Local Tissue Interactions across the Dorsal Midline of the Forebrain Establish CNS Laterality

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2Millennium Nucleus on Integrative Neurosciences
Programa de Morfología Neuroanatomica and functional asymmetries of the brain are widespread among vertebrates and play important roles in animal behavior (Rogers and Andrew, 2002). CNS asymmetries arise early in development, suggesting genetic regulation (e.g., Chi et al., 1977; Guglielmotti and Fiorino, 1999; Concha et al., 2000), but additionally, epigenetic factors can influence asymmetries both in the developing and in the adult brain (e.g., Diamond et al., 1983; Melone et al., 1984; Gurusinghe et al., 1986; Wisniewski, 1998). Although many studies have demonstrated involvement of Nodal, Bmp, Hedgehog, Fgf, and other signaling pathways in the regulation of asymmetry of the heart and viscera (reviewed in Burdine and Schier, 2000; Wright, 2001; Hamada et al., 2002), these analyses have shed little light on the mechanisms regulating brain laterality. However, recent studies in zebrafish have shown that a genetic pathway with a conserved role in the control of asymmetry of the heart and viscera regulates at least some aspects of brain asymmetry (Concha et al., 2000; Liang et al., 2000; Gamse et al., 2002).

The diencephalic epithalamus is a region of the vertebrate brain that exhibits striking neuroanatomical asymmetries. The epithalamus is constituted by the habenular nuclei and pineal complex. The habenulae form part of an evolutionarily conserved conduction system that receives input from the telencephalon via the stria medullaris and relays information to the interpeduncular nuclei of the ventral midbrain via the fasciculi retroflexus (reviewed in Sutherland, 1982). Structural asymmetries between the left and right habenular nuclei are widespread among vertebrates and involve differences in size, neuronal organization, neurochemistry, and connectivity (reviewed in Concha and Wilson, 2001). The pineal complex is formed by medial evaginations of the diencephalic roof plate that include the epiphysis (pineal organ) and parapineal organ. Both organs contain photoreceptors and projection neurons, express a similar subset of neurotransmitters, and produce melatonin, a hormonal regulator of circadian rhythms (reviewed in Ekström and Meissl, 1997; Falcón, 1999; Concha and Wilson, 2001). Asymmetries of the pineal complex are observed in lampreys, teleosts, and lizards in which the parapineal organ innervates the left habenula (Rüdeberg, 1969; Engbrecht et al., 1981; Korf and Wagner,
1981; van Veen, 1982; Yañez et al., 1996, 1999; Concha et al., 2000). In teleosts, the parapineal organ is also situated on the left side of the brain (Borg et al., 1983; Concha et al., 2000), and the pineal stalk develops subtle morphological asymmetries (Liang et al., 2000).

Epithalamic asymmetries in zebrafish are first evident as left-sided expression domains of genes encoding the Nodal ligand Cyclops (Cyc), the negative regulator of Nodal activity Lefty1 (Lft1), and the downstream effector of Nodal signaling Pitx2 (Rebagliati et al., 1998; Sampath et al., 1998; Thisse and Thisse, 1999; Bisgrove et al., 2000; Concha et al., 2000; Essner et al., 2000; Liang et al., 2000). At later stages, neuroanatomical asymmetries consist of a photoreceptive parapineal organ located on the left side and the left habenula developing more extensive neuropil (Concha et al., 2000) and more intense expression of several genes (this study and Gamse et al., 2003) than the right habenula. Zebrafish embryos with compromised Nodal signaling and/or with midline defects either lack or show bilateral expression of cyc and pitx2 in the brain (Bisgrove et al., 2000; Concha et al., 2000; Liang et al., 2000; Long et al., 2003). Neuroanatomical asymmetries still develop in both situations although laterality of the parapineal organ and habenulae is randomized (Concha et al., 2000). These results suggest that Nodal signaling is not required for the development of asymmetry per se but is required to determine the laterality of asymmetry by biasing an otherwise stochastic laterality decision to the left side of the epithalamus (Concha et al., 2000). The cellular and genetic mechanisms regulating asymmetric development per se, upon which Nodal signaling influences laterality within the brain, remain unknown.

In this study, we use fate mapping, tissue ablation, and electroporation approaches in GFP-transgenic animals to explore how laterality decisions occur within the field of asymmetric Nodal influence in the zebrafish forebrain. We show that parapineal cells originate bilaterally in the anterodorsal epithalamus from where they migrate to become positioned on the left side of the brain. Development of asymmetry in the habenular neuropil is synchronized in time and space with innervation by efferent parapineal axons, indicating that the parapineal organ and habenulae interact during the establishment of epithalamic asymmetry. By ablating different components of the epithalamus, we found that asymmetric habenular neuropil can arise independent of the parapineal organ. However, removal of ventral epithalamic habenular precursor cells from the left side of the brain affects direction of migration and connectivity of the parapineal organ and laterality of the habenulae. Furthermore, local modulation of Nodal signaling by electroporation of lefty1 can impose the direction of CNS asymmetry in embryos in which laterality is normally randomized. We propose a model by which local inhibitory interactions between the left and right epithalamus establish laterality within the forebrain. In this model, Nodal signaling imposes an initial bias that makes left/right interactions imbalanced from the outset, thereby ensuring consistent direction of laterality within the population.

Results

The Epithalamus Is Divided into Dorsal and Ventral Domains

Asymmetric expression of components of the Nodal signaling pathway is the first manifestation of asymmetry within the zebrafish forebrain and precedes asymmetric morphogenesis of the parapineal organ, habenulae (Concha et al., 2000; Gamse et al., 2003), and pineal stalk (Liang et al., 2000). Expression of the homeobox gene floating head (flh) demarcates the territory in which pineal neurons develop (Masai et al., 1997; Cau and Wilson, 2003), but the origin of other epithalamic cell groups is unknown. To enable us to address this issue, we generated transgenic zebrafish expressing GFP under the control of the flh promoter (see Experimental Procedures). Comparison of flh:eGFP expression with that of cyc, lft1, and pitx2 showed that Nodal signaling overlaps the left, anterior field of the flh expression domain (Concha et al., 2000; Liang et al., 2000) but also extends further ventral to this domain (Figures 1A–1C and data not shown). These observations define two regions of Nodal influence: the dorsal epithalamus, comprising flh-expressing cells, and the ventral epithalamus, containing cells immediately ventral to the domain of flh expression.

The Parapineal Organ Has a Bilateral Origin from the Anterior Dorsal Epithalamus

To establish the origin of epithalamic cell groups, we followed the fates of cells in the epithalamus of flh:eGFP fish. At the time of Nodal signaling, flh:eGFP expression defines a contiguous territory located at the dorsal midline (Figures 1A–1D), but between 28 and 40 hpf this splits into a large midline domain that corresponds to the pineal organ and a smaller domain located on the left side that corresponds to the forming parapineal organ (Figures 1E–1G). Expression of flh:eGFP in the parapineal organ is then gradually lost (Figures 1H and 1I) and from around 65 hpf is only detected in the pineal organ (data not shown). These results suggest that parapineal precursors express flh when located at the midline but downregulate expression as they detach from the pineal complex to become positioned on the left side of the epithalamus.

At least three mechanisms could explain the absence of a parapineal nucleus on the right side of the brain: right-sided cells could die, adopt a different fate, or could migrate across the midline to contribute to the left-sided nucleus. To resolve among these possibilities, we determined the location of parapineal precursors prior to their appearance on the left side of the pineal complex. To achieve this, we photoactivated caged fluo-
Figure 1. The Dorsal and Ventral Epithalamus Give Rise to the Parapineal Organ and Habenular Nuclei

Dorsal (A and D–O') and frontal (B and C) views of the dorsal diencephalon with anterior and dorsal toward the top of the panels, respectively. Epithalamic expression of GFP in flh:eGFP- (A–I) and flh:eGFP;foxD3:GFP- (J–O) transgenic embryos is shown in brown (A/C) or green (D–O). Stages of development are indicated in somites (s) or hours post-fertilization (h) on the bottom right for each panel.

(A–C) Relationship between the expression domains (blue) of pitx2 (A), cyc (B), and lft1 (C), and the domain of flh:eGFP expression as detected with anti-GFP antibody (brown). Arrowheads demarcate the limits of pitx2, cyc, and lft1 expression. Red asterisks and dashed lines indicate the boundary between dorsal (d) and ventral (v) epithalamus.

(D–I) Single-depth confocal images showing early phases of asymmetric morphogenesis of the parapineal organ in flh:eGFP-transgenic zebrafish. Arrows indicate the position of the parapineal organ.

(J–L and J/H11032–L/H11032) Embryos in which caged fluorescein was photoactivated by laser (pseudocolored in red) within the left (J) and right (K) dorsal epithalamus and labeling was subsequently observed in both pineal (po) and parapineal (arrowheads) organs (J' and K'). (L) and (L') are schematic representations of the labeling results.

(M–O and M/H11032–O/H11032) Embryos in which caged fluorescein was photoactivated by laser (red) within the left (M) and right (N) ventral epithalamus and labeling was subsequently observed in the left (M') and right (N') habenula, respectively. (O) and (O') are schematic representations of the labeling results. Arrowheads indicate the position of the parapineal organ.

Abbreviations: d, dorsal; LH, left habenula; po, pineal organ; RH, right habenula; v, ventral.

1J–1L and 1J'/H11032–1L/H11032 and Table 1). This data demonstrates that parapineal precursors are located bilaterally in the dorsal epithalamus within the anterior-most region of flh expression.

If anteriorly positioned flh-expressing cells are indeed the source of parapineal precursors, we reasoned that ablation of these cells should result in an absence of a parapineal organ. We therefore used laser pulses to ablate flh:eGFP cells on one or both sides of the anterior dorsal epithalamus. As flh:eGFP is a poor marker of the
mature parapineal, we performed these experiments in embryos that also expressed a foxD3:GFP transgene (Gilmour et al., 2002) that labels mature parapineal neurons (see below) but does not label epithalamic precursor cells. The use of both transgenes allowed parapineal complex cells to be followed throughout their development. A mature but small and sometimes mispositioned parapineal organ developed on the left side following ablation of flh:eGFP expressing cells on either the left side (Figures 2B′ and 2B″) or the right side (Figures 2C′ and 2C″) of the dorsal anterior epithalamus. In contrast, bilateral ablation led to complete absence of parapineal neurons (Figures 2D′ and 2D″). These results suggest that all parapineal precursors are located in the anterior dorsal epithalamus.

The Habenular Nuclei Originate from Ventral Epithalamic Cells

flh:eGFP-expressing cells do not significantly contribute to the habenular nuclei (Table 1), and so, to determine the location of habenular precursors, we photoactivated caged fluorescein in small groups of cells directly adjacent to the flh:eGFP domain at about 24 hpf and examined the position of labeling at 48 hpf. Photoactivation within the epithalamus directly ventral to the flh:eGFP-expression domain always led to staining within the ipsilateral habenula (Figures 1M–1O and 1M‴ and Table 1). These results demonstrate that, at 24 hpf, precursors of the left and right habenula are positioned within the left and right ventral epithalamus, respectively. Altogether, our fate mapping studies indicate that around 24 hpf parapineal and habenular precursors are contiguous and, on the left side of the brain, both sets of precursors are likely to be influenced by Nodal activity.

Table 1. Mapping the Location of Parapineal and Habenular Precursors within the Zebrafish Forebrain

<table>
<thead>
<tr>
<th>Position of Labelling at 22–24 hpf</th>
<th>Fate at 48 hpf</th>
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<tbody>
<tr>
<td></td>
<td>Pineal</td>
</tr>
<tr>
<td><strong>Dorsal Epithalamus</strong></td>
<td></td>
</tr>
<tr>
<td>DLA</td>
<td>87%</td>
</tr>
<tr>
<td>DLM</td>
<td>100%</td>
</tr>
<tr>
<td>DRA</td>
<td>86%</td>
</tr>
<tr>
<td>DRM</td>
<td>100%</td>
</tr>
<tr>
<td><strong>Ventral Epithalamus</strong></td>
<td></td>
</tr>
<tr>
<td>VLA</td>
<td>60%</td>
</tr>
<tr>
<td>VLM</td>
<td>20%</td>
</tr>
<tr>
<td>VRA</td>
<td>69%</td>
</tr>
<tr>
<td>VRM</td>
<td>20%</td>
</tr>
</tbody>
</table>

In the schematics, the flh:eGFP-expressing domain is outlined by a solid line in the top panel and by a dashed line in the bottom panel. Ventral epithalamic cells are positioned underneath (ventral to) the flh:eGFP domain. Abbreviations: D, dorsal; V, ventral; L, left; R, right; A, anterior; M, middle (between anterior and posterior ends of flh:eGFP expression).

Parapineal Precursors Migrate to the Left Side of the Brain and Innervate a Region of the Left Habenula that Develops Asymmetric Neuropil

We have previously shown that the laterality of parapineal and habenular asymmetries is strongly coordinated (Concha et al., 2000). In order to better understand the temporal and spatial coordination of parapineal and habenular asymmetries, we performed confocal volumetric analysis of flh:eGFP- and foxD3:GFP-transgenic fish. From about 28 hpf, parapineal precursors begin to aggregate and move away from the midline toward the left side of the epithalamus (Figures 1E–1I). This early phase of parapineal migration is followed by a second phase that involves a ventral, posterior, and medial displacement of the parapineal organ, which ends up flanking the pineal stalk (Figures 3A–3H). Efferent projections from the parapineal organ are observed from around 50 hpf and develop extensively during the late phase of parapineal migration to cover a significant fraction of the left epithalamus (Figures 3E′–3H′). The abundance of putative terminals of efferent parapineal axons within the left epithalamus is most likely due to extensive axonal branching, as the number of parapineal cells is rather small (13.5 ± 2 cells expressing foxD3:GFP at 96 hpf; n = 19).

Habenular asymmetries are characterized by the left habenula developing a larger area of neuropil than the right habenula, particularly within the medial and dorsal aspect of the nucleus (Concha et al., 2000; this study). The region of enlarged neuropil in the left habenula is evident from around 70 hpf and becomes more conspicuous contemporaneous with the escalating development of efferent projections from the parapineal organ (compare Figures 3I–3L and 3E″–3H″). Indeed, parapineal axons enter the left habenula immediately posterior to the habenular commissure and distribute extensively toward medial and dorsal regions of this nucleus to encompass the area of the left habenula that displays asymmetric neuropil (Figure 4). Altogether, these results indicate that the development of neuroanatomical asymmetry in the habenulae is coordinated both in time and space with the development of efferent parapineal connectivity.

The Laterality of Parapineal Asymmetry Is Disrupted by Selective Removal of Ventral Cells from the Left Epithalamus

By definition, the unilateral innervation of the left habenula by parapineal axons contributes to the lateralization of the habenular nuclei. Indeed, complete removal of the parapineal organ at 28–30 hpf (when it is already
located in the left epithalamus) leads to failure to upregulate the marker gene leftover (lov) in the left habenula (Gamse et al., 2003). We confirmed a requirement of parapineal cells for left-sided upregulation of lov expression by ablating parapineal precursors at 22–24 hpf (n = 9; Figures 2B″–2D″). However, despite the reduced levels of expression, we still detected left/right differences in the pattern of lov expression after parapineal ablation. The dorsomedial aspect of the left habenula retains an area of staining that is not seen in the right habenula (Figure 2D″). In addition, embryos lacking parapineal efferents through ablation of flh:eGFP+ parapineal precursors in the anterior epithalamus frequently still exhibit differences in the extent of neuropil (Figure 2D′) between left and right habenulae (Figure 2 and Table 2). Therefore, although the parapineal contributes to habenular laterality, our results suggest that there is no absolute requirement for a parapineal organ to establish differences between left and right habenular nuclei.

We next asked whether asymmetric migration and/or connectivity of the parapineal organ depends on habenular development. To test this possibility, we ablated ventral epithalamic cells that later contribute to the habenulae and assessed the subsequent direction of migration and pattern of connectivity of the parapineal organ. Ablation of cells within the ventral epithalamus led to a variable decrease in the extent of neuropil within the ipsilateral habenula and, in some cases, also affected the development of the habenular commissure (data not shown). The number of parapineal cells was also often reduced due to the close proximity between the parapineal anlage and the ventral epithalamus. As the severity of these defects was directly correlated with the quantity of cells ablated (data not shown), we focused our analyses on ablations localized to a subset of cells positioned immediately ventral to the flh:eGFP expression domain. In these cases, the overall morphology of the epithalamus was unaffected and the number of parapineal cells only mildly reduced (Table 3).

Parapineal asymmetry was disrupted in 68% of embryos following ablation of left ventral epithalamic cells...
Figure 3. Development of Asymmetric Neuropil within the Habenulae Is Spatially and Temporally Coordinated with Parapineal Innervation

Frontal (A–D) and dorsal (E–L) confocal images of the epithalamus with dorsal and anterior toward the top of the panels, respectively. Stages of development are indicated on the left for each row of images.

(A–H) Confocal volumetric projections showing late phases of parapineal migration in foxD3:GFP-transgenic zebrafish. For clarity, pineal and parapineal organs have been pseudocolored in red and green, respectively.

(E–H) Development of efferent parapineal connectivity. Images correspond to magnified views of (E)–(H). Green arrows indicate the position from which axons leave the parapineal organ, and white arrowheads delineate the area of the left epithalamus covered by these axonal projections.

(I–L) Confocal volumetric projections showing the development of asymmetric neuropil within the habenulae of wild-type zebrafish as revealed by anti-acetylated α-tubulin antibody. Large yellow arrows indicate the region of the left habenula that displays enlarged neuropil.

Abbreviations: hc, habenular commissure; LH, left habenula; pc, posterior commissure; po, pineal organ; RH, right habenula.

(Table 3). These embryos showed either a parapineal organ located at the dorsal midline (29%, Figures 5C′ and 5C″) that projected to the left (4/8), right (3/8), or both (1/8) habenular nuclei, or, most notably, a parapineal organ with a pattern of migration and efferent connectivity directed toward the right habenula (39%; Figures 5D′ and 5D″ and Table 3). Removal of an equivalent group of ventral cells from the right epithalamus had no effect on parapineal asymmetry (Table 3 and Figures 5B′ and 5B″). These results indicate that left ventral epithalamic cells are required for normal asymmetric migration and connectivity of the parapineal organ.

The Laterality of Habenular Asymmetry Is Disrupted by Selective Removal of Ventral Cells from the Left Epithalamus

To test the possibility that the left ventral epithalamus not only influences parapineal laterality but more globally affects epithalamic asymmetry, we determined whether habenular laterality was also affected by ablation of ventral epithalamic cells. For this, we examined the extent of habenular neuropil and the expression pattern of hest1 (habenularEST1), a novel genetic marker of habenular asymmetry (see Experimental Procedures). hest1 is normally expressed bilaterally in the habenulae but shows more intense expression in the dorsomedial aspect of the left nucleus (Figure 5A″).

In 48% of cases, removal of left ventral epithalamic cells led to the right dorsomedial habenular nucleus developing a region of enlarged neuropil resembling that normally found in the left habenular nucleus (Figures 5C′ and 5D′ and Table 4). In these embryos, hest1 expression was consistently reduced compared to wild-type but, in many cases, remaining expression was stronger in the right than in the left habenula (45%; Figures 5C″ and 5D″ and Table 4). When the right ha-
Figure 4. The Parapineal Organ Projects to a Region of the Left Habenula that Develops Asymmetric Neuropil

Dorsal views of the epithalamus in a foxD3:GFP-transgenic zebrafish at 96 hpf with anterior toward the top of the panels. The habenular neuropil has been labeled with anti-acetylated α-tubulin antibody (red) and the pineal complex with anti-GFP antibody. For clarity, pineal and parapineal organs have been pseudocolored in blue and green, respectively.

(A) Confocal volumetric projection. The habenular commissure (hc) normally runs between the left (LH) and right (RH) habenular nuclei but has broken during the labeling procedure.

(B–E) Single optical sections of the region encircled in (A). The depth of each section is indicated in the bottom left corner. The arrow indicates the position from which axons leave the parapineal organ, and white arrowheads delineate the area of the left habenula covered by these axonal projections.

Abbreviations: hc, habenular commissure; LH, left habenula; pineal, pineal organ; pp, parapineal organ; RH, right habenula.

benula was transformed into a nucleus with left characteristics, this was usually accompanied by migration and connectivity of the parapineal organ toward the right epithalamus (9/11 embryos; Figures 5D and 5D″). In these experiments, we did not assess whether the left habenula showed left- or right-sided character due to the reduction of neuropil after ablation. In contrast to the laterality reversals induced by left-sided removal of habenular precursors, ablation of right ventral epithalamic cells had no effect on the laterality of habenular neuropil and only minor effects on hest1 expression (Figures 5B′ and 5B″ and Table 4). Altogether, these results demonstrate that laterality is not fixed at the time of ablation and show that local tissue interactions between the left and right epithalamus are able to determine forebrain laterality.

Ventral Epithalamic Ablations Impose Handedness upon Embryos that Normally Exhibit Heterotaxia

In wild-type embryos, ablation of left-sided ventral epithalamic cells sometimes leads to reversal of epithalamic laterality. We next wished to address whether such ablations could impose laterality in embryos where the outcome of the laterality is not already biased to the left by unilateral Nodal activity (about 95% of wild-type
Table 3. Ventral Epithalamic Cell Ablation Affects the Laterality of Parapineal Migration

<table>
<thead>
<tr>
<th>Laterality of Ablation</th>
<th>Laterality of Parapineal Migration</th>
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<tr>
<td></td>
<td>Left</td>
<td>Midline</td>
</tr>
<tr>
<td>Wild-type</td>
<td>95%</td>
<td>–</td>
</tr>
<tr>
<td>right</td>
<td>91% (9 ± 3 cells)</td>
<td>–</td>
</tr>
<tr>
<td>left</td>
<td>32% (7 ± 2 cells)</td>
<td>29% (7 ± 3 cells)</td>
</tr>
<tr>
<td>ntl-MO</td>
<td>45%</td>
<td>–</td>
</tr>
<tr>
<td>right</td>
<td>82% (7 ± 2 cells)</td>
<td>–</td>
</tr>
<tr>
<td>LZOep⁻/⁻</td>
<td>52.5%</td>
<td>–</td>
</tr>
<tr>
<td>none (control)</td>
<td>74% (8.4 ± 3 cells)</td>
<td>13% (11 ± 1 cells)</td>
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Laser ablation of ventral epithalamic cells was performed in wild-type flh:eGFP;foxD3:GFP, ntl morphant (ntl-MO) flh:eGFP;foxD3:GFP, and LZOep⁻/⁻; foxD3:GFP embryos at 22–24 hpf. The laterality of parapineal migration was assessed at 96 hpf by live confocal imaging or anti-GFP immunostaining. Due to the close proximity between the ventral epithalamus and the parapineal anlage, the ablation procedure often involved some loss of parapineal cells. To take this limitation into account, we give in brackets the average number of parapineal cells in control embryos is 13.5 ± 2.

embryos show left-sided parapineal and an enlarged left habenula; Concha et al., 2000). To address this issue, we ablated ventral epithalamic cells in situations where Nodal activity within the brain is either bilaterally symmetric (embryos lacking No-tail [ntl] activity) (Blagrove et al., 2000; Concha et al., 2000; Liang et al., 2000) or absent (embryos lacking Late Zygotic activity of One-eyed-pinhead, LZOep⁻/⁻) (Concha et al., 2000; Liang et al., 2000). In both classes of embryos, parapineal and habenular laterality is normally randomized (Concha et al., 2000; Gamse et al., 2002, 2003), suggesting that there is no initial left/right bias in Nodal signaling or in the subsequent allocation of left/right laterality.

In virtually all cases, ablation of right-sided ventral epithalamic cells in ntl morphants led to left-sided migration and connectivity of the parapineal (Figures 5E⁻ and 5E⁺ and Table 3), left-sided enlargement of habenular neuropil (Figure 5E⁻, Table 4), and high levels of habenular hest1 expression on the left (Figure 5E⁺, Table 4). A shift in laterality was also observed after ablations in LZOep⁻/⁻ embryos (Figures 5F⁻–5F⁺, Tables 3 and 4), suggesting that Nodal activity is not required for the ablation-mediated imposition of laterality. These results indicate that in situations where it is likely that left and right sides of the dorsal forebrain are initially equivalent, ablation of ventral epithalamic cells leads to parapineal migration and expanded habenular development on the opposite side of the brain. As we discuss below, this suggests that a competitive mechanism between left and right epithalamic determines laterality in the dorsal forebrain.

Local Modulation of Nodal Signaling Directs Epithalamic Laterality

We have previously shown that mutations affecting Nodal signaling randomize CNS laterality (Concha et al., 2000). However, it is unclear where, when, and how Nodal signaling is acting to modulate CNS laterality. Although Nodal pathway components are asymmetrically expressed in the epithalamus (previous studies), and parapineal and habenular precursors are both likely to be influenced by Nodal signaling (this study, Figures 1A–1C), there is no direct evidence that epithalamic Nodal activity can directly influence laterality decisions. To test this possibility, we examined the consequences on laterality of unilaterally abrogating Nodal signaling locally within the epithalamus of embryos that show bilateral Nodal expression and which normally exhibit randomization of CNS asymmetries. To do this, we electroporated DNA encoding a secreted antagonist of Nodal signaling (Lefty1) together with a reporter (α-tubulin GFP) in the right epithalamus of ntl morphant embryos. Lefty1 is a very potent secreted Nodal-signaling antagonist (Solnica-Krezel, 2003), making it a good reagent to locally modulate Nodal activity. After electroporation, high levels of exogenous lefty1 mRNA expression could be detected in small numbers of cells on the right side of the epithalamus (Figure 6B), colocalizing with the site of α-tubulin GFP⁺ clones (Figure 6B, inset). As Lefty1 can function at a distance from its site of expression (Chen and Schier, 2002), then it is likely that exogenous Lefty1 activity spreads beyond the immediate vicinity of the electroporated cells.

In all cases where cells expressing α-tubulin GFP were visualized on the right side of the pineal complex (n = 10), the parapineal organ migrated to the contralateral (left) side and showed connectivity directed to the contralateral (left) habenula (Figure 6D). Conversely, coelectroporation of α-tubulin GFP⁺-DsRed DNAs in ntl morphant control embryos had no effect on laterality and parapineal migration remained randomized (L = 52%, R = 48%; n = 29) (Figure 6C). We therefore conclude that local asymmetric modulation of epithalamic Nodal signaling is sufficient to impose laterality on embryos that would otherwise develop randomized CNS asymmetries.

Discussion

In this study, we have used fate mapping, tissue ablation, and electroporation approaches in GFP-transgenic animals to elucidate the mechanisms by which laterality decisions are made in the vertebrate forebrain. We show that local tissue interactions regulate the laterality of asymmetry of the diencephalic habenular nuclei and the photoreceptive parapineal organ. Below, we discuss how both ipsilateral and contralateral organ signals expanding habenular development on the opposite side of the brain. As we discuss below, this suggests that a competitive mechanism between left and right epithalamic determines laterality in the dorsal forebrain.
Figure 5. The Laterality of Parapineal and Habenular Asymmetries Is Affected by Removal of Cells from the Left Ventral Epithalamus

Dorsal views (A–F, A′–F′, and A″–F″) and transverse sections (A‴–F‴) of the epithalamus with anterior and dorsal toward the top of the panels, respectively. Embryos are flh:eGFP;foxD3:GFP- (A–D), ntl morphant flh:eGFP;foxD3:GFP- (E), and LZoep−/−:foxD3:GFP- (F) transgenic zebrafish. (A–F) Schematics indicating the region of the ventral epithalamus ablated at 22–24 hpf (white dots) in relation to the flh:eGFP expression domain (green): control nonablated (A), ablated on the anterior right side (B) and anterior left side (C and D) in wild-type, and anterior right side in ntl morphant (E) and LZoep−/− embryos (F). (A′–F′) Confocal volumetric projections of the epithalami in embryos ablated as in (A)–(F). Embryos are labeled with anti-acetylated α-tubulin (red, habenular neuropil) and anti-GFP (green, pineal complex) antibodies. Arrowheads show the position of the parapineal organ. Large yellow arrows indicate the region of the habenulae displaying an enlarged neuropil. (A″–F″) High-magnification views of (A′–F′) focused on the region where the parapineal organ develops. Arrows indicate efferent parapineal axons. (A‴–F‴) hest1 expression in the epithalamus. There is extensive expression of hest1 in regions of the brain ventral to right (RH) and left (LH) habenular nuclei. Large arrows indicate the side of the habenulae displaying more intense hest1 labeling.

Abbreviations: po, pineal organ; ps, pineal stalk; LH, left habenula; RH, right habenula.

Coordinated Development of Habenular and Parapineal Asymmetries

Our studies have shown a remarkable degree of coordination in development of the parapineal and habenular nuclei (Figure 7A). Precursors of both nuclei are contiguous and on the left side of the brain both are likely to

be directly influenced by Nodal activity. At the stage when left-sided activation of Nodal signaling occurs, the epithalamus is symmetric, but this soon changes as parapineal cells begin to aggregate on the left side of the pineal complex. This is followed by migration to the left side of the brain and subsequently by the develop-
Table 4. Ventral Epithalamic Cell Ablation Affects Laterality of Habenular Asymmetry

<table>
<thead>
<tr>
<th>Laterrality of Ablation</th>
<th>Laterrality of Habenular Asymmetry</th>
<th>L+/R</th>
<th>L/R</th>
<th>L+/R+</th>
<th>L/R+</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>none (control)</td>
<td>a-tub/hest1</td>
<td>91%</td>
<td>1%</td>
<td>-</td>
<td>8%</td>
</tr>
<tr>
<td>right</td>
<td>a-tub</td>
<td>91%</td>
<td>9%</td>
<td>-</td>
<td>-</td>
<td>11</td>
</tr>
<tr>
<td>right</td>
<td>hest1</td>
<td>92%</td>
<td>8%</td>
<td>-</td>
<td>-</td>
<td>12</td>
</tr>
<tr>
<td>left</td>
<td>a-tub</td>
<td>24%</td>
<td>28%</td>
<td>17%</td>
<td>3%</td>
<td>29</td>
</tr>
<tr>
<td>left</td>
<td>hest1</td>
<td>10%</td>
<td>45%</td>
<td>-</td>
<td>45%</td>
<td>20</td>
</tr>
<tr>
<td>ntl-MO</td>
<td>none (control)</td>
<td>a-tub/hest1</td>
<td>44%</td>
<td>4%</td>
<td>4%</td>
<td>48%</td>
</tr>
<tr>
<td>right</td>
<td>a-tub</td>
<td>80%</td>
<td>10%</td>
<td>-</td>
<td>10%</td>
<td>11</td>
</tr>
<tr>
<td>right</td>
<td>hest1</td>
<td>100%</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>7</td>
</tr>
<tr>
<td>LZoep-/-</td>
<td>none (control)</td>
<td>a-tub/hest1</td>
<td>50%</td>
<td>-</td>
<td>-</td>
<td>50%</td>
</tr>
<tr>
<td>right</td>
<td>a-tub</td>
<td>73%</td>
<td>8%</td>
<td>4%</td>
<td>15%</td>
<td>26</td>
</tr>
<tr>
<td>right</td>
<td>hest1</td>
<td>60%</td>
<td>-</td>
<td>-</td>
<td>40%</td>
<td>15</td>
</tr>
</tbody>
</table>

Laser ablation of ventral epithalamic cells was performed in wild-type flh:eGFP;foxD3:GFP, ntl morphant (ntl-MO) flh:eGFP;foxD3:GFP, and LZoep-/-foxD3:GFP embryos at 22–24 hpf. The laterality of habenular asymmetry was assessed at 96 hpf by examination of the content of neuropil using anti-acetylated α-tubulin antibody (α-tub; Concha et al., 2000) or by hest1 mRNA in situ hybridization. Laterality of the habenulae: normal (left > right, L+/R), reversed (left < right; L/R+), left isomerism (L+/R+), right isomerism (L/R-).

ment of efferent connectivity with the left habenula. The latter is temporally synchronized and topographically coordinated with the development of asymmetric neuropil in the left habenula.

In virtually all situations, the laterality of habenular and parapineal asymmetries is coordinated (Concha et al., 2000; this study; Gamse et al., 2003). There are at least three mechanisms that could explain this: the parapineal determines the laterality of the habenular nuclei, the habenular nuclei determine the laterality of the parapineal, or a common mechanism influences laterality of both structures. We think that the first two, and probably all three, mechanisms contribute to the final morphogenesis of the epithalamic nuclei. The evidence that a common mechanism influences both nuclei is circumstantial—precursors of both nuclei are contiguous at the time at which laterality is allocated, and the only bona fide signaling pathway known to influence laterality (Nodal) is activated in both sets of precursors.

Our study provides compelling evidence that habenular precursors are asymmetric within a few hours of unilateral Nodal signaling and that this asymmetry influences parapineal migration. It is likely that the habenular nuclei continue to influence parapineal neurons at later stages, for instance, by providing guidance cues to attract parapineal axons. Conversely, unilateral parapineal innervation, by definition, affects later asymmetry of the habenular nuclei. Parapineal cells also influence lateralized gene expression within the habenular nuclei (Gamse et al., 2003). Indeed, the extent of habenular asymmetry in reptiles is strictly correlated with the presence or absence of a parapineal organ/parietal eye

Figure 6. Electroporation of lefty1 in the Epithalampus Directs Laterality of Parapineal Migration

Dorsal views of the dorsal diencephalon in ntl morphant foxD3:GFP-transgenic zebrafish, with anterior toward the top of the panels. Embryos were electroporated with α-tubulin GFP + pCS2DsRed DNAs in the left epithalamus (A and C) or α-tubulin GFP + lefty1 DNAs in the right epithalampus (B and D).

(A and B) Whole-mount in situ hybridization for lefty1 mRNA at 40 hpf. Exogenous lefty1 mRNA is detected after lefty1 DNA electroporation (arrowhead in [B]), colocalizing with α-tubulin GFP cells (arrow in inset).

(C and D) Epithalamic expression of foxD3:GFP transgene (pineal and parapineal organs), electroporated DsRed (arrow in [C]), and electroporated α-tubulin GFP (arrow in [D]) in living embryos at 48 hpf (C) and 96 hpf (D). For clarity, the pineal organ has been pseudocolored in blue, the parapineal organ in green, and electroporated cells in either red (DsRed in [C]) or green (α-tubulin GFP in [D]).

Abbreviations: DsRed, pCS2DsRed; po, pineal organ; pp, parapineal organ; tub-GFP, α-tubulin GFP.
Competitive Interactions Establish CNS Laterality

Figure 7. A Model of Tissue and Molecular Interactions Underlying Forebrain Laterality

(A) Schematics illustrating the development of epithalamic asymmetry. The earliest known manifestation of asymmetry is the activation of Nodal signaling on the left side of the brain (cyc, crosshatching), encompassing both parapineal (yellow) and habenular (pink) precursors. Shortly after this, parapineal cells aggregate on the left side of the pineal complex. Although no markers of asymmetry of the prospective habenulae at this stage have been identified, these nuclei are already likely to be lateralized. Subsequent parapineal migration to the left is dependent upon habenular precursor cells on the left side of the brain. During later development, left-sided parapineal and habenular cells continue to interact with parapineal axons innervating the left dorsomedial (LDM) neuropil of the habenulae (this study) and influencing gene expression in this nucleus (Gamse et al., 2003).

(B) Schematics illustrating a mutual inhibition model of CNS lateralization. The top row shows events occurring in the epithalamus at early stages and the bottom panels show the outcomes of these events. Central to this model is that the left and right sides of the epithalamus compete, with the outcome of the competition determining the direction of parapineal migration and the enlargement of the ipsilateral habenular nucleus.

(Bi) In the absence of Nodal signaling in the epithalamus (LZoep$^{-}$; Concha et al., 2000; Liang et al., 2000), both left and right epithalamus compete on equal terms, and laterality is determined on a stochastic basis.

(Bii) In wild-type embryos, lateralized Nodal signaling gives the left side an advantage, and the outcome of the competition is always biased to the left.

(Biii) In embryos with bilateral Nodal signaling in the brain (ntl-MO), both sides of the epithalamus receive the advantage provided by Nodal activity, and once again, the competition is equal and outcome is stochastic.

(Biv) Ablation of left-sided ventral epithalamic precursors in wild-type embryos negates the advantage provided by Nodal signaling so that, in some cases, the right side of the epithalamus wins the competition, and laterality is to the right.

(Bv) In embryos with ablation of right-sided ventral epithalamic precursors, the left side still wins the competition, and laterality is the same as in unablated embryos.

(Bvi) Ablation of right-sided ventral epithalamic precursors in embryos lacking Nodal signaling (LZoep$^{-}$) imposes an advantage to the left epithalamus, and laterality is subsequently biased to the left.

(Bvii) In embryos with bilateral activation of Nodal signaling (ntl morphants, ntl-MO), either ablation of right-sided ventral epithalamic precursors or inhibition of Nodal signaling by exogenous Lefty1 on the right restores the advantage to the left epithalamus, and laterality is subsequently biased to the left.
(Huber and Crosby, 1926; Cruce, 1974; Engbretson et al., 1981; ten Donkelaar, 1998). Whether the parapineal has any earlier role in the initial determination of habenular laterality is less clear. For instance, habenular asymmetries still develop in many vertebrate groups that apparently lack a parapineal organ (reviewed in Concha and Wilson, 2001). Furthermore, in our ablation studies, some embryos developed asymmetric habenular neuropil and asymmetric lov expression despite the apparent absence of parapineal neurons. It seems likely that reciprocal interactions between parapineal and habenular cells serve to reinforce and ensure coordination of initial molecular asymmetries, thereby guaranteeing appropriate functional development of asymmetric neuronal circuits.

Communication Occurs between Left and Right Sides of the Epithalamus
Several lines of evidence suggest that cells on the left and right sides of the diencephalon communicate across the dorsal midline of the CNS. The fact that epithalamic isomerism is rare in embryos carrying mutations affecting Nodal signaling or following disruption to axial midline tissue (Concha et al., 2000; Liang et al., 2000; Gamse et al., 2002) suggests that if one side of the CNS acquires a certain handedness (left or right), then the other is inhibited from adopting the same. Intuitively, it makes sense if this inhibitory interaction occurs locally between left and right sides of the dorsal diencephalon. However, all mutations examined to date globally affect laterality, and so we cannot be absolutely certain where, when, or how laterality in the CNS is determined in these mutant embryos.

Our fate mapping studies provide the most compelling evidence that communication occurs between left and right epithalamus. Parapineal migration is dependent upon left-sided ventral epithalamic cells, and so, signals that promote left-sided migration are able to directly or indirectly attract right-sided cells to the left. Given that right-sided parapineal precursors (which do not express the Nodal ligand Cyc) can still cross the midline in the absence of left-sided (Cyc-expressing) parapineal precursors, it seems likely that tropic signals can act across the midline. However, as midline tissue itself may be damaged in these experiments, we should not discount the possibility that left-sided parapineal precursors may promote the movement of right-sided precursors to the left. Altogether, current evidence favors the notion that signals can traverse the dorsal midline during establishment of epithalamic laterality. As we discuss below, midline-crossing signals may influence initial establishment of left/right identity as well as subsequent morphogenetic events.

Nodal Signaling in the Dorsal Diencephalon Directs Laterality
The Nodal signaling pathway has conserved roles in regulating asymmetry and laterality throughout vertebrates (Hamada et al., 2002; Boorman and Shimeld, 2002). However, this signaling pathway has many roles during early development and indeed is even used reiteratively during the establishment of asymmetry (e.g., Brennan et al., 2002; Yamamoto et al., 2003). This has made it challenging to determine where, when, and how Nodal signals influence laterality of various structures. Previous work has shown that mutations affecting Nodal signaling disrupt epithalamic laterality (Concha et al., 2000; Liang et al., 2000; Gamse et al., 2002) but have not determined whether local Nodal pathway activity in the diencephalon is responsible for regulating laterality. Indeed, recent data suggests that Nodal signaling within lateral plate mesoderm can influence laterality in the epithalamus (Long et al., 2003).

Our experiments unilaterally modulating Nodal activity in the brain provide strong evidence that diencephalic Nodal activity does have a critical role in mediating CNS laterality. In our experiments, we used Lefty1 to abrogate Nodal signaling in the right epithalamus of ntl morphants that normally show bilateral activation of Nodal pathway target genes (e.g., Bisgrove et al., 2000; Concha et al., 2000; Liang et al., 2000). In these experiments, laterality was directed to the side of the brain opposite to that of exogenous lefty1 expression. The consistency of this experimental result may be due to the fact that Nodal activity is probably equal on left and right sides of the epithalamus of ntl morphants, and so, introducing even a small bias in Nodal activity is sufficient to impose a difference between left and right sides. Furthermore, Lefty1 is a potent secreted antagonist of Nodal activity (Solnica-Krezel, 2003), and so, stable expression of exogenous lefty1 within very few cells is likely to significantly influence the ability of Nodals to signal in the vicinity of the Lefty-expressing cells.

A Mutual Inhibition Model of Laterality Determination in the CNS
We propose a model that can account for the laterality phenotypes in wild-type and mutant embryos, with or without ablation of ventral epithalamic precursor cells. Central to this model is the notion that competitive interactions between left and right epithalamus ensure lateralization by preventing symmetric development (isomerism) of the habenulae and parapineal nucleus. This interaction can be conceptualized as both left and right sides of the epithalamus producing signals that inhibit the ability of the contralateral side to differentiate with left-sided character.

We suggest that in the absence of Nodal signaling within the brain, both sides of the epithalamus are equivalent and there is a stochastic outcome to the competition (Figure 7Bi). Fifty percent of the time the left epithalamus “wins” and parapineal migration and expanded habenular development occur on the left, and fifty percent of the time there is the opposite outcome. In wild-type embryos, unilateral Nodal signaling gives the left side of the epithalamus an advantage in the competition so that laterality is consistently biased to the left (Figure 7Bii). Conversely, in embryos where Nodal signaling is bilateral, both sides of the epithalamus receive the boost provided by Nodal activity, and the competition is again balanced, resulting in stochastic allocation of laterality (Figure 7Biii). Thus, although embryos lacking Nodal signaling may be considered initially “double right-sided” (with respect to Nodal activity) and embryos with bilateral Nodal signaling “double left-sided,” in both cases, this initial unstable symmetry resolves to asymmetric differentiation of epithalamic nuclei.
In this paper, we show that ablation of ventral epithalamic cells can profoundly influence the outcome of the interactions between left and right epithalamic. We suggest that ablation of these cells on the left in wild-type embryos negates the advantage provided by ipsilateral Nodal signaling. This makes the outcome of the competition less certain, and in some cases, parapineal and habenular laterality is switched to the right (Figure 7Biv).

Ablation of equivalent cells on the right side of wild-type embryos has no consequence on laterality, as the left side is already at an advantage (Figure 7Bv). Conversely, ablation of right-sided ventral epithalamic cells in embryos lacking Nodal signaling gives an advantage to the left, and laterality is subsequently directed to this side (Figure 7Bvi). Similarly, in embryos with bilateral Nodal signaling, either ablation of right-sided ventral epithalamic cells or right-sided inhibition of Nodal signaling by exogenous Lefty1 influences the outcome of laterality decisions. In these embryos, the initial equivalence between left and right sides is disturbed with the consequence that epithalamic asymmetry is biased to the left side (Figure 7Bvii).

One outcome of consistently activating Nodal signaling on the left side of the brain is that within wild-type populations most fish will show the same direction of CNS laterality. Conversely, fish populations in which Nodal activity is not asymmetric will have equal proportions of “left-sided” and “right-sided” fish. This raises the intriguing possibility that Nodal signaling in the brain is more important for coordination of laterality between different members of the species than for the determination of CNS laterality per se. Although data is limited, coordinated CNS laterality between individuals appears to be more common in social species than in animals with solitary life styles (Vallortigara et al., 1999). It will be interesting to assess if lateralized Nodal signaling varies between species in which laterality is coordinated between individuals and those with randomly allocated CNS laterality.

Symmetry May Be Intrinsically Unstable in Many Tissues/Organs
Symmetric development (isomerism) of some organs, including the heart and gut and possibly also the brain, is not compatible with normal function. Mechanisms favoring asymmetry over symmetry may therefore have been fixed during vertebrate evolution. It is likely that redundant mechanisms ensure asymmetric development since perturbation of different pathways regulating asymmetry more often results in randomization of asymmetry than in isomerisms (e.g., Nonaka et al., 1998; Ryan et al., 1998; Meyers and Martin, 1999; Yan et al., 1999; Concha et al., 2000; Levin et al., 2002). It is intriguing that in embryos in which left/right signaling is disrupted, organs such as the lungs, for which asymmetries appear to have no obvious functional consequence, often display isomerism (Lin et al., 1999; Gaio et al., 1999; Rankin et al., 2000), whereas organs dependent on asymmetry for correct function, such as the heart and gut, exhibit heterotaxia. Symmetry may thus have evolved as an intrinsically unstable condition, and therefore an unlikely outcome, especially for structures for which asymmetry is inherently linked to function. As most such structures develop from cells on both sides of the midline, it may be that small, stochastic differences between left and right sides tip the balance in favor of left-handed or right-handed subsequent development. In each case, there would need to be a mechanism of communication across the midline to ensure coordinated development of left- and right-sided precursors.

Mechanisms Underlying Mutual Inhibition
Our model for assignment of laterality in the brain has several key features that need to be taken into account when considering the signals that might be involved. First, there is an initial bilaterally symmetric but unstable condition. This initial symmetry is broken either by a stochastic mechanism or by the influence of other factors (such as unilateral Nodal signaling). The induced asymmetry is subsequently reinforced and amplified to ensure generation of two different cell populations. Amplification and stabilization of small initial asymmetries has been described for other signaling events, such as lateral inhibition-mediated selection of cell fates (Lewis, 1998) and axon contact-dependent induction of asymmetric cell fates in worms (Troemel et al., 1999; Hobert et al., 2002). Such an outcome can also be theoretically achieved by reaction/diffusion mechanisms (e.g., Hamada et al., 2002).

If we consider a reaction/diffusion mechanism is responsible for establishing laterality in the brain, then we need to envisage a signal (or signals) capable of inducing itself and also inducing the expression of an antagonist that can spread more effectively than the signal itself. In this way, a stochastic increase in the signal on one side of the brain would lead to enhanced production of the antagonist that diffuses and suppresses contralateral expression. The Nodal pathway seems to have the criteria required for establishing a reaction/diffusion mode of activity (Chen and Schier, 2002; Solnica-Krezel, 2003). However, even if this is the case, it is unlikely that Nodal signaling constitutes the only mechanism for communication across the midline, as laterality is established perfectly well in the absence of expression of several known components of the Nodal pathway in the brain (Concha et al., 2000). One possibility is that Nodal signaling unilaterally modulates some other signaling pathway that operates between the two sides of the brain. As both Wnts and Fgfs show localized bilateral signaling, either ablation of right-sided ventral epithalamic cells can profoundly influence the outcome of the competition less certain, and in some cases, parapineal and habenular laterality is switched to the right (Figure 7Biv).

Experimental Procedures
Zebrafish Lines
Embryos and fry were obtained by natural spawning from wild-type, M2zeep (Gritsman et al., 1999), foxD3:GFP (Gilmour et al., 2002), flh:eGFPu1 (see below), and flh:eGFPu1 × foxD3:GFP fish. nif morphants (Feldman and Stemple, 2001) and LZ0e + fish (Concha et al., 2000) were obtained as previously described.

The flh gene was characterized from two zebrafish flh genomic clones, 1A and 9A, kindly provided by Will Talbot (Stanford University). These clones were isolated by screening a genomic library made in DASH-II (Stratagene) constructed by Martin Petkovich (Queen’s University, Kingston, Ontario, Canada) with the zebrafish flh cDNA (Talbot et al., 1995). Clones were restriction mapped and subcloned into pBluescript2SK+ (Stratagene). DNA sequence was
derived by primer walking using BigDye terminators (Applied Biosystems) from a 5.5 kb EcoRI subclone of 9A encompassing 1.6 kb of sequence upstream of the putative TATA box, three exons containing the entire coding region, and 1.9 kb of sequence downstream of the stop codon. At least three reads per base with at least one read per strand were performed. The genomic sequence of the zebrafish flh gene can be obtained from GenBank (accession number AV214391).

In order to generate flh:eGFP-transgenic fish, a membrane tetramer eGFP reporter (gap43eGFP, Clontech) was cloned into the 5’UTR of the 5.5 kb EcoRI flh subclone between the TATA box and the initiation methionine. Fertilized eggs were injected with linearized 5.5-flh:eGFP plasmid DNA. This construct was sufficient to recapitulate endogenous epithalamic flh expression in both Xenopus (M.G., L.S., K.G., E.A., and D.K., unpublished data) and zebrafish. Injected embryos were raised, and pairwise crosses identified a fish that passed the transgene onto its progeny. This fish was used to establish a transgenic line.

Isolation of hest1

A clone with asymmetric expression in the habenular nuclei was obtained through screening a variety of genes and ESTs for expression in sensory organs. The gene fragment has sequence homology with acs (acid-sensing ion channel) receptor encoding genes, and, until we are more certain of its orthology with related genes in other species, we have named it habenular EST1 (hest1). Sequence of the fragment is available from EMBL (accession number AJ558033).

Phenotypic Analysis

In situ hybridizations were performed using standard procedures. For antibody labeling, mouse anti-acetylated α-tubulin (Sigma), mouse anti-fluorescein (Boehringer), and rabbit anti-GFP (Chromekine) antibodies were used at 1:1000 dilutions. Light microscopy images were acquired using a Polaris 1 DMC camera attached to a Nikon upright microscope and analyzed using Photoshop 5.5 (Adobe) and Canvas 7 (Deneba) softwares. For quantification of asymmetries determined by α-tubulin and hest1 labeling, only fry that could be unequivocally scored are counted. Confocal analysis was performed on a Leica TCS SP Confocal Microscope using 25°C and 20×/40× water immersion objectives. Three-dimensional series of images were typically acquired at 1–2 μm intervals and the selected depths then projected by a combination of maximum intensity and opacity rendering methods using Velocity (Improvision) software.

Fate Mapping

flh:eGFP- or flh:eGFP;foxD3::GFP-transgenic embryos were injected at the 1 cell stage with DMNB-caged Fluorescein Dextran (D-7146, Molecular Probes), kept in the dark at 28°C until 22–24 hpf, anesthetized with 0.003% tricaine, and mounted on a custom-made chamber for experimentation. The region of photoactivation was determined relative to the flh:eGFP-expression domain using a 40× Achromplan water immersion objective on an upright Axioplan Zeiss microscope. For photoactivation of caged fluorescein, a UV-nitrogen laser microbeam (VSL-337, Laser Sciences) tuned to 365 nm by means of the BPBD-365 dye (Exciton Inc.) was focused on the selected region and laser pulses delivered at a frequency of 5–10 Hz for 20–20 s (Rohr and Concha, 2000). Embryos were kept in the dark until 48 hpf and the position of the labeling within the epithalamus examined by anti-fluorescein immunostaining.

Laser Ablation

foxD3::GFP- or flh:eGFP;foxD3::GFP-transgenic embryos were prepared as for fate mapping. For laser ablation, a UV-nitrogen laser microbeam (VSL-337, Laser Sciences) tuned to 440 nm by means of the Coumarin-440 dye (Exciton Inc.) was focused on the selected region and laser pulses delivered at a frequency of 10 Hz for 50–120 s. Ablation was performed in seven to ten consecutive neighboring regions by controlling the displacement of a motorized XY stage (Ludl) using custom-made routines written in Openlab (Improvision). Ablation was confirmed directly under DIC optics and embryos kept at 28°C until further examination.

DNA Electroporation

The electroporation technique was adapted from Haas et al. (2001) to enable the efficient transfer of DNA to a small group of cells in embryonic zebrafish CNS. ntl morphant foxD3::GFP embryos were mounted in 1.5% low melting point agarose (Sigma). Using a surgical blade (John Weiss), a small chamber of agarose was cut out to expose an area of the dorsal diencephalon. Micropipettes with a tip diameter of 1–2 μm were potted on a P-87 Micropipette puller (Sutter Instrument Company, CA) and filled with a solution containing purified plasmid DNA (Qiagen Maxiprep purification Kit) suspended in dH2O. Electroporation was performed on the right side of the epithalamus. Control embryos were electroporated with 0.5 μg/μl α-tubulin GFP and 0.8 μg/μl pCS2DsRed (final DNA concentration, 1.3 μg/μl) and experimental embryos with 0.5 μg/μl α-tubulin GFP and 0.8 μg/μl lefty1 (final DNA concentration, 1.3 μg/μl). Coelektroporation of two plasmids from the same pipette results in expression of both proteins in the same cells (>95% of cases; M.T. and J.D.W.C., unpublished data). For exogenous DNA to generate protein during the time of endogenous lefty1 expression (20–22 hpf), we performed electroporation at 10–14 somites (electroporated GFP constructs express the protein at a level that can be visualized after 10 hr, and this persists for many days [data not shown]). The following stimulation parameters were used: 1 s long trains of 1 ms square pulses at 200 Hz and a voltage of 45 V. Trains were delivered five times with 1 s interval between trains. Pulses were generated with a Grass SD9 stimulator (Grass-Telefactor, West Warwick, RI). After electroporation, embryos were cut out from the agarose and returned to embryo medium to recover.

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