III. Neural Development

Patterning the Zebrafish Central Nervous System

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1 Introduction

One of the hallmarks of the vertebrate nervous system is the enormous diversity of neurons of which it is composed. The ability of an animal to carry out its normal behavioral repertoire requires that all of these neurons develop in the appropriate numbers and at the appropriate times and positions and that they elaborate the appropriate differentiated characteristics. This process begins with patterning of the nascent nervous system, the neural plate, along the dorsoventral (DV) and anteroposterior (AP) axes of the body. In this chapter we consider what is currently known about the mechanisms involved in patterning the zebrafish neural plate. Although we discuss many of the similarities and differences between zebrafish and other vertebrates, we limit our primary focus to zebrafish, as including an exhaustive description of neural patterning in other model vertebrates would exceed the space limitations of this chapter. We apologize for any work we have overlooked and for discussion of work on other model vertebrates we direct the reader to several excellent reviews (Goulding and Lamar 2000; Jessell 2000; Patten and Placzek 2000; Wilson and Rubenstein 2000; Altmann and Brivanlou 2001; Briscoe and Ericson 2001).

2 Nervous System Morphogenesis

As for other vertebrates, the zebrafish central nervous system (CNS) begins as an ectodermal epithelium on the dorsal side of the embryo, the neural plate. A dorsomedial thickening of cells immediately after gastrulation (about 10 h; hours postfertilization at 28.5 °C) is the first morphological manifestation of neural plate formation (Schmitz et al. 1993). Within the next 1–2 hr, two lateral

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thickenings appear. Morphogenetic convergence movements (Kimmel et al. 1994) fuse the lateral and medial thickening into a neural keel around 13 h and into a neural rod around 16 h. Cavitation beginning around 17 h establishes the central canal of the hollow neural tube (Raible et al. 1992; Schmitz et al. 1993) that forms brain anteriorly and spinal cord posteriorly. Thus, the lateral edges of the neural plate form dorsal CNS and the medial neural plate forms ventral CNS, as has been shown directly by labeling studies (Papan and Campos-Ortega 1994). Much of the patterning we will discuss actually begins in the neural plate; thus, when we refer to DV patterning, we mean patterning that begins along the mediolateral neural plate axis and results in cells with specific soma positions along the DV axis of the CNS. We consider patterning of the spinal cord and brain separately, starting with the spinal cord because it is more simply organized.

The entire CNS is underlain by axial mesoderm that internalizes during gastrulation (see Kane and Adams; Kimelman and Schier, this Vol.). Anteriorly, this mesoderm forms the prechordal plate. Posterior to hindbrain rhombomere 4, this mesoderm forms the notochord which extends to the tip of the tail (Hatta and Kimmel 1993). Axial mesoderm is bounded on both sides by paraxial mesoderm that later forms somites and head mesoderm (see Kimelman and Schier, this Vol.). As we describe, signals emanating from axial mesoderm play key roles in nervous system patterning. Signals emanating from somites may also be involved in neural patterning, although considerably less is currently known about such signaling.

3 The Spinal Cord

Although the spinal cord is more simply organized than the brain, it shows both AP and DV patterning. AP patterning is most clearly manifest by the development of limb-innervating motoneurons in spinal segments adjacent to the pectoral fins (Myers 1985). There is also more fine-grained, segmental AP patterning, revealed by the soma positions of early-developing primary motoneurons that innervate myotomal muscles (Myers 1985; Eisen et al. 1986; Westerfield et al. 1986). However, the most obvious spinal cord patterning is along the DV axis such that distinct types of neurons have somata in specific DV locations.

As for other anamniote vertebrates, the zebrafish spinal cord has both early-developing primary neurons and later-developing secondary neurons (Kimmel and Westerfield 1990). Primary neurons are large, few in number, born starting about 9–10 h (Myers et al. 1986; Kimmel and Westerfield 1990), undergo axogenesis between 14–24 h and comprise all modalities, including sensory, inter- and motoneurons. Each type of primary neuron has a specific DV location within the embryonic and larval spinal cord: primary motoneurons are ventrally located, Rohon-Beard (RB) primary sensory neurons are dorsally located, and a variety of primary interneurons are located throughout the

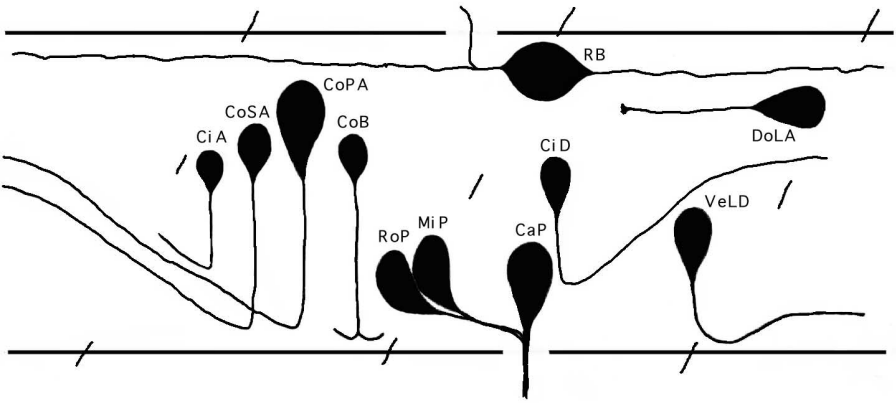


Fig. 1. Several types of primary and secondary neurons have been described in the embryonic and larval zebrafish spinal cord. Drawing shows lateral view of spinal cord, anterior to the *left*. Rohon-Beard (*RB*) sensory neurons, ventral longitudinal descending (*VeLD*) and commissural primary ascending (*CoPA*) interneurons and caudal primary (*CaP*), middle primary (*MiP*) and rostral primary (*RoP*) motoneurons are all primary neurons, whereas commissural secondary ascending (*CoSA*) neurons are secondary interneurons. Dorsal longitudinal ascending (*DoLA*), circumferential descending (*CiD*), circumferential ascending (*CiA*), and commissural bifurcating (*CoB*) neurons are all interneurons, although it is unclear whether they should be considered as primary or secondary; secondary motoneurons are not pictured here. (Adapted from Bernhardt et al. 1990)

midregion of the spinal cord. Bernhardt and colleagues (1990) used axonal morphology to classify several types of primary interneurons (Fig. 1) and Eisen and colleagues (1986) used axonal morphology to classify three types of primary motoneurons. Primary motoneurons have somata located in the medial motor column, innervate fast muscles derived from the myotomes and persist through adulthood (Westerfield et al. 1986). Persistence through adulthood is probably also the case for many primary interneurons, as cell death has not been described in this population. In contrast, RBs are a transient neuronal population. Many RBs die by 3 d (days of development at 28.5 °C); death apparently depends on signaling via TrkC1 receptors (Williams et al. 2000) and sodium-channel-mediated electrical activity (Svoboda et al. 2001).

Secondary neurons are smaller, more numerous, born later (starting about 13–14 h; Myers et al. 1986; Kimmel et al. 1994; Appel et al. 2001) and typically have finer axons than primary neurons (Kimmel and Westerfield 1990). Although secondary neurons are probably more similar to the spinal neurons studied in amniote vertebrates (Kimmel and Westerfield 1990), considerably less is known about these cells than about zebrafish primary neurons. Bernhardt and colleagues (1990) have classified only one type of secondary interneuron by axonal morphology. Myers (1985) and Pike and Eisen (Pike et al. 1992) described several types of secondary motoneurons based on axonal morphology in embryos and larvae and Westerfield and colleagues (1986)

identified several types of secondary motoneurons in adults based on innervation fields and axonal morphology. Some secondary motoneurons are located in the lateral motor column and innervate fin muscles. However, many more secondary motoneurons are located in the medial motor column and innervate myotomal muscles (Eisen 1994).

The spinal cord contains several other cell types in addition to neurons. Floor plate cells occupy the spinal cord ventral midline and it is likely that roof plate cells occupy the dorsal midline, although they have not been well described. The floor plate comprises three longitudinal columns of cells; medial floor plate forms a single column in the midline and lateral floor plate forms flanking columns on both sides of the midline column (Bernhardt et al. 1992b; Strähle et al. 1997; Odenthal et al. 2000). Lateral floor plate probably generates Kolmer-Agduhr (KA) neurons (Bernhardt et al. 1992b), a class of GABAergic neurons that contact cerebrospinal fluid and may act as proprioceptive position sensors (Dale et al. 1987a,b), although they may also generate other fates. Radial glia have been described in the zebrafish spinal cord (Appel et al. 2001), although their origin is currently unknown.

3.1 Bmp Signaling Establishes DV Pattern in the Spinal Cord

As described elsewhere (see Hammerschmidt and Mullins, this Vol.), bone morphogenetic proteins (Bmps) are key regulators of embryonic DV patterning. Within the developing ectoderm, Bmp activity has been shown to promote epidermal differentiation and inhibit neural differentiation. Because different levels of Bmp activity specify different cell fates within the mesoderm, Bmps are considered morphogens (Dosch et al. 1997; Neave et al. 1997). However, rather than establishing different levels by diffusion, it seems likely that different Bmp levels are established by at least four distinct proteins, Chordin, Follistatin, Noggin and Cerberus, that emanate from the dorsal side of the embryo and inhibit Bmp function (Dale and Wardle 1999; Piccolo et al. 1999). Of these, *noggin* (Bauer et al. 1998; Fürthauer et al. 1999), *follistatin* (Bauer et al. 1998) and *chordin* (Schulte-Merker et al. 1997) have been described in zebrafish.

Analysis of zebrafish Bmp pathway mutants reveals that Bmps participate in patterning throughout the entire mediolateral extent of the neural plate, thus the entire DV axis of the spinal cord. Studies of avian embryos have implicated Bmps in specification of dorsal neural fates and neural crest (Liem et al. 1995), a cell type that emigrates from the dorsal spinal cord, migrates along well-defined pathways and gives rise to a diverse set of derivatives including peripheral neurons and glia, pigment cells and fin ectomesenchyme (Eisen and Weston 1993; see Kelsh and Raible, this Vol.). Analysis of zebrafish embryos homozygous for mutations in Bmp pathway genes, including *swirl/bmp2b*, *snailhouse/bmp7* and *somitabun/smad5* (Nguyen et al. 1998b, 2000), provides

evidence that Bmp signaling is essential to establish neural crest as well as RB neurons that also arise from the lateral neural plate domain that generates neural crest (Cornell and Eisen 2000). However, Bmp effects are not limited to the lateral neural plate/dorsal spinal cord. Characterization of embryos with different levels of Bmp signaling provides evidence for graded effects of Bmp throughout the mediolateral axis of the neural plate (Barth et al. 1999; Fig. 2). Thus, severe depletion of Bmp signaling by overexpression of *noggin* leads to loss of RBs as well as interneurons in the midregion of the spinal cord and additionally results in expansion of ventral spinal cord fates. Slightly less severe Bmp depletion in *swirl/bmp2b* mutants leads to loss of RBs and expansion of interneurons. Mild Bmp depletion in *somitabun/smad5* mutants leads primarily to lateral displacement of RBs and interneurons.

A surprising observation of these studies was that severe Bmp depletion leads to expansion of the floor plate (Barth et al. 1999; Nguyen et al. 2000). Thus Bmp effects extend to the most medial region of the neural plate in zebrafish. Consistent with this observation, mouse embryos with augmented Bmp signaling resulting from a targeted deletion of the *noggin* gene lack floor plate and motoneurons in the caudal spinal cord (McMahon et al. 1998), suggesting that Bmp signaling suppresses formation of these ventral cell types. This effect could be direct, or it could be mediated by interactions between the Bmp-signaling pathway and other signaling pathways, such as the Hedgehog (Hh) pathway, that have been shown to specify ventral spinal cord fates (Goulding and Lamar 2000; Jessell 2000; Patten and Placzek 2000; Briscoe and Ericson 2001). Indeed, recent in vitro studies in chick embryos suggest that specification of these ventral spinal cord fates depends on integration of signals in these two pathways (Liem et al. 2000).

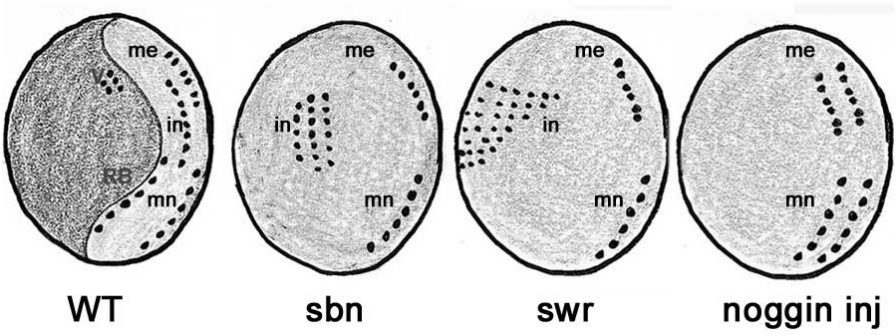


Fig. 2. Bmp signaling affects patterning of the entire neural plate. Series of embryos from wild-type (*WT*) to most severely Bmp-depleted (*noggin inj*) showing the arrangement of motoneurons (*mn*), interneurons (*in*), spinal sensory neurons (*RB*), and medial cranial neurons (*me*). *Darker gray* represents non-neural ectoderm and *lighter gray* represents neural ectoderm. (Adapted from Barth et al. 1999)

3.2 Hedgehog and Nodal Pathways Pattern the Ventral Spinal Cord

Elegant studies in avian embryos demonstrated that underlying notochord patterns the ventral neural plate (Placzek et al. 1990; Yamada et al. 1991, 1993). These studies provided evidence that, without notochord, floor plate and motoneurons do not form. However, two zebrafish mutants lacking notochord, *no tail* (Halpern et al. 1993) and *floating head* (Talbot et al. 1995), still form at least some floor plate and motoneurons, raising the possibility that the mechanisms underlying neural patterning might differ between zebrafish and amniote vertebrates.

Although one of the first studies implicating Sonic hedgehog (Shh) as the primary notochord-derived signaling molecule responsible for ventral neural tube patterning was carried out in zebrafish (Krauss et al. 1993), initial loss-of-function studies reinforced the idea that the role of Shh might be different in zebrafish and other vertebrates. In vitro studies in chick (Roelink et al. 1995) as well as targeted deletion of the mouse *Shh* gene (Chiang et al. 1996) demonstrate that Shh is both sufficient and necessary for specification of ventral spinal cord fates, including both floor plate and motoneurons. In contrast, zebrafish embryos homozygous for a deletion of the *sonic-you* (*syu*/*shh)* gene have motoneurons and medial floor plate, although lateral floor plate is absent (Schauerte et al. 1998; Odenthal et al. 2000). These results demonstrated that Shh signaling is necessary for specification of lateral floor plate, but also raised the possibility that other aspects of ventral spinal cord patterning in zebrafish do not require Shh signaling.

This controversy has been addressed in part by our current understanding that during evolution, the ancestral bony fishes apparently underwent a genome duplication with the consequence that zebrafish have an additional copy of some genes (Postlethwait et al. 1998). Thus, zebrafish have two copies of the ancestral *shh* gene, one called *shh* and the other called *tiggywinkle hedgehog* (*twhh*; Ekker et al. 1995; Zardoya et al. 1996). Initially, both genes are expressed in the dorsal embryonic shield; however, by 90% epiboly, *shh* is expressed only in presumptive notochord and *twhh* is expressed only in presumptive floor plate (Etheridge et al. 2001). During later segmentation stages, *shh* is expressed in both notochord and floor plate (Krauss et al. 1993), but *twhh* expression remains confined to the floor plate (Ekker et al. 1995). In addition, an *Indian hedgehog* homologue, *echidna hedgehog* (*ehh*; Currie and Ingham 1996; Zardoya et al. 1996), is also expressed in the notochord. Because these Hhs may function redundantly, and because mutations in all three genes are not currently available, it has been difficult to address the role of Hh signaling in zebrafish ventral spinal cord patterning.

One way the question of Hh redundancy during specification of ventral spinal cord cell fates has been addressed is by injection of morpholino antisense oligonucleotides (MOs). MOs prevent translation and thus effectively "knock down" gene function (Summerton 1999). Medial floor plate forms normally in wild-type embryos injected with *shh*-MO plus *twhh*-MO (Nasevicius

and Ekker 2000) and *syu* mutants injected with *twhh*-MO (Etheridge et al. 2001) or *twhh*-MO plus *ehh*-MO (Lewis and Eisen 2001), providing strong evidence that Hh signaling is unnecessary for medial floor plate specification.

Given that Hh signaling is unnecessary for specification of medial floor plate but necessary for specification of lateral floor plate, what about its role in specification of motoneurons? Severely reducing function of any of the three *hh* genes alone has a negligible effect on the number of primary motoneurons (Beattie et al. 1997; Schauerte et al. 1998; Lewis and Eisen 2001), suggesting that these three Hhs have redundant functions during primary motoneuron specification. Addressing the importance of each *hh* gene has been difficult because mutations in either *twhh* or *ehh* have not yet been isolated. Thus, this issue has been investigated in two ways: first by characterizing *cyclops* (*cyc*) mutants lacking floor plate, and thus diminished in Shh and Twhh signaling, *flh* mutants lacking notochord, and thus diminished in Shh and Ehh signaling, *cyc*;*flh* double mutants that should lack most Hh signaling (Beattie et al. 1997) and *cyc*;*flh*;*syu* mutants that should lack essentially all Hh signaling (Lewis and Eisen 2001), and second by injection of morpholinos for combinations of the *hh* genes (Lewis and Eisen 2001). These experiments reveal that the number of primary motoneurons is proportional to the level of Hh signaling, such that embryos lacking function of two of these Hhs have fewer primary motoneurons than wild types and embryos lacking function of all three Hhs have even fewer primary motoneurons (Beattie et al. 1997; Lewis and Eisen 2001), occasionally lacking primary motoneurons altogether (Lewis and Eisen 2001). However, a few rogue primary motoneurons typically remain, suggesting either that Hh signaling has not been entirely eliminated or that other signaling pathways may contribute to motoneuron specification.

Isolation of mutations in the *smoothened* (*smoh*) gene (*smooth muscle omitted*; *smu*) has also been extremely important in analysis of the role of Hh signaling in ventral spinal cord patterning (Barresi et al. 2000; Chen et al. 2001a; Varga et al. 2001). Just like *syu* mutants, *smu* mutants retain medial floor plate but lack lateral floor plate, again providing strong evidence that Hh signaling is not involved in specification of medial floor plate. Interestingly, though, medial floor plate begins to degenerate early in the second day of development in *smu* mutants, showing that Hh signaling is required for medial floor plate maintenance. *smu* mutants also form some primary motoneurons anteriorly, but this appears to result from the activity of maternal Smoothened (Chen et al. 2001a; Lewis and Eisen 2001). Taken together, these studies show that, in zebrafish, as in amniotes, Hh signaling is necessary for specification of at least the vast majority of motoneurons.

Because Hh signaling is unnecessary for medial floor plate specification, zebrafish must have another signaling pathway that carries out this function. Medial floor plate is absent from embryos homozygous for mutations in the *cyclops* (*cyc*; Hatta et al. 1991b) and *squint* (*sqt*) genes that encode Nodal-related members of the TGFβ family (Feldman et al. 1998), as well as in mutants for *one-eyed pinhead* (*oep*; Strähle et al. 1997; Odenthal et al. 2000), that

encodes an EGF-CFC protein that acts as an extracellular cofactor for Nodal signaling (Gritsman et al. 1999). Thus Nodal signaling is required for medial floor plate specification. Interestingly, however, medial floor plate does form later in *cyc* and *oep* mutants, suggesting that other pathways may be involved as well (Strähle et al. 1997).

Does this mean that floor plate specification in zebrafish differs from that in amniote vertebrates? Although the precise role of Nodal signaling in floor plate specification in mouse is currently unclear, in part because of the severity of the *Nodal* mutant phenotype (Zhou et al. 1993; Conlon et al. 1994), a number of recent studies suggest a link between Nodal and Shh signaling. For example, mouse embryos transheterozygous for mutations in *Nodal* and *Smad2*, a downstream effector of Nodal signaling, have many ventral CNS defects similar to mouse *Shh* mutants (Normura and Li 1998) and mutations in human TGIF, that acts as a Smad2 transcriptional corepressor, also result in defects in the ventral CNS similar to those caused by *Shh* mutations (Gripp et al. 2000). Consistent with these mammalian studies, Nodal signaling has been shown to induce *shh* expression in ventral neural tubes of zebrafish and chick embryos (Müller et al. 2000). Based on these studies, Müller and colleagues (2000) have proposed that zebrafish medial floor plate specification requires Nodal signaling to initiate expression of *shh* and *twhh* and then Shh and Twhh signaling initiate expression of downstream floor plate genes. It will be important to learn whether this model is correct and to resolve whether a similar pathway acts during floor plate specification in amniotes.

3.3 Delta/Notch Signaling Segregates Neural Fates Within Neural Plate Domains

Because different cell types arise at different mediolateral positions within the spinal cord neural plate, the neural plate can be thought of being composed of domains, each of which forms a longitudinal stripe. Several of these stripes can be defined as "proneural" domains, territories that express particular transcription factors of the bHLH class (Sommer et al. 1996; Blader et al. 1997a; Korzh et al. 1998). Two of these domains have been well studied: the lateral domain that generates neural crest and RB neurons, and the more medial domain that generates primary and secondary motoneurons. In each case, the choice of cell fate relies on signaling via the Delta/Notch signaling pathway. Thus, in the lateral neural plate, cells strongly expressing the bHLH gene, *ngn1,* become RB neurons (Blader et al. 1997a). These cells are isolated from one another and the cells between them become neural crest (Cornell and Eisen 2000). Studies in which signaling by the products of "neurogenic" genes, *delta* and/or *notch*, is blocked show that the surrounding cells are capable of becoming RBs, providing evidence that Delta/Notch mediated lateral inhibition prevents them from taking this fate (Appel and Eisen 1998; Haddon et al. 1998; Cornell and Eisen 2000). Similarly, in the medial neural plate domain,

Delta/Notch signaling prevents cells surrounding prospective primary motoneurons from taking this fate and they become secondary motoneurons instead, and possibly other cells types as well (Appel and Eisen 1998; Haddon et al. 1998). The roles of proneural and neurogenic genes are discussed in more detail elsewhere (see Appel and Chitnis, this Vol.).

3.4 Later Signals May Refine Cell Identity

Patterning of the spinal cord neural plate results in the appropriate number of neurons being specified at the right time and place. However, additional signals may be necessary to establish the final identities of these cells. For example, the neural crest cells that arise in the lateral neural plate generate both neural and non-neural derivatives. A variety of studies in zebrafish have begun to unravel both the signals and the transcriptional responses involved in specification of neural crest derivatives. As described in more detail (see Raible and Kelsh, this Vol.; Dorsky et al. 2000a), Wnt signaling is involved in establishing the pigment cell fate by regulation of *nacre*, the zebrafish gene encoding MITF (Dorsky 2000b).

Primary motoneurons also show later patterning. Initially, all of these cells express *islet1* that encodes a transcription factor shown to be required for the motoneuron fate in mouse (Pfaff et al. 1996). Later, specific primary motoneurons downregulate expression of *islet1* and express a related gene, *islet2* (Appel et al. 1995; Tokumoto et al. 1995). This dynamic change in gene expression follows somite formation and occurs in a segmental pattern within the spinal cord that mirrors the segmental pattern of the overlying somites, establishing a fine-grained AP pattern for this cell type. At least one type of ventral interneuron, VeLD, also shows this segmental pattern (Bernhardt et al. 1990; Eisen and Pike 1991). Recent studies have shown that *islet2* is required for normal development of the primary motoneurons that express it and when Islet2 function is knocked-down, these cells develop a VeLD-like morphology and express GABA, a VeLD neurotransmitter, rather than ACh, the normal primary motoneuron neurotransmitter (Segawa et al. 2001). The signals responsible for establishing the patterning of these cells are currently unknown, although circumstantial evidence from mutants with disrupted somites (Eisen and Pike 1991) and heat shock experiments (Kimmel et al. 1988) suggest that the signals could be of paraxial mesodermal origin. This idea fits well with the known role of paraxial mesoderm in determining motoneuron subtypes in chick, as demonstrated by spinal cord and somite reversals (Ensini et al. 1998). It also fits well with the results of transplantation studies (Eisen 1991; Appel et al. 1995; Fig. 3). These studies revealed that the fates of individual primary motoneurons are not fixed until after somitogenesis and that the precise AP position of each motoneuron soma within the spinal cord, and relative to the overlying somite boundaries, determines the cell's identity, as assayed by *islet* gene expression and axonal trajectory.

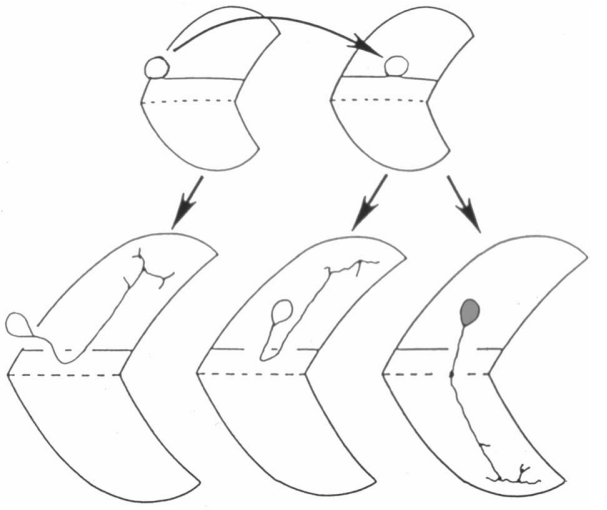


Fig. 3. Position determines the identity of primary motoneurons. An *islet1*-expressing primary motoneuron transplanted from the MiP soma position (*upper left*) to the same position (*lower left*) develops a normal MiP axonal trajectory in the overlying myotome. A similar cell transplanted to the CaP soma position (*upper right*) also develops a MiP axonal trajectory if the transplant is done within 1 hr of axogenesis (*lower middle*), but develops a CaP axonal trajectory if the transplant is done earlier (*lower right*). In this case, the cell also turns on expression of *islet2* (*gray shading*), a CaP-specific gene. (Adapted from Eisen 1991; Appel et al. 1995)

4 The Forebrain

The forebrain is the most complex region of the CNS, both in terms of its connectivity and also in terms of its morphogenesis. As with more caudal regions of the CNS, it derives from the simple neuroepithelial cell sheet of the neural plate. However, subsequent to neural tube formation, the anterior CNS undergoes a series of morphogenetic events that transform the simple neural tube and result in generation of the highly complex derivatives of the telencephalon, optic vesicles and diencephalon. It is fair to say that we know almost nothing about the mechanisms that regulate morphogenesis of the forebrain (although see Loosli et al. 2001). However, we are beginning to understand the genetic pathways that underlie allocation of regional fates within the anterior neural plate. In the following section, we review some of the progress that has been made in understanding how cells acquire their regional identities within the most rostral regions of the CNS.

The major derivatives of the anterior neural plate are the telencephalon, the optic vesicles, the hypothalamus, the ventral and dorsal thalamus and the pretectum (Fig. 4A,B). The telencephalon is the region that in mammals includes the cerebral cortex (what we normally consider to be the "thinking" part of the brain), the olfactory bulb, and the basal ganglia. Although defining the axes in

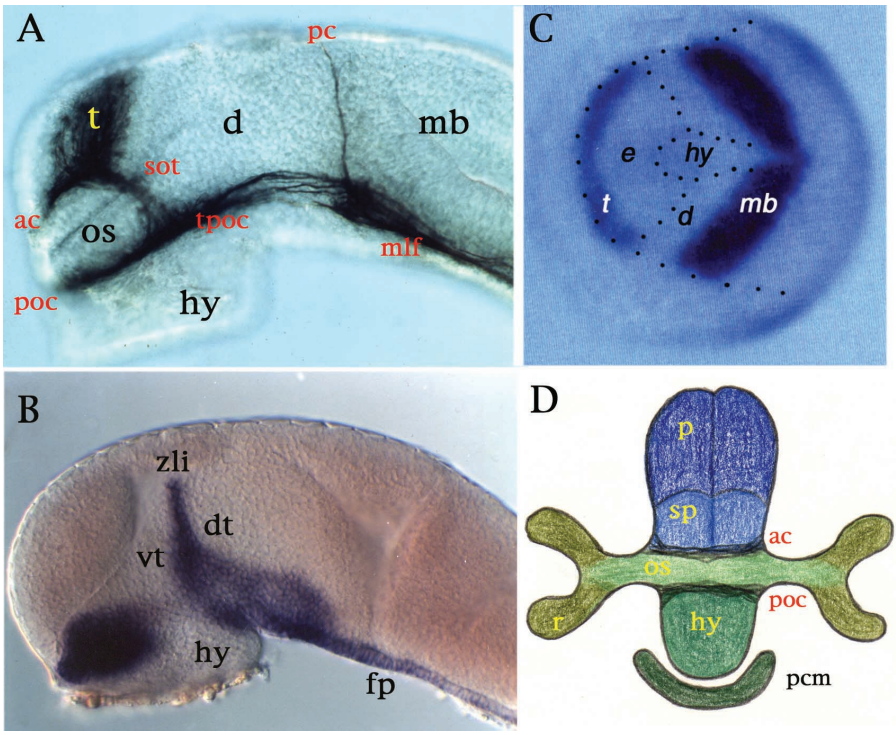


Fig. 4A–D. The embryonic zebrafish forebrain. **A** Lateral view of a 1-day-old zebrafish brain labeled with an antibody to acetylated tubulin (*black axons*). The major regions of the brain are labeled in *black* or *yellow* and the axon pathways in *red*. The eye, which is an evagination of forebrain tissue, has been removed, revealing the optic stalk positioned at the interface between telencephalon and diencephalon. Adapted from Macdonald et al. (1994). **B** Lateral view of a 1-day-old zebrafish showing expression of the *shh* gene (*blue*). *shh* expression extends into the dorsal brain along the *zli*, dividing the dorsal diencephalon into ventral thalamus and dorsal thalamus (plus pretectum – not indicated). Adapted from Barth and Wilson (1995). **C** Dorsal view of the anterior neural plate showing expression (*blue*) of *anf* in the prospective telencephalon and *pax2.1* in the prospective midbrain. The *dots* show the approximate positions of cells that will contribute to forebrain structures (see, e.g., Varga et al. 1999). At this stage, prospective hypothalamic cells are still moving rostrally within the anterior neural plate displacing eye field cells laterally (and hence into left and right eyes). Cells that remain medially in the eye field probably primarily contribute to the optic stalks (Varga et al. 1999). Adapted from unpublished work of D. Delaney and S.W. Wilson. **D** Cartoon of a frontal view of a highly schematized forebrain showing the major domains of the telencephalon (pallium and sub-pallium), the optic vesicles (retina and optic stalks) and the anterior ventral diencephalon (hypothalamus). At earlier stages, the brain is underlain by prechordal mesendoderm (sometimes called the prechordal plate). By the stage cartooned, the prechordal mesendoderm would have dispersed to other sites. *ac* Anterior commissure; *d* diencephalon; *dt* dorsal thalamus; *e* eye field; *fp* floor plate; *hy* hypothalamus; *mb* midbrain; *mlf* medial longitudinal fasciculus; *os* optic stalk; *p* pallium; *pc* posterior commissure; *pcm* prechordal mesendoderm; *poc* postoptic commissure; *r* retina; *sot* supraoptic tract; *sp* subpallium; *t* telencephalon; *tpoc* tract of the post-optic commissure; *vt* ventral thalamus; *zli* zona limitans intrathalamica

the forebrain is problematic (see below), the cortex is considered to be a dorsal, pallial telencephalic subdivision while the basal ganglia are considered to be ventral, sub-pallial derivatives (Wilson and Rubenstein 2000). The adult fish telencephalon is very different from the adult mammalian telencephalon and neuroanatomical homologies remain uncertain. However, pallial and sub-pallial telencephalic divisions are evident in all vertebrates and there is no reason to think that early stages of telencephalic development vary greatly between species. Indeed, the extensive similarities in gene expression patterns between mammals and fish during early forebrain development suggest that very similar genetic mechanisms underlie regional patterning of the telencephalon in all vertebrates.

The optic vesicles derive from cells located between telencephalic and diencephalic regions of the neural plate (Varga et al. 1999; Fig. 4C). The distal/lateral regions of the vesicles give rise to the retinal components (neural retina and pigment epithelium) of the eyes, while the proximal portions form the optic stalks that later contribute glial cells to the optic nerves. Other components of the eyes, such as the lens, cornea and sclera, are primarily derived from the surface ectoderm overlying the optic vesicles or from neural crest cells that migrate around and into the eye cups (see Easter and Malicki, this Vol.).

Several diencephalic territories are formed from cells caudal and ventral to the eye-forming regions of the neural plate. In dorsal regions of the forebrain, the diencephalon generates (in anterior to posterior sequence) ventral thalamus, zona limitans intrathalamica (zli), dorsal thalamus and pretectum. The zli is considered to be an important AP boundary within the forebrain separating chordal from epichordal regions of the CNS (Rubenstein et al. 1998; Zeltser et al. 2001) and although various mutations are known to disrupt formation of the zli in fish (e.g., Macdonald et al. 1994), little is known about the function of this prominent boundary. Indeed, with the exception of the epithalamus (see below), little work has been done on dorsal diencephalic development in zebrafish.

The most medial region of the anterior neural plate forms the hypothalamus (Fig. 4C), a brain region involved in regulation of autonomic and endocrine functions. Fate mapping studies (Varga et al. 1999; Mathieu et al. 2002; Woo, Shih and Fraser, pers. comm.) have shown that hypothalamic precursors originate in more posterior regions of the embryo and subsequently move through the neural plate to arrive at their final anterior location. This discrete origin suggests that, at least at early stages, the hypothalamus should be considered a separate compartment from the more dorsal forebrain territories that it eventually comes to underlie. The hypothalamus (or at least parts of it) may be considered an anterior extension of the floor plate and indeed the same genetic pathways are implicated in both floor plate and hypothalamus formation.

To date, studies in zebrafish have contributed very little to our understanding of the later steps of forebrain development where the greatest progress has come from analysis of transgenic and knockout mice. In contrast, the earliest

steps in patterning the forebrain are currently better understood in zebrafish than in other vertebrate model systems and it is upon these early stages of forebrain patterning in fish that we will focus.

4.1 DV Patterning of the Zebrafish Forebrain

Unlike the midbrain, hindbrain and spinal cord, the anterior ventral forebrain forms neither floor plate nor motoneurons. Despite this, the Nodal- and Hh-signaling pathways are as crucial for development of ventral tissues in the forebrain as they are elsewhere in the CNS. In embryos carrying mutations affecting these signaling pathways, defects are observed in development of the hypothalamus, patterning of the optic vesicles and establishment of ventral regions of the telencephalon. As is the case in more posterior regions of the CNS, the exact epistatic relationship between Nodal and Hh signals is not entirely clear but some inroads have been made in dissecting the relative contribution of these two pathways to ventral forebrain development.

The text-book view of ventral forebrain development suggests that mesendodermal tissues of the prechordal plate migrate underneath the developing forebrain and send signals that induce the hypothalamus and split the eye field into left and right eyes (Kiecker and Niehrs 2001; Roessler and Muenke 2001). This view is probably largely correct, but is certainly a simplification of the true situation as it has proved to be extremely difficult to dissociate the role of the prechordal plate from that of the overlying neural midline tissue. Both originate in a region of the embryo close to the shield and both express receptors and ligands of the Nodal- and Hh-signaling pathways. Thus, while we can be certain that axial tissues are crucial for patterning ventral cell types in the forebrain, the relative contribution of the axial mesendoderm versus the axial neural ectoderm remains to be determined.

4.1.1 Formation of the Hypothalamus

The embryonic hypothalamus is usually considered to equate to the ventral regions of the forebrain that lie below the tract of the postoptic commissure (Fig. 4). This definition is rather vague and includes some territories, such as posterior tuberculum, that would not classically be defined as hypothalamic. However, until better markers of specific regions and nuclei are developed, this and other working definitions will have to suffice. Defects in early hypothalamic development are usually associated with varying degrees of cyclopia (see below) and, as this is an easy phenotype to distinguish, many mutations affecting the hypothalamus have been isolated. Most of these mutations fall into either the Hh- or the Nodal-signaling pathways.

A role for the Hh-signaling pathway in hypothalamic development is supported by studies in other species which have shown that hypothalamus is missing in mice lacking Shh (Chiang et al. 1996) and that Shh can promote

hypothalamic fates in in vitro assays (e.g., Dale et al. 1997). Indeed, it is generally assumed that Hh signaling is essential for hypothalamic induction in amniotes. However, in zebrafish, some degree of hypothalamic development occurs in all known Hh pathway mutants. Most notably, in *smu* mutant embryos, early expression of the hypothalamic marker *nk2.1a* occurs and it is only later that hypothalamic tissue becomes severely reduced (Rohr and Concha 2000; Varga et al. 2001). One interpretation of these observations is that Hh signaling is required for the maintenance of the hypothalamus but not for its early induction. However, a major caveat is that residual Hh signaling is present at early stages in *smu* mutants (Chen et al. 2001a; Varga et al. 2001), and this early Hh activity may be sufficient to mediate early hypothalamic induction.

Although an absolute requirement for Hh signaling in hypothalamic induction is not established, it is clear that Hh activity is required for subsequent patterning of the ventral diencephalon. For instance, the homeobox gene *nk2.2* is expressed in a band of cells along the dorsal boundary of the hypothalamus and expression of this gene is reduced or absent in *you-too* (*yot*/*gli2*), *syu* and *smu* embryos (Karlstrom et al. 1999; Rohr et al. 2001; Varga et al. 2001). Thus, cell fates lateral (dorsal) to the ventral midline of the brain are more severely affected in Hh pathway mutants than the ventral midline tissue itself. This is highly reminiscent of the situation in more posterior regions of the CNS where medial floor plate is always present in Hh pathway mutants but more lateral fates, such as lateral floor plate and motoneurons, are reduced or absent (Odenthal et al. 2000; Chen et al. 2001a; Etheridge et al. 2001; Lewis and Eisen 2001).

In contrast to the Hh pathway, there is no doubt that the Nodal-signaling pathway is required for establishment of all hypothalamic tissue. *cyc*, *sqt*, *oep* and maternal-zygotic *schmalspur* (*MZsur/fast1*) mutant embryos all exhibit cyclopia and have reduced, or more often an absence of, hypothalamic tissue (Feldman et al. 1998; Rebagliati et al. 1998a,b; Sampath et al. 1998; Gritsman et al. 1999; Pogoda et al. 2000; Sirotkin et al. 2000a,b; Rohr et al. 2001, and references within). However, despite these striking phenotypes, where, when and how Nodal signals are required for establishment of hypothalamus is still unclear. Indeed it seems likely that Nodal signaling may act at different times and in different places to mediate different aspects of hypothalamic development. First, as mentioned above, hypothalamic precursors originate close to the shield and subsequently move anteriorly to their final position in the medial region of the anterior neural plate. This movement is compromised in *cyc* mutants (Varga et al. 1999), and presumably other Nodal pathway mutants. Therefore, one key role for Nodal signaling may be to facilitate movement of prospective hypothalamic tissue to the correct position within the neural plate for the cells to receive the signals that establish their hypothalamic identity.

Nodal signals are likely to be among those that are received by prospective hypothalamic tissue once it is positioned within the anterior neural plate. The

Nodal ligand Cyc is expressed in the prechordal plate underlying the hypothalamus and also within the prospective hypothalamus itself (Rebagliati et al. 1998a,b; Sampath et al. 1998). However, to disentangle the role of Cyc within these tissues from its earlier role in axial development is not easy. The best evidence that Nodal signals must be received by prospective hypothalamic cells has come from recent experiments in which Nodal signaling has been manipulated either in prechordal plate or in ventral brain (Matthieu et al. 2002). From these studies, it appears that cells that cannot receive Nodal signals are unable to contribute to the most ventral hypothalamus but can become part of more dorsal hypothalamic tissue. Similarly, restoration of Nodal activity in the prechordal plate but not the brain leads to recovery of dorsal but not ventral hypothalamus. Overall then, our current hypothesis is that locally acting Nodal signals are required for establishment of ventral hypothalamic fate while Hh signals are required for proper development of more dorsal hypothalamic fates. As Hh expression is lost in anterior regions of Nodal pathway mutants, both Hh activity and Nodal activity are compromised in these mutants. This disruption of both signaling pathways may explain the severity of the hypothalamic phenotypes in this class of mutants.

4.1.2 Establishment of the Optic Stalks

As prospective hypothalamic cells move anteriorly within the neural plate, they displace cells in medial regions of the eye field to more lateral positions where they will subsequently give rise to left and right retinas (Fig. 4C,D). Although fate mapping studies are incomplete, it is likely that those cells of the eye field that remain close to the midline form the optic stalks (Varga et al. 1999) which connect the retinal compartments of the eye to the brain. Mutations that disrupt the specification and/or migration of the hypothalamus or underlying axial tissues also usually affect optic stalk development. In the most severe cases, optic stalk tissue fails to be specified at all and instead medial regions of the eye field form retina resulting in cyclopia (Hatta et al. 1994; Macdonald et al. 1995). Such observations suggest that signals either from prospective hypothalamus or from underlying axial tissues promote optic stalk identity and, once again, the Hh pathway is implicated in these fate determination events.

The first indication that Hh signaling regulates early regional patterning of the optic vesicles came from analysis of embryos in which Hh signals were overexpressed. In the normal optic vesicle, *pax2.1 (no-isthmus; noi*) expression defines the compartment that will form optic stalks whereas *pax6.1* expression defines the compartment that will form retina (Macdonald et al. 1995; Macdonald and Wilson 1997). In embryos with increased Hh activity, *pax2.1* expression expands throughout the optic vesicles while *pax6.1* expression is suppressed (Ekker et al. 1995; Macdonald et al. 1995). This suggests that graded Hh activity contributes to subdivision of the optic vesicles into proximal optic

stalk tissue and distal retinal tissue. In support of this hypothesis, mice lacking Shh function lack *pax2* expression and have fused retinas (Chiang et al. 1996). In zebrafish, *shh*/*syu*) mutants have robust *pax2.1* expression within the optic vesicles (Schauerte et al. 1998) raising doubts as to the requirement for Hh signals in optic stalk induction. However, recent data has shown that *pax2.1* expression is absent in *smu* mutants (Varga et al. 2001) indicating that another Hh protein, probably Twhh, can compensate for the loss of Shh alone.

Altogether, these results are consistent with the possibility that Hh signals derived from midline tissue induce medial eye field cells to form optic stalk and not retina. However, as usual, the story is more complicated than this and several outstanding issues still need to be resolved. First, although *pax2.1* expression is lost in *smu* mutants, these embryos do not exhibit as severe cyclopic defects as Nodal pathway mutants. Therefore, there may be other optic-stalk-promoting signals (perhaps including Nodals themselves) that are lost in Nodal pathway mutants. Second, perhaps most intriguing of all, is the observation that *pax2.1* expression is restored in embryos with very severely compromised Nodal activity (Feldman et al. 2000; Masai et al. 2000). There appears to be no Hh gene expression in the anterior brain in these embryos, and so one is faced with the possibility that optic-stalk-specific gene expression can be induced in the absence of Nodal and Hh signaling. This phenomenon is not yet understood but one possibility is that Hh signaling is required to overcome repression of optic stalk identity. If the repression is alleviated in severely Nodal-depleted embryos, then *pax2.1* expression may recover, even in the absence of Hh activity. This "repression of repression" model again has parallels in more posterior regions of the CNS. In caudal regions of mouse embryos, Gli3 appears to act as a repressor of ventral fates and, if Gli3 activity is removed in embryos lacking Shh activity, then ventral fates, which are lost in *Shh* single mutants, are restored (Litingtung and Chiang 2000). Thus Hh activity is required to overcome Gli3-mediated repression of ventral cell fate identity in the spinal cord. It will be interesting to see if a similar mechanism is operating in the forebrain and eyes.

Mutations that disrupt the anteriorly directed movement of axial cells during gastrulation can also lead to reduced or absent optic stalks and cyclopia (Heisenberg and Nüsslein-Volhard 1997; Marlow et al. 1998). In these mutants, which include *silberblick* (*slb*/*wnt11*; Heisenberg et al. 2000) and *knypek* (*kny*/*glypican4/6*; Topczewski et al. 2001), signals required for optic stalk development, such as Shh and Twhh, are still present. However, the disrupted anterior movement of tissues that are the source of these signals (prechordal mesendoderm and perhaps prospective hypothalamic tissue) has the consequence that the axially derived signals fail to act at the correct place and time to properly pattern the eye field. Analysis of these mutants emphasizes the fact that the genes regulating morphogenetic events that bring tissues into proximity with each other are just as crucial to forebrain development as the genes that encode the signals that specify cell fate.

4.1.3 Establishment of Ventral Telencephalic Fates

Ventral sub-pallial telencephalic markers are reduced or absent whereas dorsal pallial markers are unaffected or expanded in zebrafish embryos carrying mutations in the Hh-signaling pathway (Rohr et al. 2001; Varga et al. 2001). Reciprocal results are observed in Hh signaling gain-of-function experiments in fish and other species (reviewed in Wilson and Rubenstein 2000). Recent studies in mice have suggested that the role for Hh signals in the telencephalon may once again be to overcome Gli3-dependent repression of sub-pallial identity (G. Fishell, pers. comm.). Thus Hh signaling appears to play comparable roles in the optic vesicles and in the telencephalon, in both cases affecting the allocation of regional identities within the compartment, without affecting the commitment of cells to a telencephalic or to an optic vesicle fate.

Ventral telencephalic markers are also absent in Nodal pathway mutants; however, this may primarily be due to the secondary loss of Hh signaling in anterior regions of the mutants (Rohr et al. 2001). Thus, if Hh signaling is restored to embryos completely lacking Nodal activity, then telencephalic cells express sub-pallial markers. This indicates that, even in the absence of Nodal activity, Hh activity is sufficient to induce ventral telencephalic identity. However, this does not imply that Hh signals are the only mediators of early DV patterning in the telencephalon. Indeed, Fgf signaling also influences the development of sub-pallial territories. In *acerebellar* (*ace/fgf8*) mutants, expression of the sub-pallial marker *nk2.1b* is reduced (Shanmugalingam et al. 2000) and, in embryos with more severely compromised Fgf signaling, expression of this marker is absent altogether (Shinya et al. 2000). How the Fgf- and Hh-signaling pathways interact to promote subpallial development will be an interesting area of investigation in the coming years.

4.1.4 Specification of Dorsal Forebrain Fates

To date, very few studies in zebrafish have addressed the mechanisms by which dorsal forebrain fates are established. However, the role of the Bmp pathway in regulating DV patterning of neural tissue appears to be conserved at all AP levels of the CNS (Nguyen et al. 1998b, 2000; Barth et al. 1999, and references cited therein). For instance, Bmp signaling appears to define the extent and position of DV subdivisions of the forebrain such as the epithalamus, the dorsal-most region of the diencephalon. *flh* is a homeobox gene required for epithalamic neurogenesis (Masai et al. 1997) and for notochord development (Talbot et al. 1995). Within the brain, *flh* expression is very tightly restricted to the epithalamic region of the dorsal diencephalon. Analysis of embryos with variably increased or decreased levels of Bmp activity has shown the medial and lateral extent of *flh* expression within the prospective epithalamic region of the neural plate is set between thresholds of Bmp activity (Barth et al. 1999). Thus, *flh* is not expressed in medial regions as Bmp activity is too low and it is not expressed beyond the limits of the neural plate where Bmp signaling is too high. Even

in embryos lacking all axial tissues, *flh* expression remains absent from medial regions of the neural plate (Concha et al. 2000) indicating that repressive signals from ventral tissue are not required to limit *flh* expression to dorsal regions. Although the Bmp pathway has a profound influence on DV patterning of neural tissue, it has little or no effect on AP patterning of the CNS. Thus, in all Bmp pathway mutants, *flh* expression remains limited to the same AP position within the embryo even though the DV extent of expression can vary enormously.

4.2 Left/Right Patterning in the Brain

Although the CNS is largely symmetrical, there are functionally important differences between left and right sides. All vertebrate, and many invertebrate, animals exhibit lateralized behaviors that are mediated by differences in neuroanatomy between the left and right brain. For instance, in humans it is well established that aspects of language processing are dominant in the left hemisphere of the cortex. Lateralization is not limited to the telencephalon and, in most vertebrates that have been studied, the epithalamic region of the diencephalon is lateralized. While the function of this lateralization is currently unknown, its presence in all classes of vertebrates indicates strong evolutionary conservation (Concha and Wilson 2001).

Neuroanatomical differences between left and right sides of the epithalamus appear relatively early in zebrafish development and are evident as two prominent asymmetries (Concha et al. 2000). The first is a left-side-specific photoreceptive nucleus called the parapineal that is located just anterior to the larger midline photoreceptive epiphysis or pineal organ (Fig. 5). The parapineal projects to a second lateralized nucleus in the epithalamus, termed the habenula. Habenular nuclei are present on both sides of the brain, but the left nucleus is larger, at least in terms of labeling of neuropil. Recent studies have shown that the Nodal-signaling pathway determines the laterality of these two asymmetries (Concha et al. 2000). This pathway also appears to influence the position of the pineal stalk in adult fish (Liang et al. 2000).

An involvement for Nodal signals in regulating brain laterality seemed likely from analysis of the expression of various genes that function in the Nodal pathway, some of which show left-side-restricted expression (Bisgrove et al. 2000; Concha et al. 2000; Liang et al. 2000; see also Wright and Halpern, this Vol.). It is likely that cells on both sides of the epithalamus are able to respond to Nodal signals, but the signals themselves may be restricted to the left side and, consequently, genes expressed in response to Nodal activity are only induced on the left side. In embryos with disrupted Nodal signaling, parapineal and habenular asymmetries are always established and so Nodal activity is not required to specify asymmetry (Concha et al. 2000). However, the laterality of these CNS asymmetries is totally randomized in mutants.

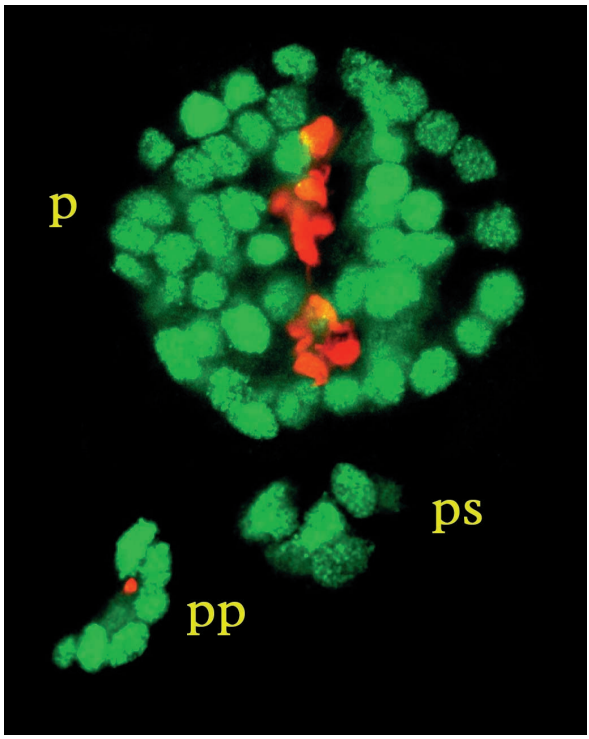


Fig. 5. Asymmetry in the epithalamus. Composite of confocal images of dorsal views looking onto the epithalamic region of the diencephalon. Neurons are labeled with an antibody to Islet1/2 (*green*) and to opsin (*red*). The opsin labeling in the pineal organ (epiphysis) indicates the position of the dorsal midline. Pineal neurons are located to both left and right sides of the midline. However, deeper into the brain, the parapineal organ is located only on the left side of the diencephalon. *p* Pineal; *ps* pineal stalk; *pp* parapineal. (Adapted from Concha et al. 2000 and courtesy of Miguel Concha)

Wheareas over 95% of wild-type fish are "left-brained", fish lacking lateralized Nodal signaling are 50% "left brained" and 50% "right-brained".

It is still early days for studies of laterality and asymmetry in the brain and one hopes that directed genetic screens should reveal many new genes that act in the pathways leading to the establishment of a lateralized CNS. Further discussion of left/right patterning in the brain and in other organ systems can be found elsewhere (see Wright and Halpern, this Vol.).

4.3 AP Patterning of the Prospective Brain

4.3.1 Establishment of Early AP Pattern in the Neural Plate

Several years ago, transplantation studies demonstrated that germ ring cells produce posteriorizing signals that inhibit development of anterior neural fates (Woo and Fraser 1997; Koshida et al. 1998). However, posteriorizing signals appear to be absent, at least at early stages, from the dorsal organizer region (shield), and indeed the organizer probably actively antagonizes such signals. These studies suggested that AP positional values within the neural

plate might be established by cells reading their positions with respect to their distance from the germ ring and shield. Our understanding of the signals that arise from the organizer and germ ring and influence AP pattern are still rudimentary. However, several recent studies have provided compelling evidence that Wnt proteins (particularly Wnt8) contribute to the posteriorizing activity and that Wnt antagonists (such as Dkk) at least partially underlie the ability of the organizer to antagonize such signals [see Hibi et al., this Vol., for a more detailed consideration of these issues in zebrafish and Yamaguchi (2001) for a discussion of Wnt activity in AP patterning in other species].

The complex activity of germ ring cells in both producing and inhibiting signals that regulate AP patterning is evident from analysis of Nodal pathway and other mutants that affect the establishment of germ ring fates (reviewed in Wilson and Rubenstein 2000; updated in Erter et al. 2001). Simplistically, most results suggest that, if induction of dorsal germ ring fates is compromised, then the activity of posteriorizing signals from more lateral and ventral germ ring cells will be increased and anterior fates, such as telencephalon, will be reduced. However, if induction of germ ring fates is more widely and severely compromised, then induction of the posteriorizing signals will be compromised and anterior neural fates will expand.

Confirmation that Wnt8 activity in vivo promotes posterior and suppresses anterior development has come from several studies in which increased or decreased Wnt8 activity has been shown to directly correlate with reduced or increased specification of anterior fates (Erter et al. 2001; Levken et al. 2001 and references cited therein). For instance, embryos that lack most or all Wnt8 activity exhibit a hugely expanded forebrain and lack hindbrain and spinal cord fates. Furthermore, suppression of Wnt activity can often restore anterior CNS fates in embryos that normally lack these structures due to mutations that directly or indirectly lead to enhanced Wnt activity in the germ ring (e.g., Fekany-Lee et al. 2000; Hashimoto et al. 2000; Kim et al. 2000a; Shinya et al. 2000; Erter et al. 2001).

Although these various studies implicate Wnts and Wnt antagonists in AP CNS patterning, it is uncertain how Wnt signals that originate in the germ ring, and are thought to diffuse poorly, could directly influence global AP pattern in the CNS. If establishment of global AP positional values is indeed a direct effect of Wnt8, then we have much to learn about the transport of Wnt proteins throughout the embryo. An alternative or additional possibility is that Wnt8 may influence the activity of other genes, possibly including Wnt pathway components that secondarily influence AP pattern. As we describe below, modulation of Wnt activity continues to influence AP patterning throughout gastrulation, and it appears that local activity of Wnt agonists and antagonists within the forming anterior neural plate influences regional fate determination in the forebrain.

4.3.2 Local Induction of the Telencephalon and Eyes

The telencephalon is generated from cells around the anterior margin of the neural plate (Varga et al. 1999; Whitlock and Westerfield 2000) and can therefore be defined both as "anterior", as it forms from cells at the front of the neural plate, and as "dorsal", as it derives from cells at the margin of the neural plate. Because of this origin, mutations that affect either the establishment of dorsal neural cell fates, such as Bmp pathway mutants, or the establishment of AP positional values both affect early development of the telencephalon. With respect to the Bmp pathway, it seems likely that induction of telencephalon can only occur between certain thresholds of Bmp activity (Barth et al. 1999). As described above, if Bmp activity is high then non-neural fates are specified, if Bmp signaling is low, then medial neural plate fates are promoted. Between these two extremes, cells at the anterior margin of the neural plate are competent to form telencephalon, if exposed to the correct inducing signals.

Cells at the anterior margin of the neural plate have the ability to non-autonomously induce telencephalic gene expression when transplanted to more posterior regions of the neural plate (Houart et al. 1998). Furthermore, when such cells are ablated, telencephalic gene expression is reduced or absent supporting the notion that anterior neural plate cells produce signals that induce the telencephalon. Among these signals is a member of the sFRP family of secreted Wnt inhibitors, termed Tlc (Houart et al. 2002). sFRPs have a structure resembling the extracellular domain of proteins of the Wnt coreceptor Frizzled family and it is thought that they bind to Wnt ligands, thereby preventing them from interacting with the Frizzled receptors (Leyns et al. 1997; Wodarz and Nusse 1998). Tlc-expressing cells have the same activity as anterior marginal cells in that they can non-autonomously induce telencephalic gene expression and suppress midbrain-specific gene expression. Furthermore, abrogation of Tlc activity leads to a reduction of telencephalic gene expression confirming an in vivo requirement for this gene. These studies imply that local antagonism of Wnt signals in the anterior neural plate promotes telencephalic identity. They also imply that Wnt ligands are present within the telencephalon-forming region of the anterior neural plate. In theory, such ligands could derive from neighboring non-neural or neural ectoderm or from the underlying mesendoderm or yolk syncytium. Among the candidate genes for being locally acting inhibitors of telencephalic identity are *wnt8b* and *wnt1* (Houart et al. 2002), both of which are expressed in the neural plate caudal to the telencephalon (Kelly and Moon 1995; Kelly et al. 1995b).

masterblind (*mbl*) is one of very few mutations known to directly affect allocation of regional fates within the anterior neural plate. In *mbl* mutant embryos, there is a transformation of telencephalic and eye fates to more posterior diencephalic identity (Heisenberg et al. 1996, 2001; Masai et al. 1997). Recent studies have shown that the *mbl* mutation affects Wnt signaling

in the anterior neural plate (Heisenberg et al. 2001; van de Water et al. 2001). The *mbl* mutant phenotype is due to a mutation in the *axin1* gene that is predicted to generate an Axin protein that cannot bind GSK3 (Heisenberg et al. 2001). Axin normally functions in an intracellular complex that includes GSK3 and several other proteins (Ikeda et al. 1998; Kishida et al. 1999). In the absence of Wnt activity, this complex is responsible for degrading β-catenin, thereby preventing activation of Wnt target genes. When Wnts bind their receptor complex, Axin interacts with the Wnt coreceptor LRP5/6 and the activity of the Axin/GSK3 complex is inhibited (Mao et al. 2001). This allows accumulation of β-catenin and activation of Wnt target genes. As the *mbl* mutation affects the ability of Axin1 to bind to GSk3, it is predicted that the Axin1 complex will not function correctly in *mbl* mutant embryos, β-catenin will accumulate and Wnt target gene activation will occur. If correct, then this suggests that telencephalon and eye-forming regions of the neural plate can be converted to diencephalon through overactivation of Wnt signaling. Thus, suppression of Wnt signaling may be important not only for induction of the telencephalon, but also for induction of the eyes. Altogether, these results raise some intriguing questions. It appears that manipulating Wnt activity either within the germ ring/organizer or within the anterior neural plate can both lead to altered specification of anterior neural plate fates. However, it remains unknown how early Wnt signaling in the germ ring is linked to later Wnt signaling in the neural plate. We clearly need to know a lot more about where and when Wnt signals are active if we are to understand how these spatially and temporally complex events are integrated.

5 The Midbrain and Hindbrain

Caudal to the forebrain lies the midbrain or mesencephalon, followed by the isthmus rhombencephali, a region considered the most rostral portion of the hindbrain or rhombencephalon. The midbrain includes dorsally the tectum, which is separated by the tectal ventricle from the ventral tegmentum and the ventrolateral torus semicircularis. The tectum and torus semicircularis derive from the alar plate of the neural tube and predominately serve as major relay centers for sensory information derived from the eyes, ears and lateral line organs, whereas the tegmentum derives from the basal plate and contains several motor nuclei, among them those of the occulomotor (III) and trochlear (IV) nerves that are involved in directing eye movements. In a 1-day-old zebrafish embryo, the isthmus forms a prominent fold, which contains dorsally the cerebellar primordium. As with other brain regions discussed so far, midbrain and isthmus development start during gastrulation and initially occur in close association. During early somitogenesis, the two territories become molecularly distinct and at late somitogenesis the sections of the neural tube giving rise to the midbrain and isthmus fold up as two clearly separable mor-

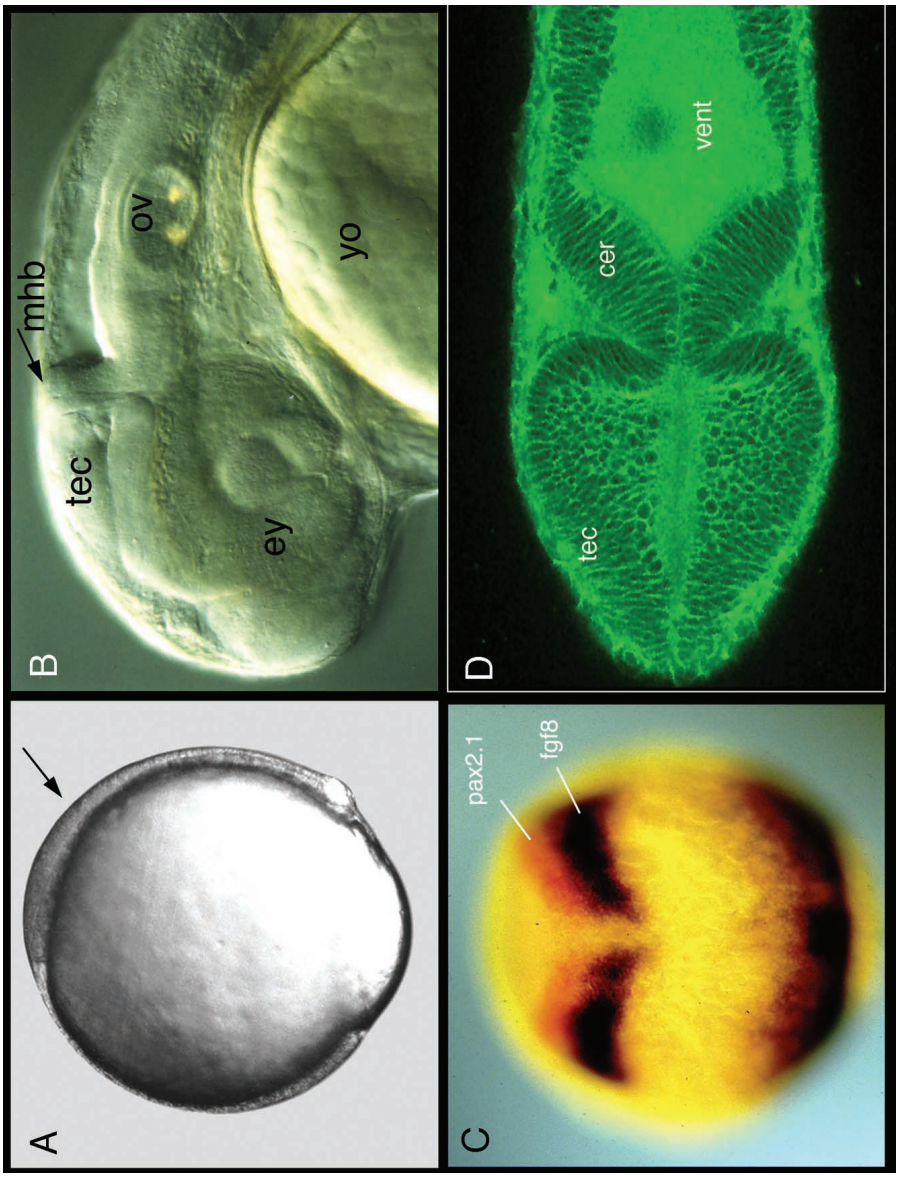
phogenetic domains, forming the prominent fold referred to as the midbrain-hindbrain boundary (MHB; Fig. 6).

Although the histological features of this area have been well described for the adult zebrafish brain (Wullimann et al. 1996), little is known so far about the connectivity, physiology or underlying function of the various midbrain and isthmic structures. As we argued above for the forebrain, the differences in adult neuroanatomy between fish and mammals make it difficult to determine homologies between brain parts at the adult stage, whereas at embryonic stages the similarities, especially in gene expression patterns, suggest a much more conserved relationship. By studying how this brain area is patterned during embryonic stages, we can therefore hope to understand mechanisms that are common to all vertebrates. For instance, studies of the zebrafish midbrain have long served as an excellent model for development of a simple visual system in vertebrates, because it becomes functional after only about 3 d (Easter and Nicola 1996; Karlstrom et al. 1997).

One major reason for interest in the midbrain-hindbrain region is that it contains a cell population acting as an organizer of cell fate, the MHB organizer or isthmic organizer, that was initially discovered by experimental manipulations in chick. When MHB tissue is transplanted into caudal forebrain, the surrounding host tissue switches fate, adopting isthmic or midbrain character; in the rhombencephalon, cerebellar fate is induced (Martinez and Alvarado-Mallart 1990; for reviews, see Rhinn and Brand 2001; Wurst and Bally-Cuif 2001). These experiments suggest that MHB tissue also acts as an organizing center in its normal location, and that differential competence of the host tissue determines the nature of the induced structures. Work on the MHB organizer has now shed light on the poorly understood mechanisms involved in AP patterning of the brain, and it may serve as a paradigm for organizers acting in other brain regions.

5.1 Midbrain and Hindbrain Development Starts in Gastrulation

As with the other CNS regions, development of the midbrain and hindbrain starts during gastrulation. Fate map studies have indicated that, by the end of gastrulation, the midbrain and hindbrain precursors occupy largely non-overlapping, bilateral, v-shaped domains in the dorsal neuroectoderm (Woo and Fraser 1995); the isthmus is not yet separate from the midbrain domain at this stage (Müller et al. 1996b). Among the earliest genes to be activated are *pax2.1*, *her5* and *wnt1* in the midbrain domain (Krauss et al. 1991, 1992; Müller et al. 1996b), and *fgf8* in the immediately abutting anterior hindbrain domain, at around 70–80% of epiboly, a time when these territories are not yet morphologically distinct (Fig. 6; Reifers et al. 1998). Genetic studies of mutants with discrete lesions in the midbrain-hindbrain domain show that, at this stage of development, the two domains do not yet influence each other.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no isthmus (*noi/pax2.1*) mutant embryos have no MHB, tectum and cerebellum at 1 d. *noi* mutant embryos fail in the earliest stages of midbrain and isthmus development to activate the *pax2.1* target genes *engrailed2* (*eng2*) and *engrailed3* (*eng3*), but have normal activation of *fgf8* in the adjacent anterior hindbrain, showing that the early hindbrain primordium develops in the absence of functional *pax2.1* (Brand et al. 1996b; Lun and Brand 1998). Conversely, the *acerebellar* (*ace/fgf8*) mutation, or *fgf*-8MO, causes absence of the MHB and cerebellum, but these embryos have normal activation of *pax2.1*, showing that midbrain development is initially normal in the absence of functional Fgf8 (Brand et al. 1996b; Reifers et al. 1998; Araki and Brand 2001; Draper et al. 2001). Furthermore, knockout studies of *Wnt1* in mice are known to cause absence of the midbrain and MHB (McMahon et al. 1992). Zebrafish *wnt1* is activated around the same time as *pax2.1* (Kelly and Moon 1995). Expression of *wnt1* is unaffected in either *noi* or *ace* mutants at the end of gastrulation (Lun and Brand 1998; Reifers et al. 1998), and, conversely, both *pax2.1* and *fgf8* are expressed normally at the end of gastrulation in a *wnt1* deletion mutant (A. Lekven, pers. comm.). These studies have suggested that at least three separate signaling pathways are activated independently of each other around the forming MHB. At later stages of midbrain development, however, these genes come to depend on each other's expression (see below).

5.1.1 Initial AP Subdivision of the Neural Plate

The spatially discrete and independent activation of *pax2.1*, *fgf8* and *wnt1* during gastrulation show that AP patterning information is already present in the neuroectoderm, raising the question of how this information is generated. Based on knockout studies in mice and expression analysis in zebrafish, the homeodomain transcription factor Otx2, which is expressed in the fore- and midbrain primordia during gastrulation, is required for correct spatial activation of MHB genes (Simeone 1998). Murine Gbx2, another homeodomain transcription factor expressed in a complementary fashion in the posterior neuroectoderm, is required for later stages of hindbrain and MHB development (Wassarman et al. 1997), though apparently not for early patterning

Fig. 6A–D. AP patterning of the midbrain and hindbrain. **A** Lateral view of a gastrulating embryo at 80% epiboly; the *arrow* points to the site of MHB formation. **B** View into the living brain of an embryo at 36 h. The various brain parts are clearly distinct from each other. **C** Similar stage embryo as in **A**, dorsal view onto the forming neural primordium. The embryo was hybridized with *pax2.1* probe (*red*), recognizing the midbrain and isthmus primordia and *fgf8* probe (*blue*), recognizing the anterior hindbrain primordium and the germ ring. **D** Confocal section of a live embryo stained with Bodipy-Ceramide to reveal the morphological folding of the brain. Dorsal view of a 30-h embryo, sectioned through the dorsal midbrain and cerebellum. Cells near the ventricular surface of the neuroepithelium are rounded up during mitosis. (Photograph kindly provided by Tobias Langenberg)

during gastrulation (A. Simeone, pers. comm.). Local misexpression of Otx2 or Gbx2 in mice causes downregulation of the other gene (Broccoli et al. 1999; Millet et al. 1999), suggesting that mutually repressive interactions contribute to establishing or maintaining the position of the MHB. For technical reasons, a possible function during gastrulation could not be tested in these studies. As in mice, zebrafish have two *gbx* genes, but, in contrast to mice, both appear to be expressed during gastrulation. *gbx1* is expressed earlier than *pax2.1* and *fgf8* in the hindbrain neuroectoderm and *gbx1* misexpression can shift *pax2.1* and *fgf8* expression to ectopic locations and convert anterior neural plate to hindbrain; *gbx2* acts in the same subdomain but later, downstream of Fgf8 (K. Lun, M. Rhinn, M. Brand, unpubl.). An additional regulator acting upstream of *pax2.1* has recently been identified. Bts-1 encodes a zinc-finger transcription factor related to the *Drosophila buttonhead* gene, and is both necessary and sufficient for *pax2.1* regulation (Tallafuss et al. 2001).

5.1.2 Wnt8 Signaling Positions the Midbrain and Hindbrain

The data in mice and zebrafish show that mutual repression of *otx2* and *gbx* is important in positioning the MHB, raising the question of how in turn the correct spatial domains of *otx2* and *gbx1* (*Gbx2* in mice) are generated. As is discussed above for the forebrain and in more detail elsewhere (see Hibi et al., this Vol.), signals derived from the germ ring, including Wnt8, may perform this function (Erter el al. 2001; Lekven et al. 2001). In this model, midbrain and hindbrain derive from neurectoderm cells that are exposed to higher concentrations of Wnt8, because they reside closer to the signal source in the germ ring than forebrain precursors, consistent with the fate map. One attractive possibility is that *gbx1* is a direct response gene to a particular concentration of Wnt8, acting to transduce the posteriorizing Wnt8 signal to specify hindbrain identity. Indeed, clones of Wnt8 misexpressing cells in the gastrula neuroectoderm can cause ectopic activation of *gbx1* in surrounding host cells, and reducing Wnt signaling in the posterior neural plate reduces *gbx1* expression, suggesting that Wnt8 might directly or indirectly control the domain of *gbx1* expression (M. Rhinn, K. Lun, M. Brand, unpubl.). It remains to be tested whether and how additional secreted factors, such as Nodals and retinoic acid or the more recently described midkine-related growth factor2 (Winkler and Moon 2001), contribute to posteriorization of the MHB domain.

5.2 Wnts and Fgfs Maintain and Pattern the Midbrain and Hindbrain

Once posteriorization and initial subdivision of the neurectoderm is achieved, the MHB organizer forms at the newly established molecular interface. At present, it is not known what, if any, additional signals at this interface allow ordered activation or maintenance of gene expression around it. Initially,

however, the midbrain and hindbrain domains appear to function independently of each other. In the midbrain, *pax2.1* is a likely direct regulator of *eng2* and *eng3* (Lun and Brand 1998), and engrailed gene-MO therefore phenocopies the midbrain/isthmus phenotype of *noi* mutants (Scholpp and Brand 2001). Fgf8 also functions at the end of gastrulation in the early hindbrain primordium (Reifers et al. 1998; Fürthauer et al. 2001; Raible and Brand 2001), but does not initially affect gene expression in the midbrain. During early somitogenesis, these genes become successively restricted in their expression toward the isthmus, where they then overlap and become mutually dependent in their expression. Around this time, isthmus-specific expression of *pax5*, *pax8* and *fgf17* is first activated, requiring both *pax2.1* and *fgf8* for their activity (Lun and Brand 1998; Pfeffer et al. 1998; Reifers et al. 1998, 2000a; M. Brand, unpubl. results).

Once established, secreted Fgf8 and Wnt1 proteins from the organizer are thought to mediate its organizing influence on surrounding neural tissue. Wnt1 functions as a mitogen and to maintain expression of En genes, but is unable to mimic organizer activity when misexpressed (Dickinson et al. 1994; Danielian and McMahon 1996). *Fgf8*, however, is expressed at the right time and place to mediate organizing activity (Reifers et al. 1998, and references cited therein). In contrast to Wnt1, ectopic application of Fgf8 protein mimics MHB organizer activity and induces isthmic-like structures and MHB-specific gene expression (Crossley et al. 1996; Liu et al. 1999; Martinez et al. 1999; M. Brand, unpubl. results). Because Fgfs can mimic each other's activity in gain-of-function experiments, loss-of-function mutants are important to support a function for Fgf8 in induction and/or patterning of the MHB region. *ace* mutants lack functional Fgf8, the MHB organizer and a cerebellum, and analysis of this mutant showed that Fgf8 is required to maintain marker gene expression in the midbrain and isthmus, but not to induce midbrain (Reifers et al. 1998; Picker et al. 1999; Araki and Brand 2001).

5.2.1 Polarization of the Midbrain

One of the crucial properties of the midbrain tectum is the ability to form a set of retinotopically ordered connections with ingrowing axons of retinal ganglion cells. When an ectopic MHB organizer forms after transplantation of MHB cells, or after bead implantation, the induced tectum is correctly polarized with respect to the ectopic organizer (Marin and Puelles 1994), suggesting that the signal required for polarization also derives from the MHB organizer. Analysis of the midbrain in *ace* mutants showed that the MHB is indeed required for AP polarization of the midbrain, including graded expression of EphrinA2 and EphrinA5 ligands in the midbrain neuroepithelium, and hence for proper retinotectal map formation (Picker et al. 1999). The expression of one likely Fgf8-receptor, Fgfr3, may mediate a possible direct activity of Fgf8 in this process (Sleptsova-Friedrich et al. 2001). Indeed, implantation

of a bead coated with Fgf8 protein into the posterior diencephalon at late somitogenesis is sufficient to reorient the highpoint of EphrinA5a graded expression (A. Picker and M. Brand, unpubl.).

5.2.2 Fgf Signaling in the Rostral Hindbrain

Fgf8 secreted from the MHB organizer is also thought to be involved in development of the chick rostral hindbrain (Irving and Mason 2000), and is required for the earliest stages of rostral hindbrain development in zebrafish (Fürthauer et al. 2001; Raible and Brand 2001). Rhombomere 1 (r1) lies closest to the MHB, and is the only rhombomere that does not express any *Hox* genes in chick. However, after transplantation to an ectopic position, r1 tissue can express *Hox* genes, and both MHB tissue and Fgf8 can inhibit this expression (Irving and Mason 2000). Thus, Fgf8 may define, directly or indirectly, the anterior limit of *Hox* gene expression. Both Fgf8 and Fgf3 are expressed initially in a wider domain encompassing the anterior hindbrain, and then become restricted to a segmental expression during early segmentation stages (Reifers et al. 1998; Raible and Brand 2001). Analysis of *erm* and *pea3*, two target genes for Fgf8 and Fgf3 signaling, suggests that Fgfs function during early hindbrain segmentation, likely in a redundant fashion (Raible and Brand 2001; Roehl and Nüsslein-Volhard 2001); however, the phenotypic consequences of this function for later hindbrain development remain to be explored. During neuronal cell type determination, Fgfs may serve yet additional roles: tyrosine hydroxylase expression in the locus coeruleus of the hindbrain is abolished in *ace* mutants. In analogy to the suggested requirement for Fgf8 in development of mammalian midbrain dopaminergic neurons (Hynes et al. 1995), it has been argued that this phenotype may represent a non-autonomous requirement for Fgf8 signaling from the MHB organizer (Guo et al. 1999a).

5.2.3 Feedback Control of Fgf Signaling

Given its potency as a signaling molecule, the activity of Fgf8 must be carefully controlled in the embryo. An emerging theme for several signaling pathways is that extracellular or intracellular inhibitors regulate their activity. *Drosophila sprouty* functions in development of the trachea and eye, as a target gene and feedback-inhibitor for Fgf and EGF signaling (Placzek and Skaer 1999). Several studies reveal a surprisingly good correlation of the expression of vertebrate *sprouty* homologues with regions of ongoing Fgf signaling, including the MHB. As in flies, vertebrate *sprouty* genes can be induced locally with recombinant Fgf8 protein (Minowada et al. 1999; Chambers and Mason 2000; Fürthauer et al. 2001). In *ace/fgf8* mutants, *sprouty4* is never activated at the MHB and anterior hindbrain, suggesting that Fgf8 regulates *sprouty4* expression. In addition, overexpression of *sprouty4* antagonizes the effects of both *fgf8* and *fgf3* injection, suggesting that zebrafish *sprouty4* is a component of an *fgf8,fgf3*-dependent inhibitory feedback loop (Fürthauer et al. 2001).

Additional observations support the existence of such a feedback loop: *fgf8* RNA is upregulated in *ace* mutants (Reifers et al. 2000a; Shanmugalingam et al. 2000) and in zebrafish *aussicht* (*aus*) mutants (Heisenberg et al. 1999); *aus* could therefore encode a component of the feedback loop. Feedback regulation could serve important functions, for instance, to maintain the MHB organizer itself, and indeed this structure is morphologically absent in zebrafish and mouse *Fgf8* mutants (Meyers et al. 1998; Reifers et al. 1998). The feedback loop also involves *Otx2* and *Gbx2*, since local expression of Fgf8 represses *Otx2* and reduction of *Otx* copy number shifts *Fgf8* and *Gbx2* expression anteriorly (Crossley et al. 1996; Simeone 1998; Liu et al. 1999; Martinez et al. 1999). The existence of the feedback loop may explain why *Fgf8* bead implantations are able to re-activate the entire genetic cascade of MHB development.

Considering the potent abilities of Fgf8, it is notable that different Fgf8 isoforms exist (Liu et al. 1999) and that additional *Fgfs* related to *Fgf8* are also expressed in the MHB organizer. *Fgf17* and *Fgf18* are turned on at the MHB after the onset of *Fgf8* (Reifers et al. 2000a; Xu et al. 2000, and references cited therein), suggesting a role in maintaining MHB organizing activity. Indeed, in zebrafish, *fgf17* injections have similar effects to *fgf8* injections; *fgf17* acts downstream of *pax 2.1* and *fgf8*, and both *fgf17* and *fgf18* can be induced ectopically in the forebrain by Fgf8. Mice carrying a null mutation in *Fgf17* have later defects in the cerebellar anlage, a phenotype that is more severe in an *Fgf8* heterozygous background (Xu et al. 2000). Thus, *Fgf8*, *Fgf17* and *Fgf18* may cooperate to maintain the organizing activity and each other's expression at the isthmus. Fgf8 is also a crucial component of the forebrain organizer located in the ANR/row1 (Shimamura and Rubenstein 1997; Shanmugalingam et al. 2000) where it is coexpressed with at least one other *fgf*, *fgf3*, suggesting a similar functional redundancy of Fgf signals (Reifers et al. 2000a; Shinya et al. 2000; Fürthauer et al. 2001; Raible and Brand 2001).

5.2.4 Controlling Competence to Respond to Fgf8 Signaling

Competence of the responding cells is a further level by which the potent abilities of Fgf8 are controlled, as recently uncovered by studying the zebrafish *spiel-ohne-grenzen* (*spg*) mutations. *spg* mutations are known for their effect on early development of the midbrain-hindbrain boundary upstream of *pax2.1* (Schier et al. 1996). Molecularly, *spg* mutations disrupt the gene encoding the POU domain transcription factor Pou2 (Belting et al. 2001; Burgess et al. 2002), which is a likely ortholog of Oct3/4 in mice, a protein thought to control stem cell and germ cell differentiation in mammals (Burgess et al. 2002, and references cited therein). Further studies of the *spg* phenotype have shown that not only is early development of the midbrain-hindbrain boundary disrupted, but also that all of the anterior hindbrain up to the rhombomere 4/5 boundary fails to be specified properly by late gastrulation (Reim and Brand 2002). Key molecules functioning in the formation of the MHB, like *pax2.1*, *spry4*, *her5*, *eng2*, and *eng3*, and in hindbrain development, like *krox20*, *gbx2*, *fkd3* and

pou2, are all abnormal in *spg* mutant embryos. In contrast, regional definition of the position of the future MHB in the neuroectoderm by complementary expression of *otx2* and *gbx1*, prior to establishment of the complex regulatory cascade at the MHB, is normal in *spg* embryos. In the hindbrain primordium, the earliest defect is a lack of *gbx2* expression in the neuroectoderm in the mutants. This corresponds to the domain of *fgf8* expression, and indeed both *fgf8* and *pou2* are absolutely required in a combinatorial fashion to activate *gbx2* and other markers (*spry4* and *fkd3*) in this neuroectodermal domain (Reim and Brand 2002; K. Lun, M. Rhinn, M. Brand, unpubl.). Importantly, injection experiments and Fgf8 bead implantations have revealed that *spg/pou2* embryos are insensitive to Fgf8 specifically in the early hindbrain neuroectoderm, although the MAP kinase pathway, through which Fgf8 is thought to signal, can function normally in these mutants. Thus, Pou2 is the first example of a factor controlling competence for Fgf8, in this case, in the gastrula neuroectoderm. Much remains to be learned about the mechanism by which Pou2 confers competence, and it will be interesting to explore potential parallels to the actions of Oct3/4 in controlling mammalian stem cell differentiation.

5.3 DV Patterning of the Midbrain and Isthmus

The midbrain and isthmus are clearly subdivided along the DV axis, but surprisingly few studies in zebrafish have addressed the basis for this. Studies of neuronal subtype specification in the chick tegmentum reveal that, as in other sections of the neural tube, Shh provides a ventral positional signal to specify cell-type identity. Likewise, Shh is sufficient to switch dorsally located tectal precursors to a ventral tegmental fate (Agarwala et al. 2001). Misexpression of *hh* genes in zebrafish similarly prevents differentiation into tectum and tegmentum (Ekker et al. 1995), presumably as a result of the overall ventralization of the neural tube. However, although inactivation of Smoothened causes defects throughout the ventral CNS, including the midbrain (Chen et al. 2001a; Varga et al. 2001), it does not affect the subdivision into tectum and tegmentum. This issue has not been addressed directly using tectum-specific markers; however, it is likely that initial subdivision occurs in response to the gradient of BMP activity that regulates DV patterning at all axial levels (Barth et al. 1999). It is currently unknown when and how the information provided by these primary gradients is converted into DV positional information in the midbrain. However, studies of an allelic series of *noi/pax2.1* and of Fgf function in the isthmus provided genetic evidence suggesting that different midbrain and isthmus genes, such as *eng2*, *eng3* and *her5*, require different levels of *noi* and Fgf activity along the DV axis during midsomitogenesis, arguing that by this time regionalization along the DV axis must be underway (Lun and Brand 1998; Carl and Wittbrodt 1999). The isolation of early tectum-specific markers should help to resolve this issue (Seo et al. 1998; Kudoh and Dawid 2001).

5.4 Later Steps of Patterning the Hindbrain

Following its initial induction during gastrulation stages, the hindbrain primordium becomes subdivided into seven segmental units, or rhombomeres, along the AP axis which are apparent at the 18-somite stage (Hanneman et al. 1988). The segmented architecture of the hindbrain is of consequence not only for the neuronal organization in this area, but also for the craniofacial elements derived from the dorsal apex of the neural tube, the neural crest (see Kelsh and Raible, this Vol.; for reviews, see Lumsden and Krumlauf 1996; Schilling and Knight 2001). Similar to the spinal cord, the hindbrain is subdivided along the DV axis into roof plate, alar plate, basal plate and floor plate, which can be further subdivided into medial and lateral regions. There is no morphological distinct border between basal and alar plates, but studies of hindbrain neuronal organization have shown that motor nuclei are predominately found in the basal plate (Trevarrow et al. 1990; Chandrasekhar et al. 1997), whereas sensory cells are predominately located in the dorsal neural tube and interneurons reside in between (Korzh et al. 1993). It is unclear how the embryonic organization relates to the more complex organization of the adult hindbrain (Wullimann et al. 1996). Unique among model vertebrates, just as in the spinal cord, it is possible in zebrafish to identify individual interneurons in the larval hindbrain, based on position, dendritic tree and axonal projection (Kimmel et al. 1985; Metcalfe et al. 1986). The concept of identified neurons has allowed great progress in unraveling the later steps of neuronal specification of identified neurons in *Drosophila* (e.g., Doe and Technau 1993), and, although comparable studies have not yet been carried out in zebrafish, progress is to be expected in this area, particularly with the advent of GFP transgenic lines labeling such identified neurons.

5.4.1 Dorsoventral Patterning

Studies of Shh pathway mutants have shown that, as in other parts of the neural tube, Shh-family members are involved in patterning ventral hindbrain. Mutations in *syu/shh* alone have only a mild effect on ventrally located branchiomotor neurons (Chandrasekhar et al. 1998; Schauerte et al. 1998), but simultaneous MO inactivation of both *shh* and *twhh* results in complete absence of these neurons (Bingham et al. 2001; Etheridge et al. 2001, and references cited therein). Inactivation of Smoothened gives the same phenotype (Chen et al. 2001a; Varga et al. 2001). Moreover, mutations in *yot/gli2* also cause defects in branchiomotor neuron development (Chandrasekhar et al. 1999; Karlstrom et al. 1999). It is not yet clear to what extent medial interneurons are affected by absence of Shh activity. However, dorsal neural tube derivatives appear to form normally, which is not surprising in light of the evidence that they are probably regulated by the BMP pathway (Barth et al. 1999; Nguyen et al. 2000).

5.4.2 Forming and Maintaining Rhombomeres

As in other vertebrates, the identity of hindbrain rhombomeres is thought to be conferred by the combination of *hox* genes they express, but how and when cells become committed to express a particular *hox* gene is still unclear. Zebrafish *hox* gene clusters are studied extensively as a paradigm for genome evolution, although we will not discuss this further here (see Amores et al. 1998; McClintock et al. 2001; Taylor et al. 2001, and references cited therein). Consistent with a role for *hox* genes in conferring identity, misexpression of *hox* genes can cause transformation of rhombomere identity (McClintock et al. 2001), and transplantation experiments between rhombomeres with differing *hox* gene profiles have shown that plasticity of *hox* gene expression correlates with plasticity in rhombomere fate (Schilling et al. 2001). In *Drosophila*, Hox proteins cooperate in a sequence-specific manner with two other homeodomain proteins, Extradenticle and Homothorax. The vertebrate homologues of the *Drosophila* genes are *pbx* and *Meis*, and recent work in zebrafish has shown that these genes have a similar role in vertebrates. The zebrafish gene *lazarus* (*lzr*) is required for proper segmentation of the hindbrain and proper expression and function of *hox* genes. *lazarus* was found to encode a novel *pbx* gene, *pbx4*, which appears to mediate the function of multiple *hox* genes (Pöpperl et al. 2000). Injecting mRNA encoding a dominant negative version of Meis results in a phenotype very similar to *lzr* mutants, arguing that it indeed functions as a transcription co-factor, as suggested by the fly work. Surprisingly, *meis* mRNA injection can in addition rescue the *lzr* mutant phenotype, suggesting that Meis acts not only at the transcriptional level, but also in some way is able to stabilize maternally derived Lzr protein (Waskiewicz et al. 2001).

5.4.3 Extrinsic Signals Controlling Segmentation

Retinoic Acid. Work in other organisms, along with transplantation experiments by Schilling et al. (2001) in zebrafish, suggested that extrinsic signals normally maintain expression of the correct combination of *hox* genes by each rhombomere. Fgfs are likely to be involved in this process, as discussed above. Studies in several vertebrates provide evidence that retinoic acid (RA) acts as a signal in hindbrain identity and *hox* gene regulation (Papalopulu et al. 1991; Gavalas and Krumlauf 2000; Schilling and Knight 2001). *raldh2* catalyzes RA production in the embryo, and knockout studies in mice or receptor blockade in chick, among other experiments, have shown that RA is indeed needed to ensure proper rhombomere formation and identity in the hindbrain (Dupé et al. 1999; Niederreither et al. 1999, 2000; Dupé and Lumsden 2001; for review, see Gavalas and Krumlauf 2000). Two independently isolated zebrafish mutations, called *neckless* and *no fin,* affect the zebrafish *raldh2* gene, and the phenotypes of these mutants support an involvement of RA in posterior hindbrain development, *hox* gene regulation and fin bud formation (Begemann et al.

2001; Grandel et al. 2002). Moreover, Begemann et al. (2001) demonstrated using mosaic analysis that *raldh2* expression is required in the paraxial mesoderm for proper *hox* gene expression in the neural plate. Notably, pharmacological inhibition of RA signaling during early stages of hindbrain development causes absence of rhombomeres posterior to rhombomere 4 (r4), while the more anterior rhombomeres expand in size (Dupé and Lumsden 2001), a phenotype also seen when mouse *raldh2* is knocked out (Niederreither et al. 1999). With later onset of inhibitor treatment, successively more posterior rhombomeres are able to form, which is similar to weaker inactivation of RA receptors, and more akin to the milder phenotype of the zebrafish mutants. It is not yet clear how these phenotypes arise, but they may reflect a further link between RA signaling and the more general problem of posteriorization of the nervous system (see above). It will be interesting to explore how and to what extent RA signaling cooperates with other posteriorizing molecules in hindbrain induction and patterning, and with molecules such as Fgf8 that signal from the anterior border of the hindbrain (Irving and Mason 2000).

valentino *and Ephrin-Dependent Cell Segregation.* The hindbrain posterior to the r3/4 boundary is also affected in *valentino* (*val*) mutants, which lack visible segmentation in this area, and have reduced expression of *krox20* in r5 (Moens et al. 1996). *val* is the zebrafish ortholog of the mouse b-zip transcription factor *Kreisler* (Cordes and Barsh 1994; Moens et al. 1998). During hindbrain formation, a double-segment morphological periodicity is apparent transiently. *val* is required for subdivision of the r5/6 territory into rhombomeres (Moens et al. 1996, 1998), thus providing the first evidence that the double-segment periodicity has a genetic basis. Moreover, *val* mutant cells are excluded from the r5/6 domain if transplanted into wild-type hindbrain, suggesting that, as rhombomere transplantation in chick had suggested previously, an adhesion mechanism controlled by *val* is involved in establishing physical segregation of cells during segmentation. Based on their expression, members of the Eph family of receptor tyrosine kinases and their ligands, the ephrins, were candidates for functioning in rhombomere boundary formation, and previous evidence indicated that an Eph receptor functions in segmentation of the hindbrain (Xu et al. 1995). In the so-called fish-ball assay, bidirectional signaling between receptor and ligand is necessary to achieve segregation of cell populations injected with mRNAs encoding different Ephs or ephrins (Mellitzer et al. 1999; Xu et al. 1999b). Moreover, recent work on Ephs and ephrins in *val* mutants has elucidated a mechanism for how boundary formation of the r5/6 domain might work (Cooke et al. 2001): expression of ephB4a coincides with *val* expression in r5/6, whereas ephrin-B2a is expressed in neighboring r4 and r7. Normal *val* function is needed to establish the mutually exclusive expression domains of these two genes. When ephB4a-expressing cells and ephrin-B2a-expressing cells are juxtaposed in the hindbrain of *val* mutants, boundary formation is restored, suggesting that interactions between ephB4a and

ephrin-B2a, possibly under the direct transcriptional control of Val, mediate cell sorting and boundary formation in this part of the hindbrain (Cooke et al. 2001). Further processes are likely to participate to ensure proper hindbrain segmentation, but their function and relative contribution is unclear. For instance, *lunatic fringe* encodes an N-acetylglucosaminyltransferase involved in modulating Notch signaling, for instance at the DV compartment boundary of the *Drosophila* wing, and participates in lineage restriction to a diencephalic segment (Zeltser et al. 2001). *lunatic fringe* is also expressed in zebrafish rhombomeres (Leve et al. 2001; Prince et al. 2001), as is a potential target gene of the Notch pathway, *her6* (Pasini et al. 2001). It will be illuminating to learn the precise functions of these molecules in hindbrain patterning.

5.5 Secondary Modification of the Ground Plan by Neuronal Migration

Although neurons are generated in particular AP and DV locations, their final locations can be dramatically different because they often migrate to other brain regions. An example of this phenomenon is provided by the GABAergic neurons of the murine neocortex, the majority of which migrate from the *Dlx1*, *Dlx2*-positive domain in the subcortical telencephalon (Anderson 1997). Migration of granular cell precursors from the upper rhombic lip has long been known to contribute extensively to formation of the cerebellum in other vertebrates (Altman and Bayer 1997). Studies addressing the mechanistic basis of such migrations have only just begun in other species (Alcantara et al. 2000). A recent time-lapse investigation of the migration path taken by cells from the upper rhombic lip through the cerebellar primordium has revealed a new, ventrally directed migration path along the MHB into the ventral brainstem (Köster and Fraser 2001b), illustrating the potential for this type of work in zebrafish.

6 Summary

We have described the formation of the zebrafish central nervous system. The spinal cord has the simplest organization and was considered first, followed by the forebrain, midbrain and hindbrain. We have discussed many studies that have revealed the molecular mechanisms, including extrinsic signals and intrinsic responses to them, underlying the establishment of nervous system regions and the wide diversity of neuronal cell types of which they are comprised. Wherever possible, we have tried to compare what has been learned from zebrafish with what is known in other vertebrate species. The simplicity of the developing nervous system makes zebrafish embryos particularly amenable to studies of nervous system development. Thus, many aspects of nervous system patterning that were unknown from other vertebrates have been revealed by studies in zebrafish. However, the relationship between

embryonic and adult nervous system morphology is still not entirely clear and remains an important avenue for further studies.

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