

# A Nodal Signaling Pathway Regulates the Laterality of Neuroanatomical Asymmetries in the Zebrafish Forebrain

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## Summary

Animals show behavioral asymmetries that are mediated by differences between the left and right sides of the brain. We report that the laterality of asymmetric development of the diencephalic habenular nuclei and the photoreceptive pineal complex is regulated by the Nodal signaling pathway and by midline tissue. Analysis of zebrafish embryos with compromised Nodal signaling reveals an early role for this pathway in the repression of asymmetrically expressed genes in the diencephalon. Later signaling mediated by the EGF-CFC protein One-eyed pinhead and the forkhead transcription factor *Schmalspur* is required to overcome this repression. When expression of Nodal pathway genes is either absent or symmetrical, neuroanatomical asymmetries are still established but are randomized. This indicates that Nodal signaling is not required for asymmetric development per se but is essential to determine the laterality of the asymmetry.

## Introduction

In vertebrates, lateralization of the CNS is evident both in terms of asymmetric behaviors and in the localization of specific cognitive abilities predominantly to one side of the brain (Bradshaw and Rogers, 1992). Differences in neural structures on left and right sides of the vertebrate brain have been described at early developmental stages (Chi et al., 1977; Guglielmotti and Fiorino, 1999), suggesting genetic regulation of CNS asymmetry. However, epigenetic factors also influence asymmetry (e.g., Rogers, 1990; Warren-Lewis and Cleaves-Diamond, 1996), and it has proved difficult to determine if there is a relationship between laterality of the CNS and handedness of the viscera and heart. Indeed, although a wide variety of studies have elucidated the pathways regulating heart and visceral asymmetry, these studies

have shed little light on the development of CNS asymmetry (reviewed in Capdevilla et al., 2000).

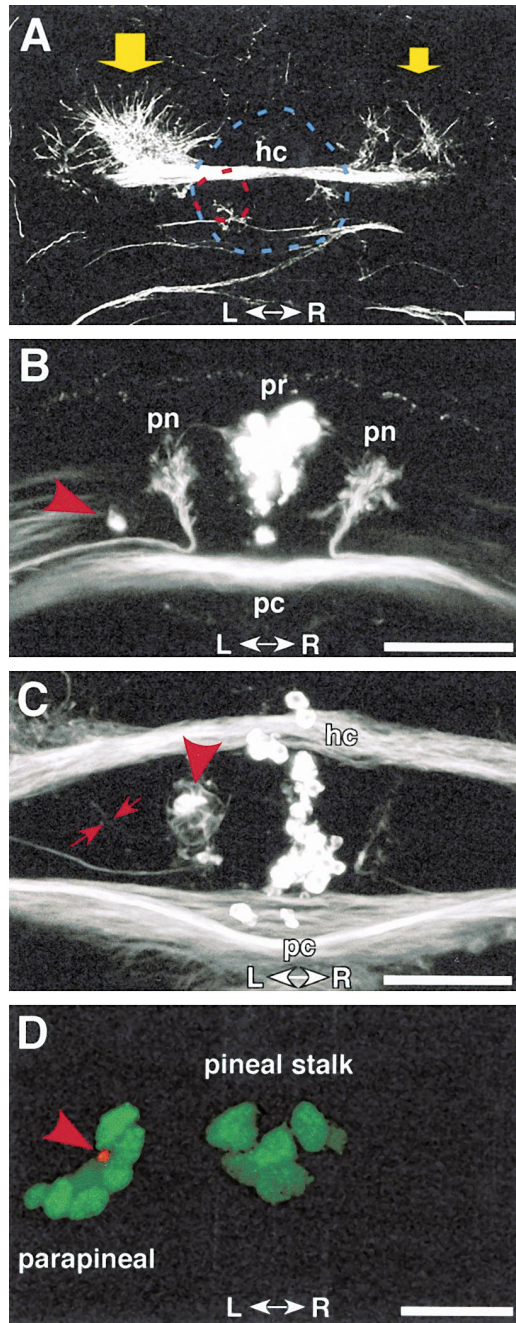
The Nodal pathway is one of several signaling pathways implicated in the establishment of organ asymmetry. *nodal* genes are expressed asymmetrically in the left lateral plate mesoderm, and their misexpression can lead to altered left/right polarity of organs (reviewed in Burdine and Schier, 2000). Further support for a role for the Nodal pathway in laterality decisions has come from analysis of proteins belonging to the EGF-CFC, *Lefty*, and *Fast* families that are implicated in Nodal signaling (reviewed in Schier and Shen, 2000). For instance, the EGF-CFC protein One-eyed pinhead (*Oep*) is a membrane attached protein that acts as an essential cofactor for the Nodal-related signals *Cyclops* (*Cyc*) and *Squint* (*Sqt*) in zebrafish (Zhang et al., 1998; Gritsman et al., 1999; reviewed in Shen and Schier, 2000). Fish lacking late activity of *Oep* and mice lacking the function of a related protein, *Cryptic*, exhibit heterotaxia (randomization of asymmetry) and fail to establish left-sided expression of genes implicated in the Nodal signaling pathway (Gaio et al., 1999; Yan et al., 1999). A very similar phenotype is observed in zebrafish *schmalspur* (*sur*) mutants (Chen et al., 1997a; Bisgrove et al., 2000), which carry mutations in *Fast1*, a transcriptional effector of Nodal signaling (Chen et al., 1997b; Watanabe and Whitman, 1999; Pogoda et al., 2000; Saijoh et al., 2000; Sirotkin et al., 2000). Moreover, mice lacking *Lefty1*, an antagonist of Nodal signaling (Bisgrove et al., 1999; Meno et al., 1999), exhibit altered patterns of asymmetric gene expression and left pulmonary isomerism (Meno et al., 1998). These results suggest that EGF-CFC/*Fast*/*Lefty*-regulated Nodal signaling is essential for the regulation of asymmetric gene expression in the lateral plate mesoderm and left-right development of internal organs. Since loss of function mutations in *hCRYPTIC* are associated with human left-right laterality defects, an essential role for Nodal signaling in left-right axis formation appears to be conserved from fish to humans (Bamford et al., 2000).

In addition to domains of expression in the lateral plate mesoderm, *oep*, *sur*, *cyc*, the *lefty* gene *antivin/lefy1* (*atv*), and the homeobox gene *pitx2* are also expressed in the zebrafish brain. *oep* and *sur* are expressed bilaterally in the diencephalon (Zhang et al., 1998; Pogoda et al., 2000; Sirotkin et al., 2000; this study). In contrast, *cyc*, *atv*, and *pitx2* are expressed exclusively on the left side of the brain (Rebagliati et al., 1998; Sampath et al., 1998; Bisgrove et al., 1999; Thisse and Thisse, 1999; Essner et al., 2000; this study). These observations suggest that Nodal signaling may have a function in the brain comparable to its role in the lateral plate mesoderm. In support of this, several mutations that affect the laterality of gene expression in the mesoderm and situs of the viscera and heart also affect expression of left-sided genes in the brain (Bisgrove et al., 2000). It has been unclear, however, if these changes in CNS gene expression have any consequences on the development of neuroanatomical asymmetries.

In order to study the genetic basis of left-right asym-

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**Figure 1. The Dorsal Diencephalon Is Asymmetric**

Dorsal (A, C, and D) and transverse (B) views of the dorsal diencephalons of 3.5- (B, C, and D) and 4- (A) day-old fry with anterior toward the top of the panels.

(A) Confocal projection of a dorsal view of the habenular nuclei labeled with antiacetylated  $\alpha$  tubulin antibody. The left habenular nucleus (large arrow) has more pronounced labeling of neuropil than the right nucleus (small arrow). The positions of the epiphysis and parapineal (dorsal to this projection) are outlined in blue and red, respectively. The habenular commissure runs between the left and right habenular nuclei.

(B) Confocal projection of a frontal view of the epiphysis and parapineal (arrowhead) labeled with antiacetylated  $\alpha$  tubulin and anti-opsin antibodies. Anti-opsin labeling of epiphysial photoreceptors is prominent at the dorsal midline. The parapineal (arrowhead) is located on the left side of the brain just anterior to the axons exiting from the epiphysis.

metry in the CNS, we have identified asymmetries in the diencephalic habenular nuclei and pineal complex of the larval zebrafish brain. The habenulae are dorsal diencephalic nuclei that receive input from the telencephalon via the stria medullaris and relay information to the interpeduncular nuclei in the ventral midbrain via the fasciculi retroflexus. These pathways are conserved in all vertebrates and are likely to represent an evolutionarily conserved output pathway from the telencephalon. The habenular nuclei are often bilaterally symmetrical in teleosts but asymmetries are described in some species (Braitenberg and Kemali, 1970; Ekstrom and Ebbesson, 1988). We find that the left habenular nucleus is considerably larger than the right nucleus in larval zebrafish.

In addition to the habenulae, the dorsal diencephalon gives rise to several evaginations that include the epiphysis/pineal organ and the parapineal organ. In adult animals, the pineal organ has endocrine roles that include secretion of melatonin, a hormonal regulator of circadian rhythms. Additionally, in many species, the epiphysis and/or parapineal organs are photoreceptive and, in some amphibia and reptiles, may form true eye-like structures. During embryogenesis, the pineal complex develops earlier than the lateral eyes (Masai et al., 1997; Ostholm et al., 1987, 1988) and may mediate early light-evoked behaviors (Roberts, 1978; Foster and Roberts, 1982; Jamieson and Roberts, 1999). The epiphysis is usually a midline structure, while, in some species, the parapineal occupies a position to the left of the midline (e.g., Borg et al., 1983). We find that the parapineal is located to the left in larval zebrafish.

In this study, we investigate the regulation of left-sided expression of genes that function in the Nodal pathway and examine how components of this pathway influence asymmetry and laterality in the zebrafish forebrain. We propose a novel model for left-right patterning in which early Nodal signaling initially represses the transcription of genes normally expressed in the left diencephalon. *Oep* and *Sur* function is subsequently required to overcome this repression and an intact midline is required to limit expression to one side of the brain. Strikingly, we find that in mutants in which expression of left-sided genes is either absent or is bilateral, anatomical asymmetry of the habenulae and parapineal is still established but laterality is randomized. These results indicate that components of the Nodal signaling pathway and the midline regulate the laterality of vertebrate brain asymmetry.

(C) Confocal projection of a dorsal view of the epiphysis and parapineal labeled with antiacetylated  $\alpha$  tubulin and anti-opsin antibodies. The parapineal nucleus (arrowhead) contains one bright spot of opsin labeling surrounded by more diffuse tubulin labeling. Axons (arrows) project from the parapineal in the direction of the left habenular nucleus.

(D) Confocal coronal section through the epiphysial stalk and parapineal of a fish labeled with anti-Islet (green labeling of nuclei) and anti-opsin (red labeling of photoreceptor outer segments). Only one or two of the parapineal neurons exhibit opsin immunoreactivity. Scale bar: 20  $\mu$ m. L $\leftrightarrow$ R indicates the position of the dorsal midline. Abbreviations: hc, habenular commissure; pc, posterior commissure; pr, epiphysial photoreceptors; pn, epiphysial projection neurons.

## Results

### The Dorsal Diencephalon Is Asymmetric

Neuroanatomical asymmetries have not previously been described in zebrafish. In order to investigate the mechanisms that underlie the generation of laterality in the CNS, we identified neuroanatomical asymmetries in the habenular nuclei and pineal complex of embryonic and larval zebrafish. Antiacetylated tubulin labeling reveals prominent asymmetry in the medial regions of the habenular nuclei by 4 days of development. In over 90% of fry, the left habenular nucleus has considerably more extensive labeling of neuropil (Figure 1A).

The most prominent component of the pineal complex in embryonic and larval zebrafish is the medially positioned epiphysis (Figure 1B; Masai et al., 1997). However, in addition to the epiphysis, we detected another photoreceptive nucleus unilaterally positioned in the left dorsal diencephalon (Figures 1B–1D). In about 15% of 3.5-day-old fry, opsin immunoreactivity was present on the left side of the brain in one or two cells lateral and ventral to the majority of midline epiphysial photoreceptors (Figure 1B). The opsin labeling was within a cluster of cells that project axons toward the left habenular nucleus (Figure 1C). The photoreceptivity and connectivity of this nucleus suggest that it is the anlage of the parapineal organ. Although opsin immunoreactivity is only detectable in a minority of 3.5-day-old fry, an antibody that recognizes the Islet1/2 homeoproteins (Korz et al., 1993) unilaterally labels a cluster of neurons on the left side (in over 95% of fry) of the diencephalon from about 2 days onwards. Double labeling with anti-Islet and anti-opsin antibodies confirmed colocalization of the parapineal photoreceptors within the Islet-expressing nucleus (Figure 1D).

### Nodal Pathway Genes Are Expressed in the Dorsal Diencephalon

The homeobox gene *floating head* (*flh*; Talbot et al., 1995) is expressed bilaterally in the dorsal diencephalon in a domain within which the majority or all Islet1-expressing epiphysial neurons are thought to be generated (Masai et al., 1997; Figures 2A and 2B). To better ascertain the locations at which Nodal pathway genes are expressed, we compared the expression of *flh* to that of *oep* (which encodes an EGF-CFC cofactor for Nodal signals), *sur* (which encodes a FAST transcription factor mediating Nodal signaling), *cyc* (which encodes a zebrafish Nodal signal), *atv* (which encodes an antagonist of Nodal signaling belonging to the Lefty family), and *pitx2* (a homeobox gene regulated by Nodal signaling; Zhang et al., 1998; Rebagliati et al., 1998; Sampath et al., 1998; Bisgrove et al., 1999; Gritsman et al., 1999; Thisse and Thisse, 1999; Bisgrove et al., 2000; Essner et al., 2000; Pogoda et al., 2000; Schier and Shen, 2000; Sirotkin et al., 2000). *cyc*, *pitx2*, and *atv* are expressed unilaterally within the diencephalon of 18-somite (18s; 18 hr postfertilization) to 26s stage (22 hr postfertilization) embryos (Figures 2G–2L and Table 1), overlapping with the anterior left domain of *flh* expression (Figures 2M and 2N and data not shown). In contrast, both *oep* and *sur* are expressed bilaterally in the dorsal diencephalon (Figures 2C–2F) with high levels of expression within the

anterior half of the *flh* expression domain and weaker expression more caudally. Expression of *atv*, *cyc*, *pitx2*, and *sur* is absent from the most dorsal medial cells and extends slightly further ventral than either *oep* or *flh* (Figure 2O). Together, these observations support and extend previous studies showing that Nodal pathway genes are expressed in the forebrain and suggest that asymmetric Nodal signaling in the dorsal diencephalon precedes the development of neuroanatomical asymmetries in the same region.

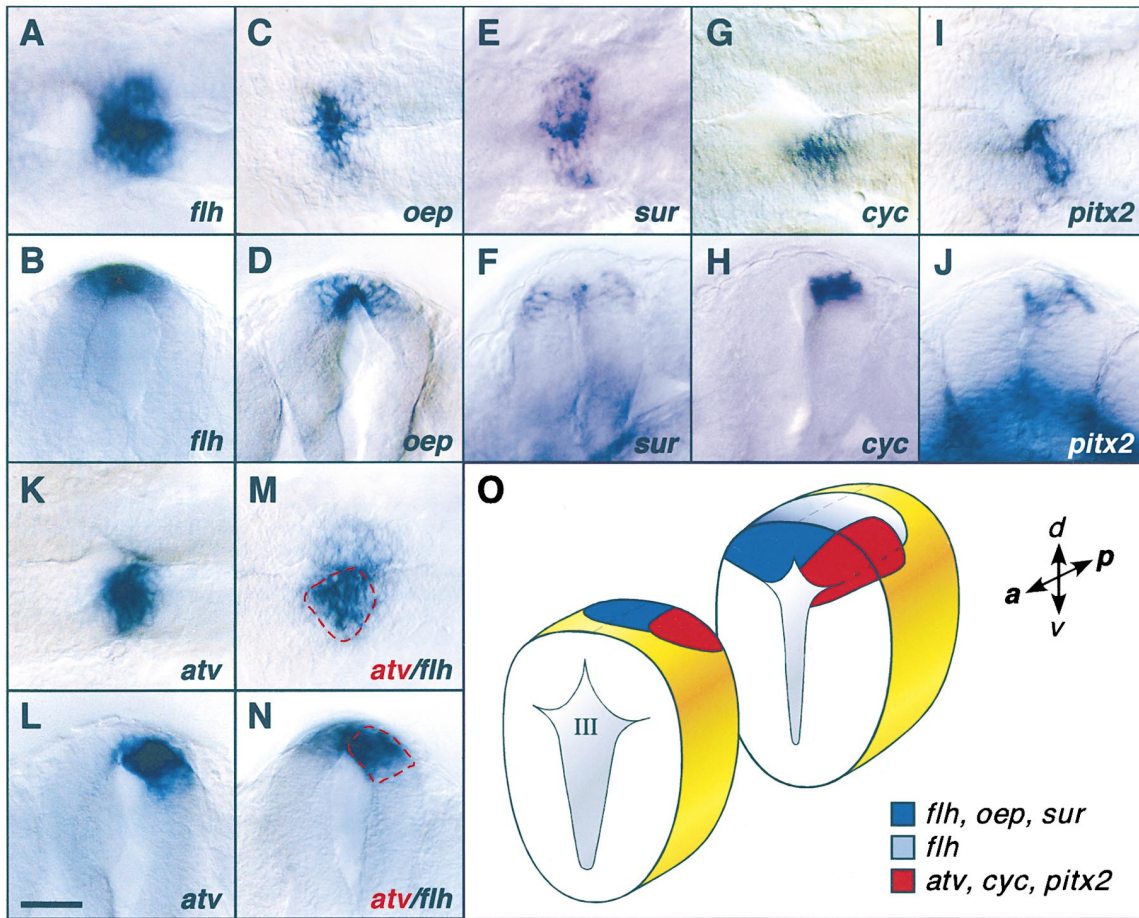
### Nodal Signaling Is Not Required for Diencephalic *cyc* and *pitx2* Expression

To address the role of Nodal signaling in the induction and regulation of genes in the dorsal diencephalon, we analyzed expression in embryos carrying mutations that compromise Nodal signaling (Table 1). In all embryos examined, expression of *oep* and *flh* remained bilateral (Figure 3 and data not shown). Nodal signaling has been implicated in the induction of left side-specific genes such as *pitx2* (reviewed in Burdine and Schier, 2000), raising the possibility that there may be an absolute requirement for the Nodal pathway in the induction of diencephalic *cyc* and *pitx2* expression. We tested this idea using double mutants for the Nodal-related signals Cyc and Sqt (Feldman et al., 1998). Unexpectedly, we found that both *cyc* and *pitx2* are expressed bilaterally in *cyc*<sup>-/-</sup>;*sqt*<sup>-/-</sup> double mutants (Figure 3 and Table 1). Bilateral expression was also observed in a majority of *cyc*<sup>-/-</sup> mutants (Figure 3 and Table 1). As an additional test of the requirement for Nodal signaling in mediating diencephalic *cyc* and *pitx2* expression, we examined maternal-zygotic *oep*<sup>-/-</sup> (*MZoep*<sup>-/-</sup>) embryos that completely lack Oep activity and that are insensitive to Nodal-related signals during gastrulation (Gritsman et al., 1999). These mutants also displayed bilateral diencephalic *cyc* and *pitx2* expression (Figure 3 and Table 1), supporting the conclusion that Oep/Cyc/Sqt-dependent Nodal signaling is not required to induce diencephalic expression of these genes but is required to restrict expression to the left side.

### Late Oep and Sur Activity Is Required for Diencephalic *cyc* and *pitx2* Expression

Our previous analysis of left–right laterality of internal organs suggested that late Nodal signaling is required for the expression of left side-specific genes in the lateral plate (Yan et al., 1999). For these experiments, we injected wild-type *oep* RNA into *MZoep*<sup>-/-</sup> embryos to provide early but not late Oep activity (Gritsman et al., 1999). In contrast to zygotic *oep*<sup>-/-</sup> mutants that have severe midline defects (Schier et al., 1997; Bisgrove et al., 2000), the transient exogenous *oep* rescues all midline structures and tissue deficiencies, and injected fish can be raised to adulthood. However, these late-zygotic *oep*<sup>-/-</sup> (*LZoep*<sup>-/-</sup>) embryos lack Oep function during somitogenesis stages, fail to express *cyc* and *pitx2* in the lateral plate, and display heterotaxia (Yan et al., 1999). To assess if CNS gene asymmetries are similarly affected, we examined diencephalic *cyc* and *pitx2* expression in *LZoep*<sup>-/-</sup> mutants and found it is indeed invariably absent (Figure 3 and Table 1). To further test the requirement for late Nodal-related signaling in dien-





**Figure 2. Nodal Pathway Genes Are Expressed in the Epiphysial Region of the Dorsal Diencephalon**  
(A–N) Dorsal (A, C, E, G, I, K, and M) and frontal (B, D, F, H, J, L, and N) views of gene expression in the dorsal diencephalon of 24s–26s and 18s (E and F) stage embryos. Genes analyzed are indicated bottom right of each panel. Labeling with combinations of probes indicates that *oep* expression lies within the domain of *flh* expression (data not shown), while *atv* expression extends slightly further ventral than *oep* or *flh* in the anterior diencephalon (M and N).  
(O) Schematic showing the relationship between the expression domains of the various genes expressed in the dorsal diencephalon.  
Abbreviations: III, third ventricle.

cephalic expression of *cyc* and *pitx2*, we analyzed zygotic *sur*<sup>-/-</sup> embryos. Early Nodal signaling is maintained in these mutants as maternally provided Sur partially compensates for the loss of zygotic Sur (Pogoda et al., 2000; Sirotkin et al., 2000). In contrast to previous studies wherein anterior midline defects were associated with the *sur*<sup>-/-</sup> phenotype (Bisgrove et al., 2000), the *sur*<sup>-/-</sup> mutants studied here exhibited mild or no axial defects. Indeed, some *sur*<sup>-/-</sup> embryos can be grown to adulthood. Similar to LZoep<sup>-/-</sup> embryos, we found that *sur*<sup>-/-</sup> mutants express *oep*, but not *cyc* and *pitx2* in the diencephalon (Figure 3 and Table 1). These results indicate that *cyc* and *pitx2* expression is bilateral when Nodal signaling is severely abrogated at all stages (MZoep<sup>-/-</sup> and *cyc*<sup>-/-</sup>;*sqt*<sup>-/-</sup>) while it is absent when Nodal signaling is present early and absent late (LZoep<sup>-/-</sup>, *sur*<sup>-/-</sup>). One interpretation of these observations is that early Oep-dependent Nodal signaling leads to repression of diencephalic *cyc* and *pitx2* expression and that late Oep and Sur activity is required to overcome this repression.

#### Midline Tissue Represses Diencephalic *cyc* and *pitx2* Expression

One reason that left-sided diencephalic expression becomes bilateral in MZoep<sup>-/-</sup>, *cyc*<sup>-/-</sup>, and *cyc*<sup>-/-</sup>;*sqt*<sup>-/-</sup> mutants may be that early Nodal signaling regulates midline development and an intact midline is required to restrict expression to one side of the embryo (reviewed in Burdine and Schier, 2000). Supporting such a role for the midline, diencephalic *cyc* and *pitx2* expression is bilateral in *ntl*<sup>-/-</sup> embryos, which lack a differentiated notochord (Figure 3 and Table 1; Bisgrove et al., 2000). However, since *ntl* is expressed in all mesodermal precursors, abnormal gene expression in the diencephalon might be caused by defects in nonmidline tissues. To directly test the role of the midline, we physically ablated the midline. Embryonic shields were removed (Saude et al., 2000), and the extent of midline deficiencies was determined by RNA in situ hybridization with the floor plate, ventral brain, and notochord marker *sonic hedgehog* (*shh*). Expression of *cyc* and *pitx2* was bilateral in the majority of embryos in which midline expres-

Table 1. Dorsal Diencephalic Expression of Genes in Wild-Type and Mutant Embryos

		Gene Expression				n
		Left	Right	Bilateral	Absent	
Wild-type	<i>pitx2</i>	92%	—	1%	7%	115
	<i>cyc</i>	95%	—	—	5%	129
	<i>oep</i>	—	—	100%	—	97
<i>cyc</i> <sup>m294</sup>	<i>pitx2</i>	9%	5%	77%	9%	22
	<i>cyc</i>	3%	—	87%	10%	31
	<i>oep</i>	—	—	100%	—	18
MZoep <sup>tz57</sup>	<i>pitx2</i>	—	—	96%	4%	49
	<i>cyc</i>	—	—	97%	3%	64
	<i>oep</i>	—	—	100%	—	66
<i>cyc</i> <sup>m294</sup> ; <i>sq</i> <sup>tc235</sup>	<i>pitx2</i>	—	—	93%	7%	15
	<i>cyc</i>	—	—	100%	—	19
	<i>oep</i>	—	—	100%	—	12
<i>sur</i> <sup>m768</sup> (+WT)	<i>pitx2</i>	70%	—	4%	26%	27
	<i>cyc</i>	73%	—	—	27%	30
	<i>oep</i>	—	—	100%	—	26
LZoep <sup>tz57</sup>	<i>pitx2</i>	—	—	—	100%	116
	<i>cyc</i>	—	—	—	100%	51
	<i>oep</i>	—	—	100%	—	25
<i>ntl</i> <sup>β160</sup>	<i>pitx2</i>	—	—	84%	16%	51
	<i>cyc</i>	4%	—	70%	26%	27
	<i>oep</i>	—	—	100%	—	14
Midline ablation	<i>pitx2</i>	19%	—	81%	—	16
	<i>cyc</i>	11%	—	89%	—	19
	<i>oep</i>	—	—	100%	—	13

For all mutant categories, except *sur*, numbers and percentages are for mutant embryos only and do not include siblings. For *sur*, the numbers refer to the whole clutch (25% should be homozygous *sur*<sup>-/-</sup> mutants). Sequencing of DNA from four of the embryos lacking expression of *cyc* or *pitx2* confirmed the embryos to be homozygous *sur*<sup>-/-</sup> mutants. Sequence analysis of six siblings with normal expression confirmed them to be wild-type or heterozygous for the *sur* mutation. Mildly affected zygotic *oep*<sup>-/-</sup> mutants showed no expression of *cyc* and *pitx2* (data not shown; also see Bisgrove et al., 2000), whereas severely affected zygotic *oep*<sup>-/-</sup> mutants displayed bilateral expression (data not shown).

sion domains of *shh* were partially or completely absent (Figure 3 and Table 1). These results demonstrate that an intact midline is essential for restricting the expression of *cyc* and *pitx2* to the left diencephalon.

#### CNS Laterality Is Disrupted in *oep*<sup>-/-</sup>, *cyc*<sup>-/-</sup>, and *sur*<sup>-/-</sup> Fish

In order to investigate the role of *oep*, *cyc*, and *sur* in the development of neuroanatomical asymmetries in the CNS, we examined the development of the parapineal and habenular nuclei in embryos carrying mutations affecting the activity of these genes. To determine the consequences of loss of late Nodal-like signaling in the dorsal diencephalon, we first focused on LZoep<sup>-/-</sup> fry. These fish are viable and have no detectable midline defects but lack *cyc* and *pitx2* expression in the dorsal diencephalon. Based on the laterality defects observed in visceral organs and the absence of left side-specific gene expression, we expected that LZoep<sup>-/-</sup> mutants would display either right isomerism (no parapineal and a mirror image of the right habenula on the left) or heterotaxia (parapineal and larger habenula either on the right or left). Consistent with the second possibility, we found that neuroanatomical asymmetry still developed in LZoep<sup>-/-</sup> fish, but it was randomized. The parapineal and enlarged habenular nucleus were detected on left or right sides of the brain at equal frequencies (Figures 4I–4K). In all cases examined, the parapineal was de-

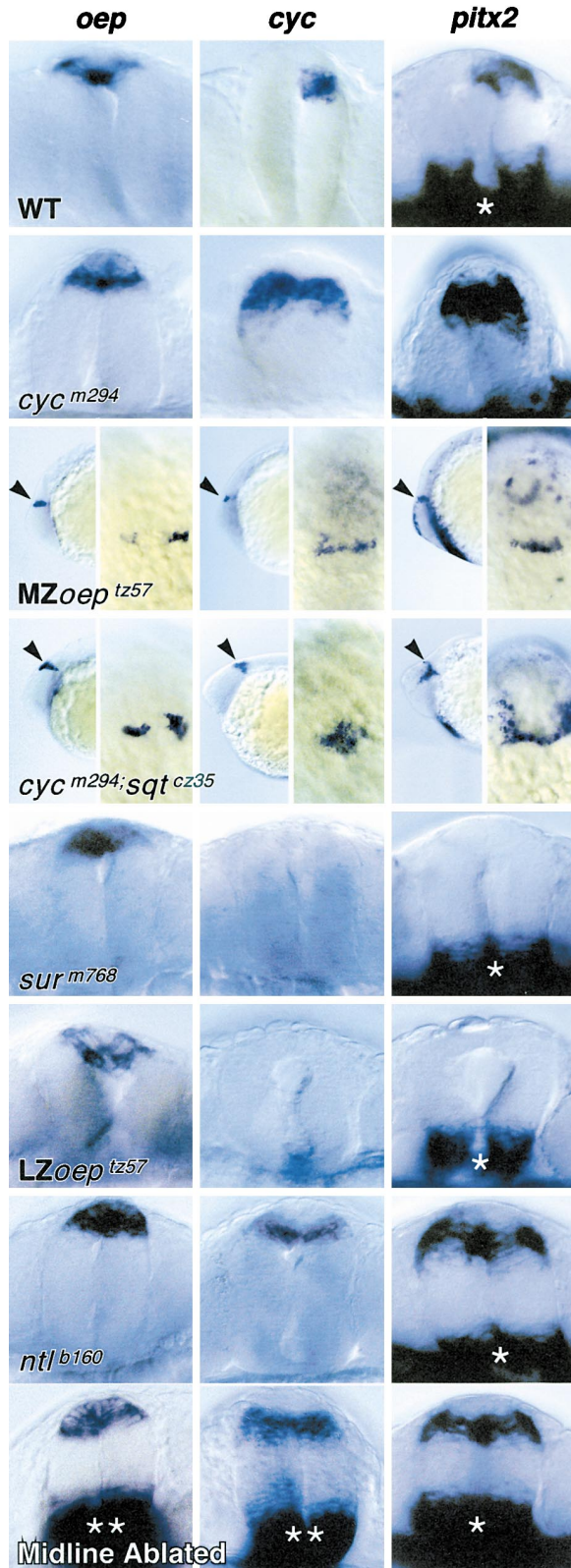
tected on the same side of the brain as the enlarged habenular nucleus (Figure 4L). Heart looping is also disrupted in LZoep<sup>-/-</sup> fish (Yan et al., 1999), but the direction of heart looping was randomized with respect to the laterality of the diencephalon (Table 2).

Expression of *cyc* and *pitx2* is also absent from the brain of zygotic *sur*<sup>-/-</sup> embryos, and diencephalic *Cyc* function is absent in homozygous *cyc*<sup>-/-</sup> embryos. In *sur*<sup>-/-</sup> and *cyc*<sup>-/-</sup> embryos, neuroanatomical asymmetries still occurred, but again laterality of the asymmetries was disrupted (n = 123 left [L] and 77 right [R] in *cyc*<sup>-/-</sup> [laterality was uncertain in six antiacetylated tubulin labeled embryos]; n = 45L and 26R in *sur*<sup>-/-</sup>; combined data for habenular and parapineal asymmetries detected by antiacetylated tubulin or anti-Islet labeling). These results indicate that *oep*, *cyc*, and *sur* are not essential for the development of CNS asymmetry per se but are required to specify the laterality of the asymmetry.

#### CNS Laterality Is Randomized in *ntl*<sup>-/-</sup> Fish

In contrast to LZoep<sup>-/-</sup> and *sur*<sup>-/-</sup> embryos, *ntl*<sup>-/-</sup> embryos exhibit bilateral diencephalic expression of *cyc* and *pitx2*. To address consequences on neuroanatomical asymmetries, we examined the parapineal and habenular nuclei in *ntl*<sup>-/-</sup> embryos. Coordinated habenular and parapineal asymmetries develop in *ntl*<sup>-/-</sup> embryos, but they exhibit completely randomized laterality (Fig-





**Figure 3.** Components of the Nodal Signaling Pathway and an Intact Midline Are Required for Asymmetric Gene Expression in the Diencephalon

Transverse (wild-type, *cyc*<sup>-/-</sup>, *sur*<sup>-/-</sup>, *LZoep*<sup>-/-</sup>, *ntl*<sup>-/-</sup>, and midline ablated), or lateral (anterior to the left; dorsal up) and dorsal (anterior

ures 4I–4K). An intact midline is therefore required to specify the laterality of neuroanatomical asymmetries, and midline disruption has similar consequences on laterality as an absence of Nodal-related signaling.

### Discussion

In this study, we describe a genetic basis for the regulation of anatomical asymmetries in the vertebrate brain. We show that the laterality of asymmetric development of the diencephalic habenular nuclei and the photoreceptive pineal complex depends on an intact midline and on *Oep*, *Cyc*, and *Sur*, proteins that function in Nodal signaling. In addition, we propose a model for left–right patterning, in which Nodal signaling initially represses left side–specific genes and then is involved in overcoming this repression.

### Development of Neuroanatomical Asymmetry

We have found that the diencephalon of larval zebrafish contains at least two neuroanatomical asymmetries—the parapineal nucleus is present only on the left side and the left habenular nucleus is more substantial than the right habenular nucleus. In no case did we observe left parapineal isomerism (i.e., a situation wherein parapineal nuclei develop on both sides of the brain). This could be explained in one of two ways. First, if parapineal development is initiated on one side of the brain, this may inhibit development on the other implying communication across the dorsal midline of the brain. In support of a unilateral origin of the parapineal, we first detect *Islet1*-expressing parapineal neurons quite laterally, and only later do these cells come to occupy a position close to the midline adjacent to the pineal stalk. A precedent for signaling across the midline comes from studies in *C. elegans*, where lateral signaling mediated by axon contact and calcium entry regulates asymmetric odorant receptor expression (Troemel et al., 1999). Alternatively, a single pool of dorsal midline parapineal precursor cells may migrate either to the left or to the right. Although the final position of the parapineal is due to a lateral to medial movement of cells, this does not discount the possibility that there is also an earlier midline to lateral migration. Indeed, most textbook descriptions of parapineal development suggest an origin from midline tissue rostral to the epiphysis. Definitive fate mapping of the parapineal precursor cells will be required to resolve this issue.

Unlike the parapineal organ, the habenular nuclei are paired, and it is a size difference that distinguishes left and right sides. In virtually all cases, the more substantial habenular nucleus is present on the same side of the

up; *MZoep*<sup>-/-</sup> and *cyc*<sup>-/-</sup>; *sqt*<sup>-/-</sup>) views of diencephalic gene expression in 24s–26s stage embryos. Genes analyzed are indicated at the top of the columns, and the class of embryo is indicated bottom left of each row. The asterisk indicates ventral diencephalic *pitx2* expression that is present in embryos without severe axial defects. The double asterisk indicates *shh* expression in the ventral brain of shield-ablated embryos. Arrowheads indicate dorsal diencephalic expression in *MZoep*<sup>-/-</sup> and *cyc*<sup>-/-</sup>; *sqt*<sup>-/-</sup> embryos. Sequence analysis confirmed that the *sur* embryos lacking *pitx2* and *cyc* were homozygous mutants.

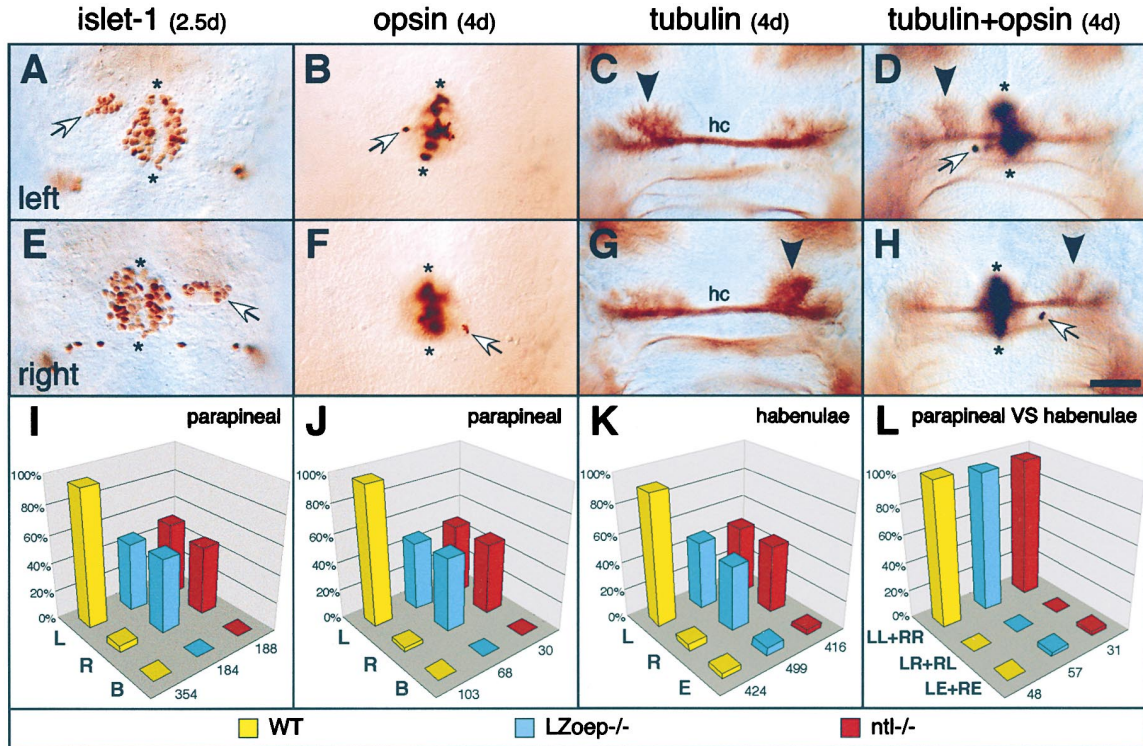


Figure 4. The Laterality of Neuroanatomical Asymmetries Is Randomized in *LZoep*<sup>-/-</sup> and *ntl*<sup>-/-</sup> Mutants

(A–H) Dorsal views of the diencephalons of embryos labeled with anti-Islet (A and E), anti-opsin (B and F), antiacetylated  $\alpha$  tubulin (C and G), or antiacetylated  $\alpha$  tubulin plus anti-opsin (D and H) antibodies. The asterisks delineate the anterior and posterior limits of the epiphysis along the dorsal midline, and hc indicates the habenular commissure

(A–D) Wild-type embryos showing left-sided parapineal nucleus ([A, B, and D], arrow) and left-sided enlarged habenular nucleus ([C and D], arrowhead).

(E–H) Examples of mutant embryos showing right-sided parapineal nucleus ([E, F, and H], arrow) and right-sided enlarged habenular nucleus ([G and H], arrowhead).

(I–L) Graphs showing laterality of brain asymmetries in wild-type (yellow), *LZoep*<sup>-/-</sup> (blue), and *ntl*<sup>-/-</sup> (red) fry. In both *LZoep*<sup>-/-</sup> and *ntl*<sup>-/-</sup> fry, brain laterality is randomized (I–K). In virtually all cases, the habenular nucleus is larger on the side containing the parapineal nucleus (L). Numbers of fry used to generate the graphs are indicated to the right of the columns.

Abbreviations: B, both sides; E, equal size; L, left side; R, right side; LL+RR, large habenula and parapineal both on left or both on right; LR+RL, large habenula and parapineal on opposite sides; LE+RE, parapineal on either side and habenulae of equal size.

brain as the parapineal, indicating that the laterality of these two structures is linked. This could be because the laterality of one nucleus depends on the other. For instance, parapineal neurons project axons toward the ipsilateral habenular nucleus (reviewed in Harris et al., 1996), and resultant innervation could regulate subsequent habenular development. Alternatively, the development of the two nuclei may be dependent on the same laterality determining event. Importantly, the analysis of

*LZoep*<sup>-/-</sup> mutants indicates that the coordinated laterality in the diencephalon is independent of cardiac laterality despite concordant absence of left side-specific gene expression in the lateral plate (Yan et al., 1999) and diencephalon (Figure 3). *LZoep*<sup>-/-</sup> fish therefore display independent lateralities of diencephalic, cardiac, and visceral asymmetry (this study and Yan et al., 1999), indicating that several independent laterality decisions are made within these fish.

The role of the diencephalic asymmetries we describe is currently unknown although zebrafish do exhibit asymmetric behaviors that are presumably driven by neuroanatomical differences between left and right sides of the brain (Miklosi et al., 1997; Miklosi and Andrew, 1999). Asymmetric behaviors in fish are often associated with eye use and direction of approach to familiar and unfamiliar objects including food, potential predators, and conspecifics including potential mates (Bisazza et al., 1998, 1999; Andrew, 2001; Andrew et al., 2000). In birds, cerebral lateralization has been shown to improve success in visual discrimination, thus highlighting the relevance of neuroanatomical and behav-

Table 2. Heterotaxia of Diencephalic Laterality and Heart Looping in *LZoep*<sup>-/-</sup> Fish

Direction of Heart Looping	Laterality of the Parapineal Organ		n
	Left	Right	
Left → right	57%	43%	54
Right → left	49%	51%	47
Uncertain	50%	50%	6

The direction of heart looping was scored in *LZoep*<sup>-/-</sup> living fry at 2 days postfertilization. Diencephalic asymmetry was subsequently detected using anti-Islet antibody.



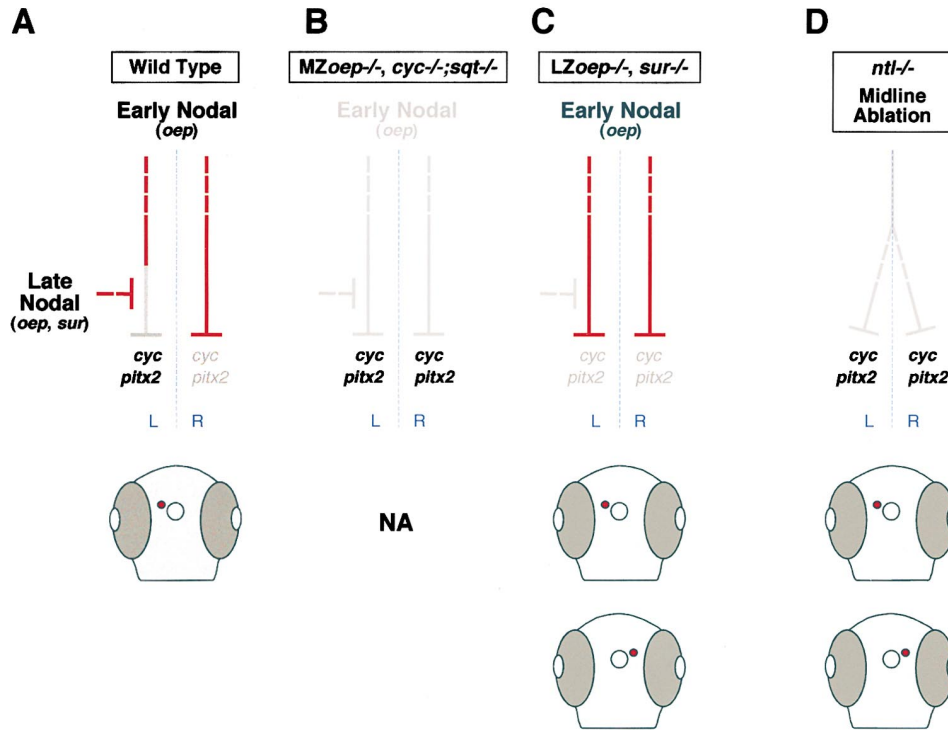


Figure 5. Summary and Model for the Genetic Control of Laterality in the Dorsal Diencephalon

(A) In the wild-type situation, *cyc* and *pitx2* are expressed only in the left diencephalon. (Top) We propose that events initiated by early Oep-dependent Nodal signaling lead to repression of dorsal diencephalic *cyc* and *pitx2* expression. We suggest that later Sur and Oep dependent signaling is required to overcome this repression on the left side. (Bottom) Left-sided expression of *cyc* and *pitx2* in the dorsal diencephalon correlates with the development of a left-sided parapineal organ (red circle) and an enlarged habenular nucleus on the left side at later stages.

(B) In *MZoep*<sup>-/-</sup> and *cyc*<sup>-/-</sup>;*sqt*<sup>-/-</sup> embryos, *cyc* and *pitx2* are expressed on both sides of the dorsal diencephalon. (Top) We suggest that in these mutants, Nodal signaling is severely or completely abrogated at early stages and so the events that lead to repression of *cyc* and *pitx2* fail to occur and gene expression is bilateral. The absence of Oep and Sur dependent inhibition of the repression has no consequence on *cyc* and *pitx2* expression. (Bottom) The effects of symmetric *cyc* and *pitx2* expression on neuroanatomical asymmetry could not be assessed due to early lethality of *MZoep*<sup>-/-</sup> and *cyc*<sup>-/-</sup>;*sqt*<sup>-/-</sup> embryos.

(C) In *LZoep*<sup>-/-</sup> and *sur*<sup>-/-</sup> embryos, *cyc* and *pitx2* expression is absent in the dorsal diencephalon. (Top) We suggest that early Nodal signaling is intact in these embryos due to the presence of exogenously provided *oep* or maternally provided *sur*, and therefore the events that lead to repression of *cyc* and *pitx2* are initiated. However, as late Oep- and Sur-dependent signaling is compromised, we suggest that the repression fails to be alleviated on the left side of the brain. (Bottom) Absence of *cyc* and *pitx2* expression in the dorsal diencephalon correlates with randomization of diencephalic laterality, with the parapineal organ developing on either the left or right side of the brain.

(D) In *ntl*<sup>-/-</sup> and midline ablated embryos, *cyc* and *pitx2* are expressed on both sides of the dorsal diencephalon. Thus, in the absence of an intact midline, repression fails to occur. As in the absence of gene expression (C), symmetric expression of *cyc* and *pitx2* in the dorsal diencephalon correlates with heterotaxial randomization of diencephalic laterality, with the parapineal organ developing on either the left or right side of the brain.

In these diagrams, there may be many as yet unresolved steps (dashes) between the genes indicated and the events illustrated. For instance, it is likely that early Nodal signaling does not directly repress genes normally expressed on the left but is required for the formation of tissues such as the midline that carry out repression. Furthermore, while it is likely that midline tissue is important for repression of left-sided genes on the right side of the embryo, it remains to be determined where within the embryo, and how, repression and inhibition of repression is mediated. For instance, although for simplicity we illustrate events occurring independently on the left and right sides of the embryo, this may not be the case in vivo.

ioral asymmetries for species survival (Güntürkün et al., 2000). The ability to generate viable “left-brained” and “right-brained” *LZoep*<sup>-/-</sup> zebrafish should help elucidate the link between neuroanatomical and behavioral laterality.

In humans, research has focused on behavioral and cognitive asymmetries, and little is known concerning the detailed neuroanatomical basis of such asymmetries. Size differences do exist between cortical regions in the left and right hemispheres, although precisely how these relate to functional asymmetries is unknown (Corballis, 1997). Telencephalic size asymmetries are detectable before birth (Chi et al., 1977), raising the

possibility that early genetic influences may globally affect asymmetries throughout the CNS. Indeed, reversal of visceral asymmetries can be associated with reversal of neuroanatomical asymmetries (Kennedy et al., 1999). However, as yet, no clear link has been made between visceral polarity and cognitive asymmetries in humans (Kennedy et al., 1999; Tanaka et al., 1999).

#### Oep, Cyc, and Sur Are Required for the Establishment of Laterality in the CNS

To determine the role of Nodal signaling in the development of neuroanatomical asymmetries, we analyzed mutations affecting the Nodal gene *cyc*, the Nodal cofactor



Oep, and the Nodal downstream transcription factor Sur. Although *LZoep*<sup>-/-</sup>, *cyc*<sup>-/-</sup>, and *sur*<sup>-/-</sup> embryos do not display asymmetric gene expression, CNS asymmetries still develop. However, the laterality of these asymmetries is altered. In *LZoep*<sup>-/-</sup> fish, laterality is completely randomized revealing a requirement for late Oep function in left–right decisions in the brain. The heterotaxic randomization of diencephalic laterality, heart looping, and pancreas positioning (this study and Yan et al., 1999) indicates that Oep-dependent Nodal-related signaling in fish does not promote left-sided development per se, but that Oep is required to bias several independent laterality decisions to the left. The failure to establish left-sided expression of *cyc* and *pitx2* in *LZoep*<sup>-/-</sup> fish suggests that the loss of function of these genes in the brain contributes to the failure to establish CNS laterality. Interestingly, *cyc*<sup>-/-</sup> embryos still show a statistically significant left bias in CNS laterality suggesting that in addition to Cyc, other ligands may depend on late Oep function to affect left–right laterality decisions in the diencephalon.

#### A Repression-Inhibition of Repression Model of Oep/Sur Function

Misexpression studies have led to a model for left–right development wherein Nodal signaling is required for left-specific gene expression (reviewed in Burdine and Schier, 2000; Capdevilla et al., 2000). However, previous studies could not assay left-specific gene expression in the absence of Nodal signaling since the tissues that would need to be assayed (e.g., lateral plate mesoderm) do not form when Nodal signaling is absent. Since the dorsal forebrain does form in the absence of Nodal signaling in zebrafish, we were able to address the requirement for Nodal signaling in left-specific gene expression. Our results demonstrate that *cyc* and *pitx2* are expressed when Oep- and Cyc/Sqt-dependent Nodal signaling is abrogated throughout development (*MZoep*<sup>-/-</sup> and *cyc*<sup>-/-</sup>;*sqt*<sup>-/-</sup> embryos). This suggests that left side-specific genes are not absolutely dependent on Nodal signals. In contrast, early presence and late absence of Oep or Sur function (*LZoep*<sup>-/-</sup> and zygotic *sur*<sup>-/-</sup> embryos) leads to complete loss of expression of asymmetric genes in the brain. These defects are not due to lack of anterior midline structures as previously suggested (Bisgrove et al., 2000), since the *LZoep*<sup>-/-</sup> and zygotic *sur*<sup>-/-</sup> mutants used in our studies form notochord and prechordal plate and can survive to adulthood. The most parsimonious explanation of these observations is that, in wild-type embryos, early Oep-dependent Nodal signaling leads to repression of asymmetric diencephalic genes and that late Sur and Oep function is required to overcome this repression (Figure 5). In this model, *MZoep*<sup>-/-</sup> and *cyc*<sup>-/-</sup>;*sqt*<sup>-/-</sup> embryos lack the repressive activity. Hence, the absence of late Oep/Sur-dependent inhibition of repression is of no consequence, and left side-specific genes are expressed. In contrast, in *LZoep*<sup>-/-</sup> and *sur*<sup>-/-</sup> embryos, early repression occurs, and this fails to be alleviated due to the absence of late Oep and Sur function. The source of the early repressive activity is not known, but the midline is a good candidate since both midline ablation and absence of Nodal signaling lead to the symmetric expression of *pitx2* and *cyc*.

Abnormal gene expression in the dorsal diencephalon following midline ablation also provides evidence for communication between the axial midline and the dorsal CNS. Although such long-distance communication is surprising, other studies investigating asymmetry have implicated the existence of signals that can act globally within the embryo or even influence neighboring embryos in the case of conjoined twins (Wood, 1997; Burdine and Schier, 2000). Determination of the nature of such signals remains an important goal in order to understand the genetic basis of left–right axis formation.

#### Experimental Procedures

##### Zebrafish Lines

Embryos and fry were obtained by natural spawning from wild-type, *cyc*<sup>m294</sup> and *cyc*<sup>tt219</sup>, *oep*<sup>tz57</sup>, *sur*<sup>m768</sup>, *cyc*<sup>m294</sup>, *sqt*<sup>cz35</sup>, and *ntl*<sup>b160</sup> fish. Homozygous *oep*<sup>-/-</sup> adults were obtained by rescuing homozygous *oep*<sup>-/-</sup> embryos with wild-type *oep* RNA (Gritsman et al., 1999). To rescue early patterning defects in *oep*<sup>-/-</sup> mutants, *MZoep*<sup>-/-</sup> embryos were injected with 25–50 pg of wild-type *oep* RNA at the one- to four-cell stage. *sur*<sup>-/-</sup> embryos were genotyped either by morphology, PCR, or by sequencing genomic DNA from suspected homozygous embryos.

##### Phenotypic Analysis

In situ hybridizations were performed using standard procedures. For antibody labeling, anti-opsin (kindly provided by Paul Hargrave) was used at 1:1000, anti-Islet (kindly provided by Vladimir Korzh) at 1:500, and antiacetylated  $\alpha$  tubulin (Sigma) at 1:1000. Confocal analysis was performed on a Leica TCS SP Confocal Microscope using a 25 $\times$  oil immersion objective. 3D series of images were acquired at 1–2  $\mu$ m intervals, and the selected depths were then projected using the brightest point method encompassing total depths of 35  $\mu$ m (Figures 1A and 1C), 10  $\mu$ m (Figure 1B), and 3  $\mu$ m (Figure 1D).

For quantitation of asymmetries determined by *islet1* in situ hybridization and anti-Islet and anti-tubulin labeling, only fry that could be unequivocally scored are counted and shown in the figures and graphs (under 5% of embryos could not be scored due to staging problems, quality of labeling, or other reasons). Less than 15% of fry had opsin-positive labeling in the parapineal of 4-day-old fry. Numbers in the results refer to opsin-positive parapineals, not the total number of fry used. For statistical analysis, a one-tailed F test was used.

To ablate midline axial tissue, embryonic shields were removed as described in Saude et al. (2000). The extent of midline deficiencies was determined by RNA in situ hybridization with the midline marker *sonic hedgehog* (*shh*). Only embryos that lacked a subset of the *shh* expression domains were scored.

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