Directional asymmetry of the zebrafish epithalamus guides dorsoventral innervation of the midbrain target

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Summary
The zebrafish epithalamus, consisting of the pineal complex and flanking dorsal habenular nuclei, provides a valuable model for exploring how left-right differences could arise in the vertebrate brain. The parapineal lies to the left of the pineal and the left habenula is larger, has expanded dense neuropil, and distinct patterns of gene expression from the right habenula. Under the influence of Nodal signaling, positioning of the parapineal sets the direction of habenular asymmetry and thereby determines the left-right origin of habenular projections onto the midbrain target, the interpeduncular nucleus (IPN). In zebrafish with parapineal reversal, neurons from the left habenula project to a more limited ventral IPN region where right habenular axons would normally project. Conversely, efferents from the right habenula adopt a more extensive dorsoventral IPN projection pattern typical of left habenular neurons. Three members of the leftover-related KCTD (potassium channel tetramerization domain containing) gene family are expressed differently by the left and right habenula, in patterns that define asymmetric subnuclei. Molecular asymmetry extends to protein levels in habenular efferents, providing additional evidence that left and right axons terminate within different dorsoventral regions of the midbrain target. Laser-mediated ablation of the parapineal disrupts habenular asymmetry and consequently alters the dorsoventral distribution of innervating axons. The results demonstrate that laterality of the dorsal forebrain influences the formation of midbrain connections and their molecular properties.

Key words: KCTD gene family, leftover gene, Left-right asymmetry, Habenular nuclei, Fasciculus retroflexus, Interpeduncular nucleus, Diencephalon

Introduction
A growing number of studies has dispelled the notion that lateralization of the brain is unique to the human cortex (see Rogers and Andrew, 2002). Even in the Drosophila brain, structural asymmetry has been linked to long-term memory (Pascual et al., 2004), and laterality has been well described in the nematode primitive sensory nervous system (Chang et al., 2004; Chuang and Bargmann, 2005). From fish to primates, evidence has accumulated for left-right (L-R) differences in brain anatomy, cellular organization, ultrastructure and neurotransmitter distribution and, in a few cases, asymmetry has been linked to behavior (Rogers and Krebs, 1996; Skiba et al., 2002; Toga and Thompson, 2003; Vallortigara et al., 1999). Despite significant progress, our understanding of the development and evolution of lateralized features of the vertebrate nervous system remains limited.

In lower vertebrates, the epithalamus of the dorsal diencephalon displays notable asymmetries (Concha and Wilson, 2001; Halpern et al., 2003). The epithalamus includes the pineal complex, which in many fish consists of the pineal organ and an asymmetrically positioned accessory organ termed the parapineal (Borg et al., 1983). Both the pineal and parapineal transcribe genes encoding melatonin biosynthetic enzymes in a circadian-regulated manner (Gamse et al., 2003; Gothilf et al., 1999), but the specific role of the parapineal is unknown. The bilateral habenular nuclei, which flank the pineal complex, can differ in size and structure (Harris et al., 1999; Concha and Wilson, 2001). In some amphibians, the left habenula contains two morphologically distinct subdomains whereas there is only a single nucleus on the right (Wehrmaker, 1969; Braitenberg and Kemali, 1970; Morgan et al., 1973). The degree of habenular asymmetry can vary seasonally in frogs, presumably correlated with the mating period (Kemali et al., 1990). In chickens, L-R habenular differences are sex-dependent and influenced by hormonal levels (Gurusinghe and Ehrlich, 1985; Gurusinghe et al., 1986).

The habenulointerpeduncular connection is poorly understood, although there is evidence from rodents that it modulates...
complex behaviors such as avoidance, reward and feeding (Sutherland, 1982).

Progress in determining how epithalamic asymmetry arises and the molecular pathways involved has come from studies in zebrafish. cyclops (cyc), which encodes a zebrafish Nodal-related Tgfβ signal (Rebagliati et al., 1998a; Rebagliati et al., 1988b; Sampath et al., 1998), and other genes functioning in this signaling pathway, are expressed transiently on the left side of the embryonic pineal anlage (Bisgrove et al., 2000; Concha et al., 2000; Liang et al., 2000). Nodal signals are known to mediate laterality of the heart and visceral organs in vertebrate embryos and play an important role in gastrulation (Schier, 2003). Zebrafish mutants for one-eyed pinhead (oep), which encodes an obligatory component of the Nodal receptor complex, can be rescued past the early requirement for Nodal signaling by injection of oep RNA (Yan et al., 1999; Zhang et al., 1998). Rescued embryos (Roep) lack asymmetric gene expression in the embryonic pineal but develop to adulthood, thus allowing Nodal pathway function in the brain to be assessed (Concha et al., 2000; Liang et al., 2000). In Roep fish the directionality of L-R differences in diencephalic anatomy is randomized (Concha et al., 2000; Liang et al., 2000; Gamse et al., 2002). The parapineal, located to the left of the pineal anlage in >95% of wild-type (WT) larvae, develops to the right (Yan et al., 2003; Gamse et al., 2003). Antisense morpholino oligonucleotides (MO) were derived from the splice acceptor site of the last intron (Spaw-MO2) (Long et al., 2002) and targets carrying the null allele oep(257) (Brand et al., 1996) were used. Embryos completely lacking maternal and zygotic Oep function (MZOep) (Yan et al., 1999) were generated from homozygous mutant adults produced by rescuing oep(257) homoyzous 1- to 2-cell stage embryos with injection of sense oep RNA (80 pg). RNA was synthesized from pCS2-oep (Liang et al., 2000) using the SP6 mMessage mMachine Kit (Ambion).

Morpholino injections

For example, the dexter (dext) gene is typically transcribed by more cells of the left habenula than the right; however, half of Roep larvae show the opposite pattern (Gamse et al., 2003). Parapineal L-R position always corresponds with the direction of habenular laterality. Moreover, following parapineal ablation, the habenulae fail to develop asymmetrically (Concha et al., 2003; Gamse et al., 2003).

Dye labeling of the habenulae

Even earlier in development, signaling by another Nodal-related factor, Southpaw (Spaw), influences asymmetric gene expression in the zebrafish diencephalon. spaw is the earliest known gene to be expressed unilaterally, with transcripts appearing in the left lateral plate mesoderm (LPM) by the 10-12 somite stage (Long et al., 2003). However, expression is not detected in the embryonic brain. Unlike the zebrafish Nodal-related signals, Cyc and Squint (Sqt), that mediate tissue specification in the early embryo (Hatta et al., 1991; Feldman et al., 1998), Spaw appears to regulate the L-R axis specifically. Embryos deficient for Spaw lack cyc, pitx2 and lft1 expression in the left diencephalon (Long et al., 2003), and thereby affect morphological asymmetry of the epithalamus. The zebrafish studies suggest that brain laterality results from a cascade of developmental events that leads from left-sided Nodal signaling in the pineal anlage to L-R assignment of parapineal position, which, in turn, directs habenular asymmetry.

Here, we provide additional evidence for molecular L-R differences in the zebrafish habenular nuclei, which extends to their efferent projections and innervation of the midbrain target, the IPN. Two genes related to lov, right on (ron) and dexter (dex), are also expressed asymmetrically in the habenular nuclei but to a greater extent on the right than the left. Consequently, Lov and Ron proteins are distributed differently in habenular subdomains and in efferent axons within the FR on the left and right sides of the larval brain. Differential dye-labeling of adult habenulae, or visualization of Lov+ and Ron+ immunoreactive axons, demonstrates that L-R habenular efferents project to different regions along the dorsoventral (DV) axis of the IPN. Reversal of habenular laterality by perturbation of Nodal signaling also reverses the L-R origin of projections onto the IPN. Ablation of the parapineal disrupts laterality and the stereotypic DV pattern of IPN connectivity. The results demonstrate that L-R differences of the dorsal forebrain influence the connections and molecular properties of innervating axons at the midbrain target.

Materials and methods

Zebrafish

Zebrafish were raised at 28.5°C and staged according to hours or days post-fertilization. AB wild-types (Walker, 1999), transgenic lines Tg(bh.GFP)y161, Tg(bh.GFP)y162 (Gamse et al., 2003) and Tg(foxd3-GFP) (Gilmour et al., 2002) and mutants carrying the null allele oep(257) (Brand et al., 1996) were used. Embryos completely lacking maternal and zygotic Oep function (MZOep) (Yan et al., 1999) were generated from homozygous mutant adults produced by rescuing oep(257) homoyzous 1- to 2-cell stage embryos with injection of sense oep RNA (80 pg). RNA was synthesized from pCS2-oep (Liang et al., 2000) using the SP6 mMessage mMachine Kit (Ambion).

Dye labeling of the habenulae

After anesthetization with tricaine (170 µg/ml), 2- to 3-month-old adults (AB) were decapitated with a razor. Brains were dissected out in cold phosphate-buffered saline (PBS), pinned dorsal side upwards on Sylgard (Molecular Probes)-coated dishes, and fixed in 4% paraformaldehyde for at least 24 hours at 4°C. Lipophilic dyes FAST DiI and FAST DiO (Molecular Probes) were dissolved in dimethylformamide at 50 mg/ml by heating at 50°C for 5 to 10 minutes. Aliquots of the dye solutions were stored at ~80°C, thawed and briefly heated at 42°C before backloading into glass needles. Prior to dye application, the prominent commissure that extends between the left and right habenulae was severed with a tungsten needle to prevent dye passage. Each habenula was impaled and dye applied using a pressure injector (Aizawa et al., 2005). Labeled brains were stored in 4% paraformaldehyde at 28°C for 18-21 days because of the distance the lipophilic dyes must travel along the habenula FR projections to reach the IPN. Dye passage was monitored under a Leica MZFLIII stereomicroscope. Labeled brains were embedded in low-melting temperature agarose (4%) in plastic molds for transverse vibratome (Leica VT1000S) sectioning. Sections (100-150 µm) were mounted on glass slides in cold Mowiol (Calbiochem) medium (480 mg/ml Mowiol in 25% glycerol with 0.1 M Tris, pH 8.5) and imaged with a Leica MZFLIII or SP2 confocal microscope.

Identification of leftover-related genes

Individual clones from an adult zebrafish kidney cDNA library containing inserts between EcoRI and XhoI sites of pBK-CMV (Stratagene) were assayed for expression during embryonic stages by whole-mount in situ hybridization. A partial ron cDNA was isolated and the transcript 5’ end identified by RNA-ligase RT-PCR.
Development of adult fish derived from on a Leica SP2 confocal microscope. For vibratome sectioning, brains single labeling. Larvae were mounted in glycerol and images collected (Jackson Immunoresearch), then blocked with unlabeled goat-anti-Probes). For double labeling with Lov and Ron antibodies, the first antibody was detected using goat-anti-rabbit:Cy3 (Jackson overnight in primary antibody diluted 1:500 in PBSTS. Primary PBS/0.1%TritonX-100/10% sheep serum (PBSTS), and incubated g/ml; Roche) for 30 minutes, refixed, blocked in /H9262 in methanol at –20°C for up to 2 weeks. Following rehydration Four-day larvae fixed overnight in 4% paraformaldehyde were stored labeling, or NBT/BCIP followed by iodonitrotetrazolium (INT) and bromo-4-chloro-3-indolyl-phosphate (BCIP) staining for single antibodies and visualized by 4-nitro blue tetrazolium (NBT) and 5-probes were detected using alkaline phosphatase-conjugated and pCRII-KCTD16a and pCRII-KCTD12b with SpeI and T7 RNA polymerase, and pCRII-KCTD16b and pCRII-dex with Xhol and SP6 RNA polymerase. Hybridized probes were detected using alkaline phosphatase-conjugated antibodies and visualized by 4-nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) staining for single labeling, or NBT/BCIP followed by iodonitrotetrazolium (INT) and BCIP staining for double labeling. Larvae were embedded in 4% low-melt agarose (Cambrex Rockland, ME) for vibratome sectioning (100-150 μm), or in London Resin Gold (Ted Pella, Redding, CA) and sectioned using a Reichert ultramicrotome (8-10 μm) followed by Basic Fuchsin (Sigma) counterstaining.

Lov and Ron antibodies

Partial lov and ron open reading frames were subcloned into pDEST17 (Invitrogen), which adds a 6-histidine tag to the amino (N) terminus. Tagged protein was not recovered at high levels from bacterial cells unless the T1 domain was also deleted. T1-deleted constructs were expressed in BL21(DE3)pLySS bacteria (Stratagene), the protein purified on nickel-NTA columns (Qiagen) and injected into rabbits (Spring Valley Laboratories) to produce polyclonal antibodies and visualized by western blotting using standard methods (Gallagher et al., 1989) and pCRII-dex with KCTD12b, 16a in controls.

Accession numbers

GenBank accession numbers: KCTD16a, AY763407; KCTD12b, AY763408; KCTD16b, AY763409; ron, AY763410.

Results

A family of lov-related genes

The leftover gene was previously identified in a screen of random cDNA clones for tissue-specific expression in zebrafish embryos and larva. lov expression was found to differ on the left and right sides of the diencephalon, in a region corresponding to the paired habenular nuclei (Gamse et al., 2003).

Upon subsequent screening, another gene, right on (ron), was found to show asymmetric expression in the habenulae. Remarkably, ron shares a high degree of sequence homology with lov, particularly in two discrete domains of the predicted protein coding sequence (Fig. 1A). Scanning of available zebrafish genomic sequence and degenerate PCR yielded four additional homologous genes (Fig. 1A). Only one, dexter (dex), is expressed in the habenulae (see below). In 4-day larvae, ron is also expressed in the gall bladder and posterior border of the optic tectum, and dex in paired groups of cells in the ventral diencephalon (data not shown).

Related genes from mammals were grouped as the potassium channel tetramerization domain containing (KCTD) gene family (Marchler-Bauer et al., 2003). Structurally, KCTD proteins contain a N-terminal sequence homologous to the T1 tetramerization domain of the Shaker class of voltage-gated potassium channels (Papazian, 1999). However, they have no other features of channel proteins, such as transmembrane domains. Phylogenetic comparison with human and mouse genes reveals that the zebrafish proteins (Fig. 1B, boxed) fall into four subclasses of the larger KCTD family: Kctd16a/b; Kctd12b; Lov/Ron/Kctd12/Pfetin and Dex/Kctd8. The zebrafish kctd12b, 16a and 16b genes are strongly expressed in the retina. Additionally, kctd16a is expressed in the dorsal forebrain and optic tectum and kctd16b in olfactory epithelium (data not shown).

In humans, KCTD12/PFET expression was detected in the fetal cochlea (Resendes et al., 2004), a structure absent in the zebrafish (Whitfield et al., 2002). We found that Kctd12/Pfet transcripts specifically localize to the medial habenulae of the adult rat brain (Fig. S1 in supplementary material).

Left-right differences in habenular gene expression patterns

Zebrafish ron and dex are expressed asymmetrically in the habenular nuclei; however, in contrast to lov, they are transcribed in more cells of the right habenula than the left. Expression, which is asymmetric from the outset, is first detected at 2 days (Fig. 1C,G), and increases significantly by 4 days (Fig. 1D,H). Parasagittal sections reveal that ron and dex are expressed in the dorsal region of the right but not the left habenula (compare Fig. 1E and I with F and J). Expression
of dex extends more laterally in both habenulae than expression of ron (Fig. 1D,H).

Double label in situ hybridization allows further refinement of habenular gene expression subdomains (Fig. 1K-N, and not shown). Only cells in the dorsal region of the left habenula express lov, while those in the ventral region express ron and dex. In contrast, cells in both dorsal and ventral regions of the right habenula express lov, ron and dex. All three genes are transcribed in medial and posterior regions of the right habenula, but only ron and dex expression is detected in anterior and lateral regions.

**Molecular L-R specialization of habenulointerpeduncular connections**

To determine whether asymmetric gene expression leads to L-R differences in protein distribution, and to evaluate subcellular localization, we generated polyclonal antibodies against Lov and Ron. Rabbit sera reacted specifically against each protein on western blots and in Lov or Ron MO-depleted embryos (Fig. S2 in supplementary material).

Confocal microscopy of Lov and Ron immunolabeled larvae demonstrated that protein levels differ between the left and right habenulae in a pattern closely reflecting mRNA distribution (Fig. 2A-C). Although transcripts were restricted to the cell bodies of habenular neurons, protein was also detected within ventrocaudally projecting axons that course through the FR, and in synaptic terminals at the midbrain target, the interpeduncular nucleus. Lov+ growth cones reach the IPN by 2 days (Fig. 2D). By 4 days, habenulointerpeduncular connections are well formed: the Lov+ axonal bundle is larger within the left FR than the right, and Lov+ (Fig. 2E,F) and Ron+ (Fig. 2G) habenular efferents project extensively along the IPN. Innervation of the target was distinguished more clearly in lateral views of the larval brain. Lov+ axons project to the dorsal and ventral IPN (Fig. 2I), while most, if not all, Ron+ axons project to the ventral IPN (Fig. 2J). Double labeling confirmed that Lov+ axons traverse the dorsal and ventral regions of the midbrain target and Ron+ axons are confined ventrally (Fig. 2K).

**Epithalamic L-R asymmetry guides DV innervation of midbrain target**

Parapineal position was shown to correlate with higher levels
of lov RNA in the adjacent habenula of normal or L-R reversed Roep brains (Gamse et al., 2003) (Fig. 3A,B). Conversely, ron and dex expression is more extensive in the habenula opposite the parapineal (Fig. 3C-F), irrespective of directionality. To determine whether projections from the habenula to the IPN are also influenced by the L-R position of the parapineal and direction of habenular asymmetry, we examined Lov and Ron immunoreactivity in Roep larvae.

In agreement with transcriptional patterns, the asymmetric distribution of Lov and Ron proteins in the habenulae and FR was similar to WT in half of Roep larvae (n=39, Fig. 3G,I). The other half (n=31) showed a L-R reversal in protein levels (Fig. 3H,J) that extended to habenular efferents within the FR.

We examined whether altered habenular laterality affected innervation along the DV axis of the IPN. Lov+ efferents project to the dorsal and ventral IPN, while Ron+ fibers innervate the ventral IPN in Roep larvae that have a left-positioned parapineal (Fig. 3K) and those that are L-R reversed, with the parapineal on the right (Fig. 3L). In such larvae, however, input to the IPN shows a mirror image reversal (Fig. 3H). Thus, when the L-R origin of habenular efferents is opposite that of WT, the DV pattern of immunoreactive connections to the IPN is still preserved.

Southpaw regulates directional asymmetry of habenulo-interpeduncular projections

Targeted depletion of Spaw in zebrafish embryos was previously shown to alter laterality of the heart and pancreas and prevent expression of Nodal pathway genes in the left LPM and diencephalon (Long et al., 2003). Because disruption of diencephalic gene expression causes L-R randomization of epithalamic asymmetry (Liang et al., 2000; Gamse et al., 2003; Concha et al., 2000; Concha et al., 2003), lack of Spaw should also affect directional asymmetry of the pineal complex and habenular nuclei.

spaw MO was injected into embryos bearing the foxD3:GFP transgene (Gilmour et al., 2002), which is highly expressed in the pineal complex at 2-3 days (Concha et al., 2003) and allows unambiguous assignment of the L-R position of the parapineal relative to the midline pineal, throughout larval stages (Fig. S3A,B in supplementary material). The direction of habenular asymmetry was determined at 4 days by assessing L-R differences in neuropil density (Fig. S3C,D in supplementary material) or lov expression (Fig. S3E,F in supplementary material). As summarized in Table 1, Tg(foxD3:GFP) controls did not show reversals in habenular laterality (n=300). However, almost half of larvae derived from spaw MO-injected embryos had L-R reversals in parapineal position and habenular asymmetry (45%, n=418). A smaller number (3%) exhibited bilaterally symmetric lov expression in the habenulae. These findings confirm that signaling by Spaw regulates directional asymmetry of the developing epithalamus.

The endocrine pancreas normally originates to the right of the midline in WT embryos (see Stafford and Prince, 2002). About twice as many spaw MO-injected larvae developed the pancreas on the right side than on the left (Fig. S3GI and HJ in supplementary material), irrespective of the
directionality of brain asymmetry. The data provide further support to the proposal that Spaw signaling coordinates directional asymmetry of the visceral organs and the brain and, that in the absence of this coordination, L-R reversal of brain and pancreas laterality occurs in a largely independent fashion.

Despite alterations in the L-R axis and presumptive heterotaxia in a significant fraction of the population, the majority of Spaw-depleted embryos hatched, developed swim bladders (>90%) and survived to adulthood (>60%). Injection of spaw MO into foxD3:GFP embryos was therefore a useful method for generating larvae with L-R randomized diencephalic asymmetry that could be screened on the basis of a left-sided or right-sided parapineal, separated, and raised to adulthood. This allowed habenular projections to be traced in the brains of adult fish that had a pre-determined parapineal position.

Habenular efferents and their connections were traced (as in Fig. 4A,B) by uniquely labeling each habenula with the lipophilic dyes DiO (left side) or DiI (right side). As was previously described for adult zebrafish (Tomizawa et al., 2001), anterograde DiI labeling of both habenulae reveals efferents that course posteriorly through the fasciculi retroflexus to the midline IPN in the ventral midbrain. Although differentially labeled efferents from the right and left habenulae of adult fish follow a similar trajectory (Fig. 4C-I), they project to different regions along the DV axis of the IPN (Fig. 4J-L). The left habenula innervates the dorsal and ventral IPN (Fig. 5A,C) and the right habenula innervates only the ventral region (Fig. 5B,C).

In adult fish derived from spaw MO-injected embryos, the brains from those that had formed the parapineal on the left side (n=4) (Fig. 5D-F) showed an IPN innervation pattern similar to WT. Brains with the parapineal on the right (n=4) exhibited a reversed pattern, in which efferents from the left habenula (green) projected solely to the ventral region of the IPN and those from the right habenula projected along the entire DV axis (Fig. 5G-I). Similar to Roep larvae, the overall DV pattern of efferents was preserved in adult brains even when the habenula of origin was L-R reversed.

The adult projection pattern onto the IPN was invariant in WT fish and closely paralleled by the distribution of Lov and Ron protein in habenular axons. Lov+ immunoreactive habenular efferents coursed throughout the DV extent of the
Asymmetry of habenular projections

adult IPN, while Ron+ axons were confined to the ventral region (Fig. 6A-C, n=3). This DV pattern of immunolabeled habenular projections was unaltered in adults derived from spaw MO-injected embryos (n=6), irrespective of the direction of epithalamic laterality (compare Fig. 6D-F and G-I).

Habenular asymmetry directs IPN connectivity

Prior studies demonstrated that habenular asymmetry is dependent on the parapineal (Concha et al., 2003; Gamse et al., 2003). Following parapineal ablation, the left habenula fails to adopt its characteristic properties, such as a larger size, expanded dense neuropil and increased lov expression relative to the right habenula. We examined whether the parapineal also influences properties associated with the right habenula by assaying ron and dex expression in larvae lacking the parapineal.

Removal of the parapineal by laser-mediated ablation was performed at 30 hours, prior to the appearance of lov, ron, and dex transcripts in the habenular region. When assessed at 4 days, the left habenula of parapineal-ablated larvae showed reduced lov expression (Gamse et al., 2003) (Fig. 7B) and expanded expression of ron and dex (compare Fig. 7C,D and E,F). The expression patterns of all genes appeared bilaterally symmetric, with both habenulae exhibiting transcriptional subdomains more typical of the right habenula.

In parapineal-ablated larvae, Lov protein levels were reduced and Ron+ domains expanded in the left habenula, relative to control-ablated larvae (compare Fig. 7G,H and I,J). Fewer Lov+ axons projected within the left FR, whose immunolabeling now resembled that of the right FR. In contrast to L-R reversal of parapineal position, ablation of the parapineal disrupted the DV distribution of habenular efferents onto the IPN. The two Lov+ domains normally observed at the dorsal IPN of control-ablated larvae

<table>
<thead>
<tr>
<th>Tg(foxD3:GFP) n=300</th>
<th>lov expression pattern</th>
<th>% of larvae</th>
<th>% Right</th>
<th>% Left</th>
<th>% Midline</th>
</tr>
</thead>
<tbody>
<tr>
<td>sinistral*</td>
<td>100†</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>spaw MO in Tg(foxD3:GFP) n=418</td>
<td>sinistral</td>
<td>52</td>
<td>31</td>
<td>18</td>
<td>3</td>
</tr>
<tr>
<td>dextral</td>
<td>45</td>
<td>25</td>
<td>17</td>
<td>3</td>
<td></td>
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<tr>
<td>symmetric</td>
<td>3</td>
<td>1</td>
<td>2</td>
<td>0</td>
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</tbody>
</table>

*Sinistral refers to the wild-type expression pattern of lov in the habenular nuclei with an expanded domain and higher levels of expression in the left habenula (Gamse et al., 2003). Dextral refers to the opposite pattern found in L-R reversed larvae (see Fig. 3).
†Absence or L-R reversal of lov expression was not observed in Tg(foxD3:GFP) control larvae. Only one larva (0.3%) had symmetric expression of lov in the habenulae and a right-sided pancreas.
(asterisk and arrowhead, Fig. 7K) were reduced to one small anterior domain (asterisk in Fig. 7L) and an increase in immunofluorescence was detected at the ventral IPN (Fig. 7L). These results are consistent with left habenular efferents adopting an IPN projection pattern more characteristic of those derived from the right habenula.

Discussion

Inroads into the understanding of the developmental and genetic basis of brain asymmetry have come from the zebrafish system. Previous work revealed that epithalamic laterality arises in a step-wise manner and that the direction of asymmetry is determined by Nodal signaling in the early embryo (Concha et al., 2000; Liang et al., 2000; Long et al., 2003; Rebagliati et al., 1998b; Sampath et al., 1998). In the vast majority of larvae, left-sided positioning of the parapineal impacts on the differentiation of an adjacent brain nucleus, the left habenula. Here, we show that left and right habenulae display asymmetric patterns of expression for three related genes, *lov*, *ron* and *dex*, which allows habenular subdomains to be distinguished molecularly in the larval brain. Antibodies directed against Lov and Ron proteins further define the trajectories and specificity of efferents from the L-R habenulae and affirm the existence of asymmetry at the level of protein distribution. Reversal of parapineal position is correlated with a mirror image reversal of habenular subdomains and of the molecular specificity of habenular efferents that course through the bilateral FR. Loss of the parapineal causes the left habenula to develop properties more similar to the right habenula. Ultimately, this alteration in diencephalic asymmetry has consequences on habenular connectivity at the midbrain target.

leftover-related gene expression defines habenular subdomains

Asymmetric specialization of the habenular nuclei has been studied extensively in amphibians (see Concha and Wilson, 2001). In species of *Rana*, the right dorsal habenula consists
Asymmetry of habenular projections

of a single nucleus, while the left habenula is composed of a medial and a lateral subnucleus (Braitenberg and Kemali, 1970; Morgan et al., 1973). The medial subnucleus can be further subdivided into medial and lateral components, on the basis of biochemical and subcellular properties. For example, only the more lateral region of the left medial habenula shows intense nitric oxide synthase activity (Guglielmotti and Fiorino, 1999). Evidence for habenular subnuclear specialization has also come from studies on cartilaginous and teleost fish. Silver staining of the adult red stingray brain enabled the identification of discrete asymmetric subdomains with differences in neuronal size, shape and density of packing (Iwahori et al., 1991a; Iwahori et al., 1991b). The left medial habenula of salmon exhibits a serotoninergic subregion that is not observed on the right (Ekstrom and Ebbesson, 1988).

In mammals, the medial habenulae are the equivalent of the amphibian dorsal habenulae (Concha and Wilson, 2001; Harris et al., 1996). In the adult rat, they consist of five discrete subdomains defined by morphological criteria, including cell packing density, neuronal shape and size, cytoplasmic volume, synaptic vesicles and degree of myelination (Andres et al., 1999). Habenular subdomains possess different neurochemical properties and project to different regions of the IPN, as evidenced by a cholinergic neuronal cluster in the ventral portion of the rat medial habenula that is distinct from dorsally situated Substance P containing neurons (Contestabile et al., 1987; Cuello et al., 1978). In contrast to lower vertebrates, these anatomical and neurochemical subdomains appear symmetric. We also did not observe prominent L-R differences in rats or mice for the habenular-specific expression of the Kctd8 or Kctd12/pfet genes. However, habenular patterns of expression for other mammalian KCTD family members have not yet been determined, nor can we rule out the presence of subtle L-R differences.

In the present study, we have shown that the zebrafish lov-related gene family includes three members, lov, ron and dex that are expressed differently by the left and right habenulae. By comparing their patterns, six asymmetric subdomains could be assigned to the left and right habenulae (designated i-vi in Fig. 8A). One distinctive feature is that the left habenula exhibits DV compartmentalization in gene expression not observed for the right. Subdivision of the right habenula is largely along the anteroposterior axis. DV regionalization of
the left habenula has been noted in other lower vertebrates. A lateral subnucleus occupies most of the dorsal left habenula in the stingray brain, but is restricted to a smaller medial posterior region on the right (Iwahori et al., 1991a; Iwahori et al., 1991b). There is also a dorsoventral difference in the distribution of the calcium-binding protein calretinin A in the left habenula of Rana esculenta (Guglielmotti et al., 2004). The significance of asymmetry and the relationship of gene expression subdomains with afferent input, habenular ultrastructure, and connectivity are important issues to pursue. For example, it is known that the parapineal selectively innervates the left habenula in several fish species (see Concha and Wilson, 2001) and may connect with neurons within a specific subdomain.

Determining the purpose of molecular specialization of the zebrafish habenulae will require a greater understanding of the functions of Lov-related proteins. The conserved N-terminal T1 domain, a protein-protein interaction motif that promotes oligomerization (Collins et al., 2001), could provide useful clues. In the Shaker family of voltage-gated potassium channels, it is required for tetramerization of alpha subunits into a functional channel and for axonal localization (Gu et al., 2003; Li et al., 1992). Preliminary data from yeast two-hybrid assays suggest that zebrafish Lov and Ron proteins form homophilic and heterophilic dimers, dependent on the presence of the T1 domain (J.T.G., unpublished observations). Since the T1 domain of Lov-related KCTD proteins is conserved with that of voltage-gated K+ channel subunits [e.g. approximately 50% amino acid identity between zebrafish Lov and Drosophila Shaker (Gamse et al., 2003)], these proteins might interact and thereby modulate channel assembly or activity. Such a role was recently proposed for KCNRG (K+ channel regulator encoding gene), which encodes a protein structurally similar to Lov-related proteins (Ivanov et al., 2003). Another potential function is in modulating neuronal responses to secreted signals from other cells. KCTD11/REN expresses signaling by the secreted protein Hedgehog by preventing the downstream transcription factor GLI from entering the nucleus (Di Marcotullio et al., 2004). Two-hybrid library screening, immunoprecipitation and MO depletion should shed more light on the cellular functions of zebrafish Lov-related proteins.

**L-R differences in zebrafish habenular projections**

The trajectory of habenular efferents along the FR and connection between the habenular nuclei and IPN has been well described and is a highly conserved feature of the vertebrate brain (Butler and Hodos, 1996; Cajal, 1966; Sutherland, 1982). Several studies have traced habenular efferents in fish, notably in the goldfish and trout (Villani et al., 1996; Yanez and Anadon, 1996), but the presence of topographic DV projections onto the IPN was not previously appreciated. Axonal tracing did reveal that neurons within particular habenular subdomains innervated specific subregions of the IPN (e.g. Herkenhaum and Nauta, 1979; Shibata et al., 1986; Villani et al., 1994), although these were presumed to be bilaterally symmetric projections. Preferential

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**Fig. 8.** Schematic of habenular domains and efferent projections in larval zebrafish. (A) Six asymmetric domains in L-R habenular nuclei of 4-day larvae are defined by lov, ron and dex expression. The left habenula is divided into one dorsal and three ventral domains, while four expression domains are organized along the anterior-posterior axis of the right habenula. (B) Normally, left habenular Lov+ Ron– neurons (domain i) project to the dorsal IPN. Ron+ efferents from both habenulae (domains ii, iii, v and vi) project to the ventral IPN. (C) In larvae with reversed epithalamic laterality, FR projections are L-R reversed, but DV innervation of the IPN is unaffected. (D) In parapineal-ablated larvae, the left habenula adopts molecular properties characteristic of the right habenula. The dorsal IPN shows a partial loss of Lov+ axons, while the ventral IPN shows increased immunoreactivity. Remaining Lov+ axons at the dorsal IPN may represent an incomplete transformation of the left habenula to right habenular fate.
innervation of the intermediate and central subregions of the IPN by a subset of habenular neurons was observed in the adult zebrafish brain (Tomizawa et al., 2001); however, differential left and right projection patterns were not described.

The discovery of molecular asymmetry in the zebrafish habenulae (Gamse et al., 2003) suggested that L-R differences could extend to habenular efferents and influence target recognition. By selective labeling with lipophilic dyes, we corroborated the recent findings of Aizawa et al. (Aizawa et al., 2005) that the left and right habenular axons project along different DV extents of the IPN. Those authors concluded that there is a “topographic projection of left-sided habenular axons to the dorsal region of the IPN and of the right-sided habenular axons to the ventral IPN.” While our data support the projection of axons from the L and R medial habenulae to topographically different domains along the DV axis of the zebrafish IPN, we find that there is substantial overlap in L and R projections within the ventral region. Thus, the assertion that left and right information is laterotopically represented onto discrete dorsal and ventral regions of the target nucleus (Aizawa et al., 2005) is an oversimplification of the pattern of connectivity documented in our experiments.

Furthermore, the finding that left habenular neurons project along the entire DV extent of the IPN and right habenular neurons project predominantly to the ventral IPN is strongly supported by their molecular specificity. Anti-Lov and anti-Ron sera label different DV domains of the IPN, domains that closely resemble the pattern of left and right habenular projections, respectively. Lov+ efferents are found throughout the DV extent of the IPN, while Ron+ efferents are confined to the ventral region.

**Directional asymmetry of the epithalamus influences target recognition**

Our results support a role for Nodal signaling in setting the direction of asymmetry in the zebrafish epithalamus that extends to epialamic projections. In Roepl larvae, the ability to respond to Nodal signaling is partially restored but is insufficient to direct brain laterality. Therefore, at a population level, the direction of brain asymmetry is L-R randomized. However, even in individuals that show a mirror image reversal of habenular asymmetry and in the L-R pattern of immunoreactive Lov+ and Ron+ habenular efferents, DV connections onto the IPN appear unaffected (Fig. 8C). Parallel studies on adult zebrafish that lacked the Nodal signal Southpaw as embryos also indicate that reversal of habenular asymmetry changes the L-R origin of inputs to the IPN, but not DV innervation of the IPN. Therefore, development of the midbrain target and its putative DV guidance cues appear intact, suggesting that the IPN is not directly modified by Nodal signals.

**Habenular asymmetry determines the dorsoventral pattern of IPN connectivity**

In contrast to genetically altered larvae, where global or early-acting effects of Nodal activity on the developing neural tube cannot be completely ruled out, selective ablation of the parapineal provides a rigorous test of the correlation between habenular laterality and IPN connectivity.

After parapineal ablation, the left habenula showed increased expression of *ron* and *dex* and reduced expression of *lov*, a molecular profile more characteristic of the right habenula. Thus, the parapineal normally functions not only to promote the acquisition of left-specific gene expression (Gamse et al., 2003), but to repress right-specific gene expression in the adjacent left habenula.

Loss of the parapineal also affects habenular connections. Lov+ axons innervating the dorsal IPN were reduced while Ron immunoreactivity increased in the ventral IPN, consistent with left habenular neurons adopting the projection pattern of right habenular neurons (Fig. 8D). However, this transformation may not be complete because some Lov+ projections persisted in the anterior region of the dorsal IPN. Dye labeling of larval or adult brains derived from parapineal-ablated embryos should resolve the L-R origin of the Lov+ axonal endings that remain at the dorsal IPN.

Parapineal ablation causes a local perturbation of diencephalic asymmetry that is not expected to affect the properties of the midbrain target directly, such as the expression of attractive or repulsive guidance cues. Therefore, we conclude that laterality of the habenular nuclei influences IPN connectivity by altering the molecular properties of habenular axons and presumably their choice of which target subdomains to innervate. How efferents from the right habenula are targeted to only the ventral IPN domain avoiding the dorsal one, while left habenula efferents innervate both, is a problem that will require more information about the types and distribution of axon guidance molecules at the IPN and neighboring brain regions. Members of the *neuropilin* gene family and the netrin 1 receptor Dcc (Deleted in colorectal cancer) are strongly expressed in the habenular nuclei of mouse embryos (Funato et al., 2000; Shu et al., 2000). Repulsive Semaphorin 3F (Sema3F) and attractive Netrin 1 signals have been implicated in directing the ventroposterior outgrowth of habenular efferents along the diencephalic neurorhyme boundary (Funato et al., 2000). Moreover, mutations of *neuropilin 2* or *Sema3F* lead to defasciculation of the FR (Giger et al., 2000; Sahay et al., 2003). Although the studies in mice provide information about FR navigation toward the midbrain, little is known about the nature of guidance cues habenular axons receive at the IPN. With the aid of GFP transgenes that highlight habenular projections (Parinov et al., 2004), forward genetic screens in zebrafish may identify such cues.

The demonstration that directional asymmetry in one region of the brain guides connectivity in a distant region has interesting implications for deciphering the origin of laterality in the mammalian cortex. Further exploration into the generation of L-R connectivity differences could also prove relevant for understanding human neurological disorders that have been associated with abnormal neuroanatomical asymmetry (Bruder, 2003; Green et al., 2003).

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Supplementary material
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