The Zebrafish Habenulae: Abundant Asymmetry in the Dorsal Diencephalon

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The animal kingdom abounds with examples of asymmetric body plans. In mammals, a gross examination of the visceral organs reveals many of these asymmetries, including the biased placement of the heart, liver, and pancreas along the Left/Right (L/R) axis. A discussion of asymmetry is easily extended to the beautifully complex organization of the brain. For instance, the human cerebral cortex has evolved specialized regions that are specific to one hemisphere; Broca's area, the locus for speech, is found specifically in the left hemisphere in the vast majority of individuals. In fact, brain asymmetry is quite common throughout the vertebrate lineage, suggesting that lateralized organization is of evolutionary merit and thus contributes adaptive advantages. Although it is difficult to correlate molecular deviations in symmetry with functional consequences in a behaving animal, careful characterization of asymmetrical brain development may eventually unveil the essential components of the nascent lateralized brain.

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The habenular nuclei, a model system for studying laterality in an emerging molecular brain, serve as relay stations of the dorsal diencephalic conduction pathway. A broad discussion of the habenulae across vertebrates is a challenging one, as functional studies are sparse, and the conservation of connectivity does not necessarily hold between divergent species. In mammals, the habenular nuclei represent a vital transit center of limbic processing, and accordingly, they have been implicated in a variety of cognitive and behavioral studies, but these limbic connections are not present in lower vertebrates. From a laterality perspective, the most intriguing aspect of the habenulae is witnessed through their asymmetric development in some fish, reptiles, and amphibians. Many studies have established the dorsal diencephalon, or epithalamus, as a premier locus of study for brain asymmetry[1]. In particular, the zebrafish has emerged as a premiere system for genetic and molecular developmental studies of the epithalamus, and these methods have revealed distinct habenular asymmetries.

VERTEBRATE DORSAL DIENCEPHALON

The dorsal diencephalon (epithalamus) of vertebrates contains a paired set of habenulae along with a photoneuroendocrine pineal organ, with the addition an accessory organ, termed the parapineal, parietal eye, or frontal organ, in fish, reptiles, and amphibians respectively[1]. There are many variations as to the specific organization of the epithalamus, but in general the pineal organ is situated at the midline of the brain, flanked by the habenulae. When present, the parapineal is often biased to one side of the brain, and this accessory nucleus provides a stark example of brain asymmetry (**Figure 1**). The parapineal organ has been shown to innervate the left dorsal habenula in trout[2], and lamprey[3], and more recently in zebrafish as well[4-6].

Research on the mammalian habenulae prompted Sutherland to describe the dorsal-diencephalic conduction pathway, in which the habenular nuclei serve as a relay center from limbic forebrain to midbrain[7]. On either side of rat brain exist distinct lateral and medial divisions of each habenular nucleus. Indeed, in all mammals, the medial (MHb) and lateral nuclei (LHb) have marked differences in their respective afferents and efferents[7-12]. It has been proposed that the medial habenula of lizards and mammals is homologous to the habenula of lampreys and teleosts[2,3,13], due largely to the fact that habenular efferents from said nuclei directly target the interpeduncular nucleus (IPN)[14-16], as do the majority of fibers from the mammalian MHb[9-11]. Thus this connection to the IPN via fasciculus retroflexus (FR) is highly conserved[8]. In fact, utilizing tritiated amino acid injections in various regions of rat habenulae, followed by autoradiography, Herkenham and Nauta conclude that no LHb projections appear to involve the IPN, in the rat[11]. Interestingly, it should be noted that afferent connections to the zebrafish habenulae are fairly homologous to those reaching the rat lateral habenulae, with many fibers originating in the eminentia thalami (EmT) or entopeduncular nucleus, respectively[12,17]. For simplicity, discussion here will be limited to the MHb in rat.

The main inputs to the medial habenula originate from the septum, and their afferent path is through the stria medullaris (SM)[7]. In fact, horse radish peroxidase (HRP) injections in the medial habenular nucleus show that only neurons in the supracommissural septal area contribute to the SM[12]. There are also ascending inputs to the medial habenula, as both serotonergic fibers from raphe nuclei[12, 18] and noradrenergic fibers from the dorsolateral tegmental nucleus and ventral central gray target the region[19]. Noradrenergic afferents travel successively through the dorsal tegmental bundle, medial forebrain bundle, and SM[19]. Thus both septal and midbrain nuclei provide afferent innervation of the MHb.

Efferents from the medial habenula project to the interpeduncular nucleus via the fasciculus retroflexus (FR)[9-11]. The rat medial habenula possesses segregated populations of neurons of both acetylcholine and substance P neurochemical nature, and these neurons retain exclusive targets in the IPN: cholinergic terminals have been found in the central core of the IPN, and substance P projections appear to innervate the periphery[20,21]. In fact, chronic exposure to nicotine causes axonal degeneration of the FR in rat, presumably through nicotinic ACh receptors[22]. Other efferents through the core of the IPN are glutamateric[23]. All habenular efferents through the fasciculus retroflexus are distinctly segregated: the core (central) processes stem from the medial habenula and the mantle (peripheral) from the lateral habenula[11]. Additionally, there may also be minor projections from the medial habenula to the ventral tegmental area as suggested from a lesion study[24].

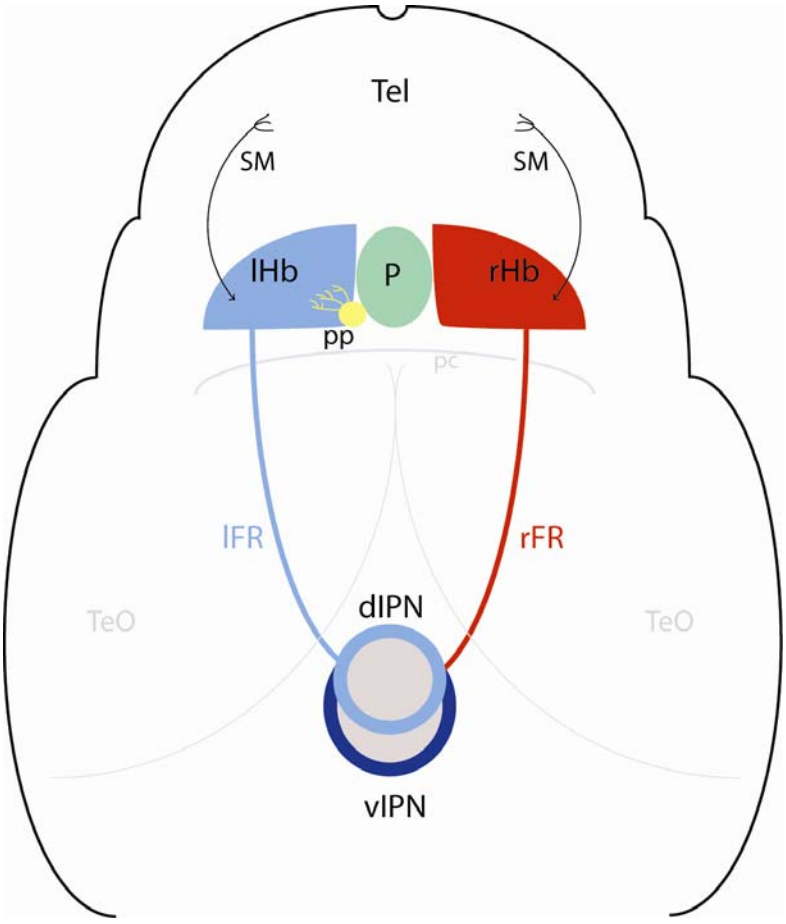

Figure 1 | **Dorsal view of a larval zebrafish at 4 days post-fertilization**. The larval epithalamus exhibits distict asymmetries. The pineal organ (P) exists as an emanation from the roofplate at the midline. The parapineal (pp) is situated both left and rostral to the pineal, and is situated just caudal to the left habenula (lHb). The habenulae themselves exhibit differences in overall size and volume of dense neuropil (yellow efferents from pp). Also shown is input from forebrain via stria medullaris (SM), and laterotopic output through the fasciculi retroflexus (FR), terminating in the interpeduncular nucleus (IPN). The ventral IPN receives input from left and right habenulae. pc, posterior commissure; TeO, optic tectum. *Adapted from* [6].

Habenular function has been implicated in a variety of behaviors, as can be deduced from their diverse connectivity, yet many of these correlations are specific to the lateral habenulae. In regard to the medial habenulae, functional studies implicate feeding and mating[7], as well as hormone secretion[25]. Additionally, several mammalian studies have implicated the lateral habenula in psychosis[26], addiction[23], avoidance learning[27,28], and as a source of negative reward signals on dopaminergic neurons[29]. These studies implicating the lateral habenula via fasciculus retroflexus likely reflect inhibitory influence on dopaminergic neurons[23], as lesions of the SM, LHb, or FR increase dopamine turnover in prefrontal cortex, nucleus accumbens and striatum[30, 31]. Even so, it should be noted that the lateral habenula does not project to the IPN, and thus these higher order cognitive functions may not be conserved in zebrafish.

As noted above, there are significant differences in the basic organization of the dorsal diencephalon across the vertebrate clade. While subtle differences between left and right habenulae have been noted in the albino rat[32] and albino mouse[33], as well as a sex-specific difference in medial habenula in chick[34], more ancient vertebrate lineages present more explicit examples of asymmetry. For instance, the hagfish, lamprey, eel, newt[35], frog[36], and lizard[37] all show dramatic habenular asymmetry. Even so, the basic organization and connectivity of this region remains comparable across the vertebrate lineage. Thus, there is likely substantial conservation of the genetic programs responsible for epithalamic development.

ASYMMETRIC HABENULAE OF THE ZEBRAFISH (*DANIO RERIO*)

The zebrafish habenular nuclei display striking asymmetries in connectivity, nuclear organization, and gene expression, all of which result from an asymmetric developmental program. The first, and most subtle, is a slight leftward bias of the pineal organ stalk from the roofplate of the dorsal diencephalon[38]. More obvious is the placement of the accessory parapineal, which arises from a common pool of progenitor cells within the pineal complex as shown by lineage labeling at 22-24 hours post fertilization (hpf)[4] and more elegantly through time-lapse imaging[39]. By 28 -32 hpf, it is a distinguishable organ, with a left-bias in around 95% of embryos[5,14].

Finally, the habenulae show several L/R differences. First, anti-acetylated tubulin labeling demonstrates more dense volume of neuropil in the left habenula[4] (**Figure 1**). Secondly, *in situ* hybridization of *cpd2*, a gene expressed bilaterally in the habenulae, shows an 18% greater area in the left habenula of larvae at 4 days post-fertilization (dpf). Finally, an additional set of marker genes, which contain a *potassium channel tetramerization domain* (*kctd*), also show distinct asymmetries. *leftover* (*kctd12.1*), is more widely expressed in the left habenula[14], whereas the remaining two, *right on* (*kctd12.2*) and *dexter* (*kctd8*), are increased on the right[15].

In recent years, the connectivity of the zebrafish dorsal diencephalon has been well characterized. Lipophilic dye tracing and has provided information on afferent connections. The majority of habenular innervation derives from migrated neurons from the EmT[17]. Innervation of the habenulae by migrated EmT neurons or the adult entopeduncular nucleus, via the stria medullaris, is conserved across species, in trout[13], goldfish[40], and rat[41]. In addition, neurons from the pallium (dorsal telencephalon) and posterior tuberculum (diencephalon) provide input[17,42]. Interestingly, in zebrafish, pallial projections are asymmetric as they terminate in the right medial habenula despite their side of origin[17]. In addition, antibody labeling against SV2, a presynaptic glycoprotein, demonstrates that neuropil density is higher in the left lateral habenula, and unveils a unique extension to the right medial subnucleus[17]. These results demonstrate that afferent innervation is also asymmetric and may contribute to the development of these lateralized nuclei.

The asymmetric habenulae of zebrafish appear to be coupled to laterotopic innervation of the IPN, the primary efferent target. Anterograde tracing studies using the lipophilic dyes DiO and DiI for left and right habenula respectively, demonstrate that left and right FR have different projection patterns and specific targets: efferents from the right habenula innervate the ventral region of the IPN, whereas the left habenula projects primarily to the dorsal region[14, 43]. Additionally, immunolabeling for Leftover and Right on proteins serve as specific tracers of left and right habenular efferents, respectively. Leftover positive axons (Lov+) target the dorsal and ventral regions of the IPN, whereas Ron+ axons are restricted to the ventral IPN[15]. In a developmental perspective, these Lov+ growth cones reach the IPN by 2 dpf, and habenulo-interpeduncular connections are well formed by 4 dpf[15].

MOLECULAR AND GENETIC CONTRIBUTIONS TO ASYMMETRIC HABENULAR DEVELOPMENT

Early patterning events in the embryonic zebrafish have a profound effect on habenular asymmetry. Mutational analysis of the Nodal genes indicate that they impact the stereotypic laterality of both visceral organs and the central nervous system[44]. In addition, misexpression of Nodals can lead to altered L/R polarity of organs[45]. The Nodal cascade is not necessary for the development of asymmetry, but is essential to determine the direction of laterality. For example, *one-eyed pinhead* mutants, which have a deficiency in the Nodal receptor complex, must be rescued past an early gastrulation requirement for Nodal by injection of *oep* RNA (these embryos are referred to as rescued *oep*, or R*oep*). These embryos are viable, yet show randomized epithalamic organization: the parapineal and ipsilateral, enlarged habenula are on the left and right sides at equal frequency[4, 38].

This randomization, as assessed by neuropil density and *leftover* expression is also seen through disruption of the earliest acting Nodal ligand, *southpaw*[46]. Targeted morpholino knockdown of *southpaw* transcript results in L/R randomization of parapineal migration, and leads to disruption of IPN targeting: when the parapineal is situated at the right efferents from left habenula project solely to ventral domain, while right habenula afferents project along entire dorso-ventral axis[15]. Early Nodal signaling is thus crucial to establish polarity of both visceral organs and the CNS.

There is substantial evidence that the asymmetric development of the habenulae in zebrafish is dependent on the parapineal. As stated previously, the parapineal is morphologically apparent at 26 hpf, while the first expression of *leftover* cannot be detected until between 38[14]. This accessory nucleus innervates a circumscribed, central region of the ipsilateral habenular nucleus[4-6]. This central habenular region is also coincident with a described zone of enlarged neuropil[4](Gamse, unpublished). In summary, the left habenula is larger, possesses denser neuropil, and is innervated by the left-sided parapineal, a situation that is reversed in embryos with right-sided parapineal placement (*spaw* morpholino injected embryos)[15].

Cell ablation studies provide a more direct examination of the relationship between the parapineal and habenular asymmetry. When the cells of the parapineal are ablated soon after beginning migration, the left habenula no longer develops asymmetrically. More specifically, the left and right habenulae now resemble each other in volume of neuropil and expression of *leftover*[5;14]. Specifically in regard to the left habenula, ablation also results in expanded expression of *ron* and *dex*[15]. In fact, in parapineal ablated larvae, all three *kctd* genes appear bilaterally symmetric, with subdomains typical of a wild type right habenula[15]. These results suggest that in the left habenula, the parapineal may be responsible

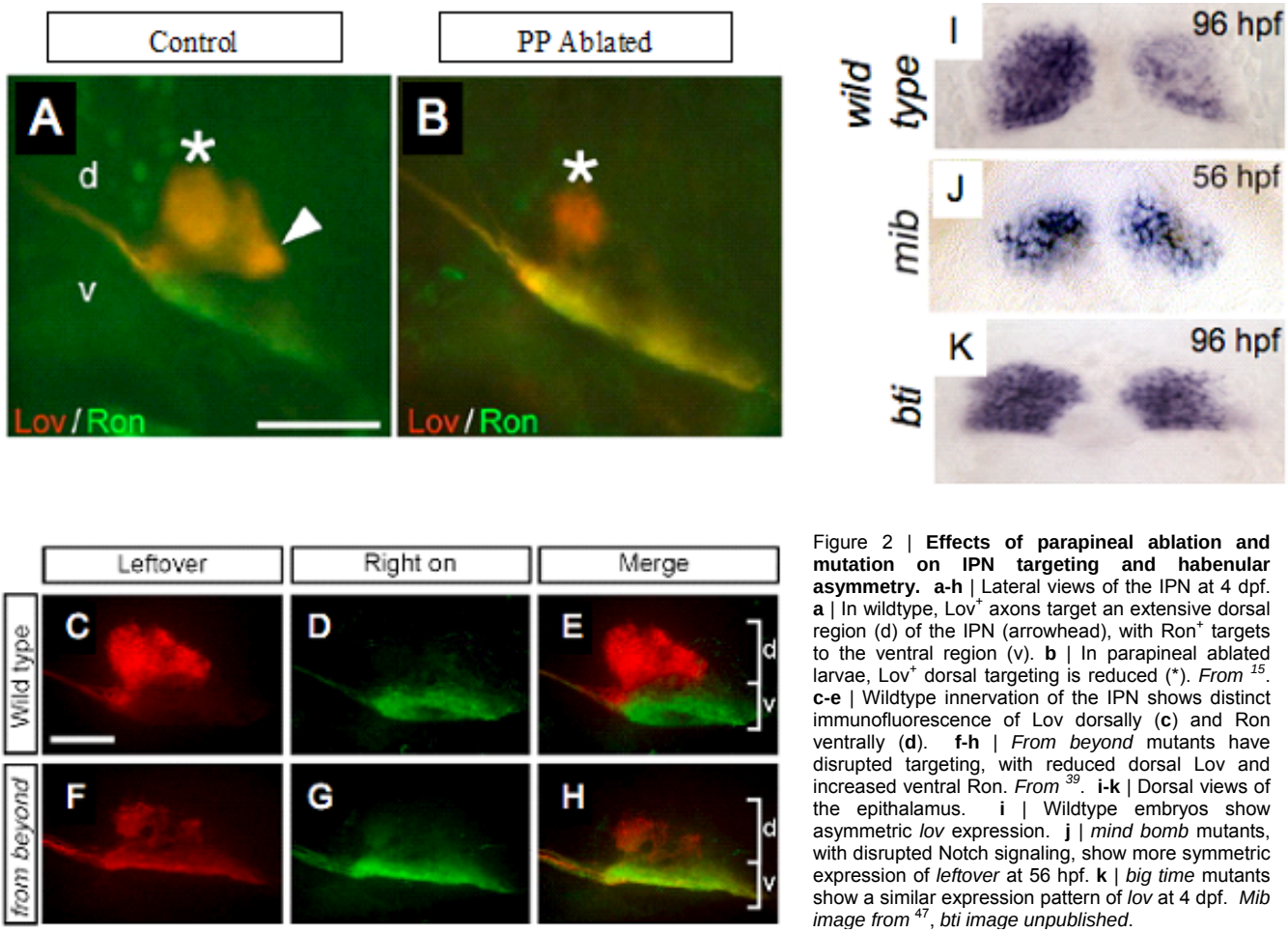

Figure 2 | **Effects of parapineal ablation and mutation on IPN targeting and habenular asymmetry. a-h** | Lateral views of the IPN at 4 dpf. **a** | In wildtype, Lov$^+$ axons target an extensive dorsal region (d) of the IPN (arrowhead), with Ron$^+$ targets to the ventral region (v). **b** | In parapineal ablated larvae, Lov$^+$ dorsal targeting is reduced (*). *From* [15]. **c-e** | Wildtype innervation of the IPN shows distinct immunofluorescence of Lov dorsally (**c**) and Ron ventrally (**d**). **f-h** | *From beyond* mutants have disrupted targeting, with reduced dorsal Lov and increased ventral Ron. *From* [39]. **i-k** | Dorsal views of the epithalamus. **i** | Wildtype embryos show asymmetric *lov* expression. **j** | *mind bomb* mutants, with disrupted Notch signaling, show more symmetric expression of *leftover* at 56 hpf. **k** | *big time* mutants show a similar expression pattern of *lov* at 4 dpf. *Mib image from* [47], *bti image unpublished*.

for specifying neurons that show greater expression of *leftover*, and may have a role in repressing right-sided gene expression[15]. In addition, these embryos have fewer Lov+ axons, and their projection within left fasciculus retroflexus (FR) now resembles that seen in the right FR. The IPN target is also affected, in that Leftover immunofluorescence is visible only in one small anterior domain in the dorsal IPN, with a concurrent increase in ventral IPN targeting[15] (**Figure 2a-b**).

Finally, a mutant analysis provides further evidence of the impact of the parapineal on asymmetric habenular development, and resultant connectivity to the midbrain. The *from beyond* (*fby*) mutation, mapped to the *tbx2b* gene, results in a nearly complete reduction in parapineal cells in embryos homozygous for the lesion. These embryos also demonstrate a habenular phenotype of symmetric expression genes *leftover*, *right on*, and *dexter*[39], as well as a reduction in Lov+ targeting to the dorsal region of the IPN, and a concordant increase in Ron+ targeting of the ventral IPN[39] (**Figure 2c-h**). This mutation, effectively blocking the formation of the parapineal organ, highlights the dependency of the habenulae on the parapineal, and shows a disruption in the dorsal diencephalic pathway through altered targeting of the midbrain nucleus, which is reminiscent of parapineal ablated larvae.

The expression of several habenula-specific genes in the zebrafish dorsal diencephalon suggests the existence of distinct medial and lateral subnuclei. It should be noted that this subnuclear division is not equivalent to the medio-lateral division in mammals, which have more distinct divisions and connectivity. In zebrafish, *leftover*, *right on*, and *dexter* visibly label different regions of the habenulae, with distinctions on dorso-ventral, medio-lateral, and antero-posterior axes[15]. In addition, the *brn3a* promoter drives expression of green fluorescent protein (GFP) specifically in the medial habenula[16]. Expression of these genes suggests that the right medial habenula is larger as compared to left. Conversely, the left lateral habenula is larger than the right[47]. In fact, these cell populations possess distinct neurogenetic programs: birth date analysis shows differential timing of neurogenesis between L/R and medio-lateral cell groups[47].

Thus far, we have witnessed the impact of early

Nodal signaling and parapineal placement on the developing asymmetric habenulae of zebrafish. There is an implied relationship between the parapineal and left habenula, yet that message has not been elucidated. Ultimately, asymmetric habenular nuclei must arise from a carefully controlled program of neurogenesis. The Okamoto laboratory took steps to characterize this neurogenesis through detailed birth date analysis, utilizing incorporation of 5-bromo-2-deoxyuridine (BrdU) in embryos expressing GFP under the *brn3a* promoter[47]. These embryos express GFP specifically in the medial habenular subnuclei, allowing medio-lateral distinction. Embryos were pulse labeled with BrdU at various developmental stages, and then allowed to develop to 5 dpf. They found that GFP- lateral habenula neural precursors were born first, beginning at 24 hpf, and peaking at 32 hpf. Neural precursors for the GFP+ medial habenula were born later, with a few visible at 32 hpf and a peak at 48 hpf. There was a significant difference in BrdU+ cells in left versus right habenula as early as 32 hpf, and significantly more medial habenular cells were born in the right habenula at 48 hpf. In short, there were more early-born lateral habenula cells on the left and more late-born medial habenula cells in the right[47], but the signaling mechanism responsible for the timing of habenular neurogenesis has yet to be determined.

One obvious candidate for such a mechanism is Notch signaling, which has been implicated in the maintenance or specification of a variety of cell types[48]. For example, oligodendrocytes are specified in a specific domain of the spinal cord, from which motoneurons also arise. The Appel laboratory has demonstrated that Notch is required for specification of oligodendrocyte progenitor cells (OPCs) within the spinal cord, as constitutively expressed Notch results in an excess formation of OPCs, at the expense of motoneurons[49]. *mind bomb* (*mib*) mutants carry a mutation in the ubiquitin ligase responsible for the internalization of Notch ligand, and thus have a drastic reduction in Notch signaling. This mutation results in excessive neurogenesis[50], and in regard to the epithalamus, these *mib* embryos show increased *leftover* expression and decreased *right on* expression in the right habenula at 56 hours post-fertilization[47] (**Figure 2j**). Thus, as witnessed in other cell types and regions of the CNS, Notch may be crucial to regulate asymmetric neurogenesis in the habenulae.

In order to begin to elucidate the molecular basis of habenular asymmetry, a chemical mutagenesis screen was performed in the Halpern laboratory, with a focus on mutations that result in altered *leftover* expression. This screen produced a mutant with symmetric *lov* expression; *big time* (*bti*) mutants have increased *lov* expression in the right habenula, such that the paired habenulae appear nearly symmetric (**Figure 2k**). The *big time* mutation was mapped to a premature stop codon within the 5th transmembrane domain of the major subunit of the vertebrate translocon, *sec61α1*. This secretory protein is localized on the endoplasmic reticulum and represents the entry point for recently translated or co-translated peptides. We have thus implicated a secretory protein in the regulation of habenular neurogenesis: one possibility is that the translocon mediates habenular asymmetry by a specific regulation of neurogenic molecular components, such as Notch receptors or ligands. Of particular interest are the effects of this mutation on targeting of the interpeduncular nucleus. With expanded *lov* expression in the right habenula, we hypothesize that both habenula now project to both dorsal and ventral regions of the IPN. Further studies investigate the role of this gene in the asymmetric development of the habenula and potential implications for connectivity.

CONCLUSIONS

Asymmetry is a common adaptation of the vertebrate brain, and is assumed to be advantageous as lateralized functions exert a unique pressure on the survival of a species. For instance, a lateralized motor response at a population level could be disadvantageous because predators would learn to predict a given response to stimuli, yet in social populations, such as zebrafish, this disadvantage could be overcome as exploration occurs in pairs or groups[51]. The habenula are an excellent model for asymmetry in organisms such as the zebrafish, which also demonstrates conservation of efferent pathway to the interpeduncular nucleus (from the MHb in mammals), as well as input from the entopeduncular nucleus. This species allows genetic characterization of this asymmetric development, of which the genes are highly conserved in mammals. It is clear that the parapineal has profound influence on the left habenula, yet what signal is it providing? Is Notch signaling necessary to maintain the right habenular neural precursors in an undifferentiated state? Further characterization of habenular neurogenesis, and the genetics that contribute to asymmetric specification and development will begin to elucidate these issues and shed light on this unique, lateralized locus of the dorsal diencephalon.

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FURTHER INFORMATION

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