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ARTICLE

Integrity of the Midbrain Region Is Required to Maintain the Diencephalic–Mesencephalic Boundary in Zebrafish *no isthmus/pax2.1* Mutants

Steffen Scholpp[1] and Michael Brand[1,2]*

Initial anterior–posterior patterning of the neural tube into forebrain, midbrain, and hindbrain primordia occurs already during gastrulation, in response to signals patterning the gastrula embryo. After the initial establishment, further development within each brain part is thought to proceed largely independently of the others. However, mechanisms should exist that ensure proper delineation of brain subdivisions also at later stages; such mechanisms are, however, poorly understood. In zebrafish *no isthmus* mutant embryos, inactivation of the *pax2.1* gene leads to a failure of the midbrain and isthmus primordium to develop normally from the gastrula stage onward (Lun and Brand [1998] Development 125:3049–3062). Here, we report that, after the initially correct establishment during gastrulation stages, the neighbouring forebrain primordium and, partially, the hindbrain primordium expand into the misspecified midbrain territory in *no isthmus* mutant embryos. The expansion is particularly evident for the posterior part of the diencephalon and less so for the first rhombomeric segment, the territories immediately abutting the midbrain/isthmus primordium. The nucleus of the posterior commissure is expanded in size, and marker genes of the forebrain and rhombomere 1 expand progressively into the misspecified midbrain primordium, eventually resulting in respecification of the midbrain primordium. We therefore suggest that the genetic program controlled by Pax2.1 is not only involved in initiating but also in maintaining the identity of midbrain and isthmus cells to prevent them from assuming a forebrain or hindbrain fate. *Developmental Dynamics 228:313–322, 2003.* © 2003 Wiley-Liss, Inc.

Key words: forebrain; midbrain; *Fgf8*; *acerebellar*; prosomere; hindbrain; isthmus; organizer; *Danio rerio*

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INTRODUCTION

The neural plate is patterned along the anterior–posterior (AP) and dorsoventral axes by distinct mechanisms (reviewed in Lumsden and Krumlauf, 1996; Rubenstein et al., 1998; Rhinn and Brand, 2001; Wilson et al., 2002). Already in gastrulation, organizing centers in the neural plate act to induce and refine local cell type identities throughout the AP axis. At present, three such signaling centers have been identified at boundaries within the embryonic neural plate: the "row-1" or anterior neural ridge organiser (Shimamura and Rubenstein, 1997; Houart et al., 1998), the isthmic organiser located at the midbrain–hindbrain boundary (MHB; reviewed in Rhinn and Brand, 2001; Wurst and Bally-Cuif, 2001), and the organiser in rhombomere 4 (Maves et al., 2002). During development of the MHB organiser, several transcription factors are required, e.g., En1 and En2 (Wurst et al., 1994; Millen et al., 1994; Scholpp and Brand, 2001), Pax2 (Brand et al., 1996; Favor et al., 1996; Lun and Brand, 1998), Pax5 and Pax8 (Urbanek et al., 1994; Pfeffer et al., 1998), and Pou2 (Belting et al., 2001; Burgess et al., 2002; Reim and Brand, 2002). Among the secreted factors, Wnt1 and Fgf8 are required for the MHB development in mice (McMa-

[1]Max Planck Institute of Molecular Cell Biology and Genetics (MPI-CBG), Dresden, Germany
[2]Department of Genetics, University of Dresden (TU), Dresden, Germany
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*Correspondence to: Michael Brand, Department of Genetics, University of Dresden (TU), Pfotenhauer Str. 108, 01307 Dresden, Germany. E-mail: brand@mpi-cbg.de

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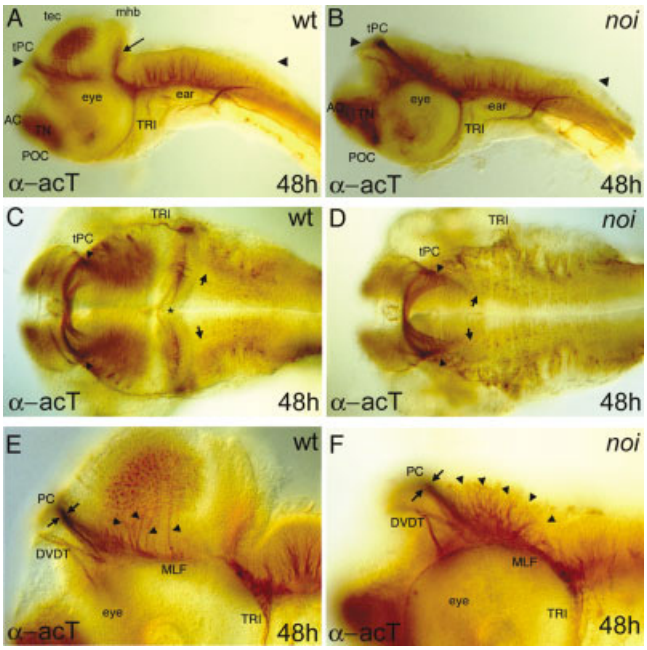

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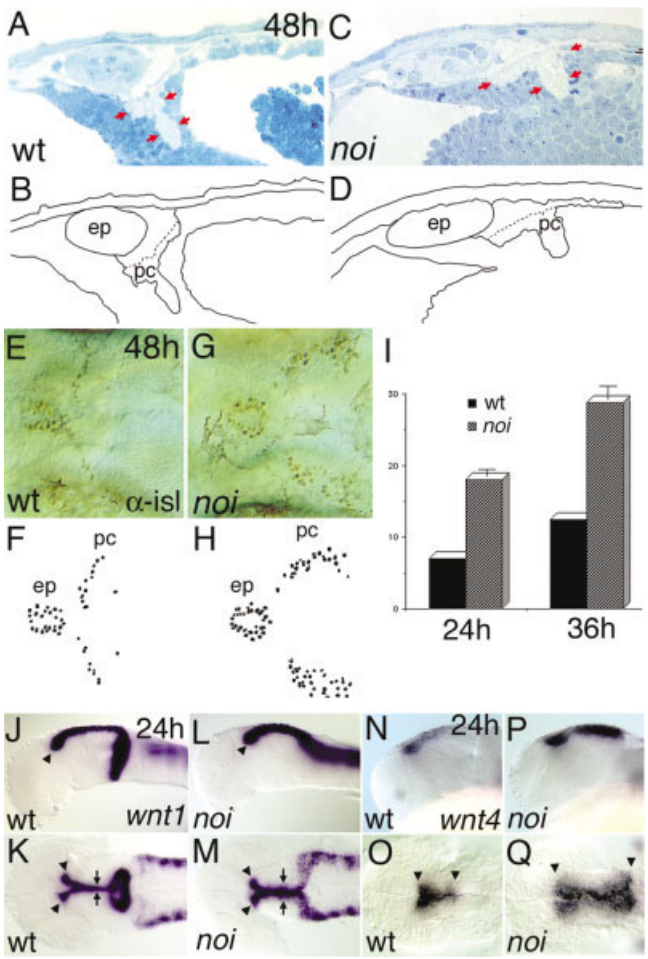

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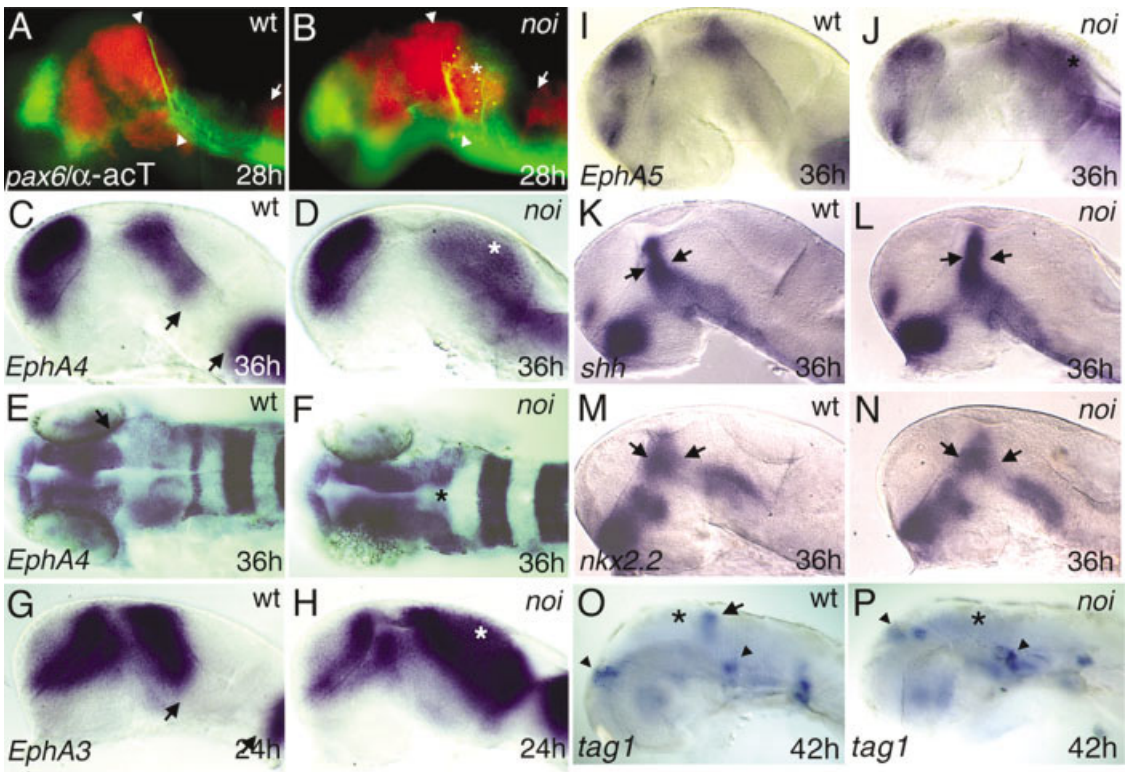


Fig. 3.

hon et al., 1992; Crossley et al., 1996) and zebrafish (Reifers et al., 1998; Lekven et al., 2003).

Based on expression patterns, it was suggested that the paired-box genes play an important role in regionalisation of the cephalic neural plate. In particular, two genes were proposed to play a crucial role during these patterning events: Pax6 in the forebrain and in the basal hindbrain domain, and Pax2 in the midbrain and MHB. Mutual repression of these genes was suggested to subdivide the neural plate into forebrain, midbrain, and hindbrain (Stoykova and Gruss, 1994; Schwarz et al., 1999). Indeed, in Pax6 mutant mice, a partial anterior expansion of markers of the midbrain territory into the presumptive forebrain is observed (Mastick et al., 1997) and cellular migration during hindbrain development is severely affected (Engelkamp et al., 1999). Pax2 plays a key role in establishment of the midbrain and the isthmus organiser in mice (Favor et al., 1996), as does the homologous gene *pax2.1* in zebrafish (Brand et al., 1996; Lun and Brand, 1998). Depending on the genetic background, inactivation of Pax2 in mice results in a variable reduction of the MHB. Full inactivation of Pax2 and Pax5, however, results in a reliably strong phenotype, suggesting a partial functional redundancy of these two factors (Pfeffer et al., 2000). The zebrafish *no isthmus* (*noi*) mutants were found during a large-scale mutagenesis screen and identified to have mutations in the *pax2.1* gene. Morphologically, lack of functional *pax2.1* protein leads to loss of the tectum, the MHB, and the cerebellum in fish (Brand et al., 1996; Lun and Brand, 1998). Downstream genes of *pax2.1*, like the homeobox transcription factors *pax5, pax8,* and the genes of the Engrailed family are no longer expressed in the midbrain and MHB of null mutant embryos. The expression of *pax2.1* is normally maintained by a transcriptional feedback loop that involves *eng2* and *eng3* (Picker et al., 2002). Morpholino-induced knockdown of these genes results in the absence of *pax2.1* at mid-somitogenesis and in a phenocopy of the *no isthmus* mutant with regard to MHB development (Scholpp and Brand, 2001). Studies of *engrailed* gene function in chick, medaka, and zebrafish, furthermore, suggested that Engrailed proteins may directly repress the forebrain marker *pax6.1* (Araki and Nakamura, 1999; Ristoratore et al., 1999; Scholpp et al., 2003).

Whereas several studies have addressed the role of Pax2/5/8 genes in the midbrain and isthmus, the consequences of absence of the midbrain and MHB organiser for the adjacent brain parts, the forebrain and the hindbrain, are less well understood. Here, we use the *noi* mutant as a tool to address this issue. Through marker gene expression and analysis of the morphology of the forebrain–midbrain region, we show that these regions are established normally but that midbrain is respecified into forebrain tissue during subsequent stages. As a consequence, the boundary between diencephalon and mesencephalon, and the posterior commissure as its neuroanatomic landmark, is shifted posteri-

Fig. 1. The posterior commissure (PC) and the medial longitudinal fasciculus (MLF) are present but modified in *noi* mutant embryos. Immunochemical labelling of acetylated tubulin was used to study axon formation in 48 hours (48h) postfertilization embryos, oriented anterior to the left. **A,B:** A lateral view of whole-mounted embryos. The tectum and the cerebellar fold, including their neurons, e.g., the nervus trochlearis, are absent in the mutant. Arrowheads in A and B mark the layer of the dorsal section in C and D, respectively. **C,D:** The PC, which demarcates the diencephalic/mesencephalic boundary, is still present. The PC looks more condensed in the mediolateral direction (arrowheads) and hindbrain neurons, visualized in small dots expand anteriorly (arrows). **E,F:** Higher magnification of the lateral view of the mutant embryo shows additional axons emerging from the dorsal midbrain area (arrowheads) and joining the medial longitudinal fasciculus (MLF). In the anteroposterior direction, the PC appears larger and defasciculated (E,F; arrows). AC, anterior commissure; DVDT, dorsoventral diencephalic tract; mhb, midbrain–hindbrain boundary; tPC, tract of the posterior commissure; POC, postoptic commissure; tec, tectum opticum; TN, telencephalic nucleus; TRI, trigeminal nerve, tro, trochlear nerve; wt, wild-type.

Fig. 2. The territory of the dorsal forebrain and midbrain is altered in *noi* mutant embryos. Histologic, immunochemical, and in situ hybridisation (ISH) analysis was used to study the dorsal forebrain and midbrain in *noi* mutant embryos. The embryos were oriented with anterior to the left. **A,B:** A parasagittal section through the dorsal part of the forebrain–midbrain region of a 48 hours postfertilization (hpf; 48h) wild-type (wt) zebrafish embryo shows the location of the epiphysis (ep) and the posterior commissure (pc). **C,D:** In the mutant, the ep and the pc seem enlarged in the anteroposterior direction marked with red arrows in C. **E–H:** A dorsal view of the nucleus of the posterior commissure interneurons stained with an α-islet (αisl) antibody indicates that the number of neurons is increased and the territory is expanded posteriorly. **I:** Already at 24 hpf and at 36 hpf, a significantly increased number of neurons is observed between wild-type siblings and in *noi* mutant embryos. **J–M:** A lateral and dorsal view of an ISH of *wnt1* shows a fusion of the midbrain and hindbrain pattern and the missing expression in the ventral part of the midbrain–hindbrain boundary. **N–Q:** The expression *wnt4* shows an expansion of these dorsal diencephalic markers into the midbrain territory (arrowheads).

Fig. 3. Marker analysis in wild-type (wt) and *noi* mutant embryos suggests a respecification of midbrain tissue. In situ hybridisation with the indicated marker genes at the given stages (h, hours postfertilisation), lateral views (except dorsal views in E, F) with anterior to the left; asterisks mark the position of the misspecified midbrain. **A:** Staining for *pax6.1*, a marker for the forebrain and basal hindbrain, combined with an acetylated tubulin staining to visualize outgrowing axons. The posterior commissure (PC, marked by white arrowheads) is located at the diencephalic–mesencephalic boundary in the *pax6.1*$^{+}$ domain. **B:** In the *noi* mutant, *pax6.1* expression is observed in the territory of the presumptive midbrain and new branches of the PC (yellow arrowheads) are visible more posterior to the endogenous position. Similar to the forebrain, the hindbrain expression domain expands anteriorly into the midbrain territory (A,B, white arrows). Markers of the Ephrin family show a similar phenomenon: *EphA4* (**C,E**), *EphA3* (**G**), and *EphA5* (**I**) respect the boundary between the diencephalon and the mesencephalon (black arrows). **D,F,H,J:** In the *noi* mutant, the expression expands into the misspecified midbrain area. **K–N:** Marker genes of the *zona limitans intrathalamica*, such as *shh* and *nkx2.2*, show no difference between *noi* mutant and wild-type siblings, suggesting a correct formation of this anterior region between the prosomeres 2 and 3 (arrows). **O:** *tag1* marks a subset of developing neurons in the cerebellar anlage (arrow). **P:** This expression domain is missing in the *noi* mutants, consistent with the loss of cerebellar identity in the *noi* mutants. However, the expression domains in the dorsal forebrain and in the anterior hindbrain are not altered (indicated by arrowheads).

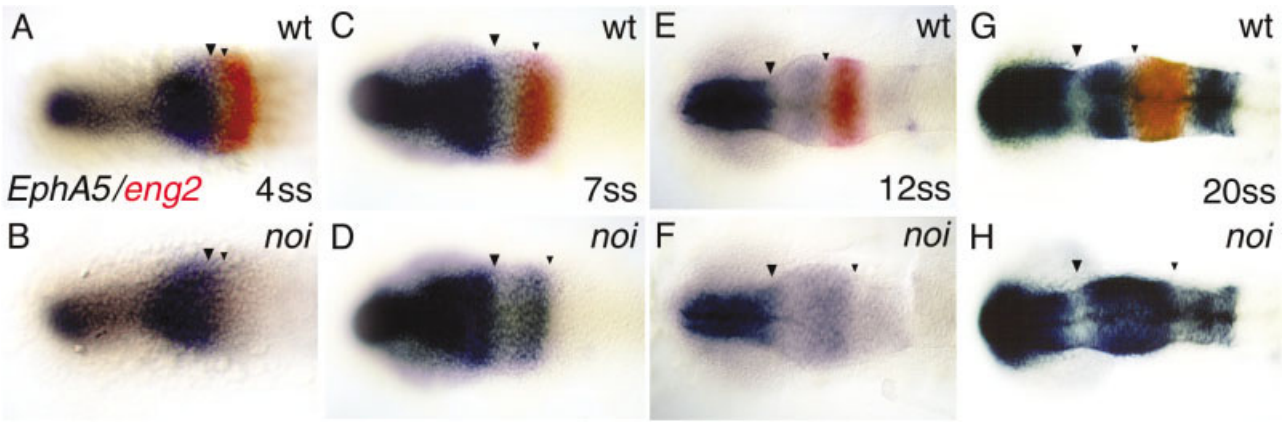
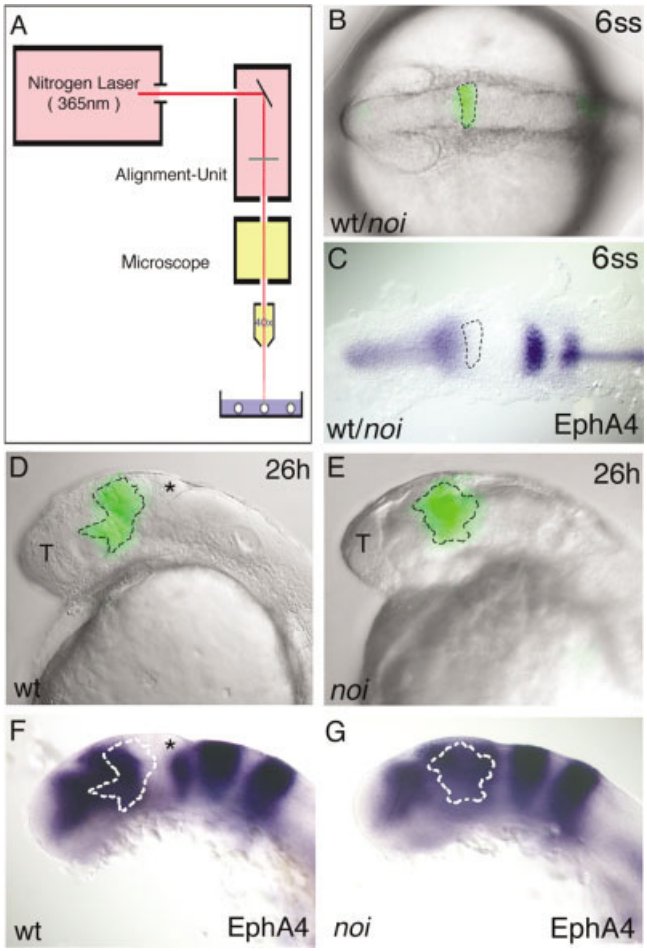


Fig. 4. Time course of marker expansion in *noi* mutant embryos. Double in situ hybridisation of wild-type (wt) and *noi* mutants with *EphA5* in blue and *eng2* in red. **A,B:** At the four-somite stage (4ss), there is no difference in forebrain marker expression between wild-type and mutants. **C,D:** At 7ss, the first change of marker expression is visible: *EphA5* expression starts in the anterior midbrain. *eng2* is used to distinguish wild-type embryos and mutant embryos, because the expression of *eng2* is absent in *noi* mutant embryos. **E,F:** At 12ss, *EphA5* is clearly expanded into the midbrain. **G,H:** The situation at 20ss in which the whole respecified midbrain expresses *EphA5*.



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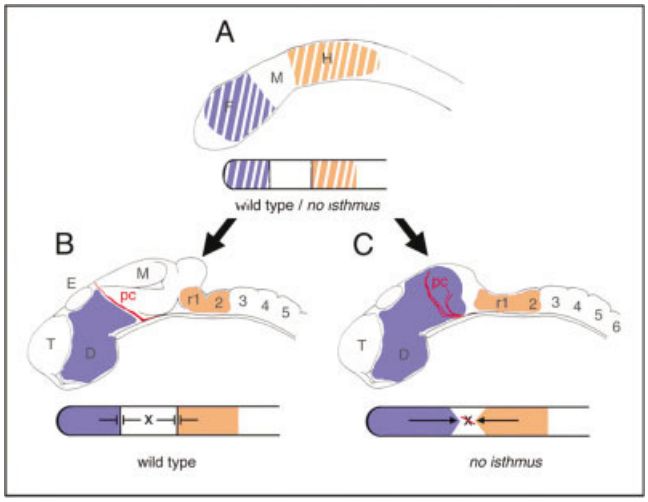

Fig. 6.

orly into the misspecified midbrain. By laser-induced uncaging of a lineage tracer dye, we show that, in *noi* mutant embryos, the midbrain is gradually transformed into forebrain fate. We therefore suggest that, although individual brain parts may be induced independently of each other, subsequent interactions are necessary to keep the identity of single brain parts. If this interaction is altered, neighbouring brain parts can take over the misspecified territory.

RESULTS

Axon Tracts of the Forebrain Are Present but Altered in the *no isthmus* Mutant

noi/pax2.1 is one of the earliest acting genes required for development of the midbrain and isthmus primordia from the gastrulation stage onward (Brand et al., 1996; Lun and Brand, 1998). *pax2.1* is initially expressed in a broad band covering

the presumptive midbrain and MHB territory at the end of gastrulation. During subsequent segmentation stages, the expression pattern of *pax2.1* condenses in its AP extent and eventually forms a narrow band across the isthmic territory (Krauss et al., 1991a; Lun and Brand, 1998). Double stainings with forebrain markers show that *pax2.1* is exclusively expressed in the midbrain and MHB territory, similar to other vertebrates (Scholpp et al., 2003).

To examine how failure to specify this part of the neural plate affects development of the neighbouring forebrain and hindbrain, we examined the nervous system of *noi* mutant embryos at 42 hpf (hours postfertilisation) with an antibody against acetylated tubulin (α-acT), which stains developing axon tracts (Fig. 1; Wilson et al., 1990; Ross et al., 1992). In comparison to wild-type embryos at the same stage, *noi* embryos lack the midbrain tectum and the tissue fold at the MHB, as well as the cerebellar commissure (Fig. 1A,B, arrow), as we reported previously (Brand et al., 1996). In a dorsal view, the cerebellar fold with the differentiating cerebellar neurons and the trochlear nerve are readily apparent in a wild-type embryo, as are differentiating neurons of the hindbrain, which appear as a regular array of dots in a dorsal view. In *noi* mutants, the cerebellar fold and trochlear nerve are absent, and the hindbrain neurons instead appear to spread to more anterior levels (Fig. 1C,D). The posterior commissure (PC) marks the boundary between diencephalon and mesencephalon (Macdonald et al., 1994), and the PC is present in *noi* mutants in its normal position relative to the axons emerging from the habenula and epiphysis (Fig. 1E,F). However, additional axonal tracts (Fig. 1F, arrowheads) appear on the mesencephalic side of the PC at dorsolateral levels, which join, similar to PC axons, the medial longitudinal fascicle (MLF) in the brainstem (Fig. 1E,F, arrows). We suggest that these additional axons derive from an increased number of neurons in the nucleus of the PC (nPC; see below).

Neurons of the Posterior Cluster Increase in Number in *noi*

The foregoing analysis showed that morphologic changes in the forebrain and hindbrain of *noi* mutant embryos are particularly pronounced in the posterior diencephalon and anterior rhombencephalon. We concentrated further on the diencephalic–mesencephalic boundary (DMB) and used histologic and immunohistochemical analysis as well as in situ hybridisation (ISH) of marker genes to examine the posterior diencephalon in the mutants.

Sections through the territory of the epiphysis and the further posteriorly located posterior commissure (PC) lying in dorsal prosomeres 2 and 1 (P2 and P1, see also Experimental Procedures section), respectively, of the posterior diencephalon show that, in *noi* mutant embryos, the size of the PC is increased and the shape of the epiphysis is different: in *noi* mutant embryos the epiphysis is more narrow compared with wild-type, however, the number of neurons seemed not to be increased (Fig. 2A–D). To study that region in more detail, we used an antibody to detect Islet homeodomain proteins, which labels interneurons of the epiphysis and of the nPC (Fig. 2E–H; Macdonald et al., 1995). At 24 and 36 hpf, we observe that the number of nPC neurons is increased in *noi* mutants by more than twofold compared with the wild-type (Fig. 2E–I; 12 wild-type embryos vs. 6 *noi* mutant embryos), whereas no major change is observed in the number of epiphyseal neurons.

wnt1 and *wnt4* expression marks the dorsal forebrain–midbrain territory, forming two prongs underneath the epiphysis that merge into a narrow dorsomedial stripe in the PC area and the tectum (Fig. 2J,K, arrowheads; Lekven et al., 2003). In *noi* mutants, this stripe remains broad throughout the dorsal tissue and the two prongs characteristic for the epiphyseal area are less distinct from the more posterior medial stripe (Fig. 2L,M, arrows). Similar to *wnt1*, *wnt4* is strongly expressed in a stripe in the posterodorsal diencephalon of the wild-type but only weakly in the anterior tectum (Fig. 2N,O). In *noi* mutant embryos, *wnt4* expression is strongly expanded posteriorly (Fig. 2P,Q, arrowheads). Further posteriorly, *wnt1* expression is normally seen in a ring at the MHB, and in differentiated neurons of the rhombic lip. As reported previously (Brand

Fig. 5. Presumptive midbrain tissue of *noi* mutant embryos is transformed into forebrain fate. Wild-type (wt) and *noi* mutant embryos were injected with caged fluorescein at the one-cell stage. **A:** At the six-somite stage (6ss), a nitrogen laser with a wavelength of 365 nm was used to activate the caged fluorescein in cells located at the position of the anterior midbrain primordium, as identified by comparison to the fate map and to gene expression data. **B:** A brightfield picture of an embryo at 6ss superimposed onto the picture of the uncaged, fluorescein-labelled cells in the midbrain primordium of the same embryo. **C:** A comparison with the expression pattern of *EphA4* in another embryo at the same stage indicates that the uncaged cell clone is located mainly in the area of the *EphA4*-negative anterior midbrain. **D:** At 26 hours postfertilisation (h), the position of the progeny of this cell clone was identified and is shown again superimposed with the brightfield picture. **F:** The same embryo was used for in situ hybridisation analysis with *EphA4*. The cell clone covers the region of the posterior forebrain (*EphA4*-positive) and the anterior midbrain (*EphA4*-negative). **E,G:** The same procedure was done with *noi* mutant embryos. The cell clone in the *noi* mutant embryos exclusively expresses *EphA4*, suggesting a transformation of cell fate. T, telencephalon.
Fig. 6. Antero-posterior patterning in the *no isthmus* mutant. **A:** After the shield stage (6 hours postfertilisation, hpf), the territory of the forebrain, midbrain, and hindbrain are generated independently of each other as is reflected in the defined expression patterns in the initiation phase. **B:** In the maintenance phase, starting during early somitogenesis stages (10–12 hpf), mutual repression of cell fate between the individual brain parts is necessary to maintain proper subdivision of the anterior neural tube. **C:** In *no isthmus* mutants, the identity of the midbrain and midbrain–hindbrain boundary territory is misspecified, causing the misspecified cells to adopt the neighbouring forebrain fate or anterior hindbrain fate in the maintenance phase. D, diencephalon; E, epiphysis; F, forebrain; H, hindbrain; M, midbrain; pc, posterior commissure; r1–6, rhombomeres 1–6.; T, telencephalon

et al., 1996; Lun and Brand, 1998), the ring of MHB expression is absent in *noi* mutants, and expression in the rhombic lip spreads to more anterior and medial levels (Fig. 2J,K).

Taken together, our marker analysis suggests that dorsoposterior diencephalon, in particular P1 and slightly P2, and anterior hindbrain tissue are expanded into the territory of the misspecified midbrain and MHB of *noi* mutant embryos.

Markers for the Posterior Diencephalon Are Expanded in *noi*

To elucidate whether an expansion holds more generally true for posterior diencephalic tissue of *noi* mutants, we compared the location of the axons of the PC, with the gene expression domain of the paired-domain transcription factor *pax6.1*. Pax6 is necessary for formation of the PC, because in Pax6 mutant mice, the PC is absent (Mastick et al., 1997). A combined ISH of *pax6.1* with an antibody staining against acT shows colocalisation of the PC with the posterior boundary of the *pax6.1* forebrain expression domain (Fig. 3A, white arrowheads), indicating that *pax6.1* labels the boundary between the forebrain and midbrain in wild-type siblings at 28 hpf. In *noi* mutant embryos, *pax6.1* expression expands into the misspecified midbrain territory, concomitant with the ectopic axons of the PC (Fig. 3A,B, yellow arrowheads). Finally, the hindbrain expression domain of *pax6.1* expands slightly anteriorly in *noi* mutant embryos (Fig. 3L, arrows).

To examine further the posterior expansion of the diencephalon, we studied the expression of other marker genes abutting the DMB and find them to be expanded as well. The posterior diencephalic expression boundary of the *Ephrin* receptors *EphA3, A4,* and *A5* lies at the DMB of wild-type embryos at the pharyngula stage (Fig. 3; Xu et al., 1994; Macdonald et al., 1994; for nomenclature, see Holder and Klein, 1999). In contrast to the situation in wild-type embryos, this expression domain is greatly increased in size and expanded toward the posterior in *noi* mutant embryos (Fig. 3C–J, asterisks). In lateral views, expression of *EphA3, EphA4,* and *EphA5* in *noi* mutant embryos occupies all of the tissue bulging in the area that would normally form the tectum (Fig. 3C–J). In its dorsal aspect, the expanded diencephalic domains of *EphA3, EphA4,* and *EphA5* now partially fuse with the domain of expression in rhombomere 1 (Fig. 3D,H,J, asterisks), which is particularly evident in a dorsal view (*EphA4*; Fig. 3F). Concomitant with this diencephalic expansion, we observe a reduction or loss of midbrain expression domains of *netrin2* and other midbrain markers, such as the *ephrin* ligands *eph5a* and *eph5b* as well as other midbrain and MHB markers (Brand et al., 1996; Lun and Brand, 1998; and data not shown).

In addition, marker genes for the presumptive metencephalon are also affected. The expression of the cell adhesion molecule *tag1*, which is expressed transiently by a subset of neurons involved in axon guidance and cell migration, is observed in the cerebellar anlage at 48 hpf (Fig. 3O; Warren et al., 1999). This expression domain is missing in the *noi* mutant (Fig. 3P). Together with the anterior expansion of the hindbrain expression domain of various markers like the Ephrins, this finding suggests a loss of posterior midbrain tissue and/or a transformation of midbrain fate into anterior hindbrain as well.

To date, our studies indicate that posterior diencephalon and probably anterior hindbrain tissue is expanded in *noi* mutant embryos, simultaneously with the reduction of midbrain/MHB tissue. This raises the question whether more anterior diencephalic domains are expanded as well. We examined expression of the signaling molecule *sonic hedgehog* (*shh*) and the homeodomain gene *nkx2.2*, which are expressed in the midline of the *zona limitans intrathalamica* (*zli*) at the boundary between the prosomeres P2 and P3 in the mid-diencephalon of wild-type embryos. In *noi* mutant embryos, these markers appear not to be expanded and occupy their normal position of expression, as is evident by comparing the position of this domain relative to the epiphysis (Fig. 3K–N, arrow). More anterior diencephalic tissue appears, therefore, not affected in *noi* mutants, consistent with the results of the α-acT and α-Isl antibody stainings.

Diencephalon Expansion Occurs Simultaneously With the Loss of the MHB Territory

Our previous analysis of gene expression in the midbrain and MHB of *noi* embryos showed that while expression of the homeodomain transcription factors *eng2* and *eng3* is already absent by the end of gastrulation, expression of other midbrain and MHB genes only becomes defective from the 7-somite stage (7 ss) onward (Lun and Brand, 1998). To study the mechanism of posterior forebrain expansion in *noi* embryos and to understand a possible link to events in the midbrain, we examined at which time forebrain expansion can first be detected in the mutants. We used double ISH staining at the 1 ss, 4 ss, 7 ss, 12 ss, and 20 ss with *EphA5* as a forebrain marker and *eng2* as a midbrain marker to identify homozygous *noi* embryos. In the wild-type, strong *EphA5* expression is seen in the diencephalic primordium, and only weak *EphA5* expression is detected overlapping with the *eng2* expression in the midbrain primordium. Until the 4 ss, no significant expansion of *EphA5* was detectable in homozygous *noi* mutants (Fig. 4A,B). Expansion of *EphA5* was first detectable at the 7 ss (Fig. 4C,D). In *noi* mutants at this stage, *EphA5* expression spreads further posteriorly (Fig. 4D, large arrowheads), thus forming a second band also in the midbrain primordium, in the approximate position normally occupied by the *eng2* domain (Fig. 4C, small arrowheads), which persists at the 12 ss (Fig. 4E,F). At the 20 ss, high levels of *EphA5* expression are observed throughout the domain normally occupied by *eng2* (Fig. 4G,H), similar to the situation seen for the pharyngula stage brain (Fig. 3A,B). Thus, the diencephalic primordium is unaffected during late gastrulation and early somitogenesis stages in *noi* embryos but begins to expand between 4 ss and 7 ss. We note that this timing does not correlate in a simple way with the initial

failure of Engrailed gene expression but with the defective maintenance of midbrain marker gene expression in *noi* mutant embryos.

Anterior Midbrain Is Transformed and Adopts Diencephalic Fate

Increased proliferation, cell death, or cell fate transformations might be mechanistically responsible for the observed expansion of posterior diencephalon. In bromodeoxyuridine incorporation experiments, we did not observe increased proliferation in the posterior diencephalon of *noi* embryos during somitogenesis stages (data not shown). Increased apoptosis can be detected with acridine orange staining in the developing midbrain and MHB of *noi* mutant embryos, but only from approximately the 20 ss onward (Brand et al., 1996). By using the more sensitive terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labelling (TUNEL) technique, we were unable to detect an earlier onset of apoptosis (data not shown).

To address the question of whether the observed expansion is due to a transformation of midbrain tissue, we labelled cell clones in the neural plate at a positions that give rise to the anterior midbrain in wild-type and *noi* mutant embryos and compared the location of the cell clone with marker gene expression at 26 hpf. To follow cell clones over time, we used a labelling technique in which a nitrogen laser with a defined wavelength of 365 nm is used to uncage a fluorescent dye, which was injected at the one-cell stage embryos (Fig. 5A; Kozlowski et al., 1997). We activated the caged fluorescein lineage tracer in the anterior midbrain in a stripe-like pattern in the embryos (Fig. 5B,C), the position of the activation was determined relative to the known wild-type fate map of the midbrain primordium at this stage (Woo and Fraser, 1995) and from comparison with marker-stained embryos at this stage. At 26 hpf, we used morphologic criteria to sort wild-type and *noi* mutant embryos and detected the new location of the labelled cells. After ISH analysis, we determined the fate of the uncaged cells. In wild-type embryos, we find that most of the cells express midbrain but not forebrain markers (Fig. 5D,F; n = 5) and only a few of the labelled cells are located in the posterior diencephalon and express *EphA4*. In contrast to wild-type siblings, we find that all of the cells express *EphA4* in the *noi* mutant at this stage (Fig. 5E,G; n = 4). Because we observe neither abnormally increased cell death in the mutants nor strong intermingling, this finding argues that, in the mutants, the expanded diencephalon is a consequence of a gradual transformation of cell fate of anterior midbrain fate into diencephalic fate.

DISCUSSION

By using the *no isthmus* (*noi*) mutant, we have examined the impact of a misspecified midbrain/MHB development onto the surrounding brain parts, the posterior forebrain and anterior rhombencephalon. Our analysis of the expression of marker genes for forebrain, midbrain, and hindbrain showed that (1) the expression of genes that normally respect the boundary of the forebrain and midbrain, and probably the boundary between midbrain and hindbrain, start to expand in the misspecified midbrain/isthmic territory. As a consequence, (2) neuroanatomic structures like the posterior commissure expand into the presumptive midbrain. (3) Lineage tracing experiments in *noi* mutants, furthermore, show that cells located at the position of the presumptive anterior midbrain adopt the fate of forebrain tissue. (4) The transformation of the tissue starts after the 7 ss. Therefore, we conclude that after a preinitiation phase (Fig. 6A), Pax2.1 is required to maintain midbrain identity (Fig. 6B) and, in absence of Pax2.1, the misspecified midbrain tissue becomes gradually transformed and adopts the fate of the surrounding brain parts (Fig. 6C).

Posterior Forebrain Expands Into Midbrain in *noi* Mutants

In 1994, Rubenstein and collaborators suggested that the anterior neural tube can be partitioned into neuromeres, so called prosomeres (Rubenstein et al., 1994), similar to rhombomeres in the hindbrain (Lumsden and Krumlauf, 1996). We report here that posterior diencephalic tissue, in particular prosomere 1 (P1), requires a signal from the midbrain or MHB to maintain its integrity during somitogenesis stages. Absence of this signal leads to expansion of P1 in *noi* mutants, but has remarkably little effect on P2. In keeping with the prosomere model, this differential susceptibility of P1 vs. P2 suggests that these cell groups already have a distinct fate at early somitogenesis stages. The results of the *EphA4* stainings suggest that a similar, rhombomere-specific susceptibility may apply to R1 vs. R2; this issue requires further investigation.

It is notable that in *noi* mutants, the forebrain does not simply fuse with the hindbrain. Other signals are likely to be important for setting the posterior forebrain boundary. One of these signals involved in maintaining forebrain identity may well be Fgf8, or another signal induced through Fgf8 protein exposure (Liu and Joyner, 2001; Scholpp et al., 2003). In the *fgf8* mutant *acerebellar*, the posterior expansion of the forebrain territory into the midbrain is weak and the mutant embryos still have a midbrain (Reifers et al., 1998; Picker et al., 1999). However, in *noi* mutant embryos, the temporal profile of expansion of posterior forebrain markers, e.g., of *EphA5*, corresponds nicely with the gradual loss of *fgf8* expression from the MHB territory at mid-somitogenesis stages. Furthermore, the results of pharmacologic inhibition of Fgf-signaling and double mutant analysis suggests that Fgf8 is directly involved in repression of forebrain fate, acting in combination with *pax2.1*-dependent Engrailed genes (Scholpp et al., 2003).

Pax2.1 and Its Requirement in Maintenance of Midbrain Integrity

In *pax2.1*-deficient zebrafish, *noi* mutant embryos and Pax2/5 mutant mouse embryos, a loss of midbrain and MHB territory was reported (Brand et al., 1996; Favor et al., 1996; Lun and Brand, 1998; Schwarz et al., 1999). Our results show that the loss

of midbrain identity causes an expansion of posterior forebrain territory, and more weakly, anterior hindbrain territory. One possibility is, therefore, that *pax2.1* (Pax2/5 in mice) may itself confer midbrain character to neuroepithelial cells and is able to suppress forebrain fate. In optic stalk development, a direct repressive function of Pax2 on the transcription of Pax6 was reported (Schwarz et al., 2000). Alternatively, in midbrain development Pax2 may function only to ensure spatially restricted activation of target genes such as the Engrailed genes. Importantly, Fgf8 and Engrailed are able to suppress forebrain fate even in a *pax2.1*-deficient condition (Scholpp et al., 2003), suggesting that *pax2.1* is indeed required for inducing a midbrain-specific program but is dispensable for repressing forebrain fate.

DMB and Lineage Restriction

Previous studies have shown that cell mixing across the DMB is restricted (Araki and Nakamura, 1999; Larsen et al., 2001), but the mechanism by which this happens is unclear. Among the marker genes we have used to follow forebrain fate were the Ephrin receptors *EphA3*, *EphA4*, and *EphA5*. The diencephalic expression domains of these markers expanded into the midbrain in *noi* mutant embryos. Ephrin receptors and their ligands have been implicated in restricting cell mixing across segment boundaries in the hindbrain (reviewed in Pasini and Wilkinson, 2002). *ephrinA2* and *ephrinA5a* are two ligands of *EphA4* that are expressed in the midbrain, complementary to the expression of *EphA4* in the forebrain and are missing in *noi* mutant embryos (Picker et al., 1999; and unpublished data). We therefore suggest that Ephrin receptors and their ligands may restrict cell mixing also 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, as mentioned above.

EXPERIMENTAL PROCEDURES

Fish Maintenance

Breeding fish were maintained at 28°C on a 14 hr light/10 hr dark cycle (Brand and Granato, 2002). Embryos were staged according to Kimmel et al. (1995) or in hours postfertilisation (hpf at 28°C for 24 hpf old or older embryos). To prevent pigment formation, some embryos were raised in 0.2 mM 1-phenyl-2-thiourea (PTU, Sigma). The data presented in this study were obtained from analysis of TL wild-type fish and of homozygous *no isthmus*^{tu29a} embryos, referred to as *noi* mutants in the following.

Histology and Immunochemistry

Procedures for the histologic analysis, sectioning, and antibody staining are described in *The Zebrafish Book* (Westerfield, 1994) and in Macdonald et al. (1994). We used a monoclonal antibody against acetylated tubulin (Sigma, T-6793) in a concentration of 1:20 to reveal neurons that have initiated axogenesis (Wilson et al., 1990; Ross et al., 1992).

Whole-Mount In Situ Hybridisation and RNA Probes

Whole-mount RNA in situ hybridisations were done as described by Reifers et al. (1998). Probe synthesis and expression patterns are described: *wnt1* (Molven et al., 1991), *wnt4* (Strähle and Blader, 1994), *pax6.1*, original clone cZK3 (Krauss et al., 1991b), *EphA3* and *EphA4* (Xu et al., 1994), *EphA5* (Holder and Klein, 1999), *shh* (Krauss et al., 1993), *axial* (Strähle et al., 1993), and *eng2* (Ekker et al., 1992).

Labelling of Cell Clones by Means of Laser-Based Activation of Caged Fluorescein

Nonfluorescent, photoactivatable (caged) fluorescein as a cell tracer for fate mapping in the zebrafish embryo was described by Kozlowski et al. (1997). Here, we use a ultraviolet-laser (Phototronic Instruments) to uncage the dye more locally. A total of 2 nl of a *solution* of 5% anionic DMNB-caged fluorescein (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 beam with 365 nm focused through a ×40 water-immersion objective was used to activate the dye 2–4 s/cell in the presumptive anterior midbrain area. The embryos were fixed at 26 hpf before ISH.

Nomenclature

The morphologic analysis of the *noi* mutant brain phenotype is based on the prosomeric model proposed by Puelles and Rubenstein (1993). Prosomere 1 (P1), with the posterior boundary to the mesencephalon, indicates the pretectum with the posterior commissure as dorsal landmark. Prosomeres 2 and 3 (P2 and P3) refer to the ventral and dorsal thalamus, respectively, including anterior and posterior parencephalon (Puelles et al., 1987); in an alternative terminology, they are referred to as D1 and D2 (Figdor and Stern, 1993) with the dorsal structure of the epiphysis in P2 and the morphologic structure of the *zona limitans intrathalamica* (*zli*) between P2 and P3.

The *pax2.1* gene was originally described as *zfpax(b)* (Krauss et al., 1991b). The zebrafish *pax6* gene was published as *zfpax(a)* and subsequently renamed to *pax6.1* after discovery of *pax6.2*, a second homologous Pax6 gene (Nornes et al., 1998). Members of the Ephrin receptors were published as *RTK1*, *RTK2*, and *RTK7* and renamed to *EphA4*, *A5*, and *A6* (reviewed in Holder and Klein, 1999).

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