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Development and Connectivity of the Habenular Nuclei

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Abstract

Accumulating evidence has reinforced that the habenular region of the vertebrate dorsal forebrain is an essential integrating center, and a region strongly implicated in neurological disorders and addiction. Despite the important and diverse neuromodulatory roles the habenular nuclei play, their development has been understudied. The emphasis of this review is on the dorsal habenular nuclei of zebrafish, homologous to the medial nuclei of mammals, as recent work has revealed new information about the signaling pathways that regulate their formation. Additionally, the zebrafish dorsal habenulae have become a valuable model for probing how left-right differences are established in a vertebrate brain. Sonic hedgehog, fibroblast growth factors and Wntless-INT proteins are all involved in the generation of progenitor cells and ultimately, along with Notch signaling, influence habenular neurogenesis and left-right asymmetry. Intriguingly, a genetic network has emerged that leads to the differentiation of dorsal habenular neurons and, through localized chemokine signaling, directs the posterior outgrowth of their newly emerging axons towards their postsynaptic target, the midbrain interpeduncular nucleus.

Keywords

habenula; fasciculus retroflexus; interpeduncular nucleus; left-right asymmetry

1. Introduction

The habenulae (Hb), are conserved, bilateral structures in the dorsal diencephalon and consist of medial (mHb) and lateral (lHb) nuclei in mammals (equivalent to dorsal and ventral nuclei, respectively, in fish and amphibians [1,2]). Habenular efferents project through the prominent fasciculus retroflexus (FR) fiber bundles, with neurons in the medial/dorsal nuclei primarily innervating the unpaired interpeduncular nucleus (IPN) in the ventral midbrain and those of the lateral/ventral nuclei predominantly targeting the raphe nuclei [3,4].

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The Hb are known to modulate diverse states such as fear and anxiety, aversion and reward, pain, sleep, and reproductive and aggressive behaviors [5–14]. Habenular activity has also been implicated in clinically relevant conditions, with dysregulation strongly associated with depressive disorders [15–17], and drug addiction [18–20]. Historically, our understanding of Hb function has come from gross lesioning experiments in the rodent brain [5,6,21,22], which can be imprecise and damage other brain regions. Such studies also fail to address the neuronal subpopulations within the Hb that mediate specific functions. State-of-the-art transgenic approaches in the mouse, however, have enabled selective manipulation of distinct cholinergic and peptidergic Hb neurons and monitoring of their activation in a variety of behavioral tests. Although increasing information is known about the identity and functions of some Hb neurons, notably the cholinergic and *tachykinin*-expressing populations, [23–25], and tracing experiments have uncovered pre- and post-synaptic partners [4,7,26,27], the neuronal complexity of the Hb and the precise circuitry underlying their diverse neuromodulatory roles are only now beginning to be appreciated. Additionally, the dorsal habenulae (dHb) of many non-mammalian vertebrates show striking L-R differences in their size, gene expression, and neuronal connections, whose formation and functional significance have become a topic of intense interest [28–31].

This review presents an overview of our current knowledge of habenular development, with a primary emphasis on the establishment of the dHb-IPN pathway in the developing zebrafish brain. The genetic network regulating development of the dHb is complex, often with a single signaling pathway involved in multiple, temporally distinct events. While some headway has been made, significant work is needed to obtain a complete picture of the regulation of Hb differentiation. Outstanding questions include how the appropriate number of neurons is generated, how neuronal diversity is achieved, and how precise connectivity with the IPN is established. A deeper molecular understanding of habenular development will not only provide fundamental insights into the diversification of brain nuclei, but will also allow the construction of more precise tools to manipulate select neuronal populations.

2. Mechanisms underlying habenular development

2.1 Specification and regionalization of the diencephalon

The posterior forebrain or diencephalon is comprised of three distinct regions referred to as prosomeres (p3–p1 along the A-P axis), which themselves can be subdivided into presumptive brain regions: p3 consists of the pre-thalamus (pTh), p2 of the epithalamus (containing the pineal complex and flanking Hb nuclei), zona limitans intrathalamica (ZLI) and thalamus, and p1 of the pretectum (PT) [32–34]. Boundaries between prosomeres are determined by gene expression and there is little mixing of cells between them or the brain regions that develop within them [35,36]. A number of regions along the A-P axis of the developing brain serve as signaling centers controlling diencephalic regionalization (Fig. 1A). Those most relevant for p2, and therefore epithalamic specification/habenular development, are the anterior neural boundary (ANB) in the very early telencephalon and the later-arising, mid-diencephalic organizer (MDO), located at the ZLI. The MDO is a source of Wnt, Fgf and Shh morphogens and is involved in setting up the p2–p3 border and

in patterning p2 [36–40]. The position, size and molecular composition of the MDO is thus critical for generation of the thalamic and epithalamic regions.

The Hb, along with the pineal gland, arise from the anterodorsal region of prosomere 2 (p2) in the roof of the developing diencephalon [33]. Consequently, formation of the Hb depends on the specification and regionalization of the diencephalon and its prosomeres. This is accomplished by input from signaling pathways along the dorsal-ventral (D-V) and anterior-posterior (A-P) axes; the former by opposing gradients of bone morphogenetic proteins (BMPs) and Sonic Hedgehog (Shh) signals and the latter by dynamic signaling centers that refine A-P subregions over time [37–40]. Wingless-INT proteins (Wnts) and Fibroblast growth factors (Fgfs) direct A-P patterning during the early subregionalization of the neural tube and establishment of the diencephalon. Canonical Wnt signals have been shown to promote posterior brain fates, such as the diencephalon, while simultaneously acting to antagonize forebrain fates [41,42]. Non-canonical Wnts and inhibitors of canonical Wnt signaling are necessary for formation of anterior brain structures and to oppose diencephalic fates [39,41,43].

Additionally, Fgf signals play important roles in the differentiation and patterning of the forebrain, influencing gene expression, neuronal identity and commissural connections [39,44,45]. The opposing action of Fgf and Wnt signaling also determines the boundary between the telencephalon and diencephalon [37,46].

Recent studies demonstrate that the adoption of thalamic or epithalamic neuronal cell fates is intimately connected. For example, in mice lacking Transcription Factor 7 Like 2 (Tcf7l2), a transcription factor in the canonical Wnt signaling pathway, post-mitotic thalamic neurons inappropriately acquire molecular properties of habenular neurons, which themselves now transmute and display features of thalamic neurons [47]. Similarly, loss of the *gastrulation brain homeobox 2* (*Gbx2*) gene results in the adoption of habenular neuronal fate at the expense of thalamic neuronal identity, apparently by indirectly altering the developmental program of proliferating thalamic progenitors [48].

2.1 From habenular progenitors to differentiated neurons

Once the epithalamic territory is defined, spatiotemporally distinct progenitor populations give rise to the vHb and dHb. This has been described in the developing zebrafish brain, where a group of cells, referred to as the thalamic-epithalamic early projecting cluster (ThEPC), was identified posterior and lateral to the developing dHb in prosomere 2 [49]. Live imaging in conjunction with photoconversion of the ThEPC population revealed that it migrates anteriorly to contribute to the vHb. Ablation of the ThEPC results in smaller Hb, as evidenced by labeling with a transgenic reporter line that labels both the vHb and dHb [49]. However, whether this was due to selective loss of vHb cells was not confirmed. The ability of ThEPC cells to migrate and contribute to the vHb is dependent on Wnt signaling. The vHb fail to form in zebrafish larvae that are homozygous mutant for the gene encoding Tcf7l2, a downstream effector of canonical Wnt signaling [49] or for *wntless* (*wls*), a gene encoding a protein required for the secretion of Wnt morphogens [50]. Although these findings demonstrate the importance of Wnt signaling in the generation of the vHb, further experiments are needed to determine whether Wnt signals have a direct impact on the

ThEPC progenitors. In contrast to the vHb of zebrafish, little is known about the origin of the homologous lateral Hb of mammals other than the timing of their neurogenesis, which occurs between E12–15 in rats [51].

Insights into the development of the medial Hb first came from the molecular characterization of the thalamus in the mouse, as some described genes were additionally expressed in the presumptive epithalamus [52]. The *developing brain homeobox (Dbx1)* gene, for example, is transcribed in a gradient in the diencephalon, resulting in high levels of Dbx1 protein in the ventricular zone adjacent to the Hb and low levels at the ZLI [52]. Short-term lineage tracing of *dbx1*⁺ cells in the mouse embryo reveals that they contribute to a number of brain regions, including neurons in the habenular region [52]. A homolog of *Dbx1*, named *dbx1b*, was identified as a marker of dHb progenitors in zebrafish [53]. Expression is detected in the developing dorsal diencephalon in the region of the developing Hb and lineage tracing confirms that *dbx1b*-expressing cells give rise to neurons throughout the larval and adult dHb [53,54].

Prior to the description of *dbx1b* expression in dHb progenitors, *chemokine (C-X-C) receptor type 4b (cxcr4b)* was thought to be the earliest gene expressed in cells of the zebrafish developing Hb [55]. However, *cxcr4b* transcripts are detected several hours after *dbx1b* expression, and in a pattern more similar to globally expressed proneural genes such as *neurogenin-1 (neurog-1)* and *achaete-scute complex-like 1b*. Because expression does not overlap with that of markers of differentiated post-mitotic neurons, the *cxcr4b*⁺ population was proposed to correspond to neural progenitors or newly born habenular neurons [55,56]. More recent work suggests that, while *dbx1b* transcripts define dHb progenitors, *cxcr4b* expression is indicative of newly generated neural precursors. Comparisons between the *dbx1b* and *cxcr4b* expression patterns indicate that *cxcr4b* transcripts are present in only the dorsal-most *dbx1b*-expressing progenitors at 36 hpf, as well as in cells positioned more dorsolaterally (Fig. 1B and [53,54]). Over time, *dbx1b* expression is downregulated in the *cxcr4b*-expressing neural precursor populations, which are positioned dorsolateral to the progenitors. Subsequently, mature dHb neurons arise dorsolateral to the *cxcr4b*-expressing precursors (Fig. 1B and [53,54]). The *dbx1b*⁺ progenitor population decreases in size, becoming progressively restricted as the dHb neurons increase in number [54]. Thus, development of the dHb proceeds from the specification of *dbx1b*⁺ progenitors to the generation of *cxcr4b*-expressing neural precursors and to the differentiation of mature neurons (Fig. 2).

2.3 Multiple signaling pathways regulate dHb formation

The ability to identify habenular progenitors enabled phenotypes to be detected at earlier stages in mutants with known defects in dHb morphology. Mouse embryos lacking the Paired Box 6 (Pax6) transcription factor show reduced *dbx1* expression in the dorsal forebrain, whereas *cxcr4b* transcripts are absent in the comparable region in zebrafish embryos injected with a morpholino against Pax6a [36,56]. Pax6 was found to genetically interact with Shh signaling to influence the formation of the Hb [36,56]; however, these studies differ in their conclusions. Chatterjee et al. concluded that expression of *pax6* is upstream of Shh signaling [36], whereas Halluin and coworkers report it is downstream [56].

Moreover, in conflicting results, Shh signaling has been reported as either inhibiting [36] or promoting [54,56] development of the dHb. In the experiments of Chatterjee and colleagues, the habenular domain was presumed to be expanded in mice homozygous for a hypomorphic allele of Shh, as well as in zebrafish lacking the Shh signaling component Smoothed (Smo; [36]). However, more recent analyses of zebrafish *smo* mutants indicate a complete loss of *dbx1b* [54] as well as *cxc4b* transcripts in the dHb region and no expression of a marker of differentiated Hb neurons [56]. The discrepancy between the zebrafish studies could be due to the use of different *smo* alleles and/or the inaccurate designation of the habenular region in earlier work.

A substantial decrease in the size of the Hb has been observed in both mouse and zebrafish embryos mutated for *fgf8* homologs [57,58]. Mouse null mutants have significant abnormalities in brain morphology that complicate the interpretation of the Hb phenotype, but smaller Hb can be clearly distinguished in *fgf8* hypomorphs [57]. Similarly, the overall morphology and patterning of the forebrain are maintained in zebrafish *fgf8a* mutants [44,53,58], but *dbx1b* expression is specifically reduced in the region of the developing dHb [53]. A transgenic reporter activated by Fgf signaling confirmed that *dbx1b*⁺ progenitors are responsive to Fgfs [53]. Furthermore, Fgf signaling is necessary not only to sustain expression of *dbx1b*, but also to maintain the *dbx1b*⁺ progenitor population by controlling expression of the *cyclin-dependent kinase inhibitor 1Ca* (*cdkn1ca*, formerly known as *kip2*) and pro-progenitor factor *hairy-related 6* (*her6*) genes [53].

Activation of the Fgf reporter is reduced in zebrafish mutant for the *mediator subunit 12* (*med12*) gene, whose product helps connect RNA Polymerase II (Pol II) to basal transcription factors [59,60]. Zebrafish *med12* mutants lack *dbx1b* and *cxc4b* transcripts and have no discernable habenulae. Expression of *dbx1b*, however, partially recovers over time; which suggests that loss of Med12 results in a delay in transcription of key genes and therefore a “missed window” for Hb specification [59]. Med12 was also shown to regulate Wnt signaling [61], which raises the interesting possibility that Med12 integrates Wnt and Fgf activity in Hb development.

Indeed, Wnt and Fgf signaling pathways interact in the establishment of the dHb (Fig. 3). Similar to *fgf8a* mutants, zebrafish embryos homozygous mutant for *wls*, which encodes a transmembrane protein essential for secretion of Wnts, develop significantly smaller dHb [50]. The production of habenular progenitors is most likely affected, as expression of *dbx1b* is delayed in the dorsal diencephalon of *wls* mutants and confined to a smaller domain compared to wild-type siblings [50]. The phenotype is more severe in *fgf8a;wls* double mutants, which completely lack *dbx1b*-expressing cells in the dorsal diencephalon [54]. Thus, Wnt and Fgf signals act in an additive manner to generate dHb progenitors.

A second and unexpected function of Wnt signaling is to restrict the domain of Fgf signaling in the dorsal diencephalon [54]. Reduction of Wnt signaling leads to expanded *fgf8a* expression in this region of the brain and, consequently, increased Fgf signaling, as evidenced by a corresponding enlargement of expression domains for genes responsive to Fgf signaling [54]. The excess Fgf signaling observed in *wls* mutants, however, is insufficient to restore normal dHb development, providing further support for an

independent requirement for Wnt signaling to regulate the size of the progenitor population. Curiously, a reciprocal regulatory interaction was described for the mouse embryo, whereby Fgf8 activity establishes the anterior boundary of Wnt expression in the diencephalon (Martinez-Ferre & Martinez, 2009).

In the zebrafish, another function of Fgf signaling is to regulate the spatial expression of genes that encode components of the Cxcr4b/chemokine signaling pathway in brain regions adjacent to the developing dHb. Fgf signaling positively regulates the expression of these genes, while Wnt indirectly controls their expression by restricting the domain of Fgf activity (Fig. 3). As discussed below in Section 2.6, chemokine signals guide the posteriorly directed axonal outgrowth of newly born habenular neurons. Therefore, an unforeseen outcome of perturbation of either the domains of Wnt, Fgf and, thus chemokine signaling, is defective dHb-IPN connectivity.

2.4 Differentiation of the dHb

Considerable progress has been made in deciphering early steps in Hb formation, yet remarkably little is known about how the seemingly homogeneous progenitor population produces the complex and diverse array of neuronal subtypes present in the mature Hb [62,63]. One clue into the control of habenular differentiation has come from the characterization of mutants for *POU class 4 homeobox 1 (pou4f1)*, a gene encoding a transcription factor (formerly known as Brn3a) that is expressed in almost all post-mitotic neurons of both the mouse mHb and zebrafish dHb [62,64]. In *pou4f1* mutant mice, expression of neurotransmitter receptors is altered in the Hb: There is a modest increase in expression of genes encoding some GABA and glycine receptor subunits and decreased expression of genes for serotonin, acetylcholine, and somatostatin receptors [62].

In rodents, the mHb can be divided into distinct dorsal and ventral subdomains on the basis of the peptidergic (i.e., Substance P) and cholinergic identity of their respective neuronal populations [62,65]. Zebrafish also possess distinct dHb subregions that have been defined by gene expression [66] and transgenic reporters [67,68], and which, for the most part, correspond with different neurotransmitter phenotypes [63]. The lateral dHb (dHbL) subregion contains neurons expressing *tachykinin1*, the precursor of Substance P, and the medial dHb (dHbM) neurons are largely cholinergic [63,69]. However, an added complexity is that the Substance P producing neurons are found in higher numbers in the left dHb, whereas the vast majority of cholinergic neurons are located in the right nucleus [63,69]. As discussed in Section 2.5, left-right (L-R) differences in neurogenesis are thought to account for the discrepancy in size and neuronal identity of the dHbL and dHbM subregions [64].

2.5 Left-right asymmetry of the zebrafish dHb

In many non-mammalian species, an intriguing feature of habenular development is the presence of neuroanatomical and molecular differences between the left and right dorsal nuclei [28,70]. Nodal-related factors, members of the transforming growth factor superfamily (TGF- β), are well known to influence the asymmetric positioning and morphology of visceral organs in vertebrate embryos, and are involved in setting up the directional asymmetry of the dHb [71,72]. In zebrafish, expression of the Nodal-related gene

southpaw (*spaw*) in the left lateral plate mesoderm (LPM) during somitogenesis has an impact on the later asymmetry of the viscera and brain [73,74]. For example, Spaw signaling in the left LPM transiently activates transcription of several other genes in the Nodal pathway in the left dorsal diencephalon [74], including *nodal related 2* (*ndr2*; also known as *cyclops* [75,76]), *lefty1* (*lft1*) which encodes a Nodal antagonist [77,78], and *paired-like homeodomain 2* (*pitx2*), which encodes a Nodal regulated transcription factor [79,80]. This diencephalic expression, the earliest L-R asymmetry detected so far in the developing neural tube, is situated in cells within the left side of the pineal organ anlage. Notably, in the adult brain, the pineal stalk emerges from the roof of the epithalamus slightly to the left of the midline [80].

Following the asymmetric expression of Nodal pathway members, a morphological asymmetry arises when a group of cells migrates leftward from the pineal anlage and coalesce to form the parapineal organ [81,82]. Shortly thereafter, expression of the *potassium channel tetramerization domain containing 12.1* gene (*kctd12.1*; previously known as *leftover*) appears in cells on the left side of the brain closely apposed to the newly formed parapineal [83]. As the dHb differentiate, the left and right nuclei diverge in their size, organization, expression of *kctd*-related genes, neuronal populations and patterns of efferent and afferent innervation [63,64,66,67,84,85].

In zebrafish, the Nodal signaling pathway does not seem to be necessary for the establishment of epithalamic asymmetry, but rather for its directionality. Loss of Nodal signaling in the left diencephalon results in randomization of the position of the parapineal [81,83] and of the base of the pineal stalk [80] along the L-R axis. Approximately half of larvae show a L-R reversal in dHb asymmetry [66,81,83]. Depletion of Spaw using antisense morpholinos also randomizes dHb asymmetry across the population [86]. The directionality of parapineal position and dHb asymmetry correspond, suggesting a causal relationship [81,83]. Indeed, after laser ablation of the parapineal, the left dHb adopts properties of the right dHb nucleus (i.e., right-isomerized dHb; [81,83]). In agreement, disruption in the specification and/or migration of parapineal cells in embryos mutant for *fgf8a* [58,87] or for the transcription factor T-box 2 (*tbx2*) gene [88] also results in bilaterally symmetric dHb with right identity.

As with Fgf signaling [89], the Wnt signaling pathway acts at multiple steps in habenular development [50,90] and plays an essential role in L-R asymmetry of the dHb (Fig. 2). Moreover, Wnts may influence the dHb in a parapineal independent manner. Loss of Axin1, a Wnt scaffolding protein required for degradation of β -catenin, results in enhanced Wnt signaling, and expression of *lft1* and *pitx2* on both sides of the pineal anlage even though *spaw* expression in the LPM is unaltered [91]. In contrast to other mutants with bilateral gene expression that later show L-R randomization of epithalamic asymmetry, *axin1* mutants develop with symmetric right isomerized dHb, irrespective of parapineal position [91]. Pharmacological over activation of the canonical Wnt pathway replicates the *axin1* mutant dHb phenotype [91]. Conversely, reduction in canonical Wnt signaling, as in *tcf712* mutants causes both dHb to adopt properties of the left nucleus [92]. In particular, the Tcf712 transcriptional regulator has been proposed to direct the formation of the asymmetrically sized dHb subregions by promoting the differentiation of dHbM fates at the expense of

lateral ones. On the left side of the brain, the parapineal is thought to override the action of Tcf7l2, thereby permitting differentiation of a larger dHbL neuronal population [92].

Just as the loss or gain of Wnt activity dictates dHbL or dHbM identity, the timing of neurogenesis regulated by Notch signaling also directs the L-R asymmetry of dHb subregions. Experiments that delay neurogenesis, such as excessive Notch signaling, inhibit differentiation of early born dHbL neurons, resulting in right isomerized dHb. In an opposite manner, premature neurogenesis in mutants defective in Notch signaling causes an excess of dHbL neurons at the expense of later born dHbM neurons on both sides of the brain (i.e. left-isomerized dHb) [64]. The outcome of the combined action of the Wnt and Notch pathways is that differing numbers of specific neuronal subtypes are produced in the left and right dHb of zebrafish. These subtypes, whether derived from the left or right nucleus, innervate the same D-V region of the IPN.

Although the precise neuronal populations have yet to be determined, L-R asymmetry of the dHb of zebrafish likely underlies the differential functions that have been attributed to them thus far. For example, in larvae, the left nucleus shows a preferential response to light [93,94], whereas the right dHb, which selectively receives bilateral input from a subset of olfactory mitral cells [85], seems to exhibit enhanced activation upon exposure to certain odorants [93,95]. Lateralization of habenular function has also been observed in social behavior, such as in aggression and conflict resolution [12] and in modulation of the response to fear [96,97].

2.6 Habenular efferent projections to the interpeduncular nucleus

Early after their birth, Hb neurons extend their axons posteriorly to join the FR, the prominent nerve bundle that projects ventrally between prosomeres 1 and 2 [98,99]. The FR of rodents is topographically organized with mHb efferents concentrated in the core and lHb efferents and afferents from the ventral tegmental area located in the surrounding sheath [3,100,101].

In zebrafish, dHb axons can be detected by immunolabeling against the related Kctd12.1 and Kctd12.2 proteins, by labeling with lipophilic dyes or using transgenic reporters, revealing that some growth cones reach the IPN between 2–3 dpf [66,102,103]. Axons from vHb neurons likely extend towards the RN much later, as the vHb are not fully formed until 4 dpf [49]. In the rat, the first habenular efferents are detected at E13 and they reach the caudal diencephalon by E14 [99]. In contrast to zebrafish, these early projections in the rat are efferents from lHb neurons, which are born between E12–15. The medial habenular neurons do not form until E14–18 [99].

Input from multiple axon guidance pathways coordinates the initial outgrowth, fasciculation, extension, and connectivity of habenular axons with their midbrain and hindbrain targets. Recent work in zebrafish implicates signaling by Cxcr4b chemokine receptors on dHb neurons in mediating the posteriorly directed emergence of their axonal processes [54]. Although *cxcr4b* transcripts are no longer detected in mature dHb neurons, fluorescently-tagged Cxcr4b protein is present and selectively localizes to the proximal region of their axons. Internalization of the fluorescently-tagged receptor and its presumed degradation

more distally is indicative of active chemokine signaling [104] at the site of axon initiation. Consistent with this hypothesis, the C-X-C motif chemokine ligand 12 (Cxcl12) homologs a and b are transcribed by cells neighboring the developing dHb: *cxcl12a* is expressed just caudal to the Hb in cells along the FR pathway; the *cxcl12b* domain is broader and includes a strip of cells adjacent to the anterior border of the dHb [54].

In the absence of the Cxcr4b receptor or the Cxcl12a chemokine, or upon misexpression of chemokine pathway components as in *wls* mutant larvae, axonal projections from the dHb can be highly aberrant. In the most severe phenotypes, almost all axons grow out in an anterior direction, often merging into a large bundle that extends up the midline toward the telencephalon and olfactory system [54]. Chemokine signals attract or create a permissive environment for posteriorly directed dHb axons, but may also prevent axons from projecting rostrally.

An interesting new study reports that the emerging Hb efferents of zebrafish navigate through the bilateral ThEPCs, which form a network of ipsilateral projections and interconnected, contralateral commissures [103]. Through this intricate network, ThEPC neurons were found to coordinate the initiation, rate of growth and synchronized elongation of Hb axons on the left and right sides of the brain [103]. Whether the ThEPCs are the source of chemokine or other signals directing outgrowth of Hb axons remains an open question.

After axon initiation, a number of pathfinding molecules are required for the successful innervation of the ventral midbrain IPN. Key among them are Semaphorin family members. In rodents, *Sema3f* and *Sema5a* are both necessary for proper fasciculation of habenular efferents in the FR [99,105–107]. *Sema3f* is expressed along the rostral border of prosomere 1 and binds to the Neuropilin-2 (Npn2) receptor, which is present on mHb neurons [99,105,108]. Loss of either *Nrp2* or *Sema3f* results in defasciculation of the FR, with the phenotype of *nnp2* mutants notably more severe [105,106,109]. Furthermore, *in vitro* assays using *sema3f*-expressing p1 and habenular explants show that *Sema3f* repels habenular axons [99].

Sema5a has been suggested to play a bifunctional role in axon pathfinding, depending on the extracellular context [107]. It acts in an inhibitory manner when presented with chondroitin sulfate proteoglycans (CSPGs) or becomes an attractive cue in the presence of heparin sulfate proteoglycans (HSPGs). HSPGs are located on the surface of extending Hb axons, while CSPGs are distributed along the path of the FR in p2. Loss of *Sema5a* results in ectopic axonal projections into p2 and the failure of Hb axons to reach their targets. Co-culture experiments of Hb and p2 explants [99] further support that that p2 is non-permissive for *Sema5a*-expressing habenular axons through contact-mediated interaction with CSPGs [107]. Moreover, the attractant HSPGs on Hb axons promote fasciculation in the FR [107]. Thus, *Sema3f* and *Sema5a* work in conjunction to create a repulsive territory surrounding the intended path of the FR, thereby preventing Hb axons from defasciculating and invading p1 or p2.

Guidance of Hb axons ventrally towards the IPN is mediated by Netrin and its receptor Deleted in Colorectal Carcinoma (DCC) [99,100]. DCC is expressed in the FR of embryonic rats [99] and in the medial habenulae of mouse embryos [62,100]. The Netrin1 ligand is concentrated in the ventral caudal diencephalon (VCD) and more caudal floor plate, where it serves to attract Hb axons ventrally and caudally towards their midbrain targets [99]. Loss of either DCC or Netrin1 results in disorganization of the FR and misdirection of Hb efferents, which, instead, project ectopically to the dorsal roof of the Hb region [100].

Other candidate guidance cues in the rodent FR have been identified through mass spectrometry and immunolabeling experiments. The Roundabout guidance receptor 3 (Robo3) and cell surface Glypican2, are found on axons of mHb neurons, and a close homolog of L1 (CHL1) protein is present in the FR sheath [100]. The Frizzled3 receptor is required for proper formation of the FR, as these axon bundles are barely detectable in *Fz3* mutants [110].

In zebrafish, a third member of the Semaphorin family, *Sema3d*, and its Neuropilin1a (Nrp1a) receptor, have been implicated in the growth cone choice of dHbL axons to innervate the dorsal rather than the ventral IPN [102]. The *nrl1a* gene is predominantly expressed in the left dHb, where the dHbL subregion is significantly larger. Translation blocking morpholinos directed against either *nrl1a* or *sema3d* transcripts disrupt innervation of the dorsal IPN, whereas ectopic overexpression of *sema3d* causes an overgrowth of dorsally directed axons [102]. *Sema3d*, produced by cells along the trajectory of the FR and dorsal to the IPN, therefore appears to act as an attractive cue in this context [102]. Nrp1 is not found in the habenular region of rats [99] nor have obvious L-R differences in IPN connectivity been detected in mice, suggesting that *Sema3d*/Nrp1a signaling is specific for connectivity of the asymmetric dHb subregions with the appropriate D-V territories of the IPN in non-mammalian species.

3. Conclusions

Converging studies on brain areas involved in nicotine addiction and on the genetic regulation of brain L-R asymmetry have led to a renewed interest in the dHb/mHb nuclei and their projections to the IPN. Understanding the many functions associated with the dHb/mHb-IPN circuitry, however, requires a greater appreciation of the development of their diverse neuronal cell types and precise connectivity. While inroads have been made regarding the identity and formation of Hb progenitor cells and the regulation of neurogenesis, far less is known about the genes that underlie the differentiation of neuronal subtypes. Transcriptional profiling and expression analyses of the Hb region [62,111,112] should expedite their identification.

More information is also needed to bridge the action of signaling pathways in early aspects of Hb development with the localization of later axon guidance cues. Restricting spatial domains of activity as in the Wnt-Fgf-Cxcr4 signaling hierarchy in zebrafish [54], or by the Wnt transcriptional effector Tcf7l2 that imparts Hb neuronal identity critical for formation of the mouse FR [47], are but two examples of the effects early patterning events can have on later axonal projections.

The application of a variety of methods for labeling Hb neurons in the zebrafish and mouse brain, and for tracing their efferents to the IPN, have revealed that distinct subregions or neurotransmitter expressing populations innervate specific IPN territories [4,63,65,96]. However, little is known about the guidance mechanisms that precisely shape the Hb-IPN connectivity map.

Complementary experimental approaches in zebrafish and mice have shown that key aspects of Hb development are well conserved, such as the Dbx1-expressing progenitor populations and the involvement of Shh, Fgf and Wnt signaling in their generation. However, the modes of action of other signaling pathways may have diverged in evolution. For example, in more ancestral fish species, such as the catshark and the lamprey, Nodal signaling is necessary for asymmetric development of the epithalamus [113], and not just for its directionality, as in zebrafish and medaka [81,83,114]. The Hb of amphibians and reptiles [28,115,116] also display prominent L-R asymmetry, but how this arises developmentally is unknown.

In birds and mammals, while there is some evidence for neuroanatomical differences between the Hb [28,117,118], these may be subtle or less widespread across species, and a role for Nodal signaling in their formation has not been found. From recent histological analyses of human brain samples, and reconstruction of habenular shape and volume, the mHb were determined to be symmetric, but the volume of the lateral nucleus is significantly larger on the left than the right [119]. Such findings coupled with studies demonstrating that the bilateral lHb nuclei of mammals are differentially activated [16,101,120,121], suggest that other, as yet undiscovered, developmental processes must be in place to confer L-R asymmetry and functional lateralization on the habenular region.

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Abbreviations

ANB	anterior neural boundary
A-P	anterior-posterior
BMP	Bone Morphogenetic Protein
CSPG	chondroitin sulfate proteoglycans
d	dorsal
dpf	days post fertilization
D-V	dorsal-ventral
Fgf	Fibroblast growth factor
FR	fasciculus retroflexus
GABA	gamma aminobutyric acid

Hb	habenula
hpf	hours post fertilization
HSPG	heparin sulfate proteoglycans
IPN	interpeduncular nucleus
l	lateral
LPM	lateral plate mesoderm
L-R	left-right
m	medial
MDO	mid-diencephalic organizer
p(1–3)	prosomeres 1–3
PT	pretectum
pTh	prethalamus
RN	raphe nucleus
Shh	Sonic hedgehog
ThEPC	thalamic-epithalamic early projecting cluster
v	ventral
Wnt	Wingless-type MMTV integration site family member
ZLI	zona limitans intrathalamica.

References

1. Kemali M, Guglielmotti V, Gioffre D. Neuroanatomical identification of the frog habenular connections using peroxidase (HRP). *Exp Brain Res.* 1980; 38:341–347. [PubMed: 6154592]
2. Amo R, Aizawa H, Takahoko M, Kobayashi M, Takahashi R, Aoki T, Okamoto H. Identification of the zebrafish ventral habenula as a homolog of the mammalian lateral habenula. *J Neurosci.* 2010; 30:1566–1574. [PubMed: 20107084]
3. Herkenham M, Nauta WJ. Efferent connections of the habenular nuclei in the rat. *J Comp Neurol.* 1979; 187:19–47. [PubMed: 226566]
4. Quina LA, Harris J, Zeng H, Turner EE. Specific connections of the interpeduncular subnuclei reveal distinct components of the habenulopeduncular pathway. *J Comp Neurol.* 2017; 525:2632–2656. [PubMed: 28387937]
5. Rodgers C, Law OT. The effects of habenular and medial forebrain bundle lesions on sexual behavior in female rats. *Psychon Sci.* 1967; 8:1–2.
6. Modianos DT, Hitt JC, Flexman J. Habenular lesions produce decrements in feminine, but not masculine, sexual behavior in rats. *Behav Biol.* 1974; 10:75–87. [PubMed: 4592636]
7. Sutherland RJ. The dorsal diencephalic conduction system: a review of the anatomy and functions of the habenular complex. *Neurosci Biobehav Rev.* 1982; 6:1–13. [PubMed: 7041014]

8. Haun F, Eckenrode TC, Murray M. Habenula and thalamus cell transplants restore normal sleep behaviors disrupted by denervation of the interpeduncular nucleus. *J Neurosci.* 1992; 12:3282–3290. [PubMed: 1494957]
9. Hikosaka O. The habenula: from stress evasion to value-based decision-making. *Nat Rev Neurosci.* 2010; 11:503–513. [PubMed: 20559337]
10. Aizawa H, Cui W, Tanaka K, Okamoto H. Hyperactivation of the habenula as a link between depression and sleep disturbance. *Front Hum Neurosci.* 2013; 7:826. [PubMed: 24339810]
11. Wang D, Li Y, Feng Q, Guo Q, Zhou J, Luo M. Learning shapes the aversion and reward responses of lateral habenula neurons. *Elife.* 2017; 6
12. Chou MY, Amo R, Kinoshita M, Cherng BW, Shimazaki H, Agetsuma M, Shiraki T, Aoki T, Takahoko M, Yamazaki M, et al. Social conflict resolution regulated by two dorsal habenular subregions in zebrafish. *Science.* 2016; 352:87–90. [PubMed: 27034372]
13. Flanigan M, Aleyasin H, Takahashi A, Golden SA, Russo SJ. An emerging role for the lateral habenula in aggressive behavior. *Pharmacol Biochem Behav.* 2017
14. Li Y, Wang Y, Xuan C, Li Y, Piao L, Li J, Zhao H. Role of the Lateral Habenula in Pain-Associated Depression. *Front Behav Neurosci.* 2017; 11:31. [PubMed: 28270756]
15. Savitz JB, Nugent AC, Bogers W, Roiser JP, Bain EE, Neumeister A, Zarate CA Jr, Manji HK, Cannon DM, Marrett S, et al. Habenula volume in bipolar disorder and major depressive disorder: a high-resolution magnetic resonance imaging study. *Biol Psychiatry.* 2011; 69:336–343. [PubMed: 21094939]
16. Lawson RP, Nord CL, Seymour B, Thomas DL, Dayan P, Pilling S, Roiser JP. Disrupted habenula function in major depression. *Mol Psychiatry.* 2016
17. Liu WH, Valton V, Wang LZ, Zhu YH, Roiser JP. Association between habenula dysfunction and motivational symptoms in unmedicated major depressive disorder. *Soc Cogn Affect Neurosci.* 2017
18. Antolin-Fontes B, Ables JL, Gorlich A, Ibanez-Tallon I. The habenulo-interpeduncular pathway in nicotine aversion and withdrawal. *Neuropharmacology.* 2015; 96:213–222. [PubMed: 25476971]
19. Velasquez KM, Molfese DL, Salas R. The role of the habenula in drug addiction. *Front Hum Neurosci.* 2014; 8:174. [PubMed: 24734015]
20. Molas S, DeGroot SR, Zhao-Shea R, Tapper AR. Anxiety and Nicotine Dependence: Emerging Role of the Habenulo-Interpeduncular Axis. *Trends Pharmacol Sci.* 2017; 38:169–180. [PubMed: 27890353]
21. Murray M, Murphy CA, Ross LL, Haun F. The role of the habenula-interpeduncular pathway in modulating levels of circulating adrenal hormones. *Restor Neurol Neurosci.* 1994; 6:301–307. [PubMed: 21551761]
22. Murphy CA, DiCamillo AM, Haun F, Murray M. Lesion of the habenular efferent pathway produces anxiety and locomotor hyperactivity in rats: a comparison of the effects of neonatal and adult lesions. *Behav Brain Res.* 1996; 81:43–52. [PubMed: 8950000]
23. Hsu YW, Wang SD, Wang S, Morton G, Zariwala HA, de la Iglesia HO, Turner EE. Role of the dorsal medial habenula in the regulation of voluntary activity, motor function, hedonic state, and primary reinforcement. *J Neurosci.* 2014; 34:11366–11384. [PubMed: 25143617]
24. Hsu YW, Morton G, Guy EG, Wang SD, Turner EE. Dorsal Medial Habenula Regulation of Mood-Related Behaviors and Primary Reinforcement by Tachykinin-Expressing Habenula Neurons. *eNeuro.* 2016; 3
25. Han S, Yang SH, Kim JY, Mo S, Yang E, Song KM, Ham BJ, Mechawar N, Turecki G, Lee HW, et al. Down-regulation of cholinergic signaling in the habenula induces anhedonia-like behavior. *Sci Rep.* 2017; 7:900. [PubMed: 28420875]
26. Klemm WR. Habenular and interpeduncularis nuclei: shared components in multiple-function networks. *Med Sci Monit.* 2004; 10:RA261–273. [PubMed: 15507867]
27. Turner KJ, Hawkins TA, Yanez J, Anadon R, Wilson SW, Figueira M. Afferent Connectivity of the Zebrafish Habenulae. *Front Neural Circuits.* 2016; 10:30. [PubMed: 27199671]
28. Concha ML, Wilson SW. Asymmetry in the epithalamus of vertebrates. *J Anat.* 2001; 199:63–84. [PubMed: 11523830]

29. Duboué ER, Halpern ME. Genetic and transgenic approaches to study zebrafish brain asymmetry and lateralized behavior. *Neuromethods*. in press.
30. Aizawa H. Habenula and the asymmetric development of the vertebrate brain. *Anat Sci Int*. 2013; 88:1–9. [PubMed: 23086722]
31. Duboc V, Dufourcq P, Blader P, Roussigne M. Asymmetry of the Brain: Development and Implications. *Annu Rev Genet*. 2015; 49:647–672. [PubMed: 26442849]
32. Wullimann MF, Puelles L. Postembryonic neural proliferation in the zebrafish forebrain and its relationship to prosomeric domains. *Anat Embryol (Berl)*. 1999; 199:329–348. [PubMed: 10195307]
33. Hauptmann G, Soll I, Gerster T. The early embryonic zebrafish forebrain is subdivided into molecularly distinct transverse and longitudinal domains. *Brain Res Bull*. 2002; 57:371–375. [PubMed: 11922991]
34. Puelles L, Rubenstein JL. Forebrain gene expression domains and the evolving prosomeric model. *Trends Neurosci*. 2003; 26:469–476. [PubMed: 12948657]
35. Staudt N, Houart C. The prethalamus is established during gastrulation and influences diencephalic regionalization. *PLoS Biol*. 2007; 5:e69. [PubMed: 17341136]
36. Chatterjee M, Guo Q, Weber S, Scholpp S, Li JY. Pax6 regulates the formation of the habenular nuclei by controlling the temporospatial expression of Shh in the diencephalon in vertebrates. *BMC Biol*. 2014; 12:13. [PubMed: 24528677]
37. Wilson SW, Houart C. Early steps in the development of the forebrain. *Dev Cell*. 2004; 6:167–181. [PubMed: 14960272]
38. Scholpp S, Lumsden A. Building a bridal chamber: development of the thalamus. *Trends Neurosci*. 2010; 33:373–380. [PubMed: 20541814]
39. Cavodeassi F, Houart C. Brain regionalization: of signaling centers and boundaries. *Dev Neurobiol*. 2012; 72:218–233. [PubMed: 21692189]
40. Hagemann AI, Scholpp S. The Tale of the Three Brothers - Shh, Wnt, and Fgf during Development of the Thalamus. *Front Neurosci*. 2012; 6:76. [PubMed: 22654733]
41. Heisenberg CP, Houart C, Take-Uchi M, Rauch GJ, Young N, Coutinho P, Masai I, Caneparo L, Concha ML, Geisler R, et al. A mutation in the Gsk3-binding domain of zebrafish Masterblind/Axin1 leads to a fate transformation of telencephalon and eyes to diencephalon. *Genes Dev*. 2001; 15:1427–1434. [PubMed: 11390362]
42. van de Water S, van de Wetering M, Joore J, Esseling J, Bink R, Clevers H, Zivkovic D. Ectopic Wnt signal determines the eyeless phenotype of zebrafish masterblind mutant. *Development*. 2001; 128:3877–3888. [PubMed: 11641213]
43. Cavodeassi F, Carreira-Barbosa F, Young RM, Concha ML, Allende ML, Houart C, Tada M, Wilson SW. Early stages of zebrafish eye formation require the coordinated activity of Wnt11, Fz5, and the Wnt/beta-catenin pathway. *Neuron*. 2005; 47:43–56. [PubMed: 15996547]
44. Shanmugalingam S, Houart C, Picker A, Reifers F, Macdonald R, Barth A, Griffin K, Brand M, Wilson SW. Ace/Fgf8 is required for forebrain commissure formation and patterning of the telencephalon. *Development*. 2000; 127:2549–2561. [PubMed: 10821754]
45. Walshe J, Mason I. Unique and combinatorial functions of Fgf3 and Fgf8 during zebrafish forebrain development. *Development*. 2003; 130:4337–4349. [PubMed: 12900450]
46. Houart C, Caneparo L, Heisenberg C, Barth K, Take-Uchi M, Wilson S. Establishment of the telencephalon during gastrulation by local antagonism of Wnt signaling. *Neuron*. 2002; 35:255–265. [PubMed: 12160744]
47. Lee M, Yoon J, Song H, Lee B, Lam DT, Yoon J, Baek K, Clevers H, Jeong Y. Tcf7l2 plays crucial roles in forebrain development through regulation of thalamic and habenular neuron identity and connectivity. *Dev Biol*. 2017; 424:62–76. [PubMed: 28219675]
48. Mallika C, Guo Q, Li JY. Gbx2 is essential for maintaining thalamic neuron identity and repressing habenular characters in the developing thalamus. *Dev Biol*. 2015; 407:26–39. [PubMed: 26297811]
49. Beretta CA, Dross N, Bankhead P, Carl M. The ventral habenulae of zebrafish develop in prosomere 2 dependent on Tcf7l2 function. *Neural Dev*. 2013; 8:19. [PubMed: 24067090]

50. Kuan YS, Roberson S, Akitake CM, Fortuno L, Gamse J, Moens C, Halpern ME. Distinct requirements for Wntless in habenular development. *Dev Biol.* 2015; 406:117–128. [PubMed: 26116173]
51. Altman J, Bayer SA. Development of the diencephalon in the rat. IV. Quantitative study of the time of origin of neurons and the internuclear chronological gradients in the thalamus. *J Comp Neurol.* 1979; 188:455–471. [PubMed: 489803]
52. Vue TY, Aaker J, Taniguchi A, Kazemzadeh C, Skidmore JM, Martin DM, Martin JF, Treier M, Nakagawa Y. Characterization of progenitor domains in the developing mouse thalamus. *J Comp Neurol.* 2007; 505:73–91. [PubMed: 17729296]
53. Dean BJ, Erdogan B, Gamse JT, Wu SY. Dbx1b defines the dorsal habenular progenitor domain in the zebrafish epithalamus. *Neural Dev.* 2014; 9:20. [PubMed: 25212830]
54. Roberson S, Halpern ME. Convergence of signaling pathways underlying habenular formation and axonal outgrowth. *Development.* 2017; 144:2652–2662. [PubMed: 28619821]
55. Roussigne M, Bianco IH, Wilson SW, Blader P. Nodal signalling imposes left-right asymmetry upon neurogenesis in the habenular nuclei. *Development.* 2009; 136:1549–1557. [PubMed: 19363156]
56. Halluin C, Madelaine R, Naye F, Peers B, Roussigne M, Blader P. Habenular Neurogenesis in Zebrafish Is Regulated by a Hedgehog, Pax6 Proneural Gene Cascade. *PLoS One.* 2016; 11:e0158210. [PubMed: 27387288]
57. Martinez-Ferre A, Martinez S. The development of the thalamic motor learning area is regulated by Fgf8 expression. *J Neurosci.* 2009; 29:13389–13400. [PubMed: 19846726]
58. Regan JC, Concha ML, Roussigne M, Russell C, Wilson SW. An Fgf8-dependent bistable cell migratory event establishes CNS asymmetry. *Neuron.* 2009; 61:27–34. [PubMed: 19146810]
59. Wu SY, de Borsetti NH, Bain EJ, Bulow CR, Gamse JT. Mediator subunit 12 coordinates intrinsic and extrinsic control of epithalamic development. *Dev Biol.* 2014; 385:13–22. [PubMed: 24184636]
60. Yin JW, Wang G. The Mediator complex: a master coordinator of transcription and cell lineage development. *Development.* 2014; 141:977–987. [PubMed: 24550107]
61. Rocha PP, Scholze M, Bleiss W, Schrewe H. Med12 is essential for early mouse development and for canonical Wnt and Wnt/PCP signaling. *Development.* 2010; 137:2723–2731. [PubMed: 20630950]
62. Quina LA, Wang S, Ng L, Turner EE. Brn3a and Nurr1 mediate a gene regulatory pathway for habenula development. *J Neurosci.* 2009; 29:14309–14322. [PubMed: 19906978]
63. deCarvalho TN, Subedi A, Rock J, Harfe BD, Thisse C, Thisse B, Halpern ME, Hong E. Neurotransmitter map of the asymmetric dorsal habenular nuclei of zebrafish. *Genesis.* 2014; 52:636–655. [PubMed: 24753112]
64. Aizawa H, Goto M, Sato T, Okamoto H. Temporally regulated asymmetric neurogenesis causes left-right difference in the zebrafish habenular structures. *Dev Cell.* 2007; 12:87–98. [PubMed: 17199043]
65. Contestabile A, Villani L, Fasolo A, Franzoni MF, Griabudo L, Oktedalen O, Fonnum F. Topography of cholinergic and substance P pathways in the habenulo-interpeduncular system of the rat. An immunocytochemical and microchemical approach. *Neuroscience.* 1987; 21:253–270. [PubMed: 2439945]
66. Gamse JT, Kuan YS, Macurak M, Brosamle C, Thisse B, Thisse C, Halpern ME. Directional asymmetry of the zebrafish epithalamus guides dorsoventral innervation of the midbrain target. *Development.* 2005; 132:4869–4881. [PubMed: 16207761]
67. Aizawa H, Bianco IH, Hamaoka T, Miyashita T, Uemura O, Concha ML, Russell C, Wilson SW, Okamoto H. Laterotopic representation of left-right information onto the dorso-ventral axis of a zebrafish midbrain target nucleus. *Curr Biol.* 2005; 15:238–243. [PubMed: 15694307]
68. Agetsuma M, Aizawa H, Aoki T, Nakayama R, Takahoko M, Goto M, Sassa T, Amo R, Shiraki T, Kawakami K, et al. The habenula is crucial for experience-dependent modification of fear responses in zebrafish. *Nature neuroscience.* 2010; 13:1354–1356. [PubMed: 20935642]

69. Hong E, Santhakumar K, Akitake CA, Ahn SJ, Thisse C, Thisse B, Wyart C, Mangin JM, Halpern ME. Cholinergic left-right asymmetry in the habenulo-interpeduncular pathway. *Proc Natl Acad Sci U S A*. 2013; 110:21171–21176. [PubMed: 24327734]
70. Braitenberg V, Kemali M. Exceptions to bilateral symmetry in the epithalamus of lower vertebrates. *J Comp Neurol*. 1970; 138:137–146. [PubMed: 4189834]
71. Duboué, E., Halpern, ME. Genetic and transgenic approaches to study zebrafish brain asymmetry and lateralized behavior. In: Vallortigara, G., Rogers, L.J., editors. *Lateralized brain Function: Methods in Human and Non-Human Species*. Humana Press; 2017. p. 553–589.[Walz W (Series Editor): *Neuromethods*, Springer Protocols, vol 122.]
72. Signore IA, Palma K, Concha ML. Nodal signalling and asymmetry of the nervous system. *Philos Trans R Soc Lond B Biol Sci*. 2016; 371
73. Rebagliati MR, Toyama R, Fricke C, Haffter P, Dawid IB. Zebrafish nodal-related genes are implicated in axial patterning and establishing left-right asymmetry. *Dev Biol*. 1998; 199:261–272. [PubMed: 9698446]
74. Long S, Ahmad N, Rebagliati M. The zebrafish nodal-related gene southpaw is required for visceral and diencephalic left-right asymmetry. *Development*. 2003; 130:2303–2316. [PubMed: 12702646]
75. Rebagliati MR, Toyama R, Haffter P, Dawid IB. cyclops encodes a nodal-related factor involved in midline signaling. *Proc Natl Acad Sci U S A*. 1998; 95:9932–9937. [PubMed: 9707578]
76. Sampath K, Rubinstein AL, Cheng AM, Liang JO, Fekany K, Solnica-Krezel L, Korzh V, Halpern ME, Wright CV. Induction of the zebrafish ventral brain and floorplate requires cyclops/nodal signalling. *Nature*. 1998; 395:185–189. [PubMed: 9744278]
77. Bisgrove BW, Essner JJ, Yost HJ. Regulation of midline development by antagonism of lefty and nodal signaling. *Development*. 1999; 126:3253–3262. [PubMed: 10375514]
78. Thisse C, Thisse B. Antivin, a novel and divergent member of the TGFbeta superfamily, negatively regulates mesoderm induction. *Development*. 1999; 126:229–240. [PubMed: 9847237]
79. Concha ML, Burdine RD, Russell C, Schier AF, Wilson SW. A nodal signaling pathway regulates the laterality of neuroanatomical asymmetries in the zebrafish forebrain. *Neuron*. 2000; 28:399–409. [PubMed: 11144351]
80. Liang JO, Etheridge A, Hantsoo L, Rubinstein AL, Nowak SJ, Izpisua Belmonte JC, Halpern ME. Asymmetric nodal signaling in the zebrafish diencephalon positions the pineal organ. *Development*. 2000; 127:5101–5112. [PubMed: 11060236]
81. Concha ML, Russell C, Regan JC, Tawk M, Sidi S, Gilmour DT, Kapsimali M, Sumoy L, Goldstone K, Amaya E, et al. Local tissue interactions across the dorsal midline of the forebrain establish CNS laterality. *Neuron*. 2003; 39:423–438. [PubMed: 12895418]
82. Snelson CD, Burkart JT, Gamse JT. Formation of the asymmetric pineal complex in zebrafish requires two independently acting transcription factors. *Dev Dyn*. 2008; 237:3538–3544. [PubMed: 18629869]
83. Gamse JT, Thisse C, Thisse B, Halpern ME. The parapineal mediates left-right asymmetry in the zebrafish diencephalon. *Development*. 2003; 130:1059–1068. [PubMed: 12571098]
84. Bianco IH, Carl M, Russell C, Clarke JD, Wilson SW. Brain asymmetry is encoded at the level of axon terminal morphology. *Neural Dev*. 2008; 3:9. [PubMed: 18377638]
85. Miyasaka N, Morimoto K, Tsubokawa T, Higashijima S, Okamoto H, Yoshihara Y. From the olfactory bulb to higher brain centers: genetic visualization of secondary olfactory pathways in zebrafish. *J Neurosci*. 2009; 29:4756–4767. [PubMed: 19369545]
86. Facchin L, Burgess HA, Siddiqi M, Granato M, Halpern ME. Determining the function of zebrafish epithalamic asymmetry. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences*. 2009; 364:1021–1032. [PubMed: 19064346]
87. Clanton JA, Hope KD, Gamse JT. Fgf signaling governs cell fate in the zebrafish pineal complex. *Development*. 2013; 140:323–332. [PubMed: 23250206]
88. Snelson CD, Santhakumar K, Halpern ME, Gamse JT. Tbx2b is required for the development of the parapineal organ. *Development*. 2008; 135:1693–1702. [PubMed: 18385257]
89. Neugebauer JM, Yost HJ. FGF signaling is required for brain left-right asymmetry and brain midline formation. *Dev Biol*. 2014; 386:123–134. [PubMed: 24333178]

90. Husken U, Carl M. The Wnt/beta-catenin signaling pathway establishes neuroanatomical asymmetries and their laterality. *Mech Dev.* 2013; 130:330–335. [PubMed: 23022991]
91. Carl M, Bianco IH, Bajoghli B, Aghaallaei N, Czerny T, Wilson SW. Wnt/Axin1/beta-catenin signaling regulates asymmetric nodal activation, elaboration, and concordance of CNS asymmetries. *Neuron.* 2007; 55:393–405. [PubMed: 17678853]
92. Husken U, Stickney HL, Gestri G, Bianco IH, Faro A, Young RM, Roussigne M, Hawkins TA, Beretta CA, Brinkmann I, et al. Tcf7l2 is required for left-right asymmetric differentiation of habenular neurons. *Curr Biol.* 2014; 24:2217–2227. [PubMed: 25201686]
93. Dreosti E, Vendrell Llopis N, Carl M, Yaksi E, Wilson SW. Left-right asymmetry is required for the habenulae to respond to both visual and olfactory stimuli. *Curr Biol.* 2014; 24:440–445. [PubMed: 24508167]
94. Zhang BB, Yao YY, Zhang HF, Kawakami K, Du JL. Left Habenula Mediates Light-Preference Behavior in Zebrafish via an Asymmetrical Visual Pathway. *Neuron.* 2017; 93:914–928 e914. [PubMed: 28190643]
95. Krishnan S, Mathuru AS, Kibat C, Rahman M, Lupton CE, Stewart J, Claridge-Chang A, Yen SC, Jesuthasan S. The right dorsal habenula limits attraction to an odor in zebrafish. *Curr Biol.* 2014; 24:1167–1175. [PubMed: 24856207]
96. Agetsuma M, Aizawa H, Aoki T, Nakayama R, Takahoko M, Goto M, Sassa T, Amo R, Shiraki T, Kawakami K, et al. The habenula is crucial for experience-dependent modification of fear responses in zebrafish. *Nat Neurosci.* 2010; 13:1354–1356. [PubMed: 20935642]
97. Duboue ER, Hong E, Eldred KC, Halpern ME. Left Habenular Activity Attenuates Fear Responses in Larval Zebrafish. *Curr Biol.* 2017; 27:2154–2162 e2153. [PubMed: 28712566]
98. Figdor MC, Stern CD. Segmental organization of embryonic diencephalon. *Nature.* 1993; 363:630–634. [PubMed: 8510755]
99. Funato H, Saito-Nakazato Y, Takahashi H. Axonal growth from the habenular nucleus along the neuromere boundary region of the diencephalon is regulated by semaphorin 3F and netrin-1. *Mol Cell Neurosci.* 2000; 16:206–220. [PubMed: 10995548]
100. Schmidt ER, Brignani S, Adolfs Y, Lemstra S, Demmers J, Vidaki M, Donahoo AL, Lillevall K, Vasar E, Richards LJ, et al. Subdomain-mediated axon-axon signaling and chemoattraction cooperate to regulate afferent innervation of the lateral habenula. *Neuron.* 2014; 83:372–387. [PubMed: 25033181]
101. Ichijo H, Toyama T. Axons from the medial habenular nucleus are topographically sorted in the fasciculus retroflexus. *Anat Sci Int.* 2015; 90:229–234. [PubMed: 25145706]
102. Kuan YS, Yu HH, Moens CB, Halpern ME. Neuropilin asymmetry mediates a left-right difference in habenular connectivity. *Development.* 2007; 134:857–865. [PubMed: 17251263]
103. Beretta CA, Dross N, Guglielmi L, Bankhead P, Soulika M, Gutierrez-Triana JA, Paolini A, Poggi L, Falk J, Ryu S, et al. Early Commissural Diencephalic Neurons Control Habenular Axon Extension and Targeting. *Curr Biol.* 2017; 27:270–278. [PubMed: 28065605]
104. Venkiteswaran G, Lewellis SW, Wang J, Reynolds E, Nicholson C, Knaut H. Generation and dynamics of an endogenous, self-generated signaling gradient across a migrating tissue. *Cell.* 2013; 155:674–687. [PubMed: 24119842]
105. Giger RJ, Cloutier JF, Sahay A, Prinjha RK, Levengood DV, Moore SE, Pickering S, Simmons D, Rastan S, Walsh FS, et al. Neuropilin-2 is required in vivo for selective axon guidance responses to secreted semaphorins. *Neuron.* 2000; 25:29–41. [PubMed: 10707970]
106. Sahay A, Molliver ME, Ginty DD, Kolodkin AL. Semaphorin 3F is critical for development of limbic system circuitry and is required in neurons for selective CNS axon guidance events. *J Neurosci.* 2003; 23:6671–6680. [PubMed: 12890759]
107. Kantor DB, Chivatakarn O, Peer KL, Oster SF, Inatani M, Hansen MJ, Flanagan JG, Yamaguchi Y, Sretavan DW, Giger RJ, et al. Semaphorin 5A is a bifunctional axon guidance cue regulated by heparan and chondroitin sulfate proteoglycans. *Neuron.* 2004; 44:961–975. [PubMed: 15603739]
108. Giger RJ, Pasterkamp RJ, Holtmaat AJ, Verhaagen J. Semaphorin III: role in neuronal development and structural plasticity. *Prog Brain Res.* 1998; 117:133–149. [PubMed: 9932406]
109. Chen H, Bagri A, Zupicich JA, Zou Y, Stoeckli E, Pleasure SJ, Lowenstein DH, Skarnes WC, Chedotal A, Tessier-Lavigne M. Neuropilin-2 regulates the development of selective cranial and

- sensory nerves and hippocampal mossy fiber projections. *Neuron*. 2000; 25:43–56. [PubMed: 10707971]
110. Hua ZL, Jeon S, Caterina MJ, Nathans J. Frizzled3 is required for the development of multiple axon tracts in the mouse central nervous system. *Proc Natl Acad Sci U S A*. 2014; 111:E3005–3014. [PubMed: 24799694]
 111. Wagner F, Bernard R, Derst C, French L, Veh RW. Microarray analysis of transcripts with elevated expressions in the rat medial or lateral habenula suggest fast GABAergic excitation in the medial habenula and habenular involvement in the regulation of feeding and energy balance. *Brain Struct Funct*. 2016; 221:4663–4689. [PubMed: 26888156]
 112. Wagner F, French L, Veh RW. Transcriptomic-anatomic analysis of the mouse habenula uncovers a high molecular heterogeneity among neurons in the lateral complex, while gene expression in the medial complex largely obeys subnuclear boundaries. *Brain Struct Funct*. 2016; 221:39–58. [PubMed: 25244943]
 113. Lagadec R, Laguerre L, Menuet A, Amara A, Rocancourt C, Pericard P, Godard BG, Celina Rodicio M, Rodriguez-Moldes I, Mayeur H, et al. The ancestral role of nodal signalling in breaking L/R symmetry in the vertebrate forebrain. *Nat Commun*. 2015; 6:6686. [PubMed: 25819227]
 114. Signore IA, Guerrero N, Loosli F, Colombo A, Villalon A, Wittbrodt J, Concha ML. Zebrafish and medaka: model organisms for a comparative developmental approach of brain asymmetry. *Philos Trans R Soc Lond B Biol Sci*. 2009; 364:991–1003. [PubMed: 19064351]
 115. Kemali M, Guglielmotti V, Fiorino L. The asymmetry of the habenular nuclei of female and male frogs in spring and in winter. *Brain Res*. 1990; 517:251–255. [PubMed: 2375994]
 116. Guglielmotti V, Cristino L. The interplay between the pineal complex and the habenular nuclei in lower vertebrates in the context of the evolution of cerebral asymmetry. *Brain Res Bull*. 2006; 69:475–488. [PubMed: 16647576]
 117. Gurusinghe CJ, Ehrlich D. Sex-dependent structural asymmetry of the medial habenular nucleus of the chicken brain. *Cell Tissue Res*. 1985; 240:149–152. [PubMed: 3995537]
 118. Jacinto LR, Mata R, Novais A, Marques F, Sousa N. The habenula as a critical node in chronic stress-related anxiety. *Exp Neurol*. 2017; 289:46–54. [PubMed: 27940019]
 119. Ahumada-Galleguillos P, Lemus CG, Diaz E, Osorio-Reich M, Hartel S, Concha ML. Directional asymmetry in the volume of the human habenula. *Brain Struct Funct*. 2017; 222:1087–1092. [PubMed: 27155991]
 120. Hennigan K, D'Ardenne K, McClure SM. Distinct midbrain and habenula pathways are involved in processing aversive events in humans. *J Neurosci*. 2015; 35:198–208. [PubMed: 25568114]
 121. Hetu S, Luo Y, Saez I, D'Ardenne K, Lohrenz T, Montague PR. Asymmetry in functional connectivity of the human habenula revealed by high-resolution cardiac-gated resting state imaging. *Hum Brain Mapp*. 2016; 37:2602–2615. [PubMed: 27038008]

Figure 1. Schematic of diencephalic and habenular development

(A) Development of the zebrafish dorsal diencephalon from 15 to 42 hours post fertilization (hpf, lateral view, anterior left). At 15 hpf, Wnt (purple), Fgf (light blue), and Shh (yellow) signals pattern the forebrain. Shh expression and signaling has not yet expanded dorsally at the presumptive ZLI. By 24 hpf, Wnt, Fgf and Shh all influence the presumptive epithalamus, and expression of *dbx1b* (green) can be detected. By 36 hpf, expression of *cxc4b* (red) has initiated and the first dHb neurons appear (dark blue). At 42 hpf, the number of dHb neurons has increased, and their efferent axons project towards the midbrain target, extending between the border of p2 and p1 (Th and PrT, respectively). In zebrafish, the vHb have not yet differentiated. Abbreviations: Epithalamus (Epi), Prethalamus (pTh), Thalamus (Th), Pre-tectum (PrT), Zona limitans intrathalamica (ZLI). Drawings are adapted from [40]. (B) Frontal view of the developing zebrafish dHb drawn from live images at the indicated stages [54]. The *dbx1b*-expressing dHb progenitors (green) lie ventromedial to *cxc4b*-expressing neural precursors (red) and dHb neurons (blue). As dHb development proceeds, the transition from progenitors to mature neurons results in progressive restriction

of *dbx1b* expression to a smaller, ventromedial domain, with *cxcr4b* transcripts concentrated in cells positioned between the progenitor and neuronal populations.

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Figure 2. Timeline of zebrafish dorsal habenular development

Asymmetric expression of genes in the Nodal pathway is detected in the developing diencephalon as early as 18 hpf. Canonical Wnt signaling also helps specify the L-R axis, however, the precise time of action is unknown (dashed box). Transcripts of *dbx1b* are present in dHb progenitors by 26 hpf, just prior to the emergence and leftward migration of cells from the pineal anlage that will eventually coalesce into the parapineal. Neural precursors expressing *cxcr4b* appear at approximately 32 hpf. Efferents emerge from dHb neurons and reach the IPN by 48 hpf. Throughout these later stages, L-R differences in neuronal number and identity are further elaborated under the influence of Notch signaling and the parapineal. Formation of the zebrafish vHb begins at 48 hpf and continues to 4 dpf (not shown). Events relevant to the establishment of dHb L-R asymmetry (purple) and development (blue) are indicated in hpf.

Figure 3. Signaling pathways in habenular development

Shh, Wnt and Fgf signaling promotes development of *dbx1b*-expressing dHb progenitors. These give rise to *cxcr4b*-expressing neural precursors that differentiate into mature dHb neurons. It is unclear whether Shh functions directly to generate habenular progenitors or acts upstream of Wnt and/or Fgf signaling. Outgrowth of efferents from Hb neurons is posteriorly directed by transient chemokine signaling. Expression of multiple components in the Cxcr4b-chemokine signaling pathway is positively regulated by Fgf and, indirectly, negatively regulated by Wnt signaling. Proper formation of the fasciculus retroflexus fiber bundles and innervation of specific target regions require additional repulsive and attractive axon guidance cues.