Erratum

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An error in this article was not corrected before going to press.

Throughout the paper, *efna4* 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 patterning 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 Pax2/5/8 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 chicken, mice and zebrafish (Wurst et al., 1994; Araki and Nakamura, 1999; Scholpp and Brand, 2001). Mis-expression of *En1* in the anterior neural tube represses *Pax6* expression, resulting in a rostral shift of the DMB (Araki and Nakamura, 1999). Similarly, mis-expression of the Medaka *eng2* gene can repress forebrain fate, including optic vesicle formation (Ristoratore et al., 1999). The phenotype of *En1* knockout mice demonstrates its importance for proper development of the mesencephalon (Wurst et al., 1994; Liu and Joyner, 2001). Zebrafish *eng2* and *eng3* (*eng2a* and *eng2b* – Zebrafish Information Network) are the functional orthologues of the murine *En1* gene and are expressed in similar spatial domains during late stages of gastrulation and early somitogenesis (Force et al., 1999). A knock-down of these genes causes a loss of *pax2.1* expression and severe defects in midbrain development, similar to those observed in mice (Scholpp and Brand, 2001). 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* knockout 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*^{tu29a} embryos, '*noi*-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 TTT TG-3′; and reverse, 5′-TCT TTG GAC TTC AGC ATG GA-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/nl. For a control, randomized mis-priming morpholinos (con-MO) were used, which showed no effect on embryos injected at 15 ng/nl but cause unspecific effects at a concentration of 30 ng/nl. Morpholino-injected embryos (morphants) were fixed at given stages prior to in situ hybridization or antibody staining.

Sequences were as follows:

eng2-MO, 5′-CGC TCT GCT CAT TCT CAT CCA TGC T-3′;

eng3-MO, 5′-CTA TGA TCA TTT TCT TCC ATA GTG A-3′;

fgf3-MO, 5′-CAG TAA CAA CAA GAG CAG AAT TAT A-3′;

fgf3-4bp-mismatch, 5′-CA**C** TAA CAA GAA GAC CA**C** AAT TAT A-3′;

fgf4-MO, 5′-GCC GAC TGG ACA CTC ATC CTT CTA A-3′;

fgf8-MO, 5′-GAG TCT CAT GTT TAT AGC CTC AGT A-3′; and

con-MO, 5′-CCT CTT ACC TCA GTT ACA ATT TAT A-3′.

Inhibition of Fgf signalling

At 90% epiboly, an inhibitor of Fgf signalling, SU5402 (Calbiochem) was added to the medium at a concentration of 8 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, photoactivatable (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 DMNB-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 40× 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). Digoxygenin- 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: *efna4* (Xu and Brulet, 1984), *gbx1* (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* (Pfeffer 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. 1I) (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

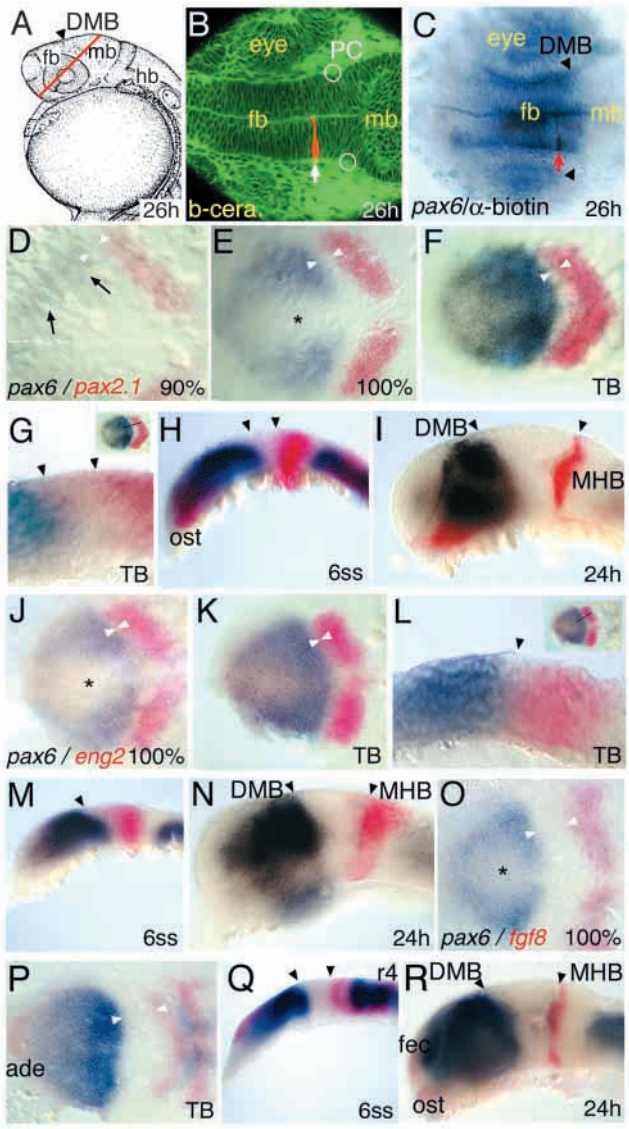

Fig. 1. Anteroposterior patterning in the early neural tube. Morphological analysis and whole-mount double in situ hybridization of wild-type embryos with indicated markers and stages. (A-C) The transition between forebrain and midbrain is marked by altered morphology, the posterior *pax6.1* gene expression boundary and location of the posterior commissure (PC). (A-C) Morphology of the diencephalic-mesencephalic boundary (DMB): confocal microscopy of a living Bodipy-FL-ceramide C5 stained embryo at 26 hpf (B); (A) scanning plane. The white arrow indicates the position of the biotinylated rhodamine-dextran labeled cell and white circles indicate the position of the PC as a morphological landmark of the DMB. (C) The same embryo after in situ hybridization with a *pax6.1*-probe and antibody staining against biotin to visualize the position of the labeled cell (red arrow). (D-R) Analysis of expression patterns at the territory of the DMB: expression pattern of *pax6.1* and *pax2.1*. *pax6.1* is first detectable at 90% of epiboly in a 'salt and pepper' distribution (D; black arrows). A horseshoe-like pattern is visible at 100% (E). (G) Cross-section of the embryo in F visualizes the gap between *pax6.1* and *pax2.1* at tailbud stage (arrowheads). (H,I) Pattern of gap at 6 ss and 24 hpf. (J-N) Expression pattern of *pax6.1* and *eng2*. From the onset (J) until midsomitogenesis (M) there is no gap between the expression domains of *pax6.1* and *eng2*. At 24 hpf (N), the expression of *eng2* is much broader on the MHB compared with *pax2.1* (I) and fades out into the midbrain. (O-R) Expression pattern of *pax6.1* and *fgf8*. *fgf8* is first expressed in a wide band spreading from the MHB primordium over to the anterior hindbrain (O). At tailbud stage the expression is confined to the MHB territory and the presumptive rhombomere 4 (P). In the early phase of neural patterning, ~10 cell wide gap is shown between the expression domains (O,P). At 6 ss the gap increases in size to 20 cells (Q). At 24hpf, the expression of *fgf8* is refined to the posterior part of the MHB (R). Arrowheads demarcate the gap between the expression domains of *pax6.1* and the indicated marker (D-R). Asterisks mark the *pax6.1* free territory (E,J,O). dd, dorsal diencephalon; eye, eye anlage; fb, forebrain; fec, facial ectoderm; DMB, diencephalic-mesencephalic boundary; mb, midbrain; MHB, midbrain-hindbrain boundary; ost, optic stalk; PC, posterior commissures, somites per stage; tb, tailbud stage; tec, tectum opticum. Percentage specifications are percent of epiboly indicated in the figures.

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 *efna4* (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

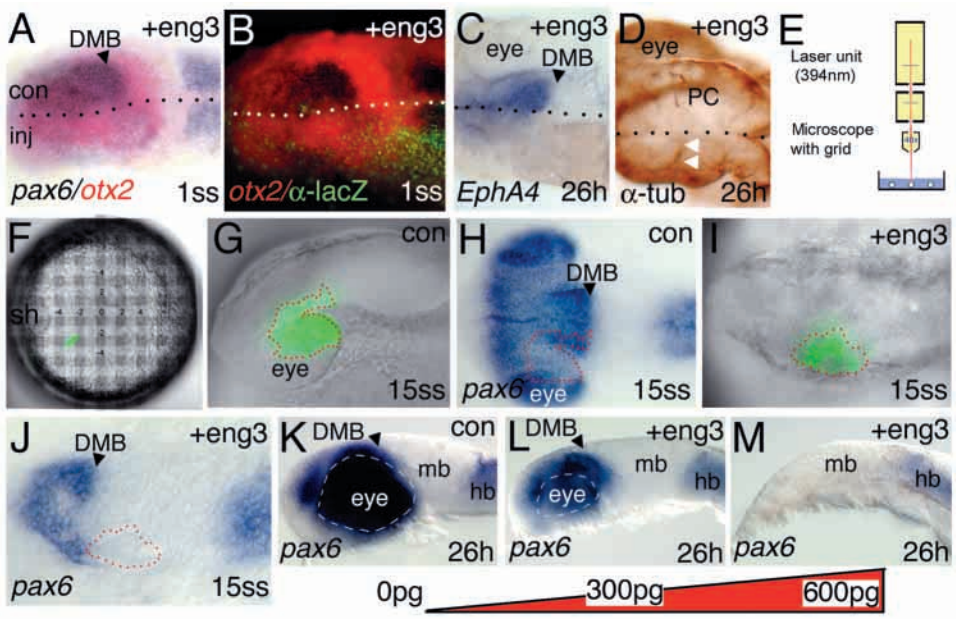

Fig. 2. Mis-expression of *eng3* results in an anterior shift of the position of the DMB. Mis-expression of 500 pg *eng3* mRNA in one cell at the two blastomere stage (A-D). The broken line indicates the midline; the upper side is the control side (con) and the lower side is the injected side (inj). 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, out of 84) or in a loss of the posterior commissure (*n*=23, out of 84; 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 an 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 (I,J). (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.

the embryo (Fig. 2K-M). At the highest injected amount of mRNA (600 pg), development of the presumptive diencephalons was completely blocked, as seen by total loss of the *pax6.1* expression (Fig. 2M). The same amount of *lacZ*-mRNA has no effect (not shown).

The absence of the diencephalon and the suppression of the formation of the eye could either be the result of cell death or of transformation of forebrain into midbrain tissue or a combination of both. We therefore examined small cell clones in the territory of the presumptive forebrain neural plate of *eng3*-misexpressing and of control embryos, and mapped the location of the clones relative to marker gene expression at 15 ss. For this, we adopted a recently described labeling technique (Kozlowski et al., 1997). Embryos were injected at the one-cell stage with a caged-fluorescent dye. At the appropriate stage, a nitrogen laser emitting a wavelength of 365 nm was used to uncage the dye in a small group of cells (Fig. 2E). At shield stage, we activated the dye in both control embryos and in embryos co-injected with 500 pg *eng3*-mRNA at a position which gives rise to the diencephalon or its derivatives according to the fate map of Woo and Fraser (Woo and Fraser, 1998) (Fig. 2F). In control embryos, we found that all of the labeled cells were located in either the eye anlage or the diencephalon, and all labeled cells expressed *pax6.1* (Fig. 2G,H; *n*=9). By contrast, in the *eng3*-injected embryos, the majority of labeled cells were located in the presumptive midbrain territory, and only a few labeled cells were located in the posterior diencephalon, 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*

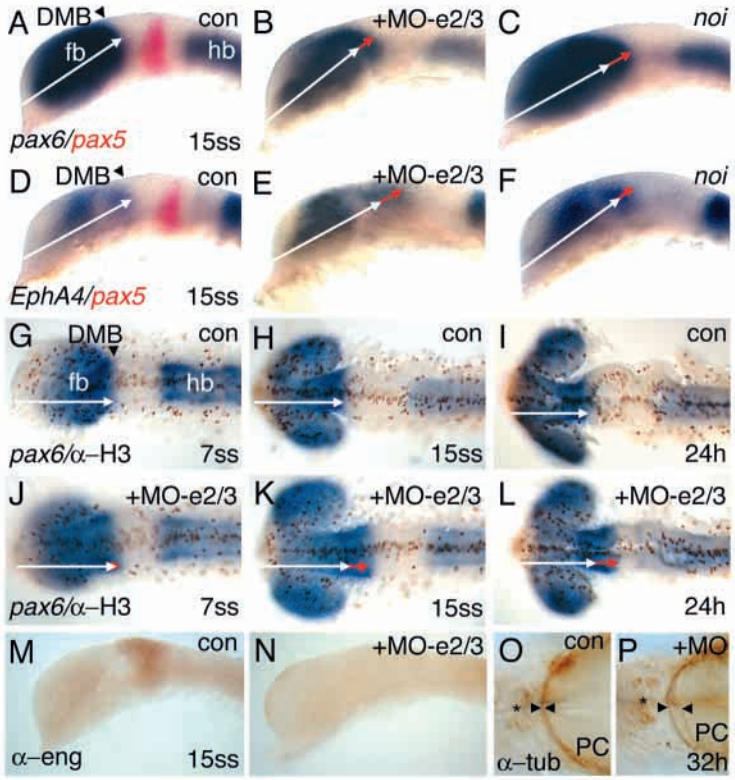

Fig. 3. The DMB shifts posteriorly in embryos deficient for *eng2/eng3*. The forebrain expression domain of *pax6.1* expands posteriorly in embryos deficient for *eng2/eng3* at 15 ss (A,B). *noi* mutants are recognized by the absence of *pax5* expression (C). The normal AP extent of the forebrain is marked by a white arrow from the anterior tip of the telencephalon to the posterior border of the forebrain; this arrow is of the same length in A,B,D,E. The red arrow indicates the expansion of *pax6.1* expression observed in embryos lacking *eng2/eng3* or *noi* function. A comparable phenotype is observed in the *noi* mutant embryos (C,F). Diencephalic expression of *efna4* is similarly expanded in *eng2/eng3* morphant embryos (D,E) and *noi* mutant embryos (F). Proliferation of forebrain cells is unaffected, as visualized by α-Phosphohistone 3 antibody and in situ hybridization with *pax6.1* probe of wild-type and *eng2/eng3* morphant embryos at the indicated stages (G-L). The expanded *pax6.1* territory is marked by red arrows. (M,N) In *eng2/eng3* morphants Engrailed protein is not detectable by the α-Engrailed antibody 4D9. (O,P) The expansion is paralleled by a posterior shift of the posterior commissure (arrowheads) at 32 hours, stained with antibodies against acetylated tubulin. Asterisks mark the epiphysis. DMB, diencephalic-mesencephalic boundary; fb, forebrain; hb, hindbrain.

to expand posteriorly, first detectable at the 7 ss and becomes more prominent at later stages such as the 15 ss (Fig. 3A,B,D,E). This expansion correlates with a caudal shift of some branches of the posterior commissure into the presumptive midbrain territory at 32 hpf (Fig. 3O,P). *pax5* expression is not detectable in the MO-*eng2* and MO-*eng3* injected embryos (Fig. 3I,J). The observed phenotype resembles that of the *pax2.1* mutant *noi* (Fig. 3C,F) (Scholpp and Brand, 2001). The lack of a functional *pax2.1* protein leads to the absence of expression of the Engrailed genes, except for a very faint and transient expression of *eng2* at the tailbud stage (Lun and Brand, 1998). The repression of the Engrailed mRNA translation via morpholino injection did not produce a more pronounced phenotype than the *noi* mutant, arguing that *pax2.1* largely exerts its function via *eng2* and *eng3* (Scholpp and Brand, 2001). *eng2* and *eng3* are therefore both necessary and sufficient to restrict the posterior forebrain boundary in the cephalic neural plate. In keeping with the transformation of cell type identity in the neural plate that we observed after *eng3* overexpression (Fig. 2), loss of *eng2* and *eng3* function does not cause overproliferation of forebrain cells at the 7 ss, 15 ss and 24 hour stage, as detected in α-Phosphohistone 3 antibody and Pax6 double-stained embryos (Fig. 3G-L).

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 with a non-functional Fgf8 protein or in MO-*fgf8* injected 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 the 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 Pax2.1 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 Pax2.1 as an upstream regulator of Engrailed gene expression.

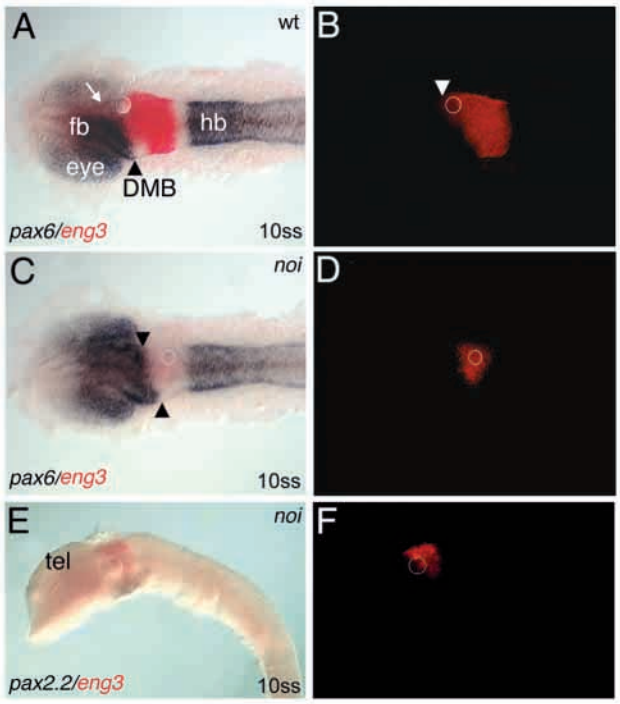

Fig. 4. FGF8-bead represses *pax6.1* expression in the anterior neural plate. (A) FGF8-soaked unilaterally implanted beads repress *pax6.1* expression in the posterior diencephalon of a wild-type embryo (arrowhead). An ectopic induction of *eng3* expression was observed around the bead (B). In the *noi* mutant embryos, *pax6.1* is also repressed by FGF8 (C) and *eng3* is ectopically activated in the absence of functional Pax2.1 protein. In the *noi* mutant embryos *pax2.2*, a *pax2.1*-independent marker at the MHB, is not induced in the same timeframe (E,F). All pictures show dorsal views with anterior to the left, (B-D) show darkfield pictures of embryos in (A,C,D); stages as indicated; arrowheads indicate the posterior border of the expression domain of *pax6.1* in the forebrain, circles indicate the position of the bead. DMB, diencephalic-mesencephalic boundary; eye, eye anlage; fb, forebrain; hb, hindbrain.

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

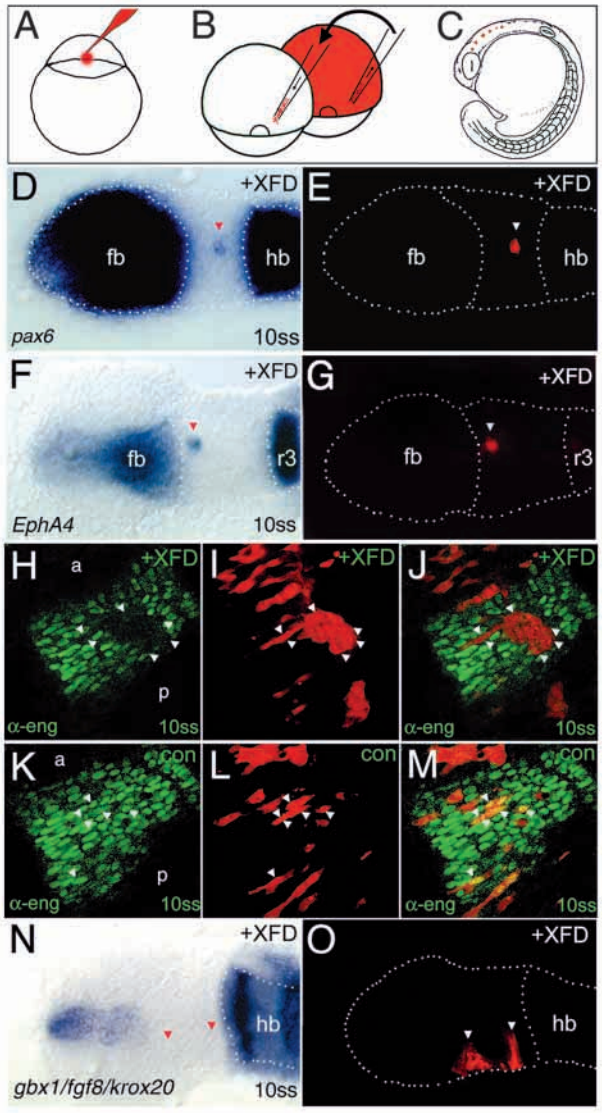

Fig. 5. Midbrain cells that cannot respond to Fgf signalling acquire forebrain character. Embryos were injected with 400 pg *XFD*-mRNA (A), grafts of these donor embryos were transplanted to a wild-type host embryos (B) and fixed at indicated stages prior to staining procedures (C). Arrows indicate transplanted cells. (D,F) Cell clones in the midbrain express the forebrain markers *pax6.1* and *efna4*. Transplanted cells contain rhodamine dextran (E,G), but do not express the midbrain marker Engrailed, as visualized by α-Engrailed antibody 4D9 staining (H-J). Control transplantations without *XFD* mRNA show co-localization of rhodamine dextran and α-Engrailed staining (K-M). (J,M) Superimposed pictures of H,K and I,L. Transplanted cells do not express any of the hindbrain markers *gbx1*, *fgf8* or *krox20* (N,O). a, anterior; fb, forebrain; hb, hindbrain; p, posterior; r3, rhombomere 3.

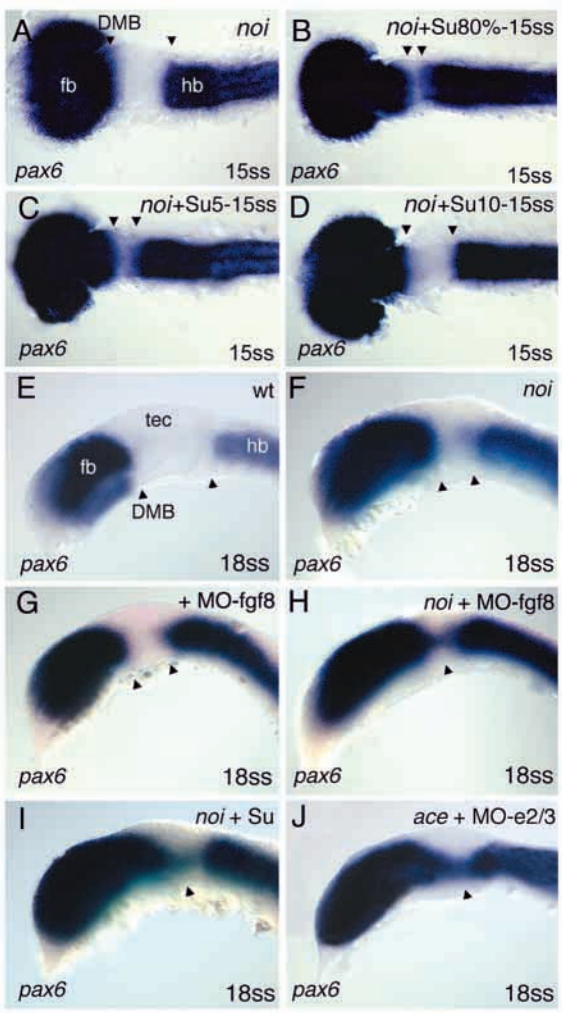

Fig. 6. Eng2/Eng3 and Fgf8 act synergistically in positioning of the DMB. The positioning of the DMB is dependent on Fgf signalling in early somitogenesis. Embryos were orientated dorsal side upwards and anterior towards the left. Inhibition of Fgf signalling via the pharmaceutical Fgfr inhibitor SU5402 (8 μm) in *noi* mutant embryos from 90% of epiboly to 15 ss leads to a strong expansion of the anterior *pax6.1* domain (A,B). A weaker expansion is visible when the treatment starts at 5 ss and lasts until 15 ss (C). After 10 ss, inhibition of Fgf signalling does not lead to a posterior shift of the forebrain expression of *pax6.1* (D). (H,J) Eng2/Eng3- and Fgf-deficient embryos show a stronger expansion of the forebrain expression of *pax6.1* than do single deficient embryos (F,G). (E-G) Embryos were orientated laterally and anterior is towards the left. The forebrain expression domain of *pax6.1* expands in wild-type embryos (E), *noi* mutant embryos (F) and in MO-*fgf8* morphant embryos (G). In *noi* mutant embryos, injected with MO-*fgf8*, the forebrain and hindbrain expression domain fuses (H). A comparable phenotype is observed in *noi* mutant embryos treated with Fgfr inhibitor SU5402 from 90% of epiboly until 15 ss (I). In *ace* mutant embryos injected with MO-*eng2/eng3* forebrain and hindbrain domain fuses similarly (J). DMB, diencephalic-mesencephalic boundary; fb, forebrain; hb, hindbrain.

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*

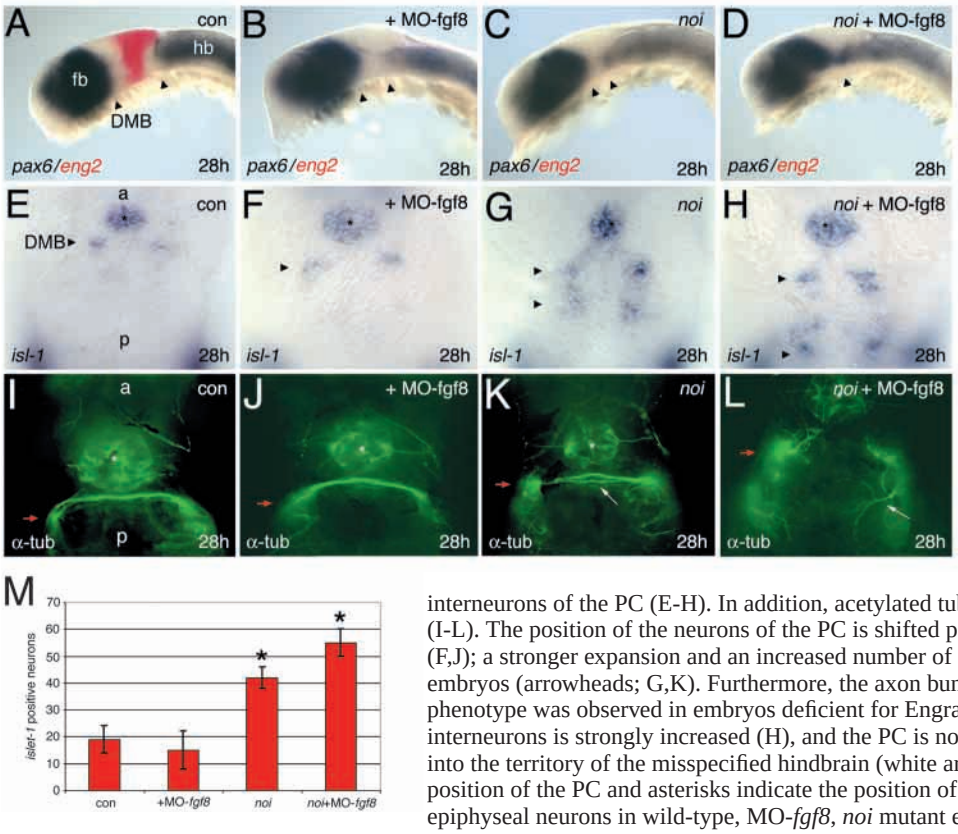

Fig. 7. Morphological analysis of the function of *fgf8* and *eng2/eng3* at the DMB at late stages. In comparison with the control embryo at 26 hpf (A), the expression of *eng2* is absent in *fgf8*-MO morphant embryos (B), in *noi* mutant embryos (C) and in *fgf8*-MO-injected *noi* mutant embryos (D). The expression domain of *pax6.1* weakly expands in *fgf8*-MO embryos (B), strongly in *noi* mutant embryos (C) and it fuses in the double treated embryos (D). The border of the *pax6.1* expression is marked by arrowheads (A-D). The position of the posterior commissure (PC) is altered in *eng2/eng3*- and *fgf8*-deficient embryos (E-L). Embryos were orientated dorsal side upwards and anterior towards the top. At 28 hpf the *isl1* staining labels the neurons of the epiphysis and interneurons of the PC (E-H). In addition, acetylated tubulin is marking the outgrowing axons (I-L). The position of the neurons of the PC is shifted posteriorly in *fgf8*-MO-injected embryos (F,J); a stronger expansion and an increased number of neurons were found in the *noi* mutant embryos (arrowheads; G,K). Furthermore, the axon bundle of the PC is fanned out. The strongest phenotype was observed in embryos deficient for Engrailed and Fgf8 (H,L). The number of interneurons is strongly increased (H), and the PC is not formed at all (L). Single branches project into the territory of the misspecified hindbrain (white arrows). Red arrows indicate the most lateral position of the PC and asterisks indicate the position of the epiphysis. Number of *isl1* positive epiphyseal neurons in wild-type, MO-*fgf8*, *noi* mutant embryos and *noi* mutants injected with MO-*fgf8* (M). Values 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. 6H,I). 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 *acerebellar* (*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 *isl1*-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 *isl1* 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-K). 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 Pax2/5 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 [Pax2/5 (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 *pax2.1* 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 *pax2.1*-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.,

2001), which may also involve long-distance signalling by Fgf8 in the forming tectum.

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, FGF8-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 the suppression of forebrain and support of midbrain fate by FGF8-bead implantation, without a need to induce an ectopic MHB organizer. This explanation is supported by the fact that FGF8-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 *efna4*, 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 excluded in the dorsal part of the telencephalon and hindbrain 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.

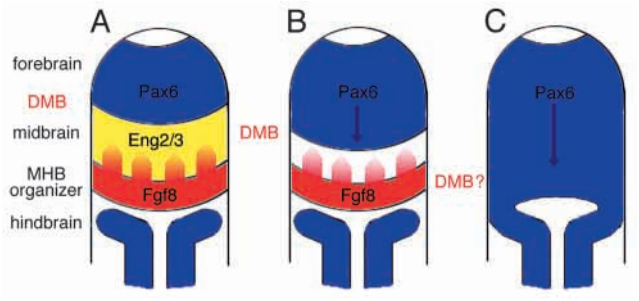

Fig. 8. Function of *eng2/eng3* and *fgf8* in AP patterning of the anterior neural plate. Pax6.1 (blue) demarcates the forebrain and hindbrain, whereas expression of *eng2/eng3* (yellow) and *fgf8* (red) can be observed in the midbrain/MHB organizer territory (A). Eng2/Eng3 displays the cell-autonomous signal maintaining midbrain fate, whereas Fgf8 acts non-autonomous in the midbrain territory visualized by red arrows. (B) Loss of Eng2/Eng3 causes a posterior shift of the DMB (blue arrow). Fgf8 expression is unaffected at least until 10 ss, suggesting a partial repression of the forebrain expansion. Loss of both signal Eng2/Eng3 and Fgf8 causes in a loss of midbrain identity and in a fusion of forebrain and hindbrain domain (C). Only a dorsal patch of cells remains free of *pax6.1* expression. DMB, diencephalic-mesencephalic boundary.

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 morpholino-mediated 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 rhombomere boundaries, like tenascin and vimetin, are expressed also at the DMB in chick and zebrafish (Larsen et al., 2001; Cerdá 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 *efna4*, 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 Efna4 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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