Erratum

Engrailed and Fgf8 act synergistically to maintain the boundary between diencephalon and mesencephalon
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An error in this article was not corrected before going to press.

Throughout the paper, efn4 should be read as EphA4, as the authors are referring to the gene encoding the ephrin A4 receptor (EphA4) and not to that encoding its ligand ephrin A4.

We apologise to the authors and readers for this mistake.
Engrailed and Fgf8 act synergistically to maintain the boundary between diencephalon and mesencephalon

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Summary

Specification of the forebrain, midbrain and hindbrain primordia occurs during gastrulation in response to signals that pattern the gastrula embryo. Following establishment of the primordia, each brain part is thought to develop largely independently from the others under the influence of local organizing centers like the midbrain-hindbrain boundary (MHB, or isthmic) organizer. Mechanisms that maintain the integrity of brain subdivisions at later stages are not yet known. To examine such mechanisms in the anterior neural tube, we have studied the establishment and maintenance of the diencephalic-mesencephalic boundary (DMB). We show that maintenance of the DMB requires both the presence of a specified midbrain and a functional MHB organizer. Expression of pax6.1, a key regulator of forebrain development, is posteriorly suppressed by the Engrailed proteins, Eng2 and Eng3. Mis-expression of eng3 in the forebrain primordium causes downregulation of pax6.1, and forebrain cells correspondingly change their fate and acquire midbrain identity. Conversely, in embryos lacking both eng2 and eng3, the DMB shifts caudally into the midbrain territory. However, a patch of midbrain tissue remains between the forebrain and the hindbrain primordia in such embryos. This suggests that an additional factor maintains midbrain cell fate. We find that Fgf8 is a candidate for this signal, as it is both necessary and sufficient to repress pax6.1 and hence to shift the DMB anteriorly independently of the expression status of eng2/eng3. By examining small cell clones that are unable to receive an Fgf signal, we show that cells in the presumptive midbrain neural plate require an Fgf signal to keep them from following a forebrain fate. Combined loss of both Eng2/Eng3 and Fgf8 leads to complete loss of midbrain identity, resulting in fusion of the forebrain and the hindbrain primordia. Thus, Eng2/Eng3 and Fgf8 are necessary to maintain midbrain identity in the neural plate and thereby position the DMB. This provides an example of a mechanism needed to maintain the subdivision of the anterior neural plate into forebrain and midbrain.

Key words: CNS, Engrailed, Fgf8, Forebrain, Midbrain, Isthmus, Zebrafish, Danio rerio

Introduction

Pattern formation in the CNS primordium at the neural plate stage is controlled by distinct mechanisms along the anterior-posterior (AP), left-right and dorsal-ventral axes (reviewed by Lumsden and Krumlauf, 1996; Rubenstein et al., 1998; Wilson et al., 2002). Studies in amphibia have suggested an ‘activation and transformation’ model of AP patterning, whereby early neural tissue is initially anterior in character and later on becomes transformed into more posterior character by signals originating from more caudal regions of the embryo (Nieuwkoop and Nigtevecht, 1954) (see also Foley et al., 2000). Among the postulated posteriorizing signals are Fgfs, retinoic acid, Nodals and Wnt proteins (Wilson and Rubenstein, 2000; Wilson et al., 2002). Wnt proteins are likely to be one of the earliest posteriorizing signals acting in the forming neural primordium. The zebrafish mutants headless (hdl) and masterblind (mbl) carry mutations in the genes encoding the components of the Wnt pathway tcf3 and axin, respectively, and exhibit severe anterior patterning defects (Heisenberg et al., 1996; Kim et al., 2000; Heisenberg et al., 2001). In these embryos, the forebrain and the eye anlage are reduced or absent and the midbrain expands forward into the tip of the neural tube. Following this initial patterning step, further mechanisms must exist that maintain and refine regional identity in the neural tube. Organizing centers are probably crucial for maintenance processes, e.g. the organizers located at the midbrain-hindbrain boundary (MHB) and at the anterior neural border (reviewed by Rhinn and Brand, 2001; Wurst and Bally-Cuif, 2001; Wilson et al., 2002).

The paired-domain proteins Pax6 in the diencephalon and Pax2/5/8 in the midbrain and MHB organizer serve an important function during the maintenance phase and subdivide the anterior neural plate into forebrain, midbrain and hindbrain domains [Pax6 (Walther and Gruss, 1991; Stoykova et al., 1996; Grindley et al., 1997) and Pax2/5/8 (Urbanek et al., 1994; Favor et al., 1996; Schwarz et al., 1997; Schwarz et al., 1999; Lun and Brand, 1998; Pfeffer et al., 1998; Scholpp and Brand, 2003)]. Loss-of Pax6 function in mice and in chicken cause a fate change of the caudal diencephalon into mesencephalic tissue (Stoykova et al., 1996; Matsunaga et al., 2000). Conversely, ectopic Pax6 expression in the chick midbrain causes a downregulation of Pax2 and Engrailed gene expression and
posterior enlargement of the diencephalon (Matsunaga et al., 2000). Similarly, posterior forebrain expression of Pax6 expands into the presumptive midbrain in Pax2 and Pax5 double deficient mutant mice (Pax2/5) (Schwarz et al., 1999) or pax2.1 mutant zebrafish (Scholpp and Brand, 2003) with concomitant enlargement of the posterior commissure as the anatomical landmark of the diencephalic-mesencephalic boundary (DMB) (Macdonald et al., 1994; Mastick et al., 1997). These experiments show that Pax6 plays an essential role in determining forebrain fate, whereas Pax2/5 is essential for development of the midbrain and MHB territory.

In addition to Pax genes, the Engrailed homeodomain transcription factors are necessary to maintain midbrain fate in development of the midbrain and MHB territory. Mis-expression chicken, mice and zebrafish (Wurst et al., 1994; Araki and Nakamura, 1999) demonstrates its importance for proper development of the midbrain and MHB territory. Furthermore, in Pax2/5 deficient mice and fish, Engrailed expression is strongly reduced or eliminated (Favor et al., 1996; Lun and Brand, 1998; Pfeffer et al., 1998; Schwarz et al., 1999).

Little is known about intercellular signals regulating the formation of the DMB. In addition to Pax6 and En, the secreted factor Fgf8 is involved during establishment and maintenance of the anterior CNS. Fgf8 knock-out studies in mice and zebrafish showed that Fgf8 is essential to maintain the MHB, to induce the cerebellum and to pattern the midbrain (Crossley et al., 1996; Brand et al., 1996; Reifers et al., 1998; Picker et al., 1999; Chi et al., 2003). These observations raised the possibility that Engrailed expression is maintained in the midbrain through Fgf signaling, but so far evidence for direct action by Fgf is lacking (Reifers et al., 1998; Liu and Joyner, 2001).

Although the mechanisms involved in establishing AP patterning in the anterior neural tube are well studied, it is unclear how the AP subdivisions are maintained, and how this relates to organizer function in the anterior neural plate. We study the formation and maintenance of the zebrafish diencephalic-mesencephalic boundary to understand such maintenance mechanisms. Based on expression studies and functional analysis during DMB formation, we find that eng2 and eng3 genes play a crucial role in maintenance of the DMB. In addition, we find that Fgf signalling molecules, in particular Fgf8, act synergistically with eng2 and eng3 as non-autonomous signals to maintain midbrain identity and hence position the DMB.

Materials and methods

Maintenance of fish

Breeding fish were maintained at 28°C on a 14 hour light/10 hour dark cycle (Brand and Granato, 2002). Embryos were staged according to Kimmel et al. (Kimmel et al., 1995) or in hours post-fertilization (hpf) at 28°C for 24-hour-old or older embryos. To prevent pigment formation, some embryos were raised in 0.2 mM 1-phenyl-2-thiourea (PTU, Sigma). The data we present in this study were obtained from analysis of TL wild-type fish and of homozygous no isthmus (nisi) embryos, ‘not-embryos’ in the following (Brand et al., 1996).

Injections

For preparation of mRNA the complete ORF for eng3 was amplified. The following primer were used: forward, 5'-TTC CCG TTC GTT TCT TT T G-3'; and reverse, 5'-TCT TGG GAC TTC AGC A TG-3'. We subcloned the cDNA into the vector pCS2+ (Rupp et al., 1994) and used the SP6 message machine kit (Ambion) for transcription. The amount of injected mRNA was estimated from the concentration and volume of a sphere of mRNA injected into oil at the same pressure settings. mRNA was dissolved in 0.25 M KCl with 0.2% of Phenol Red and back-loaded into borosilicate capillaries prepared on a Sutter puller. During injection, mRNA was deposited into the cytoplasm of one- to two-cell stage embryos. Typically, 500 pg eng3 RNA was injected. The embryos were fixed at appropriate stages prior to in situ hybridization and antibody staining.

For transient knock-down of gene expression, Morpholino-antisense oligomers (Morpholinos; MO; by GeneTools) were prepared targeting eng2 and eng3 (Scholpp and Brand, 2001), fgf3 (Raible and Brand, 2001), fgf4 and fgf8 (Araki and Brand, 2001) by dissolving in 5 mM HEPES-buffer with 0.2% Phenol Red. Morpholinos were injected into the yolk cell close to the blastomeres between the one- and eight-cell stages at a concentration of 4 ng/μl. For a control, randomized mis-priming morpholinos (con-MO) were used, which showed no effect on embryos injected at 15 ng/μl but cause unspecific effects at a concentration of 30 ng/μl. Morpholino-injected embryos (morphants) were fixed at given stages prior to in situ hybridization or antibody staining.

Sequences were as follows:

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>eng2-MO</td>
<td>5'-CGC TCT GCT CAT TCT CAT CCA TGG T3'</td>
</tr>
<tr>
<td>eng3-MO</td>
<td>5'-CTA TGG TCT AAG TTT TTC TCC ATG AAG A-3'</td>
</tr>
<tr>
<td>fgf3-MO</td>
<td>5'-CAG TAA CAA GAA GAC CA</td>
</tr>
<tr>
<td>fgf3-MO</td>
<td>Mismatch</td>
</tr>
<tr>
<td>fgf3-MO</td>
<td>5'-CAT TAA CAA GAA GAC CAC AAT TAT A-3'</td>
</tr>
<tr>
<td>fgf4-MO</td>
<td>5'-GCC TGC TCC ATA CTA GTC TAT TG</td>
</tr>
<tr>
<td>fgf4-MO</td>
<td>5'-GAG TCT CAT GTT TAT AGC CTC AGT A-3'</td>
</tr>
<tr>
<td>FGf4-MO</td>
<td>5'-GCT ACC TCA GTT ACA ATT TAT A-3'</td>
</tr>
</tbody>
</table>

Inhibition of Fgf signalling

At 90% epiboly, an inhibitor of Fgf signalling, SU5402 (Calbiochem) was added to the medium at a concentration of 1 μM as described previously (Reifers et al., 2000). Embryos were incubated for different time periods and fixed directly after treatment.

Implantation of FGF soaked beads

Heparin-coated acrylic beads (Sigma) were prepared as described previously (Reifers et al., 2000). The beads were implanted unilaterally into the region of the presumptive anterior midbrain of wild-type and no isthmus mutant embryos at the 10 somite stage (10 ss). Embryos were incubated for 2 hours at 28°C and fixed at the 15 ss for further examination.

Transplantation

Embryos were injected with 400 pg mRNA of a truncated FGF receptor (XFD) as described previously (Launay et al., 1996). Rhodamine-dextran (Mini Ruby, Molecular Probes) was co-injected for tracing cells after injection. At shield stage, cells from the region of the presumptive midbrain region were taken from a donor embryo and implanted into a non-labeled host embryo. At 10 ss, embryos were fixed prior to in situ hybridization and antibody staining.
Labeling of cell clones via laser-based activation of caged fluorescein
Non-fluorescent, photocatalytically (caged) fluorescein as a cell tracer for fate mapping in the zebrafish embryo was described by Kozlowski et al. (Kozlowski et al., 1997). We used a UV laser (Phototronic Instruments) to uncage the dye more locally. A solution of 5% anionic DMBN-caged fluorescein (2 nl) (Molecular Probes, D-3310, 0.25 M KCl, 0.25% Phenol Red and 40 mM HEPES-NaOH (pH 7.5) was injected in embryos at the one-cell stage and for development the embryos were kept in a dark humid chamber at 28°C. At the 6 ss, embryos were oriented in a viewing chamber dorsal up and a laser with 365 nm focused through a 40x water-immersion objective was used to activate the dye 2-4 seconds/cell in the presumptive anterior midbrain area. The embryos were fixed at 26 hpf prior to in situ hybridization.

In vivo imaging of the development of the DMB
At shield stage, dechorionated wild-type host embryos containing transplanted donor cells (see above) were stained with 100 μm Bodipy-FL-ceramide C5 (Molecular Probes) in Ringer medium for 30 minutes. At 26 hpf embryos were scanned by confocal microscopy and fixed prior to in situ hybridization and antibody staining.

Whole-mount in situ hybridization
Whole-mount mRNA in situ hybridization were carried out as described by Reifers et al. (Reifers et al., 1998). Digoxigenin- and fluorescein-labeled probes were prepared from linearized templates using an RNA labelling and detection kit (Roche). Stained embryos were dissected and mounted in glycerol. Embryos were photographed on a Zeiss Axioskop and assembled using Adobe Photoshop. Expression patterns have been described previously: efn4 (Xu and Brulet, 1984), gbxl (Rhinn et al., 2003), pax6.1 (original clone cZK3) (Krauss et al., 1991b; Krauss et al., 1991a), eng2 and eng3 (Ekker et al., 1992), otx2 (Mori et al., 1994), pax2.2 (Pfeiffer et al., 1998), and isl1 (Okamoto et al., 2000).

Antibody staining
We visualized the Engrailed proteins with a monoclonal antibody 4D9 (Patel et al., 1989) using the protocol described by Holder and Hill (Holder and Hill, 1991). A monoclonal antibody against acetylated tubulin (Sigma, T-6793) was used at 1:20 dilution to reveal neurons that have started axogenesis (Wilson et al., 1990).

Results
Morphology of the forebrain-midbrain territory
Development of the DMB can be visualized using confocal microscopy of living embryos stained with the lipophilic dye Bodipy-FL-ceramide C5 which detects plasma membranes and extracellular spaces (Fig. 1A,B). At 26 hpf an indentation of the neuroepithelium is visible at the diencephalic-mesencephalic boundary (DMB) that corresponds to the position of the posterior commissure (Fig. 1B), which serves as an anatomical landmark of the DMB (Macdonald et al., 1994; Mastick et al., 1997). Forebrain neuroepithelial cells stretch from the basal to the ventricular side (Fig. 1B, labeled cell), and round up during division at the ventricular surface. Through strong proliferation, cells of the midbrain tectum form a broadened neural tube. In situ hybridization analysis with pax6.1 of the same embryo reveals that the posterior expression boundary and the position of the PC both lie at the DMB, as shown previously in fish and chicken (Macdonald et al., 1994; Matsunaga et al., 2000; Scholpp and Brand, 2003).

Gene expression at the diencephalic-mesencephalic boundary
To determine when the DMB is first established, we examined the expression of pax6.1, pax2.1, fgf8 and eng2 at earlier stages of development. Expression of pax6.1 starts within the anterior neural plate at the end of gastrulation, at 90% epiboly, in close proximity to, but already separate from, pax2.1 expression at the MHB (Fig. 1D). At 100% epiboly, pax6.1 is expressed in two almost triangular patches anterior to the pax2.1 expression domain (Fig. 1E). The midline expression is not yet detectable at this stage. At the end of epiboly, the two lateral expression domains of pax6.1 in the forebrain fuse at the most rostral part at the midline. In the more caudal part, midline expression is still not detectable, leading to a horseshoe-like pattern. This pattern is known for many genes expressed in the anterior forebrain, for example for emx1 (Shanmugalingam et al., 2000). The posterior parts of the bilateral pax6.1 domains fuse along the midline at tailbud stage (Fig. 1F). Anteriorly, pax6.1 is weakly expressed, whereas expression in the posterior forebrain is comparatively strong, indicating a graded expression (Fig. 1C) (Grindley et al., 1997). At tailbud stage, expression of pax6.1 starts in the presumptive hindbrain region, specifically in two stripes adjacent to the midline, which will later be located in the basal plate (data not shown). At 24 hpf, the anterior expression domain of pax6.1 is restricted to the diencephalon (Fig. 1D) (Macdonald et al., 1994). Thus, pax6.1 is one of the earliest markers of the presumptive forebrain and its posterior expression boundary marks the DMB. The early pax2.1 expression marks the future midbrain and isthmus (Krauss et al., 1991a; Lun and Brand, 1998). The expression of pax2.1 starts in two wings at midgastrula stage, 80% of epiboly, which fuse into one band at the tailbud stage (Fig. 1F) (Lun and Brand, 1998). Surprisingly, double in situ hybridization experiments with pax6.1 and pax2.1 revealed a gap between these two expression domains (Fig. 1D-I); that is 4-5 cells wide on cross-sections at tailbud stage (11 sections examined, Fig. 1G). At mid-somitogenesis stage (6 ss) the gap between pax2.1 and pax6.1 widens, particularly on the dorsal side (Fig. 1H). With progressive restriction of pax2.1 to the isthmic zone, the midbrain emerges free of expression of either pax6.1 or pax2.1. At 24 hpf, expression of pax2.1 is restricted to the area of the isthmus proper (Fig. 1I).

In contrast to pax2.1, mapping of the expression of pax6.1 relative to the Engrailed genes does not reveal a gap (Fig. 1J-M). Expression of eng2 starts at 90% of epiboly, at the same position as the pax2.1 expression domain (Fig. 1J) and generally follows the pattern of pax2.1. However, eng2 expression is detectable more anteriorly than pax2.1 expression at 100% of epiboly (Fig. 1J). From this stage onwards, the posterior boundary of pax6.1 and the anterior boundary of eng2 are immediately adjacent to each other (Fig. 1K,L), with a slight overlap in one or two cell rows. eng3 expression starts around tailbud stage as a transverse band in the forming midbrain. Neither eng2 nor eng3 is detectable elsewhere in the presumptive forebrain. At 24 hpf, all Engrailed genes, including eng1 (eng1a – Zebrafish Information Network) and eng4 (eng1b – Zebrafish Information Network) are strongly expressed at the isthmus, and anterior and posterior to the pax2.1 expression domain in the midbrain (Fig. 1N). Thus, in contrast to pax2.1, the eng2 and eng3 genes are expressed in the future midbrain territory and are posteriorly adjacent to
the pax6.1 domain starting at late gastrulation until mid-somitogenesis stages (Fig. 1M). This close proximity of Engrailed gene and pax6.1 expression suggests a regulative interaction during formation of the DMB.

At the onset of pax6.1 expression, fgf8 is expressed in a more posterior and wider stripe than pax2.1 (Fig. 1O). The fgf8-expressing region covers the prospective MHB and continues into the fourth rhombomere, but leave the midbrain primordium free (Reifers et al., 1998). At tailbud stage, a gap of ~15 cell rows is visible between the posterior limit of pax6.1 expression and the anterior limit of fgf8 expression. The distance between these two expression domains remains similar until mid-somitogenesis (Fig. 1P,Q). After this period, the distance increases owing to the strongly proliferating midbrain primordium between the pax6.1- and the fgf8-positive domains (Fig. 1R).

Engrailed-dependent repression of pax6.1 during formation of the diencephalic-mesencephalic boundary

Our gene expression study of pax6.1 and Engrailed genes suggests that eng2/eng3, but not pax2.1, might interact with pax6.1 during formation of the DMB. To investigate this further, we mis-expressed eng3-mRNA unilaterally by injecting into one blastomere at the two blastomere stage.

We find that eng3 mis-expression causes a repression of pax6.1 in the anterior part of the embryo from the onset of its expression, whereas hindbrain expression is unaffected (Fig. 2A,B). Otx2, another gene expressed in the prosencephalon and midbrain, is not affected, indicating that change of cell fate from forebrain/midbrain to hindbrain fate does not occur (Fig. 2A,B). In a later phase at 26 hpf, eng3 mis-expression leads to an alteration in gene expression and structure of the DMB, visualized by repression of pax6.1 and efn4 (previously known as ephA4), a further marker gene respecting the DMB (Macdonald et al., 1994). In addition absence of the posterior commissure is observed on the injected side (Fig. 2D; white arrowheads). We also found that pax6.1 dependent structures such as the eyes (Bally-Cuif and Wassef, 1994; Halder et al., 1995) were reduced or absent in eng3-mis-expressing embryos (Fig. 2C,D,M) (Ristoratore et al., 1999). The injection of different amounts of eng3 mRNA led to a repression of pax6.1 in a dose-dependent manner specifically in the anterior part of
Fig. 2. Mis-expression of eng3 results in an anterior shift of the position of the DMB. Mis-expression of 500 pg eng3 mRNA in one cell at the two blastomere stage (A-D). The broken line indicates the midline; the upper side is the control side (con) and the lower side is the injected side (inj). At 1 ss, pax6.1 expression is strongly diminished in the injected side (black arrowheads). The expression of otx2 is not altered. To localize the experimental side, lacZ mRNA was co-injected and detected with a secondary, FITC-coupled antibody (B). At 26hpf, efna4 is suppressed at the injected side (C). The mis-expression of eng3 results in an altered formation \((n=36, \text{out of } 84)\) or in a loss of the posterior commissure \((n=23, \text{out of } 84)\; \text{D, white arrowheads). To follow the fate of small cell clones in eng3-mRNA-injected embryos, caged fluorescein was co-injected with lacZ-mRNA in control embryos or together with eng3-mRNA (E-J). For this experiment, a microscope was used that was provided with a nitrogen laser unit (E) and a grid (raster size 0.05 mm). At shield stage, the embryos were orientated dorsal side up with the shield to the left. The fluorescein dye was uncaged with a laser beam (365 nm) at the position of the grid: \(x\)-axis, \(-4; y\)-axis, \(-3\) (F). A picture of a living embryo at shield stage was superimposed on a dark field picture to visualize the clone, and a grid was overlaid. ‘sh’ marks the position of the shield. At 15 ss, the cell clones are visible in the eye or in the posterior diencephalon visualized by a superimposed pseudo lateral picture of the same living wild-type embryo with a darkfield-fluorescence picture (G). In situ hybridization for pax6.1 expression on the same embryo shows that the clone is located in the pax6.1-positive domain (encircled by red dots, H). Small cell clones that were uncaged in embryos injected with 500 pg eng3-mRNA are located in the pax6.1-negative region, suggesting a transformation of cell identity (IJ). (K-M) Injection of different amounts of eng3-mRNA (300 pg and 600 pg, indicated by the red triangle below) show concentration-dependent reduction of pax6.1 expression and of the size of the eye anlage (arrowheads). After the injection of 600 pg eng3-mRNA, the forebrain expression of pax6.1 is completely abolished and the eye anlage was not detectable (M). DMB, diencephalic-mesencephalic boundary; eye, eye anlage; hb, hindbrain; mb, midbrain; PC, posterior commissure; sh, shield.

In the case of the DMB, as marked by pax6.1 expression (Fig. 2I,J; \(n=7\)). In addition, we do not observe an increase of cell death in the labeled clones by Acridine Orange staining. We therefore conclude that in the eng3-injected embryos cells located in the position of the presumptive diencephalic neural plate, including the eye anlage, become transformed into a midbrain fate. These results show that eng3 is capable of repressing forebrain identity in the cephalic neural plate.

Inactivation of Engrailed gene expression causes forebrain expansion

To determine whether Engrailed genes are also required to suppress forebrain identity in the cephalic neural plate, we examined the development of embryos deficient for eng2 and eng3. To inactivate the Engrailed genes, we injected morpholino antisense oligomers (Nasevicius and Ekker, 2000) to prevent translation of the early expressed genes eng2 and eng3 (Scholpp and Brand, 2001). We compared the phenotype of morpholino-injected embryos to no isthmus (noi) mutant embryos that lack functional Pax2.1 protein. In the noi mutant embryos, the pax5 transcripts are not detectable (Lun and Brand, 1998).

With regard to development of the DMB, the repression of either eng2 or eng3 did not lead to a phenotype different from the control embryos (Scholpp and Brand, 2001). In contrast to this, double knock-down of both eng2 and eng3, caused the expression of the diencephalic marker genes pax6.1 and epha4...
Effect of a second transgenic line, Ptc1Δ11, was injected at the 8-cell stage and expanded postnatally to the midbrain. The midbrain expansion was more prominent in embryos deficient for ptc1Δ11 at 15 ss (A,B). noi mutants were recognized by the absence of ptc1Δ11 expression (C). The normal AP extent of the forebrain is marked by a white arrow, and the DMB, diencephalic-mesencephalic boundary. (D) The normal AP extent of the forebrain is marked by a white arrow, and the DMB, diencephalic-mesencephalic boundary. (E) The DMB, diencephalic-mesencephalic boundary. (F) The expanded pax6.1 territory is marked by red arrows. (G,L) In eng2/eng3 mutants, Engrailed protein is not detectable by the α-Engrailed antibody 4D9. (O,P) The expansion is paralleled by a posterior shift of the posterior comissure (arrowheads) at 32 hours, stained with antibodies against acetylated tubulin. Asterisks mark the epiphysis. DMB, diencephalic-mesencephalic boundary; fb, forebrain; hb, hindbrain.

Effects of ectopic FGF8 on the diencephalon and the midbrain

In noi mutant embryos or embryos depleted of eng2 and eng3, the DMB did not expand to the hindbrain, nor was the presumptive midbrain completely absent. This result suggests that an additional signal might maintain the midbrain and/or posterior forebrain, possibly via interaction with pax6.1. Previous experiments have demonstrated that Fgf8 is able to suppress posterior diencephalic fates, assumed to be due to its isthmus-inducing ability (Sato et al., 2001). In ace mutant embryos, the pax6.1 expression domain in the forebrain expands slightly into midbrain territory (Fig. 6J). This raised the possibility that midbrain induction (via induction of Engrailed genes) and forebrain suppression might be two separate events. We therefore examined the effect of Fgf signalling on posterior forebrain development.

To determine whether Fgf8 has a direct effect on pax6.1 expression, we introduced an ectopic source of Fgf8 protein near the DMB. Heparin-coated beads soaked with the FGF8b protein isoform (MacArthur et al., 1995) provide a local source of functional protein that can diffuse over a few cell diameters (Storey et al., 1998). We implanted FGF8b-coated beads into the anterior part of the prospective midbrain of wild-type embryos at 5 ss. Two hours after implantation, we observed repression of pax6.1 in the implanted side of the embryos (n=11), whereas eng3 expression was expanded anteriorly (n=5) (Fig. 4A,B). To determine whether Fgf8-dependent repression of pax6.1 requires functional P2x2.1 or Engrailed proteins, we implanted the FGF8-soaked beads into the prospective midbrain of noi mutants, which normally lack both. We recognized noi homozygous embryos by simultaneously staining the experimental embryos with eng3 probe. On the implanted side, the FGF8-bead caused a repression of pax6.1 in the forebrain, as described above for wild-type embryos. To our surprise, we found that on the implanted side the FGF8-bead was able to induce eng3 expression around the implantation site, although in a pax2.1-deficient situation. Thus, FGF8-bead implantation could by-pass the requirement for P2x2.1 as an upstream regulator of Engrailed gene expression.
To examine whether the repression of pax6.1 is due to the induction of an ectopic isthmus-like structure within a relatively short time period, we stained these noi mutant embryos carrying an FGF8 bead for pax2.2 and eng3 expression. We used pax2.2 as a marker for the MHB, because it is regulated independently of pax2.1 at midsomitogenesis stages (Pfeffer et al., 1998). In noi mutants, pax2.2 is downregulated at this stage but it is still present in a ventral expression domain. Similar to the previous experiments, we were able to induce expression of eng3 but not expression of pax2.2. This finding suggests that FGF8-bead implantation might cause forebrain repression independent of MHB-organizer induction (Fig. 4E,F; n=4). Interestingly, beads coated with FGF4 showed a similar effect, i.e. induction of eng3 and repression of pax6.1 (n=6) (not shown).

We conclude that Fgfs are involved in maintaining eng3 expression in the midbrain territory independent of functional Pax2.1 protein. Furthermore, Fgfs are able to repress forebrain fate, as monitored by expression of pax6.1.

**Fgf-blind cells in the midbrain acquire forebrain fate**

Our findings raise the possibility that cells located in the presumptive midbrain neural plate might require Fgf to keep them from following a forebrain fate. To test this idea, we blocked reception of Fgf signalling in small cell clones in the midbrain by expressing the dominant negative Fgf-receptor (Fgfr) XFD (Amaya et al., 1991). We co-injected XFD-mRNA with a fluorescent lineage tracer into the one blastomere stage. At shield stage we transplanted small cell clones from the injected embryo into the territory of the presumptive midbrain of a wild-type embryo, i.e. before the onset of pax6.1 and pax2.1 expression (Fig. 5A-C). The resulting chimaeras were examined for fluorescent cell clones located in the midbrain and subjected to in situ hybridization at the 10 ss. Transplanted cells autonomously expressed the forebrain markers pax6.1 and efna4 in the presumptive midbrain (Fig. 5D,G; n=5), whereas surrounding midbrain cells express neither pax6.1 nor efna4.

To examine if the transplanted cells also lack midbrain identity, we stained chimaeric embryos with monoclonal antibody 4D9, which detects all Engrailed proteins, as a midbrain marker (Patel et al., 1989). Cells expressing XFD, which were unable to transduce the Fgf signal did not co-express Engrailed and pax6.1 (n=4) (Fig. 5H-J). As a control, we transplanted cells that are labeled only with the fluorescent marker. These cells display normal expression of Engrailed protein, visible as co-localization of the red fluorescent lineage tracer and the green fluorescent labeled antibodies in the nuclei (Fig. 5K-M). Expression of marker genes for the anterior hindbrain (gbx1), for the MHB territory (fgf8) and for rhombomeres 3 and 5 (krox20) were not observed in such clones (Fig. 5N,O; n=10). Together, these results indicate that XFD-expressing cells in the midbrain change their identity cell-autonomously and acquire forebrain character, but not hindbrain character, if they are inhibited from transducing an Fgf signal. The neighboring host cells are unable to by-pass the block to Fgf signalling and rescue the midbrain phenotype. We conclude that reception of a direct Fgf signal is necessary to prevent forebrain fate and to maintain midbrain fate.

**Fgf signalling is necessary to maintain the position of the DMB until 10 ss**

The above results lead us to determine when Fgf signalling is required to suppress forebrain fate, and to compare this with the onset of gene expression in the forming DMB. We treated embryos with the pharmaceutical Fgfr inhibitor SU5402 (Mohammadi et al., 1997) at different developmental time points and for varying time periods. Wild-type embryos treated with SU5402 showed only a weak posterior expansion of the diencephalic pax6.1 expression, similar to the phenotype observed in ace mutant embryos (not shown). We then repeated the SU5402 inhibition with noi mutant embryos, which lack eng2 and eng3 expression (Lun and Brand, 1998) and therefore provided an opportunity to study the role of Fgf signalling in a sensitized background. We applied the inhibitor from 80% epiboly, i.e. before the onset of pax6.1 expression, until 15 ss. This treatment causes a strong expansion of pax6.1 expression into the territory of the presumptive midbrain around 15 ss in noi embryos (Fig. 6A,B). Inhibition between 5 ss to 15 ss led to a weaker expansion of pax6.1 expression (Fig. 6C), and with inhibition from the 10 ss onwards, posterior expansion of pax6.1 is no longer observed (Fig. 6D), even when the treatment was continued until 24 hpf (not shown). We conclude...
that Fgf signalling is necessary in combination with eng2/eng3 for positioning of the DMB until about the 10 ss.

**Fgf8 and Engrailed act in a synergistic fashion to position the DMB**

To determine which Fgf is required for correct positioning of the DMB, we blocked the translation of fgf3, fgf4 and fgf8, all of which are expressed at the MHB, via injection of morpholino (MO) antisense oligos, alone or in combination. (Araki and Brand, 2001; Raible and Brand, 2001). The embryos were injected with 4 ng of MO and showed gene-specific defects. MO-fgf8 injection phenocopies the ace
hindbrain domains of expression (Fig. 6J), arguing that pax6.1 (ace) expansion in the MO-pax6.1 morphants, was able to increase the fgf formation of the DMB (not shown). In addition, no single Eng gene expression in fused ventrally (Fig. 6H,I). To distinguish whether the lack of fgf8 phenotype of MO-injected We next compared the. These experiments that among the Fgfs tested, Fgf8 is the fgf8 MO- injected pax6.1 domains fuse ventrally, and only a dorsal patch of cells with unknown identity remains free offgf8 (M). V alues are the average of the total number of neurons of 10 embryos per treatment. Error bars show the standard deviation and asterisks indicate significant differences (*P=0.01) when compared with wild-type siblings.

mutant in 79% of the injected embryos (not shown) (Araki and Brand, 2001). The injected embryos showed a weak expansion of the forebrain marker pax6.1 into the presumptive midbrain, a loss of eng2 expression from the 15 ss onwards, and failure of MHB formation (Fig. 6G). The same phenotype is observed in ace mutant embryos (Reifers et al., 1998). The injection of MO-fgf8 into noi embryos caused a stronger expansion of pax6.1 expression into the presumptive midbrain than that observed after inactivation of either fgf8 or pax2.1 alone (compare Fig. 6H, 6F and 6G). In the injected embryos, we observe a fusion of the forebrain and hindbrain expression domains of pax6.1. By contrast, the injection of MO-fgf3 and MO-fgf4 did not lead to any changes with regard to the formation of the DMB (not shown). In addition, no single injection of the other fgf morpholinos, or a combination of them, was able to increase the pax6.1 expansion in the MO-fgf8 injected morphants (data not shown). We conclude from these experiments that among the Fgfs tested, Fgf8 is the primary signalling molecule that restricts pax6.1 expression to the forebrain cephalic neural plate. We next compared the phenotype of MO-fgf8 injected noi mutant embryos with that of SU5402-treated noi mutant embryos. In both situations, the forebrain and hindbrain expression domains of pax6.1 were fused ventrally (Fig. 6HJ). To distinguish whether the lack of Eng gene expression in noi mutants, or the lack of some other function controlled by pax2.1, is responsible for forebrain repression, we injected MO-eng2/eng3 into the acerbellar (ace) mutant. We again observe the fusion of the forebrain and hindbrain domains of pax6.1 expression (Fig. 6J), arguing that the Eng genes are responsible for forebrain repression in noi mutants.

To determine the neuroanatomical consequences of pax6.1 expression in embryos lacking both fgf8 and eng2/eng3-dependent forebrain restriction, we examined the phenotype of 28-hour-old embryos. We examined in particular the formation of isll-positive neurons in the epiphysis (Masai et al., 1997) and the location of the posterior commissure with an antibody against acetylated tubulin, in relation to pax6.1 and eng2 expression. Expanded forebrain expression of pax6.1 into the midbrain was visible in the MO-fgf8 injected embryos, when compared to the noi mutant embryos (Fig. 7B,C). A significantly enhanced phenotype is shown in embryos lacking both fgf8 and eng2/eng3 (Fig. 7D). As observed during mid-somitogenesis stages, the forebrain and hindbrain expression domains fuse ventrally, and only a dorsal patch of cells with unknown identity remains free of pax6.1 expression at 28 hpf. Expression of the Engrailed genes was absent in the MO-fgf8 injected embryos, in the noi mutants and in MO-fgf8/noi double mutants (Fig. 7B-D).

In noi mutant embryos the nuclei of posterior commissure neurons stretch further posteriorly into the presumptive midbrain, compared with the control situation and compared with wild-type embryos injected with MO-fgf8 (Fig. 7E-G). This phenotype becomes significantly stronger after MO-fgf8 injection into noi mutant embryos (Fig. 7H). Compared with the wild-type, we found an increase of about 50% of neurons expressing isll in the dorsal midbrain (Fig. 7M). The posterior commissure, visualized by anti-acetylated tubulin staining,
stretches further posteriorly also in the noi mutant embryos, and some fascicles of the commissure were not restricted to the territory of the DMB and crossed into the presumptive midbrain (Fig. 7I-L). In MO-fgf8-injected noi mutants, the PC axons lost their path completely, leading to a disorganized pattern (Fig. 7L).

Discussion

We studied the formation of the zebrafish diencephalic-mesencephalic boundary (DMB) as a model to help us understand how neural tube subdivisions are maintained. We find that with regard to the DMB: (1) eng3-mis-expression is sufficient to repress diencephalic fate; (2) knock-down of leads to a caudal shift of the DMB at the expense of the presumptive midbrain; (3) Fgf8 protein is necessary and sufficient to repress forebrain identity and to maintain midbrain identity, independently of functional Pax2.1 protein; (4) pax6.1 repression by Fgf8 is necessary between the tailbud and 10 ss (14 hpf) to maintain the position of the DMB; and (5) a lack of both eng2/eng3 and fgf8 expression results in a fused forebrain and hindbrain domain, and to loss of a proper DMB. We suggest that both midbrain autonomous mechanisms (via Eng2/Eng3) and non-autonomous signals (Fgf8) maintain the distinction between fore- and midbrain subdivisions of the neural plate.

Positioning of the DMB requires eng2/eng3 function

Previous work in mice had suggested that the DMB may be generated by mutual repression of Pax6 and Pax2 plus Pax5 (Schwarz et al., 1999). Loss of Pax2.2 in mice or Pax2.1 in fish leads to expansion of the forebrain, and loss of Pax6 leads to expansion of the midbrain into forebrain territory [Pax2.2 (Schwarz et al., 1999), Pax2.1 (Scholpp and Brand, 2003), Pax6 (Stoykova et al., 1996)]. The nature of these interactions is not understood during the early embryonic period when the DMB division is established. In particular, because Pax2.5 controls the expression of En1 and En2 transcription factors, it was not clear whether Pax2.5 act directly onto Pax6 transcription, as suggested for Pax2 during optic stalk development (Schwarz et al., 2000), or if regulation occurs indirectly via En1 and En2. Our expression analysis shows that pax6.2 and pax6.1 are initially not adjacent or overlapping at the DMB, which would be a prerequisite for mutual repression. pax6.2 is a duplicate Pax6 gene in teleosts with a potentially similar function as pax6.1. However, because expression of pax6.2 is restricted to the mid-diencephalon, it is unlikely to contribute to formation of the DMB (Nornes et al., 1998). In contrast to pax2.1, eng2 and eng3 (the functional zebrafish orthologues of the mammalian En1 gene) (Force et al., 1999; Scholpp and Brand, 2001) abut or overlap with the pax6.1 domain already at the onset of expression, suggesting that eng2 and eng3 may serve this repressive function. Evidence from mis-expression of En1 and En2 in chick and OL-eng2 in Medaka previously suggested that En can suppress Pax6 expression in the forebrain neural plate (Araki and Nakamura, 1999; Ristoratore et al., 1999). We find that mis-expression of eng3 has the same effect in zebrafish, causing suppression of forebrain development and expansion of midbrain fate. In loss-of-function conditions, the En genes function redundantly in mice and zebrafish (Hanks et al., 1995; Scholpp and Brand, 2001), and although we have not tested eng2 mis-expression, we expect this to give the identical result as eng3 mis-expression. These findings suggest that repression of pax6.1 by eng2/eng3 operates during establishment of the DMB at neural plate stages. Our laser-uncaging experiments in eng3-injected embryos support the idea that the crucial interactions take place during early neural plate stages, and thus argue that transformation of the forebrain neural plate into a midbrain identity is the likely basis of the observed neuroanatomical alterations at later stages embryos. An interesting side aspect is that pax6.1 expression is specifically affected in the forebrain, but not the hindbrain neural plate in such embryos. Expression of pax6.1 in the hindbrain and spinal cord may therefore be under different genetic control, and indeed murine Pax6 is thought to act during dorsoventral, but not anteroposterior patterning processes in the spinal chord (Goulding et al., 1993). A direct action of Eng2/Eng3 onto forebrain pax6.1 expression is also supported by our loss-of-function studies for eng2/eng3. Nevertheless, it remained possible that Pax2.1 would confine pax6.1 expression at the DMB both directly and indirectly, via regulating Eng2/Eng3. We think this is unlikely, because FGF8-soaked beads can suppress pax6.1 expression and forebrain identity even in a pax6.1-mutant genetic background, when implanted into noi mutant embryos (see below). Together, these results argue that eng2/eng3 expression is both necessary and sufficient as the key determinant for restricting pax6.1 expression and forebrain identity at the DMB.

Non-autonomous repression of forebrain fate by Fgf signalling

A key observation of our work is that, as well as eng2/eng3, Fgf signalling is also necessary for normal formation of the DMB. Morpholino inactivation suggests that among the Fgfs expressed in the early neural plate, Fgf8, but not Fgf3 or Fgf4, perform this function, a notion that is further confirmed by our studies of the ace (fgf8) mutant. The closest source for Fgf8 during the crucial phase of development is the forming midbrain-hindbrain organizer, located at the junction between the midbrain and hindbrain primordia. The temporal requirement for Fgf signalling, as seen by pharmacological inhibition with the Fgfr inhibitor SU5402, is consistent with the time of Fgf8 expression in the forming MHB organizer. Beyond 10 ss, Fgf signalling is no longer required. Wnt signalling during this period is thought to subdivide the forebrain domain (Heisenberg et al., 2001; Kudoh et al., 2002) (reviewed by Wilson et al., 2002). Interestingly, the likely source for Fgf8 at the MHB does not directly abut the cells at the DMB, suggesting a possible long-range effect of Fgf8 signalling. We suggest that long-range signalling may occur directly: our results show that an Fgf signal needs to be directly received by midbrain cells, as midbrain cells expressing a dominant-negative Fgf receptor construct lose midbrain identity, and switch to a pax6.1-positive forebrain fate. This finding predicts that Fgf8 can signal over a considerable distance through the forming midbrain neural plate, consistent with its role as a secreted factor. During later stages of midbrain development, it is thought that Fgf8 polarizes ephrin ligand expression to allow proper formation of the retinotectal projection of retinal axons (Lee et al., 1997; Picker et al., 1999; Yates et al.,
Fgf8 can occur even in the absence of pax2.1 expression, and is linked to pax6.1 (Fig. 4). Similarly, in mice, pax6.1 expression is concomitantly reduced in En1 in an implantation of an Fgf8 bead causes repression of Pax6 even in response to eng2/eng3 expression also indirectly via pax6.1 in the forebrain, and Fgf8 may therefore repress bead implantation also causes activation of eng3 expression (Reifers et al., 1998). Moreover, Fgf8-bead implantation also causes activation of eng3 expression in the forebrain, and Fgf8 may therefore repress pax6.1 expression also indirectly via eng2/eng3 expression. Importantly, the activation of eng3 expression in response to Fgf8 can occur even in the absence of pax2.1, and is linked to concomitant reduction of pax6.1 (Fig. 4). Similarly, in mice, implantation of an Fgf8 bead causes repression of Pax6 even in an En1+/−/En2−/− background (Liu and Joyner, 2001). Together, these findings provide evidence that Fgf8 can act directly on the DMB. In chick, FGFR-soaked beads can activate En1 and Pax2 expression in the forebrain, which was suggested to reflect induction of an ectopic MHB organizer (Garda et al., 2001). Based on our studies of DMB formation, an early step in these events may be the suppression of forebrain and support of midbrain fate by FGF8-head implantation, without a need to induce an ectopic MHB organizer. This explanation is supported by the fact that FGFR-soaked beads do not induce later markers of the MHB such as pax2.2 or pax5 in noi mutant embryos under these circumstances (not shown).

Notably, isl1 staining of Eng2/Eng3- and Fgf8-deficient embryos revealed that posterior parts of the diencephalon are strongly expanded caudally, in particular the nucleus of the posterior commissure in dorsal prosomere 1 (p1), whereas the epiphysis in dorsal prosomere 2 (p2) appears less affected. The posterior commissure, as the anatomical landmark of the DMB, is likewise expanded caudally, as was noted also in Pax2/5 knockout mice and in zebrafish noi mutants (Schwarz et al., 1999; Scholpp and Brand, 2003). Because of a lack of markers distinguishing p1 and p2, we cannot resolve whether a similar preferential sensitivity of p1 also applies at ventral neural tube levels. However, forebrain markers abutting the DMB, like pax6.1 or efn4, also expand at ventral levels in midbrain-deficient embryos, suggesting that this may be the case.

Even with the most extreme loss of Eng2/Eng3 and Fgf8, a small group of cells located in the dorsal midbrain does not acquire pax6.1 expression. These cells express the dorsal markers wnt1, wnt4 and the dorsal midbrain marker pax7c (not shown), and are therefore most likely neural tube cells with a dorsal identity. Why do these cells not acquire forebrain character? One possibility is that an unknown signal can suppress pax6.1-positive dorsal diencephalic identity, or promote dorsal midbrain identity. This signal is unlikely to be Wnt1 or Wnt4 itself, because these are normally co-expressed in dorsal p1 with pax6.1. Furthermore, knock-down of wnt1 and the partially redundant wnt10b do not lead to predominately dorsal neural tube defects (Lekven et al., 2002). Promising candidates are bone morphogenetic proteins (Bmps), which can repress Pax6 in the hindbrain and dorsal spinal cord (Goulding et al., 1993; Timmer et al., 2002). Indeed, in mice, Bmp6 and Bmp7 are expressed in the roof plate of the telencephalon, the midbrain and in the hindbrain (Furuta et al., 1997). Interestingly, pax6.1 expression becomes already during midsomitogenesis stages (Fig. 1J,K). However, expression of zebrafish bmp7 has not been detected in the roof plate of the midbrain (Schmid et al., 2000), suggesting that another member of the Bmp family, e.g. Bmp6, might perform this function.

Synergistic repression by Eng2/Eng3 and Fgf8

A key finding of our work is that the most extreme disruption of DMB formation and concomitant reduction of midbrain formation is seen only after inactivation of both Eng2/Eng3 and Fgf8 function, arguing that both have parallel, independent functions in maintaining the DMB (Fig. 8). We observed this in various genetic situations, e.g. when Eng2/Eng3 function is knocked-down either through morpholino injection, or in noi mutant embryos, in combination with SU5402 inhibition of Fgf signalling, after more specific loss of Fgf8 in ace mutants, or after morpholino-inhibition of Fgf8 (Fig. 6). However, Fgf8 is apparently also involved in Engrailed maintenance in the midbrain primordium, because ace mutants fail to maintain eng2/eng3 expression (Reifers et al., 1998). Moreover, Fgf8-bead implantation also causes activation of eng3 expression in the forebrain, and Fgf8 may therefore repress pax6.1 expression also indirectly via eng2/eng3 expression. Importantly, the activation of eng3 expression in response to Fgf8 can occur even in the absence of pax2.1, and is linked to concomitant reduction of pax6.1 (Fig. 4). Similarly, in mice, implantation of an Fgf8 bead causes repression of Pax6 even in an En1+/−/En2−/− background (Liu and Joyner, 2001). Together, these findings provide evidence that Fgf8 can act directly on the DMB. In chick, FGFR-soaked beads can activate En1 and Pax2 expression in the forebrain, which was suggested to reflect induction of an ectopic MHB organizer (Garda et al., 2001). Based on our studies of DMB formation, an early step in these events may be the suppression of forebrain and support of midbrain fate by FGF8-head implantation, without a need to induce an ectopic MHB organizer. This explanation is supported by the fact that FGFR-soaked beads do not induce later markers of the MHB such as pax2.2 or pax5 in noi mutant embryos under these circumstances (not shown).

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Pax2 and its transient requirement in midbrain development and forebrain suppression

pax2.1-deficient zebrafish noi mutant embryos and Pax2/5 mutant mouse embryos lack Engrailed expression and later the midbrain territory (Favor et al., 1996; Lun and Brand, 1998; Schwarz et al., 1999). One possibility is therefore that pax2.1 (Pax2/5 in mice) may itself confer midbrain character to neuroepithelial cells. Alternatively, Pax2 function might only be needed to ensure spatially restricted activation of Engrailed-type genes, which in turn repress forebrain fate and control a midbrain specific program. An important finding of our study in favour of the latter possibility is that pax2.1 is not necessary to achieve repression of forebrain fate, because Fgf8-soaked beads implanted into the forebrain primordium can suppress pax6.1, even in the absence of functional pax2.1. Likewise, the normally stringent requirement for pax2.1 for eng2/eng3 activation (Lun and Brand, 1998) can be circumvented if expression is directly activated by implantation of an Fgf8-soaked beads into the midbrain primordium. We therefore suggest that the main, and perhaps only, function of pax2.1 in the midbrain may be to activate eng2/eng3 expression. This
finding is consistent with our observation that morpholinemediated knock-down of eng2 and eng3 gives an exact phenocopy of the noi mutant phenotype in the midbrain (Scholpp and Brand, 2001).

The DMB and lineage restriction

Neither the mutual repression model of pax6.1 and eng2/eng3, nor the non-autonomous repression of Pax6 genes by Fgf8 explains cellular behavior at the DMB. Previous studies have shown that cell mixing across the DMB is restricted (Araki and Nakamura, 1999; Larsen et al., 2001), and classical markers of rhombomeric boundaries, like tenascin and vimetin, are expressed also at the DMB in chick and zebrafish (Larsen et al., 2001; Cerdà et al., 1998); however, the mechanism that restricts mixing is not clear. Among the marker genes we have used to follow forebrain fate was the ephrin receptor efn4, the expression of which expanded into the midbrain in embryos with disrupted midbrain development, e.g. after knock-down of eng2/eng3. Efn receptors and their ligands have been implicated in restricting cell mixing across segment boundaries in the hindbrain (Wilkinson, 2000), and ephrin A2 and ephrin A5a are two ligands of Efn4 that are expressed in the midbrain, complementary to the receptor expression. These ligands are not only important for mediating retinotectal projection in zebrafish, but are also expressed in earlier somitogenesis stages of midbrain development, prior to ingrowth of retinotectal axons (Brennan et al., 1997), and expression is missing in noi and ace mutants (Picker et al., 1999) (S.S., C.L. and M.B., unpublished) We therefore suggest that Eph receptors and their ligands may also perform a similar function at the DMB. One implication of these findings is that segmentation in the hindbrain may be mechanistically related to a potentially neuromeric organisation of the more rostral neural plate.

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