The parapineal mediates left-right asymmetry in the zebrafish diencephalon

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SUMMARY

The dorsal diencephalon (or epithalamus) of larval zebrafish displays distinct left-right asymmetries. The pineal complex consists of the pineal organ anlage and an unpaired, left-sided accessory organ – the parapineal. The neighboring brain nuclei, the left and right dorsal habenulae, show consistent differences in their size, density of neuropil and gene expression. Mutational analyses demonstrate a correlation between the left-right position of the parapineal and the laterality of the habenular nuclei. We show that selective ablation of the parapineal organ results in the loss of habenular asymmetry. The left-sided parapineal therefore influences the left-right identity of adjacent brain nuclei, indicating that laterality of the dorsal diencephalon arises in a step-wise fashion.

Key words: Habenula, Epithalamus, Epiphysis, Pineal organ, Brain laterality

INTRODUCTION

The left and right hemispheres of the mammalian brain have long been known to possess anatomical and functional differences. Notably, the planum temporale, a region within the temporal lobe of the human cerebral cortex, occupies a greater area on the left side of the brain than on the right (Geschwind and Levitsky, 1968). The Sylvian fissure, which borders the planum temporale, is longer and less steep on the left side (LeMay and Culebras, 1972). Studies of cortical injuries and modern methods for imaging brain function have linked the left temporal lobe to the generation and comprehension of language for the majority of the population (LeMay, 1999).

Left-right (LR) asymmetries have also been described in the forebrains of lower vertebrates. Examples include the left hemisphere specialization of rodents in the production and perception of vocalizations (Ehret, 1987; LeMay, 1999), and the preferential eye use in food-searching behavior and predator recognition exhibited by amphibians, fish and birds (Deng and Rogers, 1997; Miklosi et al., 2001; Miklosi et al., 1998; Rogers, 2000; Vallortigara et al., 1998). The wide phylogenetic distribution of asymmetric behaviors suggests that functional lateralization of the brain is not a novel feature of the human cerebral cortex, but arose early in vertebrate evolution. Thus, it has been argued that asymmetry of the brain is a consequence of the cortex evolving from an already asymmetric neural template (Trevarthen, 1996).

The dorsal diencephalon displays striking anatomical asymmetries in many species (reviewed by Concha and Wilson, 2001), providing a promising entry point to study the developmental origin of forebrain laterality. The pineal complex of fish, reptiles and amphibians consists of the pineal organ (or epiphysis) and an unpaired accessory organ (the parapineal, parietal eye or frontal organ, respectively), which is frequently situated on the left side of the brain. In several species of fish, including stickleback, lamprey and trout, the parapineal preferentially innervates the left dorsal habenula (van Veen et al., 1980; Yanez and Anadon, 1994; Yanez and Anadon, 1996), one of a bilateral pair of brain nuclei that directly neighbor the pineal complex. The left and right dorsal (or designated medial for some species) habenular nuclei of several amphibians and reptiles show notable differences in their size, structure and molecular composition. In the frogs Rana esculenta and Rana temporaria, for example, the medial habenula consists of two compartments on the left side and only one on the right (Kemali and Guglielmotti, 1977; Morgan et al., 1973). Ultrastructural asymmetries have also been noted in the habenular nuclei, including LR differences in cell morphology and packing density, synaptic vesicle specializations and neuropil organization (Kemali and Guglielmotti, 1977). Accordingly, anti-acetylated tubulin labeling of the larval zebrafish brain reveals a denser region of neuropil in the left dorsal habenula compared with the right (Concha et al., 2000). The major efferent fiber tract projecting from the dorsal/medial habenula, the fasciculus retroflexus, shows LR differences in its cross-sectional diameter in the brains of some fish (Braford and Northcutt, 1983). In addition, the habenulae can be distinguished by their biochemical properties: the left habenula of dogfish expresses the Ca²⁺-binding protein calbindin more abundantly (Rodriguez-Moldes et al., 1990) and, in Rana esculenta, it exhibits higher nitric oxide synthetase activity than the right (Guglielmotti and Fiorino, 1999). LR differences in levels of monoamine neurotransmitters have also been observed (Ekstrom and Ebbesson, 1988).

The mechanisms that underlie the development of epithalamic asymmetry are starting to be understood in the zebrafish system, where many molecular and genetic tools are available. The earliest LR difference detected in this region of...
the brain is a transient asymmetry in gene expression found during embryonic development. Genes that encode components of the Nodal signaling pathway, including the Nodal-related TGFβ signal Cyclops (Cyc/Ndr2), the Nodal antagonist Antivin/Lefty1 (Lft1) and a Nodal-activated transcription factor Pitx2, are expressed on the left side of the presumptive pineal organ during midsomitogenesis (Bisgrove et al., 2000; Concha et al., 2000; Essner et al., 2000; Liang et al., 2000; Rebagliati et al., 1998a; Sampath et al., 1998; Thisse and Thiss, 1999). Mutations in genes that act at gastrulation can affect the later gene expression asymmetry of the epithalamus. For example, in no tail (nil) and floating head (flh) mutant embryos, in which formation of the axial mesoderm is perturbed, gene expression in the pineal anlage is later bilateral rather than left-sided (Bisgrove et al., 2000; Concha et al., 2000; Liang et al., 2000). The mutation casanova (cas), which affects the initial differentiation of endoderm and other early embryonic structures (Alexander et al., 1999), causes cyc expression to become LR randomized or bilateral in the brain (Liang et al., 2000). Although the results of such mutant analyses are indirect, they suggest that LR specification of the zebrafish nervous system is already under way during gastrulation.

As Nodal signaling is essential for early tissue patterning, and mutants defective in this signaling pathway are lethal (Feldman et al., 1998; Rebagliati et al., 1998b; Sampath et al., 1998; Zhang et al., 1998), it is difficult to evaluate the specific function of the transient, asymmetric gene expression in the brain. However, it is possible to rescue the early requirement for Nodal activity using embryos mutated at the one-eyed pinhead (oep) locus, which encodes an essential co-factor for the reception of Nodal signals. Injection of oep RNA at the one-cell stage is sufficient to rescue Nodal signaling during gastrulation (Yan et al., 1999; Zhang et al., 1998), but does not restore asymmetric gene expression (cyc, flh1 or pitx2) in the developing brain (Concha et al., 2000; Liang et al., 2000). Using this approach, it was determined that the Nodal pathway is required for normal positioning of the pineal organ stalk, which in the adult zebrafish brain typically emanates from the roof of the diencephalon with a subtle left bias (Liang et al., 2000). In rescued oep mutants, the position of the pineal stalk becomes displaced along the LR axis (Liang et al., 2000), and parapineal sidedness and the asymmetry in habenular neuropil are LR randomized (Concha et al., 2000; Gamse et al., 2002). These findings suggest that transient activation of the Nodal signaling pathway is involved in regulating the directionality of diencephalic asymmetry.

In the present study, we describe a molecular marker of LR identity of the dorsal habenular nuclei. The leftover (lov) gene is expressed at high levels in many cells of the left dorsal habenula, but at reduced levels and in a smaller number of cells in the right habenula. In mutants with disrupted midline development or defective Nodal signaling, laterality of the lov expression pattern is LR randomized. However, the habenula that shows stronger expression is always found on the same side of the brain as the parapineal. We show that laser-mediated ablation of the parapineal results in the loss of the lov expression asymmetry, with both habenulae adopting the weaker pattern characteristic of the right side. Our data indicate that the LR molecular identity of the habenulae is influenced by the unpaired parapineal organ, suggesting that lateralization of the diencephalon occurs through a step-wise accumulation of asymmetries and biases in cellular interactions.

**MATERIALS AND METHODS**

**Zebrafish**

Zebrafish were raised at 28.5°C on a 14/10 hour light/dark cycle and staged according to hours (h) or days (d) postfertilization. The wild-type AB strain (Fritz et al., 1996); the transgenic lines Tg(flh:eGFP)161 and Tg(flh:eGFP)162; and mutants carrying the ethyl nitrosourea-induced alleles oepm134 (Stemple et al., 1996) and casna56 (Chen et al., 1996), the gamma-ray induced allele ntb110 (Halpern et al., 1993) and the spontaneous allele flh11 (Talbot et al., 1995) were used. To produce mutants completely lacking maternal and zygotic Oep function (MZoep), we injected sense oep RNA (40 pg), synthesized from pCS2-oep (Liang et al., 2000; Yan et al., 1999; Zhang et al., 1998) using the SP6 mMessage mMachine Kit (Ambion), into the yolk of one- or two-cell embryos derived from homozygous mutant adults. Because rescued MZoep (Roep) embryos are presumed to lack Oep function by somitogenesis (Liang et al., 2000; Concha et al., 2000), they are also referred to as late zygotic oep mutants (LZoep) (Concha et al., 2000).

To produce the flhe:eGFP fish lines, 5.5 kb of genomic DNA sequence upstream of the flh transcriptional start site was fused in frame to DNA encoding enhanced GFP (eGFP; from the EGFP-1 plasmid, Clontech). Linearized DNA was injected into one-cell stage embryos, which were grown to adulthood and assayed for germine transmission of the transgene. Two stable transgenic founder fish were independently isolated. Fluorescent labeling of transgenic embryos resembles endogenous flh expression; however, eGFP is not detected in pineal precursors until the 18-20 somite stage. Labeling in Tg (flh:eGFP)162 is similar to Tg (flh:eGFP)162, except for an additional domain of fluorescence in the rostral hindbrain, probably caused by enhancer trapping at the site of transgene integration.

**Identification of the lov and cpd2 genes**

Individual clones were derived from an adult zebrafish kidney cDNA library that contained inserts between the EcoRI and XhoI sites of pBK-CMV (Stratagene). Expression of cDNAs was assayed during embryonic stages by a whole-mount in situ hybridization screen. Partial fspondin2 (Higashijima et al., 1997), lov and cpd2 cDNAs were isolated from this screen. The theoretical translation of the partial zebrafish Cpd2 cDNA is 63% identical to mouse Cpd2 (GenBank accession number NP_694803). The 5’ end of the lov transcript was identified by RNA-ligase mediated RT-PCR (RLM-RACE kit, Ambion) using total RNA from 4 day-old larvae. RLM-RACE products were cloned into the pCRII vector (Invitrogen) and sequenced. All products were identical in sequence, indicating that there is a single transcriptional start site for lov.

**RNA in situ hybridization and immunofluorescence**

Whole-mount in situ hybridization was performed as described previously (Gamse et al., 2002) with reagents obtained from Roche Molecular Biochemicals. To synthesize antisense RNA probes, pBK-CMV-lov was linearized with EcoRI and transcribed with T7 RNA polymerase and pBK-CMV-fspondin2 and pBK-CMV-cpd2 were linearized with SaI and transcribed with T7 RNA polymerase. Probes were labeled with UTP-digoxigenin or UTP-fluorescein and incubated with embryos at 70°C in hybridization solution containing 50% formamide. 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 cross-sections, embryos were embedded, sectioned and counterstained as described previously (Liang et al., 2000). For combined in situ hybridization and immunofluorescence,
the lov probe was visualized using the Fast Red (Roche) substrate, followed by labeling with an anti-acetylated α-tubulin primary antibody (Sigma) and an Alexa 488-conjugated secondary antibody. Images were captured using a Kontron ProReg 3012 or a Zeiss AxiosCam HRm camera mounted on a Zeiss Axiophot microscope, and with a Leica TCS-NT confocal microscope.

Laser ablation
Transgenic embryos were dechorionated, anesthetized with tricaine (Sigma), and positioned dorsal side upwards in 1.2% molten agar layered on bridged cover slips. GFP labeling at 28-32 h confirmed parapineal location. Ablation was performed with the 440 nm beam from a Photonic Instruments MicroPoint laser system mounted on a Zeiss Axiophot microscope using a 40x water immersion objective. Laser output was calibrated to the same level for each experiment. Approximately 15-20 cells were ablated using 5-10 pulses per cell. Control embryos received a similar treatment, except that the ablated cells were situated contralateral to the parapineal. Loss of cell integrity during laser treatment was assessed by a change in contrast under Nomarski optics.

Accession numbers
GenBank accession numbers are: lov, AY120891; zebrafish cpd2, AY120892.

RESULTS

LR asymmetry of the zebrafish epithalamus
As in other fish species (Borg et al., 1983; Concha and Wilson, 2001), the unilateral parapineal organ lies to the left of the pineal organ in the zebrafish epithalamus (Concha et al., 2000; Gamse et al., 2002; Gothilf et al., 1999). Development of the zebrafish pineal complex can be monitored by expression of the otx5 gene. Transcripts localize to the paired domains of the presumptive pineal during somitogenesis and are first detected in the parapineal at ~28 h (Gamse et al., 2002). As larval development proceeds, the position of the otx5-expressing parapineal cellular cluster changes relative to the pineal anlage, becoming progressively more caudal (Fig. 1A,C). The parapineal also shifts from a superficial position to a more ventral one, deeper within the brain (compare Fig. 1B with 1D,E). The parapineal persists in the adult brain near the base of the pineal stalk and closely apposed to the left dorsal habenula (Fig. 1F).

LR differences are also found in the dorsal habenular nuclei of larval zebrafish. The size difference is not as dramatic as in some amphibian brains (see Harris et al., 1996); however, the left habenula is typically larger in 4 d zebrafish larvae (an approximately 18% greater area expressing cerebellum postnatal development associated protein 2 (cpd2) and other markers; see Fig. 7B,F and data not shown). A significant molecular asymmetry was discovered in this region of the diencephalon by screening the gene expression patterns of random cDNA clones during embryonic and larval development. The lov gene shows a high level of expression within a localized domain on the left side of the brain, but only weak expression and in fewer cells in the corresponding region on the right side (Fig. 2A). The vast majority of wild-type embryos (97%, n=232) exhibited this pattern. Double labeling (Fig. 2C,D,E) confirmed that the left-sided domain of lov expression corresponded to the previously described region of the left dorsal habenula associated with denser neuropil (Concha et al., 2000). Expression of lov is restricted to the habenulae at 4 d, although transient expression in the pituitary anlage and caudal blood island is found between 1 and 2 d (not shown). In contrast to genes that encode components of the Nodal signaling pathway, which are expressed transiently, asymmetric expression of lov persists in the dorsal habenulae throughout larval development and is retained in the adult diencephalon (Fig. 2B).

The lov cDNA encodes a 288 amino acid protein of unknown function that possesses a conserved domain near the N terminus (Fig. 2F,G) that is similar to the oligomerization site of the Shaker voltage-gated K+ channel (Papazian, 1999). This protein-protein interaction domain is also found in members of the POZ/BTB family of transcription factors (Collins et al., 2001). The Lov protein shares no other structural features of ion channel proteins or transcription factors.

Correlation between the parapineal and habenular laterality
Several zebrafish mutations have been shown to perturb LR patterning of the epithalamic region, including those that affect the development of specific tissues at gastrulation (i.e. casanova, no tail and floating head), and those that block the Nodal signaling pathway (i.e., one-eyed pinhead, cyclops and schmalspur). Asymmetric expression of cyc, ltd1 and pitx2 in the pineal anlage becomes bilateral or is absent in the majority of mutant embryos and the parapineal position is LR randomized (Bisgrove et al., 2000; Concha et al., 2000; Essner et al., 2000; Gamse et al., 2002; Liang et al., 2000; Rebagliati et al., 1998a). To determine whether molecular asymmetry of the dorsal habenulae is similarly affected, lov expression was examined in cas, nil, flh and rescued MZoep mutant larvae. LR reversal of the asymmetric lov expression pattern was found in approximately 50% of mutant larvae examined (Table 1; Fig. 3A-H). Less frequently (3-7% of mutants, Table 1), equivalent levels of lov expression were observed on both sides of the brain (Fig. 3I,J).

We assessed the correspondence between parapineal position and habenular laterality in wild-type and mutant larvae by double in situ hybridization. In the majority of larvae, lov was expressed at higher levels and more extensively in the dorsal habenular nucleus that was adjacent to the parapineal (Table 2 and Fig. 4A,B,D,E,G,H). In the few instances in which

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*Data summed from wild-type (AB) larvae and wild-type siblings of mutants.

The leftover habenular expression pattern is LR reversed in about half of mutant larvae. Transcripts were detected by in situ hybridization of 4 d mutant and wild-type sibling larvae (as in Fig. 3). High levels of lov expression were scored in the L (left habenula), R (right habenula), B (both habenulae) or N (neither habenula). Data are shown as the percentage of total larvae with the indicated phenotype.
high levels of expression were found in both habenulae, two otx5 expressing parapineal organs were detected, one to the left and one to the right of the pineal anlage (Fig. 4C,F). Conversely, weak lov expression on both sides of the brain always correlated with the lack of a detectable parapineal (Table 2). In flh mutants, pineal development is disrupted, resulting in a greatly reduced pineal anlage with fewer photoreceptors and projection neurons (Masai et al., 1997). Although LR randomized, parapineal formation appears largely unaffected in flh larvae (Gamse et al., 2002). As in the other mutants, directionality of the habenular lov always correlated with the LR position of the parapineal in flh larvae (Fig. 4G-I).

The temporal relationship between parapineal formation and lov expression in the diencephalon was examined in 26-48 h embryos at 2 hour intervals. A distinct parapineal cell cluster could be recognized by 28 h (Fig. 5). However, lov transcripts were not detected in the habenular region until 38-40 h and were asymmetrically distributed from the onset (Fig. 5). Therefore, the appearance of the parapineal (as assayed by otx5 expression) precedes asymmetric lov expression in the dorsal habenular nuclei by ~12 hours.

**LR asymmetry of the dorsal habenulae requires the parapineal**

On the basis of their spatial and temporal relationship, the parapineal could play an instructive role in regulating the LR molecular identity of the dorsal habenulae. To test this hypothesis, we selectively destroyed the parapineal by laser-mediated cell ablation. As the parapineal is difficult to visualize in living embryos, laser ablation was performed using two independently isolated transgenic lines, which carry the flh promoter driving expression of the green fluorescent protein reporter gene (GFP). Transgenic flh:GFP embryos exhibit labeling in the developing midline and pineal organ (Fig. 6A,B,E,F and data not shown) similar to endogenous flh expression (Masai et al., 1997). A notable exception is the transient appearance of fluorescence in the parapineal organ, a structure in which flh transcripts have never been detected (Gamse et al., 2002). In 28-30 h transgenic embryos, the GFP-positive parapineal organ is found anterior and to the left of the pineal anlage (Fig. 6A,E), consistent with the position of the emerging otx5-positive parapineal domain (Fig. 5).

Fluorescent labeling of the parapineal in flh:GFP transgenic larvae served as a useful guide for targeted laser-mediated ablation of this structure. Approximately 15-20 cells corresponding to the labeled parapineal were destroyed in 28-32 h transgenic embryos (Fig. 6C,D) using five to ten pulses of a focused laser beam. The embryos were subsequently allowed to develop to 4 d, at which time ablation of the parapineal was confirmed by the absence of the parapineal-specific otx5 expression domain (refer to Fig. 7J-M). In these larvae, the otx5-expressing pineal anlage appeared normal, indicating that only the parapineal was affected. In control larvae, either a comparable region on the right side of the pineal anlage (n=31; Fig. 6G,H) or cells adjacent to the parapineal were ablated (n=9; data not shown).

The denser neuropil characteristic of the left dorsal habenula was present in control-ablated larvae (Fig. 7A,E). However, this neuropil density was absent in the left habenula of parapineal-ablated larvae (n=12/12, Fig. 7I). To confirm that the habenular nuclei were intact after laser-mediated destruction of the parapineal, we examined expression of two markers. The cpd2 and f-spondin2 genes are transcribed at equal levels in the L and R habenular nuclei. Expression of

Table 2. Correspondence between habenular and parapineal laterality

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*Data summed from WT (AB) larvae and WT siblings of mutants.

**Fig. 1.** Laterality of the pineal complex. The zebrafish pineal complex consists of the pineal organ and the parapineal (black arrowhead), which both express otx5 (blue). (A,B) The parapineal lies to the left of the pineal anlage at 2 d, at the same dorsal level as the pineal, but (C,D) becomes more caudally and ventrally located during larval development. (E) In a frontal view of a 15 d larval brain, the parapineal lies in a more ventral position relative to the pineal. (F) In the adult brain, the parapineal is found at the base of the pineal stalk (arrow), next to the left habenula (Lh). (A,C,F) Dorsal views; (B,D) cross-sections counterstained with basic fuchsin (pink). Rh, right habenula. Scale bars: 50 μm for A-E; 150 μm for F.
these genes was unaffected in all parapineal-ablated larvae (n=18/18 for cpd2, 8/8 for f-spondin2, Fig. 7J,K), and indistinguishable from untreated or control ablated larvae (Fig. 7B,C,F,G), indicating that the habenulae were not directly damaged by the laser treatment.

The habenulae of control-ablated and untreated sibling larvae displayed the typical asymmetric pattern of lov expression (n=18/18 for control ablated, Fig. 7D,H). Destruction of the pineal organ, with the parapineal intact, also had no effect on lov expression (n=4/4, Fig. 7N). By contrast, both the left and right habenulae of larvae in which the parapineal organ was selectively destroyed prior to the onset of lov transcription (Fig. 7L,M) showed the pattern of lov expression normally found on the right side (weaker expression in a subset of cells, n=24/24). Thus, elimination of the parapineal specifically abolished the neuropil and molecular LR asymmetry of the adjacent brain nuclei.

DISCUSSION

The left and right sides of the vertebrate brain exhibit characteristic anatomical differences, yet how these arise and their significance is not well understood. The present study describes a molecular LR asymmetry of the zebrafish forebrain. The newly identified lov gene is strongly expressed in the habenular nuclei. (A) The left habenula (white arrowhead) expresses lov (blue) at higher levels and in more cells than the right. (B) Asymmetric lov expression persists in the adult zebrafish brain. (C-E) lov expression in the left habenula coincides with a region of dense neuropil (black arrowhead on left side in D, compare with right side) labeled with anti-acetylated tubulin antibody. (F) The lov gene encodes a 288 amino acid protein that contains a conserved oligomerization domain (T1-like domain) at the N-terminus. (G) The Leftover oligomerization domain is 47% similar to the T1 tetramerization domain of the Drosophila Shaker K⁺ channel (Papazian, 1999) and 41% similar (not shown) to the POZ/BTB domain of human promyelocytic leukemia zinc finger protein (PLZF) (Collins et al., 2001). Scale bars: 50 µm for A,C-E; 150 µm for B.

Parapineal mediates diencephalic asymmetry

**Fig. 2.** Asymmetric expression of leftover in the habenular nuclei. (A) The left habenula (white arrowhead) expresses lov (blue) at higher levels and in more cells than the right. (B) Asymmetric lov expression persists in the adult zebrafish brain. (C-E) lov expression in the left habenula coincides with a region of dense neuropil (black arrowhead on left side in D, compare with right side) labeled with anti-acetylated tubulin antibody. (F) The lov gene encodes a 288 amino acid protein that contains a conserved oligomerization domain (T1-like domain) at the N-terminus. (G) The Leftover oligomerization domain is 47% similar to the T1 tetramerization domain of the Drosophila Shaker K⁺ channel (Papazian, 1999) and 41% similar (not shown) to the POZ/BTB domain of human promyelocytic leukemia zinc finger protein (PLZF) (Collins et al., 2001). Scale bars: 50 µm for A,C-E; 150 µm for B.

**Fig. 3.** LR randomization of habenular laterality. The laterality of lov expression is altered in mutant larvae. (A-D) Approximately half of casanova (casta56), no tail (ntl b160), floating head (flh n1) and rescued one-eyed pinhead (oep m134) homozygotes showed the same lov expression pattern as wild type at 4 d. (E-H) The pattern was reversed in the other half. (I-J) Infrequently, both habenulae showed equivalent levels of expression (and refer to Fig. 4C,F,I). Scale bar: 50 µm. See also Table 1.
throughout the left habenula of the dorsal diencephalon, but is expressed at lower levels and in only a subregion of the right habenula. We find that this asymmetry is dependent on the presence of the left-sided parapineal organ. Three lines of evidence support this finding. First, changes in the lov expression pattern in wild-type and mutant larvae always correlate with changes in the LR position of the parapineal. Second, the parapineal is detectable prior to the onset of asymmetric lov expression, closely apposed to the left habenula. Most importantly, the LR difference in lov expression and of the neuropil is abolished after selective ablation of the parapineal. We conclude that the asymmetric pineal complex influences the laterality of the adjacent diencephalic region.

Progression of LR asymmetry in the zebrafish epithalamus

The epithalamus had previously been identified as a site of molecular and morphological LR differences in the zebrafish brain. These differences appear in a progressive manner during embryonic and larval development. The earliest manifestation of brain asymmetry is the transient expression of components of the Nodal-related Cyc signaling pathway on the left side of the presumptive pineal organ during somitogenesis (Bisgrove et al., 2000; Concha et al., 2000; Essner et al., 2000; Liang et al., 2000; Rebagliati et al., 1998a; Sampath et al., 1998; Thisse and Thisse, 1999). Shortly afterwards, the parapineal appears to the left side of the presumptive pineal organ (Gamse et al., 2002). Fluorescent labeling of the parapineal in flh:GFP larvae suggests that it

Fig. 4. Correspondence between parapineal and habenular laterality. (A) In the majority of wild-type 4 d larvae, the parapineal (black arrowhead, otx5 expression) is on the left, beside the habenula with high lov expression (red). (B) LR reversal of parapineal and habenular laterality is found infrequently (n=1/395). (D) Almost half of rescued MZoep larvae show the wild-type pattern, while the other half (E) show a LR reversal in laterality. (F) Bilateral expression of lov in rescued MZoep mutants also occurs when two parapineals form (partially obscured by intense otx5-expressing pineal). (G) flh mutants have a greatly reduced pineal anlage (Masai et al., 1997). The majority of otx5-expressing cells in flh mutants are presumed to be parapineal because of their lack of flh expression (Gamse et al., 2002). In half of flh mutants, the parapineal and stronger lov expression are found on the left side, and (H) in the other half, laterality is reversed. (I) The parapineal is sometimes found medially, adjacent to both habenulae (5/84). In these flh mutants, lov is expressed strongly in both habenulae. Scale bar: 50 μm. See also Table 2.

Fig. 5. Parapineal formation precedes asymmetric expression of lov. Expression of otx5 (blue) and lov (red) in wild-type sibling embryos at the indicated times post-fertilization. The otx5-expressing parapineal (blue arrowhead) can be distinguished at 28 h. Expression of lov (red arrowhead) is first detected at 40 h and is always found at higher levels on the left side of the brain. Scale bar: 50 μm.
Parapineal mediates diencephalic asymmetry

derives from the same precursor population as the pineal, and results from the persistence of GFP in lineally related cells. Laser ablation of the pineal anlage prior to parapineal formation supports this idea (K. Cygnar and J. T. G., unpublished); however, direct fate-mapping analysis is required to confirm the precise cellular origin of the parapineal.

Adjacent to the pineal complex lie the paired habenular nuclei, which exhibit LR differences in their size, neuropil density (Concha et al., 2000) and (as shown in this study) in expression of the \( \text{lov} \) gene as early as 2 d. Lateralized features of the

**Fig. 6.** Selective ablation of the parapineal in \( \text{flh:GFP} \) transgenic embryos. (A-D) Integrity of the parapineal before and after laser-mediated cell ablation. (A) The pineal anlage and parapineal can be recognized by their specific fluorescent labeling in 30 h \( \text{Tg(flh:eGFP)} \) embryos, and (C) visualized by Nomarski optics (black arrowhead; parapineal). The parapineal was destroyed by laser pulses, as evidenced by (B) the loss of fluorescence and (D) abnormal morphology immediately after ablation. Cell death was not observed in neighboring regions after the laser treatment. (E-H) As a control, an equal number of cells were destroyed to the right of the pineal organ (white arrowhead in H), but the parapineal was left intact (black arrowhead in G and H). Scale bar: 25 \( \mu \text{m} \).

**Fig. 7.** Parapineal mediates habenular laterality. After ablation of the parapineal (pp) or contralateral (control) region at 28-32 h, larvae were allowed to develop and assayed at 4 d for expression of diencephalic markers by in situ hybridization as indicated or (A,E,I) by labeling with an acetylated tubulin antibody (white) and an \( \text{otx5} \) RNA probe. Under fluorescence microscopy, the pineal and parapineal (outlined in white) appear black because of quenching by the NBT/BCIP precipitate. Wild-type larvae showed (A) dense neuropil labeling in the left habenula (white arrowhead), an \( \text{otx5} \)-labeled parapineal (white asterisk in A, black arrowhead in B), (B,C) bilateral expression of \( \text{cpd2} \) and \( \text{f-spondin2} \), and (D) the characteristic asymmetric \( \text{lov} \) expression pattern in the dorsal habenular nuclei. (E-H) Control-ablated larvae (see Fig. 6G,H) were indistinguishable from wild type. (I-M) Parapineal-ablated larvae. Destruction of the parapineal was confirmed by the later absence of the \( \text{otx5} \) parapineal domain (black arrowhead in J), and resulted in (I) the absence of dense neuropil in the left habenula (\( n=12/12 \)). (J,K) Bilaterally expressed habenular markers were unaffected (\( \text{cpd2}, n=18/18; \text{f-spondin2}, n=8/8 \)), but (L-M) the laterality of \( \text{lov} \) expression was lost. Instead, weak expression resembling the right side was found in both habenulae (\( n=24/24 \)). (N) By contrast, ablation of the pineal anlage, with the parapineal intact, did not affect habenular asymmetry (\( n=4/4 \)). Scale bar: 50 \( \mu \text{m} \).
epithalamus persist throughout larval stages and are found in the adult brain. The mature pineal organ consists of the photoreceptive pineal end vesicle and the pineal stalk, with the stalk emanating from the diencephalic roof at a left-of-medial position (Liang et al., 2000). The otx5-expressing parapineal remains a rudimentary structure at the base of the pineal stalk adjacent to the left habenular nucleus. In the adult, the left habenula continues to express lov at high levels relative to the right side.

Analyses of zebrafish mutants have shed some light on how the LR differences of the diencephalon are regulated. Although the initial symmetry-breaking event is unknown, early patterning processes, including the formation of midline tissues at gastrulation, influence the later laterality of the epithalamic region. Activation of the Nodal signaling pathway in the left pineal anlage during segmentation stages appears to be an essential cue for setting the position of both the parapineal (Concha et al., 2000) and pineal outgrowth along the LR axis (Liang et al., 2000). In mutants defective in Nodal signaling (or those that show bilateral activation of the pathway), we presume that this cue is lost in the developing epithalamus. As a consequence, placement of the pineal stalk and parapineal is determined by a stochastic mechanism, and at the populational level, sidedness of the pineal complex becomes LR randomized.

Our data reveal that the lateralized features of the habenular nuclei are associated with the parapineal. A discrete parapineal cellular cluster is detected in the left dorsal diencephalon almost 12 hours before the onset of lov expression in the dorsal habenulae. Notably, lov expression is asymmetric from the outset, with the stronger left-sided domain closely apposed to the parapineal. In all mutant and wild-type larvae, the direction of parapineal sidedness (L versus R) corresponds with the neuropil density of the dorsal habenulae (left-biased versus right-biased; this study) (see also Concha et al., 2000) and the sidedness of the lov expression pattern. Furthermore, in the few larvae that develop bilateral parapineal organs, both the left and right habenulae show high levels of lov expression. The findings indicate that parapineal formation precedes and strictly correlates with habenular asymmetry.

The parapineal is required for habenular laterality

One interpretation of the data is that the parapineal plays an instructive role in the generation of habenular asymmetry. An alternative hypothesis is that habenular asymmetry is not dependent on the parapineal; rather, the laterality of both structures is coordinately regulated through a common signal. The latter hypothesis, however, is not supported by the results of parapineal ablation experiments. In all larvae in which the parapineal was selectively destroyed before the onset of lov transcription, equivalent low levels of lov expression were later detected on both sides of the brain. The loss of lov expression and dense neuropil characteristic of the left habenula was specific to parapineal ablation. Moreover, only these asymmetric features were lacking, as other habenular markers that are normally expressed at equal levels on the right and left were unaffected. Removal of the pineal anlage had no effect on habenular asymmetry as long as the left-sided parapineal organ was preserved. The analysis of flh mutant larvae, which show a greatly reduced pineal anlage (Masai et al., 1997) and an intact parapineal (Gamse et al., 2002), further suggests that the parapineal is not only necessary but is sufficient to influence habenular laterality. Whether an earlier event(s) establishes the LR asymmetry of the habenulae, and the parapineal serves to maintain or reinforce this lateralization cue(s), remains to be determined. It is also possible that loss of the parapineal might delay the appearance of rather than inhibit habenular asymmetry. This is currently under investigation by examining the persistence of bilateral symmetry in the habenulae of juvenile and adult fish that have been reared after parapineal ablation (J. T. G and M. E. H., unpublished).

At present, the nature of the interaction between the parapineal and left habenula is unknown. Preferential innervation of the left habenula by the parapineal has been described for some fish species, including trout (Yanez and Anadon, 1996), lamprey (Yanez and Anadon, 1994) and stickleback (van Veen et al., 1980). Connectivity between the parapineal and diencephalon has not yet been rigorously demonstrated for zebrafish, although axons from opsin-immunoreactive parapineal neurons do selectively project towards the left habenula (Concha et al., 2000). Direct cell–cell signaling is another potential mechanism, as the close apposition of the developing parapineal and left habenula cellular domains appears to be essential for generating habenular asymmetry. Known mutations of candidate signaling molecules, including Ace/Fgf8 (Reifers et al., 1998), Syu/Shh (Schauerte et al., 1998) and Snh/Bmp7 (Dick et al., 2000; Schmid et al., 2000), do not disrupt the lov expression pattern in a specific manner (J. T. G and M. E. H., unpublished). To determine how the parapineal exerts its influence on the brain, an unbiased genetic screen is under way to identify zebrafish mutations that block the parapineal/left habenula interaction.

Implications of epithalamic asymmetry

On the basis of their work on amphibia, Braithberg and Kemali (Braithberg and Kemali, 1970) proposed that asymmetry of the parapineal complex imposes a LR bias on the brain that could extend beyond the habenular region. The major efferent projection of the medial/dorsal habenula nuclei, the fasciculus retroflexus (FR), terminates on the unpaired interpeduncular nucleus as part of an evolutionarily conserved conduction pathway that extends from telencephalic nuclei to the midbrain (Butler and Hodos, 1996). The number of axons can differ between the left and right FR (Braford and Northcutt, 1983), but this is not a consistent feature of vertebrate brains (Kemali and Guglielmotti, 1982). Thus, it will be important to determine whether the asymmetry of the zebrafish habenulae extends to the axonal tracts of the FR and influences the laterality of other brain regions.

Epithalamic asymmetry has been described in fishes, amphibians, reptiles and birds, and in a few mammalian species (see Concha and Wilson, 2001); however, the significance of LR differences in this region of the brain is unknown. Despite a high degree of anatomical conservation (Sutherland, 1982), the habenular region and habenulo-interpeduncular circuit are poorly understood at the functional level. LR differences in the habenular nuclei have been linked to seasonal variation, reproductive behavior and sexual dimorphism in some vertebrates (Harris et al., 1996; Bisazza et al., 1998). Characterization of the Lov protein, its subcellular localization, multimeric structure and binding partners may...
provide insight into potential asymmetric functions of the habenular nuclei. Preliminary experiments to deplete Lov protein using morpholino antisense oligonucleotides have so far been uninformative, but genetic redundancy could be a complicating factor. The lov gene is a member of a multigene family with homologues identified so far in frog, mouse, rat and humans (J. T. G., C. Brösamle and M. E. H., unpublished). Examination of gene expression in other species will reveal to what extent the lov molecular asymmetry is preserved in the vertebrate diencephalon.

Many species of fish display lateralized behaviors at both the individual and populational level (reviewed by Vallortigara and Bisazza, 2002). Zebrafish, for example, prefer to use the right eye when fixating on foreign objects and the left eye for viewing familiar ones (Miklosi and Andrew, 1999; Miklosi et al., 1998), properties presumed to play a role in feeding, schooling and escaping behaviors (see Andrew, 2002). Adult zebrafish have also been found to show biases in their turning behavior while swimming (Heuts, 1999). Whether there is a connection between epithalamic asymmetry and the lateralized motor behaviors elicited by the LR discrimination of visual stimuli remains unclear. Intriguingly, activity of the pineal gland has been found to modulate the swimming behavior of Xenopus tadpoles under some conditions (Jamieson and Roberts, 1999; Jamieson and Roberts, 2000), although it is not known whether this behavior has a lateralized component. The molecular genetic approaches afforded by the zebrafish system will permit the perturbation of epithalamic asymmetry so that the relevance of this asymmetry can be explored in a behavioral context.

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