have revealed that this part of the limbic system in the dorsal diencephalon is involved in reward, addiction, and other behaviors and its impairment is associated with various neurological conditions and diseases. Since the initial description of the dorsal diencephalic conduction system (DDC) with the habenulae in its center at the end of the nineteenth century, increasingly sophisticated techniques have resolved much of its anatomy and have shown that these pathways relay information from different parts of the forebrain to the tegmentum, midbrain, and hindbrain. The first part of this review gives a brief historical overview on how the improving experimental approaches have allowed the stepwise uncovering much of the architecture of the habenula circuit as we know it today. Our brain distributes tasks differentially between left and right and it has become a paradigm that this functional lateralization is a universal feature of vertebrates. Moreover, task dependent differential brain activities have been linked to anatomical differences across the left–right axis in humans. A good way to further explore this fundamental issue will be to study the functional consequences of subtle changes in neural network formation, which requires that we fully understand DDC system development. As the habenular circuit is evolutionarily highly conserved, researchers have the option to perform such difficult experiments in more experimentally amenable vertebrate systems. Indeed, research in the last decade has shown that the zebrafish is well suited for the study of DDC system development and the phenomenon of functional lateralization. We will critically discuss the advantages of the zebrafish model, available techniques, and others that are needed to fully understand habenular circuit development.

Keywords: habenula, asymmetry, zebrafish, DDC, epithalamus, neural circuit, 2PM imaging

ANATOMY AND CONNECTIVITY OF THE DORSAL DIENCEPHALIC CONDUCTION SYSTEM

The function of the brain has fascinated people for thousands of years and indeed the brain was alluded to as early as 1700 BC in an Egyptian papyrus describing cases of brain injuries and recommended treatments (Breasted, 1980). After Herophilus, the "father of anatomy," predicted the brain as being the source of intelligence around 300 BC, analyses focused on descriptions of brain compartmentalization and anatomy (von Staden, 2007). This approach reached a peak during the renaissance when Leonardo da Vinci designed models of brain structures and introduced the general concept of asymmetry (Bell and Sons, 1897). 150 years ago, Paul Broca demonstrated that the brain is used asymmetrically across the left–right axis through analyses of patients with deficits on one or the other side of the brain (Broca, 1861). Since then it has become apparent that the phenomenon of functional brain lateralization holds true for numerous sensory and cognitive processes and is evolutionary conserved across vertebrate species (Bisazza et al., 1998; Vallortigara et al., 1999; Vallortigara and Rogers, 2005). In particular neural circuits of the limbic system are involved in processing cognitive and sensory information. The limbic system is a complex of brain structures that connects several neurotransmitter pathways involved in behavior, emotion, memory, and olfaction (Hikosaka, 2010). Two major neural pathways of this system can be distinguished: The medial forebrain bundle (MFB) interconnects the anterior olfactory areas with the lateral preoptic, lateral hypothalamic, and ventral tegmental area. In the diencephalic conduction system (DDC) system, the bilaterally formed habenulae in the epithalamus receive information through the stria medullaris from the anterior portion of the medial forebrain and relay it into the ventral midbrain via axons of the fasciculus retroflexus (Sutherland, 1982; Morgane et al., 2005).

HABENULAR NUCLEI

The bilaterally established habenulae are often referred to as "relay station." They are at the center of the DDC system, just dorsal to the posterior part of the thalamus with which it shares various axonal connections (Guillery, 1959; Cragg, 1961; Morgane et al., 2005). One of the first reports on anatomical differences in the habenulae across the left–right axis came from the study of bone fish in the late nineteenth century (Goronowitsch, 1883; Guglielmotti and Cristino, 2006). Thirty years later, the first functional studies were reported by Brown (1914–1915) who discovered

that electrical stimulation of the habenulae evoked respiration patterns in chimpanzee. During this period the chromate staining technique was one of the earliest methods to visualize axonal connections and allowed Cajal to compile a first general description of the DDC neuronal connectivity and to uncover its main components (Cajal, 1911). In mammals, each habenula can grossly be subdivided into a medial and a lateral subnucleus, each of which is innervated by afferent axons of often different origins (**Figure 1A**). Injections of horseradish peroxidase in combination with autoradiographic methods identified prominent afferent projections to the lateral habenula nucleus from the globus pallidus but also from other areas of the fore- and midbrain running mostly within the stria medullaris (Herkenham and Nauta, 1977). In addition, anterograde fiber degeneration techniques and electron microscopy revealed secondary afferent projections, which have been reviewed elsewhere (Sutherland, 1982; Bianco and Wilson, 2009). These latter techniques also identified projections innervating the medial habenula subnucleus from mainly the hypothalamic area and the precommisural septum but also the lateral preoptic area, the interpeduncular nucleus (IPN), the dorsal raphe nucleus, and the superior cervical ganglion (Mitchell, 1963; Zyo et al., 1963; Powell, 1968; Smaha and Kaelber, 1973; Pierce et al., 1976).

The fasciculus retroflexus is the most prominent efferent habenula axon bundle connecting each habenula subnuclei with the raphe nuclei and the IPN, which is differentially innervated from three different groups of medial habenular efferent projections (Kuhar et al., 1975; Bianco and Wilson, 2009). The dorsal part of the medial habenular nuclei projects to the lateral IPN, the medial part to the ventral IPN, and the lateral part to the dorsal IPN. Early work of Cajal and later electron microscopy observations from Lenn describe the unusual phenomenon of habenular fibers traversing the entire width of the IPN several times making various synaptic connections with the IPN neurons (Cajal, 1911; Lenn, 1976). Indeed, very recent DNA electroporation studies in teleosts corroborate this remarkable discovery (Bianco et al., 2008). Autoradiographic experiments identified a second group of lateral (and to a minor extend also the medial) habenular efferent projections terminating in the ventral area of Tsai and the dorsal and median raphe (Sutherland, 1982), which makes this part of the habenula being directly connected to the dopaminergic as well as the serotonergic system respectively. Additionally, the lateral habenula projects into the rostromedial tegmental nucleus, which in turn relays information into monoaminergic regions of the midbrain (Jhou et al., 2009a,b).

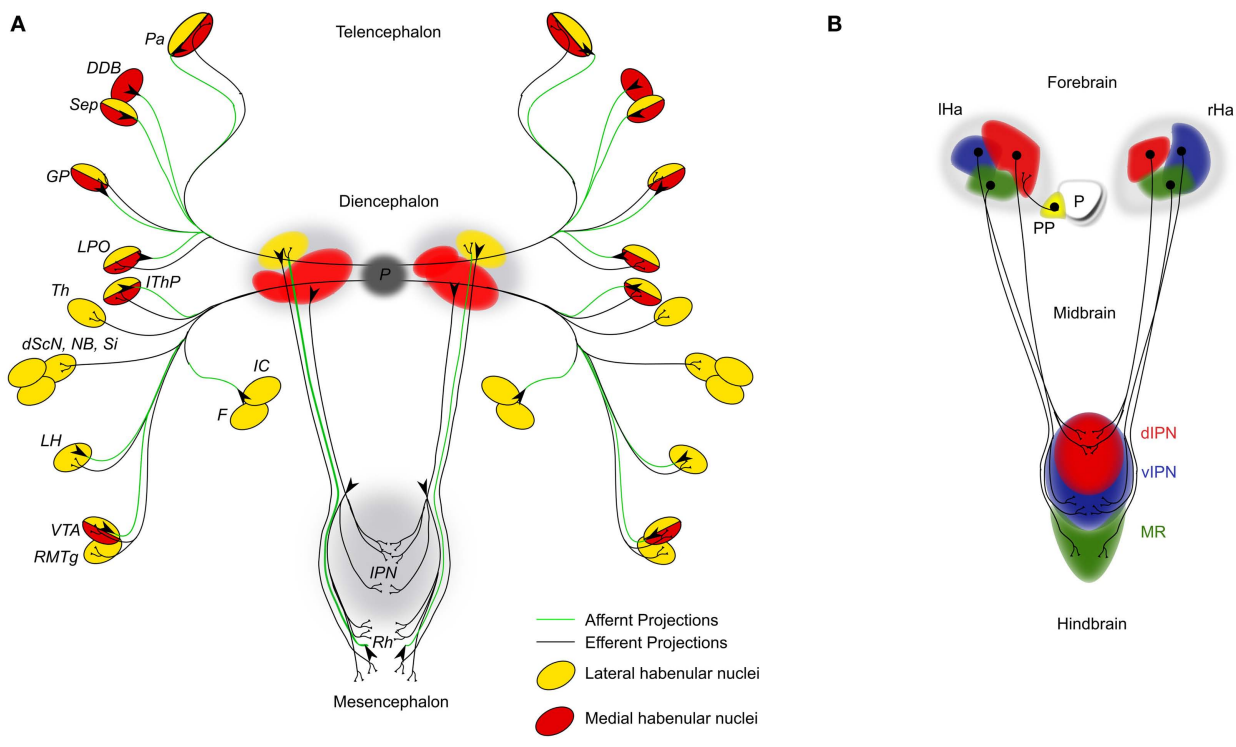

FIGURE 1 | (A) Simplified schematic diagram of the main afferent (green lines) and efferent connections (black lines) of the dorsal diencephalic conduction system in the mammalian brain. Targets and origins of axons related to the lateral habenulae are highlighted in yellow; those of the medial habenulae in red. DDB, nucleus of diagonal band; dScN, dorsal superchiasmatic nucleus; F, fornix; GP, globus pallidus (primate homolog of the teleost entopeduncular nucleus); IC, internal capsule; IThP, inferior thalamic peduncle; LH, lateral hypothalamic area; LPO, lateral preoptic area; NB, nucleus basalis; P, pineal; Pa, pallium; RMTg, rostromedial tegmental nucleus; Sep, septum; Si, substantia innominate; Th, thalamic nuclei; VTA, ventral tegmental area of Tsai. **(B)** Schematic of the habenular cell composition and efferent axonal projections innervating the interpeduncular nucleus (IPN) in 4 day old zebrafish embryos. The habenular nuclei are subdivided into lateral (red), medial (blue), and ventral (green) domains. D, dorsal; Ha, habenula; IPN, interpeduncular nucleus; l, left; MR, median raphe; P, pineal organ; PP, parapineal organ; r, right; v, ventral.

INTERPEDUNCULAR NUCLEUS

The IPN was described for the first time by the early histological observation of Forel (1872) as an unpaired structure astride the midline in the posterior part of the midbrain (Forel, 1872). The first detailed description of the "*nucleus interpeduncularis*" was carried out by Cajal (1911) using silver chromate labeling methods in different species to show the axonal connections between the IPN and the habenular nuclei and within the IPN. In the middle of the twentieth century electron microscopy techniques allowed the identification of three different types of synapses between the habenulae and the IPN in cat (Milhaud and Pappas, 1966), a number that was corrected to four, 10 years later (Lenn, 1976). Based on anatomical features and neurotransmitter expression, at least seven different IPN subnuclei can be distinguished and these are divided into two main categories: unpaired and paired. The unpaired subnuclei are divided in three subdomains, the rostral, central, and dorsal, while the four paired subdomains are classified in intermediate, lateral, interstitial, and dorso-lateral (Hamill and Lenn, 1984).

Most axons that enter the IPN originate in the habenulae and form the fasciculus retroflexus that show characteristic high levels of acetylcholine, choline acetyltransferase, and acetylcholine esterase (Hattori et al., 1977). Measuring the levels of neuropeptides and enzymes following lesion experiments Staines and colleagues identified IPN afferent connections other than those derived from the habenula, which express additional neurotransmitters such as GABA, monoamines, and neuropeptides (Staines et al., 1980; Bianco and Wilson, 2009). These comprise axons originating in the medial frontal cortex, the nucleus of the diagonal band, the substantia innominata, the preoptic and the hypothalamic nuclei, and the supramammillary nucleus. Similar to the habenulae, the IPN is well embedded into the serotonergic and dopaminergic system as its efferent axons target the raphe nuclei and the ventral tegmental nucleus respectively. Other targets comprise the central medial region of the brain stem, the tegmental nuclei of Gudden, the hypothalamus, the septum, and the diagonal band of Broca (Hamill and Lenn, 1984).

ZEBRAFISH AS A MODEL TO STUDY THE BRAIN ASYMMETRY

Linking anatomical asymmetries to the lateralization of brain functions is a challenging task for neuroscientists. Starting a decade ago, the DDC system of teleosts has nourished hopes to unravel this mystery. Both the bilaterally formed habenulae and the parapineal complex exhibit distinct anatomical asymmetries that are linked to behavior (Concha and Wilson, 2001; Barth et al., 2005; Snelson et al., 2008; Bianco and Wilson, 2009; Facchin et al., 2009; Gutiérrez-Ibáñez et al., 2011). Most of the initial studies on DDC circuit development focused on unraveling the genetics underlying laterality of the asymmetries found in this structure (Halpern et al., 2003). The zebrafish is a well established model organism suitable to combine the advantages of its transparency and external development with genetics and transgenesis to follow the effect of genetic manipulations in the living animal. The most unbiased approach to uncover genes involved in developmental processes in vertebrates is the forward genetic approach of mutagenesis screens. This technique has been used extensively in zebrafish and has uncovered a vast number of genes involved in embryonic development (Driever et al., 1996). The discovery that mutants carrying mutations in genes of the Nodal signaling pathway exhibit randomized laterality of internal organs and neuroanatomical asymmetries has been a breakthrough in this field of research (Concha et al., 2000). Indeed, several Nodal pathway components such as *cyclops*, *pitx2*, and *lefty1* are expressed asymmetrically in the dorsal diencephalon in a narrow time window during late somitogenesis before the appearance of neuroanatomical asymmetries. Elegant DNA electroporation approaches in transgenic zebrafish corroborated that the evolutionarily highly conserved Nodal signaling pathway is a major determinant of brain laterality in the dorsal diencephalon (Concha et al., 2003; Gamse et al., 2003). In fact, studies in numerous species ranging from sea urchins to mammals demonstrated that the function of Nodal in determining laterality in the developing embryo is a highly conserved mechanism (Hamada et al., 2002).

How does Nodal signaling confer laterality to the fish brain? Nodal influences the directionality of a group of migrating cells that detach from the medially located pineal organ (Concha et al., 2000, 2003; Gamse et al., 2003). Fate map experiments using caged fluorescein have demonstrated that these parapineal cells originate from both sides of the pineal complex and migrate exclusively to the left side of the brain, where they end up in the immediate vicinity of the left habenula (Concha et al., 2003). The analysis of mutants allowed to uncover that the t-box containing transcription factor *tbx2b* is essential for this migratory event as well as for the development of the correct number of parapineal cells (Snelson et al., 2008). In mammals, neither *tbx2* nor the closely related *tbx3* gene is expressed in the dorsal diencephalon. This led Snelson and colleagues hypothesize that the loss of *tbx* gene expression in the epithalamus of higher vertebrates may be the reason why mammals do not form overt parapineal structures.

Combinations of forward and reverse genetics and transgenesis allowed to reveal another pathway involved in the migration of parapineal cells (Regan et al., 2009). Implantations of FGF8 protein coated microbeads into mutant brains being otherwise devoid of FGF8 mediated signaling identified FGF signaling being crucial in this process. Moreover, transient inactivation of the FGF signaling pathway using drug (SU5402) treatments of embryos revealed that FGF signaling is required at the onset of parapineal cell migration. The FGF pathway appears to be necessary for the parapineal cells to migrate and acts in concert with Nodal signaling to allow their migration into the correct location. As tbx mediated signaling and FGF signaling are known to interact during developmental processes (Rodriguez-Esteban et al., 1999) it is tempting to speculate that also in the dorsal diencephalon tbx2/3 and FGF signaling are epistatically related to facilitate the migration of parapineal cells.

In fish, amphibians, reptiles, birds, and possibly other vertebrates, the bilaterally formed habenulae display side typical characteristics both on anatomical and morphological level (Concha and Wilson, 2001; **Figure 1B**). The laterality of these asymmetric characteristics and the location of parapineal cells are always concordantly established (Concha et al., 2000). For instance, in wild type embryos with left sided Nodal signaling the parapineal cells will migrate to the left and the left and right habenula will show

left and right specific characteristics respectively. This scenario is inverted in manipulated embryos, in which Nodal signaling on the right side of the diencephalon results in a right sided migration of the parapineal cells, leading to the right habenula exhibiting left specific features and vice versa.

The parapineal organ and the habenulae are physically connected through ipsilateral axonal projections from the parapineal cells into the left habenula. Evidence is accumulating that both of these asymmetrically formed structures communicate with one another. Transgenic embryos with labeled pineal complex allow the targeting and photoablation of parapineal cells just prior to the onset of migration (Concha et al., 2003; Gamse et al., 2003; Bianco et al., 2008). In the absence of parapineal cells, the habenulae develop largely symmetric suggesting that signals derived from these cells are important for asymmetric development of the habenulae. In line with this, *tbx2b* mutants, in which parapineal cell development and migration are impaired, exhibit a loss of habenula asymmetry (Snelson et al., 2008). Conversely, ablations of cells forming the left habenula result in parapineal cell migration defects (Concha et al., 2003). These findings suggest that so far unknown signals derived from either structure establish the communication required for asymmetric DDC system development.

A first clue as to the signaling pathways involved in this communication comes from our previous work on Wnt/beta-catenin signaling (Carl et al., 2007). The upregulation of this pathway in *axin1/masterblind* mutant embryos (Heisenberg et al., 2001) results in a loss of habenula asymmetry similar to the phenotype of wild type embryos, in which parapineal cells have been removed. Intriguingly, the parapineal cells in most *axin1* mutants do migrate normally to the left side of the brain suggesting that Wnt/beta-catenin signaling is involved in the communication between the parapineal cells and the habenula. However, it remains to be determined by which mechanism and in which brain structure Wnt signaling acts in this process.

Besides uncovering these communication signals it will be important to investigate whether parapineal cells misplaced to the right side of the brain have an effect on the development of the right habenula. This would provide an answer to the question whether permissive factors present only on the left side of the brain are required to establish the communication. However, transplantation of cells or tissues at advanced stages of zebrafish development is technically challenging and has so far hampered progress to resolve this issue.

The current availability of five teleost genome sequences facilitates comparative studies between evolutionary distant related species such as zebrafish and medaka to find out about conserved genetic elements underlying DDC network formation (Carl et al., 2007). This comparative approach can also be used to identify conserved and species specific axonal pathways as well as their targeting within the teleost lineage (Signore et al., 2009). It is however equally important to unravel such homologies between higher and lower vertebrates (Aizawa et al., 2011). This will allow us to relate functional consequences of network alterations in lower vertebrates to mechanisms in higher vertebrates. For instance, the asymmetries in the habenulae of mammals are rather subtle when compared to the clear neuroanatomical asymmetries in lower vertebrates (Wree et al., 1981). The habenulae in teleosts can grossly be divided into a dorsal and a ventral domain. The dorsal habenula consists of a lateral subnucleus being larger on the left side and the medial subnucleus being larger on the right, while the ventral domains show no signs of asymmetries (**Figure 1B**). Recently, significant progress regarding the identification of homologies has been made through the analysis of cell population specific genes and by using anterograde and retrograde dye-labelings of axonal habenula tracts (Amo et al., 2010). The dorsal habenulae in fish were found homologs to the medial habenula of mammals, while the ventral habenula in fish shows homology to the mammalian lateral habenula.

Much of the work mentioned to understand the DDC neural network is based on techniques applied to fixed embryos. These studies are starting to be successfully complemented by the use of live embryos. Combinations of transgenic techniques, DNA electroporation and the use of photoconvertible proteins have helped to identify mainly conserved elements of the DDC system such as afferent projections from different areas of the forebrain like the eminentia thalami, posterior tuberculum, and pallium (Hendricks and Jesuthasan, 2007; Miyasakan et al., 2009). Other findings were rather surprising as for example unlike in mammals, olfactory bulb neurons as well as pallium derived axons were discovered to send their axons asymmetrically into the habenulae. One theory now is that such asymmetric connections may be involved in the habenula laterality dependent differential eye use of zebrafish (Barth et al., 2005; Facchin et al., 2009) as no direct connections between the visual system and the habenulae have been discovered to date.

Like in all vertebrates analyzed to date the fasciculus retroflexus in fish connects the habenulae with the IPN and median raphe. A number of studies used lipophilic dyes to label efferent habenula axons and show that the differential targeting of left versus right sided habenula axons convert the left–right asymmetry in the dorsal diencephalon into a dorso-ventral asymmetry in the ventral midbrain (Gamse et al., 2005; Aizawa et al., 2006; Carl et al., 2007; Bianco et al., 2008). Focal DNA electroporation was used to fluorescently label single habenula efferent axons (Bianco et al., 2008). These experiments have shown that axons not only segregate laterotopically in the IPN but differ significantly in their morphology and arborization. It remains a challenging task to understand the functional significance of these results as well as the mechanism allowing these axons to cross the midline multiple times.

It has become apparent that at least habenula efferent axons receive much of their targeting information within the habenula, while information within their targets appears important for their fine tuning (Bianco et al., 2008; Roussigne et al., 2011). Axons derived from the lateral habenula cell subpopulation will target preferentially the dorsal IPN, while medial habenula cell derived axons predominantly innervate the ventral side (Roussigne et al., 2011). Axons exiting the ventral habenula domain target the median raphe nuclei (**Figure 1B**). However, also intermediate targets innervated by axons derived from the medial habenula subpopulation have been proposed. This suggests the presence of habenula cells or cell clusters within the three subpopulations with divergent characteristics (Aizawa et al., 2006). It appears that the currently favored subdivision into three habenula cell populations is by far oversimplified. In fact, only a very few genes found to

be expressed asymmetrically across the left–right axis seem to be specifically localized to one particular habenula cell population. The *kctd12.1/left over* gene is expressed exclusively in lateral habenula cells (Gamse et al., 2005). Conversely *kctd12.2/right on* gene as well as the tg(brn3a:GFP) transgene are markers for the medial cell population (Aizawa et al., 2007). Other asymmetrically expressed genes are mostly so called right sided markers with greater expression on the right side such as *kctd8/dexter* and *tag1* (Gamse et al., 2005; Carl et al., 2007). However, these genes are not restricted to only one subpopulation of habenula cells. This indicates that different groups of cells express different sets or combinations of genes at a given time point. Thus, the different content of information of cells or cell clusters within one habenula cell subpopulation may allow the generation of far more distinct habenula cell populations across the anterior–posterior (A–P) and the dorso-ventral (D-V) axes. These may provide different cues to efferent and possibly afferent axons and allow the establishment of stereotype axonal projection pattern important for behavior.

But how is habenula asymmetry established in the first place? Recent evidences suggest that Nodal signaling is not only required for brain laterality decisions but also for differences in neurogenesis across the midline (Roussigne et al., 2009, 2011). Furthermore, Aizawa et al. (2007) identified a time sequence of early habenula asymmetry using mutant, transgenic, and BrdU-labeling approaches on fixed embryos. Cells of the initially equally large pools of precursors start to proliferate earlier on the left side. During subsequent differentiation steps influenced by light induced melatonin production (de Borsetti et al., 2011) more cells of the early born lateral habenula cell subpopulation differentiate on the left side, possibly under the influence of Nodal signaling (Roussigne et al., 2009), while habenula cells on the right side increase their proliferation rate resulting in similar total numbers of habenula cells. Later during development, the remaining habenula precursor cells differentiate and give rise to the medial habenula cell subtype, which is consequently larger on the right side. A pathway regulating the differentiation events is the Notch signaling pathway (Aizawa et al., 2007). Using heat shock inducible constructs to transiently influence Notch signaling activity and mutant analyses Aizawa and colleagues show that Notch signaling delays habenula cell differentiation resulting in equal numbers of the later born medial cell subtype.

Mutant analyses and drug treatment experiments implicated also the Wnt/beta-catenin pathway in the establishment of habenula asymmetry (Carl et al., 2007). Wnt signaling as well as transcription factors such as *Six3* (Inbal et al., 2007) are crucial regulators of asymmetric Nodal gene expression and act as early as gastrulation stages. At subsequent mid-somitogenesis stages Wnt signaling is again involved in Nodel gene expression and embryos with increased Wnt signaling develop less lateral habenula cells on the left side resulting in symmetric habenulae. Still, nothing is known as to the timing or the mechanism, by which Wnt signaling is involved in this process. However, the striking resemblance of habenula phenotypes resulting from inappropriate upregulation of either the Notch or the Wnt pathway may give a hint regarding a possible interaction during the establishment of habenula asymmetry.

REMAINING CHALLENGES AND HOW WE CAN ADDRESS THEM

FOLLOWING DDC NETWORK FORMATION

Much of the current understanding of morphological changes during embryonic brain development is based on data collected from labelings in fixed samples at different developmental stages. Thus, it is rather difficult to draw conclusions regarding the highly dynamic processes of brain development and neural network formation, because continuity is lacking when considering the course of events. The zebrafish has proven to be an excellent model to follow neural network formation during embryonic development (Aramaki and Hatta, 2006). Although the DDC system is well accessible in the zebrafish, it spans several hundred micrometers in both the A–P and the D-V directions, and following the entire DDC system development over time requires time-lapse imaging for at least 72 h.

Conventional confocal laser scan microscopy (CLSM) is commonly used for time-lapse imaging. However, the effect of photobleaching of the fluorescence requires increasing laser power over time, which in turn results in phototoxicity (Squirrell et al., 1999). In fact, our attempts to perform time-lapse recordings of GFP-transgenic embryos for more than 40 h resulted in the death of the embryo.

Two-photon microscopy uses a very restricted excitation volume and longer excitation wavelengths, which circumvents the problem of photobleaching and photodamage (Helmchen and Denk, 2005; Svoboda and Yasuda, 2006). We used this technique to image the novel zebrafish enhancer trap line *Et(-1.0otpa:mmGFP)hd1*, which expresses GFP in possibly all cells of the habenulae and its efferent projections starting as early as 32 hours post fertilization (hpf; **Figure 2** and data not shown). Applying a similar approach to that previously used to image developing blood vessels (Kamei and Weinstein, 2005), no detectable harm to the animal or a decrease in GFP intensity was observed. The pictures show GFP expressing habenula cells in the dorsal diencephalon at 38 and 47 hpf and habenula afferent projections entering the midbrain at 57 hpf, eventually innervating the IPN about 300 μm away from their source in the ventral midbrain (**Figures 2A–D**).

We next compared the efficacy of 2PM versus CLSM to image deep into the brain and record the axonal innervation of the IPN (not shown). CLSM was never able to reach the depth attained by 2PM, being limited to a penetration of ∼100–120 μm in comparison to >400 μm achieved in two-photon excitation (2PE), where the final limitation was due to the absence of tagged cells. **Figure 3** shows the comparison between images of the IPN of embryos recorded by CLSM (**Figures 3A–D**) and 2PM (**Figures 3E–H**) at different depths using partial maximum intensity projections of the whole Z-stack. Note that IPN structures are still visible reaching the maximum imaging depth using the CLSM (**Figure 3D**).

These experiments show the obvious advantages of 2PM over CLSM when working *in vivo* on whole animals: besides the possibility of long-term time-lapse imaging without harming the specimens, the imaging depth of 2PM in thick samples or tissues is far superior to that of CLSM.

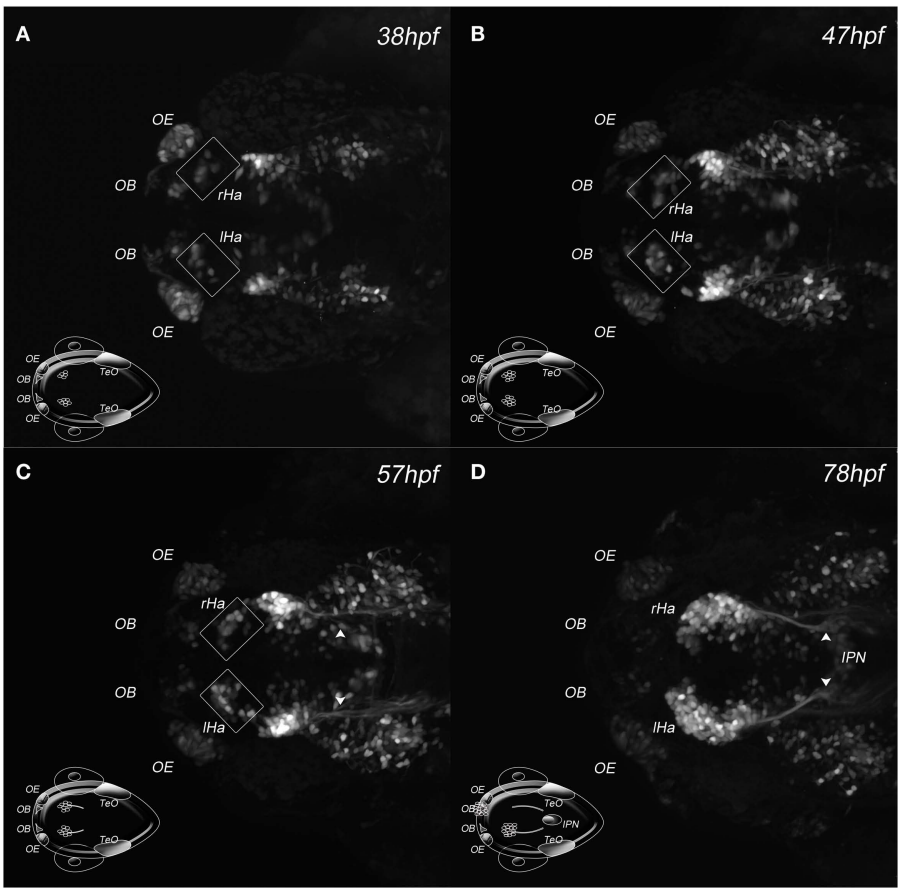

FIGURE 2 | Two-photon excitation of DDC system architecture. (A–D) Maximum intensity projections of four developmental stages acquired by *in vivo* 2PM of a *Et(-1.0otpa:mmGFP)hd1* transgenic zebrafish embryo; dorsal views, anterior left; developmental stages are indicated. The cartoon inserts show regions of GFP expressing cells for orientation. **(A–C)** Squares frame GFP expressing habenular cells. **(C)** The arrowheads mark habenula efferent axons. **(D)** The arrowheads mark habenula efferent axons entering the IPN. Ha, habenula; IPN, interpeduncular nucleus; l, left; OB, olfactory bulb; OE, olfactory epithelium; r, right; TeO, optic tectum.

The number of transgenic zebrafish lines that label cells of the DDC system has increased exponentially during the last years. Using 2PM time-lapse imaging we can now analyze the spatio-temporal formation of the entire asymmetric DDC system. We have the exciting possibility to learn all about the origins of habenula cells, timing of events during DDC system development and axonal targeting and shaping. These are the essential prerequisites if we are to analyze the consequences of genetic or laser manipulations in their full spectrum.

FUTURE GOALS

Progress has been made as to the genetic network underlying the asymmetric formation of the DDC system. Several pathways including Wnt/beta-catenin, Notch, Fgf, and Nodal have been identified but their epistatic relationship remains unknown. Which factors are involved in the communication of parapineal cells and the habenulae? Which are the molecules important for guidance of habenula projections, their targeting and shaping? Some of these questions can readily be addressed while others need creativity and the development of new resources and techniques. A variety of tools to manipulate genetic pathways in time are already available ranging from signal cascade specific drugs to transgenic animals harboring heat shock inducible constructs. For instance, the established infrared laser based "laser evoked gene operator (LEGO)" for focal activation of such constructs offers the exciting possibility to study the consequences of manipulating genes in one particular region of the brain at a given time point (Deguchi et al., 2009; Kamei et al., 2009). Also the application of the Gal4–VP16 system in combination with tetanus toxin or nitroreductase to specifically ablate particular structures of the DDC system have been used successfully (Agetsuma et al., 2010; Lee et al., 2010). Other tools are still missing such as for instance promoters that drive gene expression in defined areas of the circuit to not only visualize or ablate these structures but as well to misexpress genes of interest and analyze the consequences on DDC system development. Also techniques for simultaneous timely and spatially controlled activation of genes are rather limited in the zebrafish.

The targeting of axonal projections exiting the habenulae is a good read out as to the nature of the habenula cell types. Based on this targeting and on the expression of marker genes the habenulae

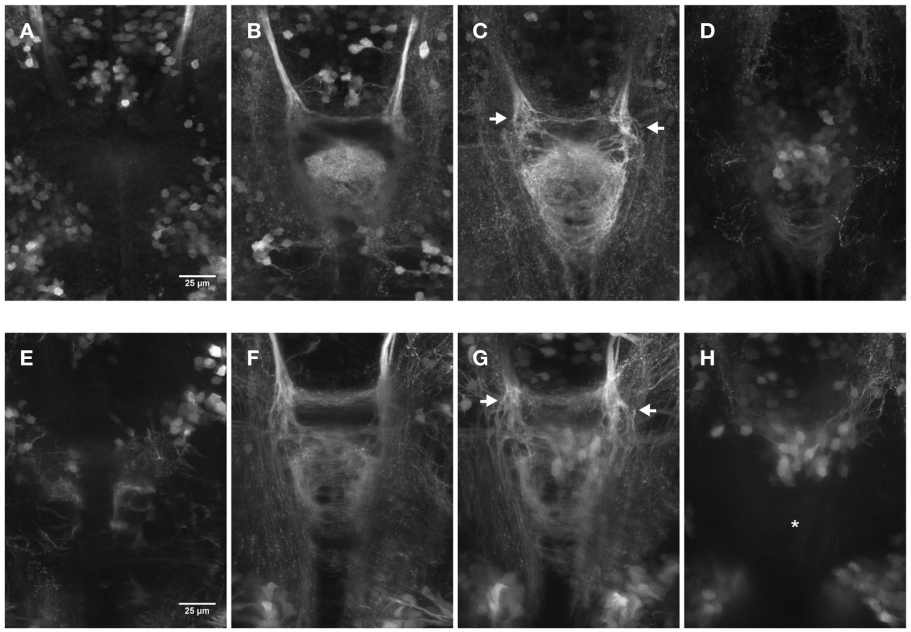

FIGURE 3 | Comparison of dorsal views (150 × 200 µm) of two 4 day old embryos focused on the IPN. The pictures show partial maximum intensity projections for CLSM **(A–D)** and 2PM **(E–H)**. **(A–D)** Maximum intensity projections of a range of 15 µm each for a total *Z*-height of 60 µm. **(E–H)** Maximum intensity projections of a range of 25 µm each for a total Z-height of 100 µm. Different focal planes are pictured due to the differences in range. Laser power correction was used in both cases to compensate for increasing depth. The display range is kept constant within the series and the gamma was corrected to 0.5 using FiJi for display purpose. **(C–G)** The arrows highlight the axons entering the IPN area more dorsal on the left and more ventral on the right side. Asterisk in **(H)**: less structures can be observed compared to **(D)** due to the fact that **(H)** shows a deeper range past the IPN that view **(D)** does (9–34 µm versus 2–17 µm after the "end" of the IPN).

have been divided into lateral, medial, and ventral cell subpopulations. However, as outlined above the situation appears much more complex and one can easily imagine that many more cell subtypes could be distinguished. For instance, what makes some cells of the medial habenula cell subpopulation send their axons into an intermediate area of the IPN rather than into the ventral part (Aizawa et al., 2006)? Focal electroporation techniques have been applied to follow single axons innervating the IPN. Although this being a very informative method, it is very difficult and laborious to apply. The same holds true for the tracking of single axons in networks of such complexity. With the development of reliable multicolor labeling techniques on fixed as well as living animals we may be able to approach this complex task. For instance, one promising tool to map habenula axonal projections that has been used to study retino-tectal projections in mammals is the brainbow technique (Livet et al., 2007). The unique, randomly generated combinatorial expression of various fluorescent proteins allows researchers to label cells and their axons in various colors (over 100) to discriminate between them. This technique (Pan et al., 2011) requires the identification of genes with appropriate promoters, which drive gene expression in habenula precursor cells and their descendants such as the *cxcr4b* gene (Roussigne et al., 2009). Moreover, this labeling technique will have to be combined with super-resolution light microscopy to allow the unique identification of neurites (Helmstaedter et al., 2008).

The use of *in vivo* long-term time-lapse 2PM to study DDC system development in the living embryo will give us many clues as to the timing of neural circuit developmental processes. Moreover, we will be able to compare DDC system development between normal and mutant embryos and study the impact of the mutated gene in great detail. Although progress has been made, the lack of appropriate transgenic lines or vital dyes to mark specific embryonic structures to be used as landmarks is currently hampering the rapid detailed description of axonal targeting. It also remains difficult to actually trace single axons in embryos in which the entire circuit is labeled. Promoters are needed that drive for instance the photoconvertible Kaede protein (Ando et al., 2002) or the recently reported photoswitchable monomeric orange (PSmOrange) protein in habenula precursor cells (Subach et al., 2011). Photoconversion of the Kaede protein switches its fluorescence from green to red while the PSmOrange is converted from orange into far-red fluorescence. Photoconversion of single cells or cell clusters in such a line followed by long-term multiphoton time-lapse microscopy would be a promising tool to learn more about the development of the DDC system architecture.

We have now acquired the knowledge, tools, and resources to visualize the DDC system in the living animal. Moreover we can manipulate habenula laterality and asymmetry either genetically or by leaving the embryo genetically unchanged. We even have the means to introduce rather subtle changes in neural activities using a variety of optogenetic tools that have been reviewed elsewhere (Del Bene and Wyart, 2011). But before we manipulate neural activity we need to visualize it for comparison between normal and manipulated embryos. Increasing numbers

of genetically encoded reporters of neural activity have been generated to study the locomotor, olfactory, and visual systems. The usefulness of genetically encoded calcium indicators (GECIs) for visualizing neural activity upon electrical stimulation or touch escape responses in the living zebrafish was first demonstrated in 2003 (Higashijima et al., 2003). Since then, a multitude of reporters have been developed which can also be stimulated by odors (Li et al., 2005) or light (Sumbre et al., 2008; Del Bene et al., 2010; Naumann et al., 2010). Although these reporters are promising tools, they only indirectly measure neural activity as they visualize changes in Ca2+-levels. Recently, the improved voltage sensor Mermaid has been reported to allow recordings of spikes in the fish similar to action potentials (Tsutsui et al., 2010). It will be exciting to generate transgenic animals, which express such reporters under the control of habenula cell population specific promoters to investigate neural activity upon stimulation. This will allow to unravel the functional properties of the DDC system.

The next step then is to use readily available and constantly improving tools to selectively inhibit or activate neural activity of habenula neurons (Del Bene and Wyart, 2011) and to investigate the effect on larval behavior. This approach can be refined by for instance making use of the differing excitations spectra of microbial opsins used to modulate neural activity and to perform a combinatorial analysis of the effect of activating neurons of different habenula subpopulations in the same animal. One caveat of using zebrafish is the existence of only a few reliable behavior assays that correlate habenula laterality or asymmetry with behavior and in these cases there has been no direct demonstration that habenular circuits are involved in the described behaviors (Barth et al., 2005; Facchin et al., 2009). The habenulae have been reported to be involved in syndromes such as depression and schizophrenia but we are far from having the possibility to use the fish as a disease model for such syndromes. One successfully applied test perhaps closest to a human depression syndrome is an escape assay, which implicated that afferent habenula projections derived from the telencephalon are important in this process (Lee et al., 2010). The usefulness for automated behavior and drug testing makes the zebrafish an exquisite model for large scale screens (Gerlai, 2010; Zhong and Lin, 2011). Furthermore, the vast variety of techniques and resources available for the zebrafish together with the enormous potential of a research field such as neurological diseases should be encouraging enough for scientists to think about novel readout systems.

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