Isthmus-to-midbrain transformation in the absence of midbrain-hindbrain organizer activity

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Summary

In zebrafish *acerebellar* (*ace*) embryos, because of a point mutation in *fgf8*, the isthmic constriction containing the midbrain-hindbrain boundary (MHB) organizer fails to form. The mutants lack cerebellar development by morphological criteria, and they appear to have an enlarged tectum, showing no obvious reduction in the tissue mass at the dorsal mesencephalic/metencephalic alar plate. To reveal the molecular identity of the tissues located at equivalent rostrocaudal positions along the neuraxis as the isthmic and cerebellar primordia in wild-types, we undertook a detailed analysis of *ace* embryos. In *ace* mutants, the appearance of forebrain and midbrain specific marker genes (*otx2*, *dmbx1*, *wnt4*) in the caudal tectal enlargement reveals a marked rostralized gene expression profile during early somitogenesis, followed by the lack of early and late cerebellar-specific gene expression (*zath1*/*atoh1*, *gap43*, *tag1*/*cntn2*, *neurod*, *zebrin II*). The *Locus coeruleus* (LC) derived from rostral rhombomere 1 is also absent in the mutants. A new interface between *otx2* and *epha4a* suggests that the rostralization stops at the caudal part of rhombomere 1. The mesencephalic basal plate is also affected in the mutant embryos, as indicated by the caudal expansion of the diencephalic expression domains of *epha4a*, *zash1b*/*ashb*, *gap43* and *tag1*/*cntn2*, and by the dramatic reduction of *twhh* expression. No marked differences are seen in cell proliferation and apoptotic patterns around the time the rostralization of gene expression becomes evident in the mutants. Therefore, locally distinct cell proliferation and cell death is unlikely to be the cause of the fate alteration of the isthmic and cerebellar primordia in the mutants. Dil cell-lineage labeling of isthmic primordial cells reveals that cells, at the location equivalent of the wild-type MHB, give rise to caudal tectum in *ace* embryos. This suggests that a caudal-to-rostral transformation leads to the tectal expansion in the mutants. Fgf8-coated beads are able to rescue morphological MHB formation, and elicit the normal molecular identity of the isthmic and cerebellar primordium in *ace* embryos. Taken together, our analysis reveals that cells of the isthmic and cerebellar primordia acquire a more rostral, tectal identity in the absence of the functional MHB organizer signal Fgf8.

Key words: *ace*, *acerebellar*, Fgf8, Midbrain, Hindbrain, Cerebellum, *isthmus rhombencephali*, MHB, Rhombomere 1, Rostralization, Transformation, Patterning, Lineage analysis, Bead implantation, Plasticity, Modularity, Zebrafish, *D. rerio*

Introduction

It is an intriguing question, how neuronal diversity is ultimately generated in the developing nervous system. Work on several model systems has revealed that the initial crude anteroposterior subdivision of the vertebrate neuraxis into prosencephalon, mesencephalon, hindbrain and spinal neural tube is refined by local organizing centers. The best characterized local organizers involved in the refinement of the patterning of the nervous system are the floor plate and roof plate, the anterior neural ridge/row1, and the isthmic (midbrain-hindbrain) organizer (reviewed by Altmann and Brivanlou, 2001; Briscoe and Ericson, 2001; Liu and Joyner, 2001; Rhinn and Brand, 2001; Simeone, 2002; Wilson et al., 2002).

During gastrulation, the boundary between the prospective midbrain and hindbrain can be defined as the interface of a rostral *Otx2* and a caudal *Gbx2* (*gbx1* in zebrafish) expression domain in the neural plate of both amniotes and zebrafish (Broccoli et al., 1999; Millet et al., 1999; Rhinn et al., 2003) (reviewed by Simeone, 2000). Later on at this interface, activation of a genetic network composed of various transcription factors triggers localized expression of a secreted organizer signal (Fgf8), which in turn determines the development of the surrounding tissue (reviewed by Liu and Joyner, 2001; Rhinn and Brand, 2001; Wurst and Bally-Cuif, 2001). Molecularly, expression of *fgf8* is controlled by distinct regulators, a combinatorial interaction between inductive and modulatory factors (Reifers et al., 1998; Lun and Brand, 1998; Ye et al., 2001). Analysis of *noi/pax2a* mutants in zebrafish demonstrates that induction of *fgf8* is independent of *pax2a* (Lun and Brand, 1998). In spite of the fact that the isthmic organizer develops at the interface of the *otx2* and Gbx expression domains (Broccoli et al., 1999; Millet et al., 1999; Rhinn et al., 2003) (reviewed by Simeone, 2000), these factors are only involved in the maintenance and refinement of *fgf8* expression and not in its induction (Ye et

al., 2001; Martinez-Barbera et al., 2001; Li and Joyner, 2001). Beside positive autoregulatory circuits, Fgf8 triggers expression of the Fgf target gene *sprouty*, a negative feedback modulator of Fgf signaling at the MHB (Fürthauer et al., 2001). Many of the MHB cascade genes are initially induced independently of Fgf8, and become dependent on Fgf8 activity only around the mid-somitogenesis stages (Reifers et al., 1998). By contrast, expression of the ETS transcription factors *erm*, *pea3* and *gbx2* are tightly dependent on Fgf8, and may mediate Fgf8 responses during early MHB development (Raible and Brand, 2001; Roehl and Nusslein-Volhard, 2001; Rhinn et al., 2003). Fgf8 thus fulfills multiple functions during development. Emitted from a perpendicular narrow stripe in the rostral hindbrain *gbx* expression domain, Fgf8 is required for self-maintenance of the MHB domain (Reifers et al., 1998; Lun and Brand, 1998). Fgf8 also controls the morphogenetic events leading to the formation of the anatomical isthmic constriction (*isthmus rhombencephali*) that separates the midbrain and hindbrain domains macroscopically (Brand et al., 1996; Reifers et al., 1998). Fgf8 has a crucial role in polarizing the midbrain tectum and defining ordered ingrowth of retinotectal axons (Lee et al., 1997; Picker et al., 1999). It also strongly influences the patterning of the dorsal metencephalon, a part of which eventually gives rise to the cerebellum (Reifers et al., 1998).

Zebrafish *acerebellar* (*ace*) mutants have a point mutation in the *fgf8* gene (Brand et al., 1996; Reifers et al., 1998; Araki and Brand, 2002). In *ace* mutants a number of important regulatory genes prefiguring the position of the future anatomical isthmic constriction are initially present, but their expression is later abrogated. Consequently, proper patterning of the midbrain/hindbrain along the rostrocaudal axis is disturbed in *ace* mutants. The isthmic constriction fails to form between the midbrain and rhombomere 1 (r1), and a separate cerebellar anlage is not recognizable in the mutants. Interestingly, the mutants have no obvious truncation along the rostrocaudal extent of the mesencephalic/hindbrain alar plate. Rather, the mutants appear to have a caudally enlarged tectum in place of the cerebellum. This raises the question of whether the cells in *ace* located at equivalent rostrocaudal positions along the neuraxis as the isthmic and cerebellar primordia in wild types, retain their original fate, or adopt a new one in both domains in mutant embryos. In the present study we investigated whether the special morphological features of the *ace* mutants are due to a simple dysmorphology or whether they are associated with fate alteration. To distinguish between these scenarios we analyzed the molecular and positional identities of the morphologically reorganized tectal compartment of mutant embryos by comparing tectal/cerebellar specific gene expression patterns, neuronal subtypes, and cell proliferation, cell death and cell lineage characteristics of both wild-type and mutant embryos. We provide evidence that the primordial cells of the rhombencephalic isthmus undergo marked cell fate changes, demonstrated by their rostralized gene expression pattern and by cell-lineage analysis. Implantation of Fgf8-protein coated beads suggests that the observed cell fate transformation and lack of cerebellar development is due to the missing polarizing and patterning activity of the organizer signal Fgf8.

Materials and methods

Obtaining fish embryos

Zebrafish (*Danio rerio*) were raised and kept under standard conditions at 27°C (Westerfield, 1994; Brand et al., 2002), and carriers heterozygous for the *ace* locus were identified by random intercrosses. To obtain homozygous *ace* mutants, heterozygous carriers were crossed. Freshly laid fertilized eggs were harvested into E3 embryo medium. Embryos were raised at 28.5°C, occasionally with 1-phenyl-2-thiourea (PTU) to prevent melanization. Embryos were staged according to Kimmel et al. (Kimmel et al., 1995).

Whole-mount in situ hybridization and whole-mount detection of Eph receptor tyrosine kinase ligands with Epha3-AP fusion protein

Embryos were fixed in 4% paraformaldehyde (PFA) at the required stage of development. After dechorionation, embryos were dehydrated in 100% methanol and stored at –20°C until use. Whole-mount in situ hybdridization was performed as described (Reifers et al., 1998).

Membrane-bound ephrin A molecules were detected with an Epha3-AP fusion protein as described (Picker et al., 1999).

In situ hybridization and immunohistochemistry on sections

Zebrafish larvae were anaesthetized and, after fixation in 4% PFA, were cryoprotected in 30% sucrose and embedded in OCT compound (Sakura). Sections were then cut at 15 μm thickness on a Microm cryostat. Sections were mounted onto positively charged microscope slides (Superfrost Plus). In situ hybridization was performed according to a protocol established by Henrique (Henrique et al., 1995).

For zebrin II/aldolase C immunolocalization, sections were incubated with a 1:1000 dilution of the primary antibody (anti-zebrin II mouse monoclonal; kindly provided by M. Mione, UCL, London) for 48 hours at 4°C. The imunoreactivity was detected with a biotinylated horse anti-mouse antibody (Vector), avidin-biotin-peroxidase complex (ABC Elite Vectastain kit, Vector) and DAB chromogen.

Cell proliferation and cell death detection

Cell proliferation was detected by whole-mount immunohistochemical detection of phosphorylated histone H3, using a rabbit polyclonal (IgG) antibody, according to the manufacturer's instructions (Upstate Biotechnology). To detect apoptotic cells an in situ nick-end labeling procedure was performed using a commercial kit (Roche).

SU5402 inhibitor treatment

To inhibit Fgf signaling a pharmacological inhibitor, SU5402 (Calbiochem), was used at a final concentration of 24 μM as described previously (Reifers et al., 2000a).

Bead implantation and cell lineage tracing

Bead implantation was carried out as described by Reifers et al. (Reifers et al., 2000a). Cell lineage analysis was as follows: *ace* mutant embryos were distinguished from their siblings by morphology at the 5-somite stage (Brand et al., 1996) and manually dechorionated. Mutant and wild-type embryos were fixed in the desired position by attaching them with one side to a stripe of 3% methyl cellulose on a microscope cover slip in Ringer's embryo solution. A glass capillary was pulled, covered with crystalline DiI (Molecular Probes), and inserted into the embryo at the level of the midbrain-hindbrain boundary. The capillary was left inside for not more than 5 seconds. The first pictures were taken at the age of 10 somites, after the embryos had completely recovered from the labeling procedure. Images were captured on an Olympus BX61 microscope equipped with a Spot RT camera and Metamorph imaging software.

Measurements were made using Metamorph. The system was calibrated using a micrometer calibration slide. Images were taken at 10× or 20× magnification. Fluorescent images were captured using a standard rhodamine filter set.

Results

The initial molecular identity of the isthmic domain is not maintained in *ace*

The anatomical features of the *ace* mutants are easily recognizable on living embryos (Brand et al., 1996) (Fig. 1A,B). In ace mutants the isthmic indentation fails to form and, as we demonstrated previously, although several markers of the molecular MHB are initially induced, their expression is not maintained (Brand et al., 1996; Reifers et al., 1998). To illustrate development of the isthmic primordium in *ace* mutants, we demonstrate expression of another set of genes that also prefigure the position of the future anatomical isthmic constriction during normal development and that behave in a similar fashion to the previously described ones (Brand et al., 1996; Reifers et al., 1998). A previously uncharacterized zebrafish homolog of the *Drosophila* region specific homeotic zinc-finger gene *spalt* (*spa1a*; GenBank Number AJ293862) (E. M. Camp and M. T. Lardelli, unpublished), similar to medaka and mouse *Spalt* homologs, specifically labels the MHB (Koster et al., 1997; Carl and Wittbrodt, 1999; Ott et al., 2001) during normal development. In *ace* mutants, the MHB expression domain of *spalt1a* (*spa1a*) is completely missing, as revealed at the 9- to 10-somite stage (Fig. 1C,D). Expression of *pax8*, a member of the *pax2/5/8* subgroup of Pax genes (Pfeffer et al., 1998), is initiated weakly at the MHB at early somite stages in the mutant embryos (Fig. 1E,F) and later lost in *ace* mutants (Fig. 1G,H). *sprouty4* (*spry4*) is one of the vertebrate homologs of the *Drosophila* receptor tyrosine kinase (FGFR, EGFR) inhibitor *sprouty* (Fürthauer et al., 2001). As *spry4* is a direct target of Fgf8, its expression is not initiated in the mutant embryos (Fig. 1I,J). The above examples combined with our previous observations suggest that, in *ace* mutants, preceding the lack of the isthmic indentation, the cells of the isthmic primordium change their gene expression profile at around early- to mid-somitogenesis stages.

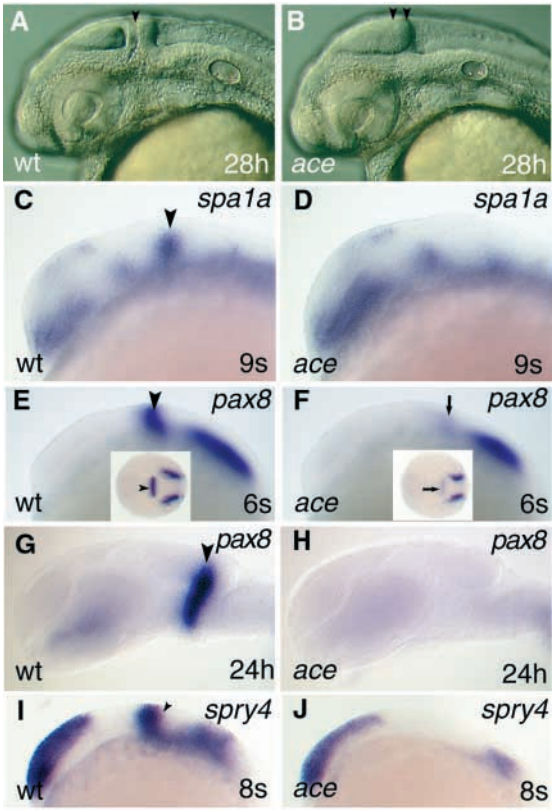

Fig. 1. The molecular identity of the isthmic domain is not maintained in *ace*. All views are rostral to the left, and are lateral apsects, apart from the insets in E and F, which are dorsal views. (A,B) Captures of living embryos illustrating the special anatomical features (lack of isthmus and separate cerebellar anlage) of the *ace* mutants. The black arrowhead in panel A points to the isthmic constriction; the two black arrowheads in panel B mark the caudal tectal expansion. (C,D) Analysis of *spa1a* expression reveals the lack of the isthmic expression domain in the mutant embryos in comparison with wild type. The black arrowhead labels the MHB expressing *spa1a* in wild-type embryo. (E,F) Only a low level of *pax8* expression can be detected at the MHB in the mutant embryos (arrows; F,F inset) in comparison with wild types (arrowheads; E,E inset). (G,H) Later on, expression of *pax8* is abolished from the prospective MHB region in *ace* mutants. (I,J) In contrast to *pax8*, expression of *spry4* is not initiated in the mutants. The arrowhead (I) marks the MHB expression domain of *spry4* in the wild-type embryo.

Caudal expansion of fore- and midbrain markers in *ace* mutants

The gradual loss of MHB domain markers in *ace* mutants (Brand et al., 1996; Reifers et al., 1998) (present study) raises the question of how identities of the confronting midbrain (Otx expressing) and hindbrain (Gbx expressing) domains react to the loss of isthmic identity. To test this, we first analyzed the expression of markers that are restricted to the mesencephalic side of the (molecular) boundary, which are therefore not expressed in the cerebellar anlage of wild-type embryos. In vertebrates, *Otx*2, a well-characterized homolog of *Drosophila orthodenticle* (*otd*), marks the caudal limit of the developing mesencephalon (Millet et al., 1996), abutting the expression domain of another homeodomain protein, *Gbx*2 (Millet et al., 1999). [The latter function is exerted by *gbx1* in zebrafish (Rhinn et al., 2003)]. *Otx*2 is expressed from early gastrulation and its expression is maintained through embryogenesis (Simeone et al., 1993). In *ace* embryos, *otx2* expression is expanded caudally (Fig. 2B,H,J), whereas its expression stops at the caudal end of the mesencephalon in wild-type embryos (Fig. 2A,G,I). The caudal expansion of *otx2* remains prominent throughout the somitogenesis period (Fig. 2F,H). By applying a pharmacological inhibitor of Fgf signaling, SU5402, aspects of the *ace* mutant phenotype can be phenocopied. SU5402 blocks the ATPase domain of Fgfr1 (Mohammadi et al., 1997). In wild-type embryos treated with SU5402 from tailbud to 7-somite stage, the caudal expansion of *otx2* expression can be recapitulated (Fig. 2C,D,I,J). *dmbx1* (*diencephalic-mesencephalic homeobox 1*) is an evolutionarily conserved homolog of *Drosophila aristaless* that has recently been described (Martinez-Barbera et al., 2001; Kawahara et al., 2002; Ohtoshi et al., 2002; Gogoi et al., 2002). As in amniotes (mouse and chick), the caudal limit of *dmbx1* expression coincides with the caudal end of the

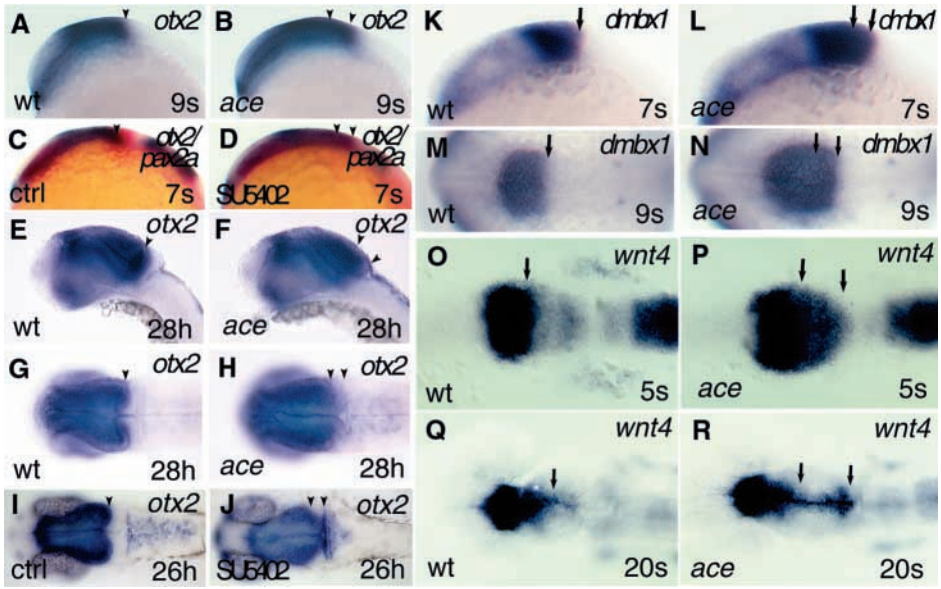

Fig. 2. Caudal expansion of fore- and midbrain markers in *ace* mutants and SU5402 inhibitor-treated embryos. All views are rostral to the left; (A-F,K-L) lateral views; (G-J,M-R) dorsal views. (A-J) Analysis and comparison of *otx2* expression by whole-mount in situ hybridization in wild-type, *ace* mutant and SU5402-treated embryos, at early somitogenesis (A-D), and at the pharyngula period (E-J). (A,B) In the mutant embryos, expression of *otx2* is caudally shifted well before the period when the anatomical isthmic constriction should form. (C-F) In SU5402-treated embryos, expression of *otx2* (blue) is markedly shifted, similar to *ace* mutants, whereas expression of pax2a (red) is reduced. The expanded *otx2* territory is also characteristic for later developmental stages, both in *ace* mutants (E-H) and SU5402 inhibitor-treated embryos (I-J), as illustrated at 28 hpf and 26 hpf, respectively. The black arrowhead (A,C,E,G,I) points to the caudal expression limit of *otx2* at the MHB of wild-type embryos. The two arrowheads (B,D,F,H,J) mark the caudally expanded territory expressing *otx2* in *ace* mutant and SU5402-treated embryos. (K-N) As with *otx2*, expression of *dmbx1* is markedly expanded at early somitogenesis in the mutant embryos, as illustrated at the 7- and 9-somite stage. The single arrow (K,M) labels the caudal limit of *dmbx1* expression in the developing wild-type mesencephalic tectum. The two arrows (L,N) indicate the expanded expression of *dmbx1* in *ace* mutant siblings. (O-R) Analysis of *wnt4* expression reveals an early upregulation, and expansion of the fore-midbrain territory toward caudal coordinates of the mesencephalic alar plate in *ace* mutants. (O-P) The initially broad, expanded expression domain of *wnt4* narrows to the dorsomedial parts of the alar plate, and is extended to the caudally enlarged tectal compartment at later somitogenesis stages, as illustrated at the 20-somite stage. The black arrows (O,Q) indicate the caudal limit of the enriched expression of *wnt4* at the fore-midbrain region in wild-type embryos. The two arrows (P,R) show the markedly upregulated and expanded expression of *wnt4* in *ace* mutants.

mesencephalic anlage, excluding the anterior portion of the MHB fold (posterior mesencephalic lamina) (Fig. 2K,M; and data not shown). *dmbx1* behaves in a similar way to *otx2* in *ace* embryos: its expression is dramatically expanded caudally by the 7-somite stage, the earliest stage investigated (Fig. 2K-N). The expansion of *otx2* and *dmbx1* is coincident with the fading of the MHB markers (see also Brand et al., 1996; Reifers et al., 1998) at the position relative to where the isthmic constriction should form later on. In vertebrates, members of the Wnt family, such as *wnt1* or *wnt4*, are expressed in overlapping domains along the mesencephalic tectum (Hollyday et al., 1995; Ungar et al., 1995). However, their expression does not expand to the hindbrain side of the isthmic constriction. In wild-type zebrafish embryos, *wnt4* expression is enriched between the fore- and midbrain domains at the 5-somite stage (Fig. 2O). By the 20-somite stage, *wnt4* expression gradually extends along the dorsal midline toward the caudal end of the mesencephalic tectum (Fig. 2Q). However, in *ace* mutant embryos, there is a marked caudal shift in *wnt4* expression toward the mesencephalic tectum by the 5-somite stage (Fig. 2P). Later on, expression of *wnt4* narrows down to the dorsal midline of the mesencephalic tectum, and extends toward the caudal end of the enlarged tectal compartment (Fig. 2R).

Taken together, our results reveal a marked rostralization in gene expression at the dorsal MHB primordium and cerebellar anlage in *ace* mutants.

Lack of cerebellar development in *ace*

Having seen that *dmbx1*, *otx2* and *wnt4* appear at ectopic caudal locations, we analyzed the expression of genes that mark the upper rhombic lip where the cerebellar anlage forms in wild-type embryos. *zath1* (*zebrafish atonal homologue 1*; *atoh1* – Zebrafish Information Network) is a marker of the upper rhombic lips (Koster and Fraser, 2001). In *ace* mutants, we consistently find that *zath1* is missing from the upper rhombic lip region, when compared with wild-type siblings (Fig. 3A-D).

As the upper rhombic lips showed a marked rostralized gene expression profile in *ace* embryos, we analyzed whether cerebellar development could still be initiated in spite of this. To monitor cerebellar development, we used markers expressed by cerebellar granule and Purkinje cells. Of these genes, *gap43* (Reinhard et al., 1994) and *tag1/axonin* (*cntn2* – Zebrafish Information Network) are expressed by migrating cerebellar granule cell precursors (Fig. 3E,G) (Console-Bram et al., 1996; Wolfer et al., 1994; Lang et al., 2001). In mutant embryos, no expression of these markers can be detected in the region that would give rise to the cerebellum, at any of the stages investigated (Fig. 3F,H).

Likewise, staining for the bHLH transcription factor *neurod*, a marker expressed by cerebellar granule cells (Fig. 3I,K) (Miyata et al., 1999; Lee et al., 2000; Mueller and Wullimann, 2002), fails to detect a cerebellar compartment in mutant embryos (Fig. 3J,L). In addition, Purkinje cells of the cerebellum cannot be detected by anti-zebrin II/aldolase C (Brochu et al., 1990) immunostaining of mutant embryos (Fig. 3M,N). Thus, complementary to the caudal expansion of gene expression domains normally excluded from the dorsal metencephalon, specific markers for the developing cerebellar

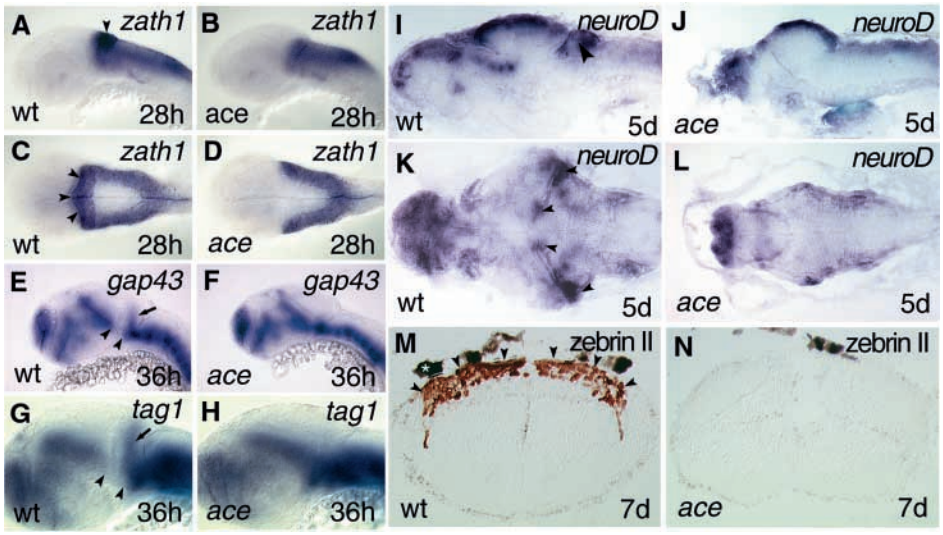

Fig. 3. Lack of cerebellar development in *ace* mutants. (A-H) Whole-mount in situ hybridisations. (I-L) In situ hybridisation of sections. (M-N) Immunohistochemistry of sections. (A-L) rostral to left; (A,B,E,H) lateral views; (C,D) dorsal views; (I,J) lateral views of sagittal sections; (K,L) dorsal views of horizontal whole brain sections; and (M,N) transversal hindbrain sections. *zath1* expression is not detectable in the upper rhombic lips of *ace* mutants (B,D) in comparison with wild-type (A,C) embryos. Arrowheads (A,C) point to the upper rhombic lips expressing *zath1* in wild-type embryos. No migrating granule cell precursors can be detected by analysing *gap43* (E,F) and *tag1* (G,H) expression in *ace* mutants. Arrows (E,G) mark the migrating granule cell precursors in the developing cerebellar anlage. Note that the ventral mesencephalic expression domain of both *gap43* and *tag1* are fused to the hindbrain expression domain. Arrowheads mark the gap between the rostral and caudal expression domains of *gap43* (E) and *tag1* (G). (I-L) Expression analysis of *neurod* fails to detect a cerebellar compartment containing granule cell precursors in the mutants. Arrowheads (I,K) point to the cerebellar anlage expressing *neurod* mRNA. (M,N) Immunohistochemical visualization of zebrin II, the evolutionarily conserved marker of Purkinje cells, fails to detect any of these cells in *ace* mutants. Arrowheads (M) point to the cerebellar plate loaded with zebrin II-expressing cells (brown staining). The white asterisk above the hindbrain section (M) marks a pigment granule.

primordium, or for later granule and Purkinje cells, are not expressed in *ace* embryos.

Rhombomere 1 behaves as a bipartite structure in *ace* embryos

Rhombomeres are transiently appearing segmental structures during hindbrain development. Of the 7 (8 in amniotes) rhombomeres, the rostral rhombomere 1 (r1) gives rise to the cerebellum and other important structures of the vertebrate central nervous system, such as the *Locus coeruleus* (LC) (Morin et al., 1997). As our results revealed that the cerebellar plate derived from rostral r1 lost its identity, we investigated the expression of further specific marker genes characteristic of r1 in wild-type embryos. Expression of *epha4a* (formerly known as *rtk1*) marks caudal r1 (Fig. 4A,C), and its expression is not diminished in *ace* embryos (Fig. 4B,D; small arrow). However, the orientation of its expression domain is altered: instead of being perpendicular to the rostrocaudal axis of the hindbrain, it becomes slanted (Fig. 4B). Moreover, double detection of *otx2* and *epha4a* (Fig. 4E,F) reveals that their expression domains now abut, forming a new interface in *ace* embryos (Fig. 4F).

Precursors of the LC catecholaminergic neurons expressing *phox2a* are born in the rostral r1 (Morin et al., 1997; Guo et al., 1999). Subsequently, descendants of these cells occupy a ventrolateral position in that rostral segment expressing tyrosine hydroxylase (TH), the rate-limiting enzyme of catecholamine synthesis (Fig. 4G). In *ace* embryos, TH message is not detectable at the pontine flexure, indicating that the LC is absent (Fig. 4H) (Guo et al., 1999).

The presence of *epha4a* in caudal r1, and the abutting of the *epha4a* and *otx2* domains in conjunction with the lack of cerebellar and LC development, suggest that the lack of Fgf8 in *ace* mutants affects the rostral and caudal part of r1 unequally. The effect of the loss of organizer-derived Fgf8 function is restricted to rostral r1. Our analysis supports the notion raised by C. Moens' and I. Mason's laboratories that r1 is a bipartite structure, where the rostral part of this rhombomere should be considered as a separate entity designated as rhombomere 0 (Waskiewicz et al., 2002; Walshe et al., 2002). In *ace* mutants, rhombomeres rostral or caudal to r4 appear to be often reduced in width along the rostrocaudal axis of the hindbrain, as judged by rhombomere marker analysis (Maves et al., 2002) (Fig. 4A-H). This is most probably due to the impaired r4 signaling activity in *ace* (Maves et al., 2002), rather than to the loss of isthmic organizer function. However, beyond the smaller rostrocaudal extent of certain rombomeres in *ace*, the generation of rhombomere-specific branchiomotor neuron patterns, including r2 and r3 trigeminal motoneurons, is largely normal (Maves et al., 2002).

Rostralization can also be detected at the ventral part of the MHB primordium

As previously shown, the narrow perpendicular stripe of the isthmic organizer region emits rostrocaudal patterning signals along the whole extent of the MHB region. The lack of functional Fgf8 signaling dramatically alters the morphology of the isthmic and dorsal metencephalic alar plate region in *ace* embryos. We also examined how the basal plate (tegmental) region is affected in *ace* mutants. To address this, we analyzed expression of *zash1b* (*ashb* – Zebrafish Information Network), *gap43*, *tag1*/*cntn2* and *epha4a*. These genes are expressed at ventral aspects of the diencephalon and hindbrain leaving a gap at the tegmental part of the MHB and midbrain. In *ace* embryos we observe a fusion of the diencephalic and hindbrain expression domains of *zash1b*, *gap43* and *tag1*/*cntn2*, filling the above mentioned gap (Fig. 4I,J, Fig. 3E,F and Fig. 3G,H, respectively). In the case of *epha4a*, a caudal expansion of the

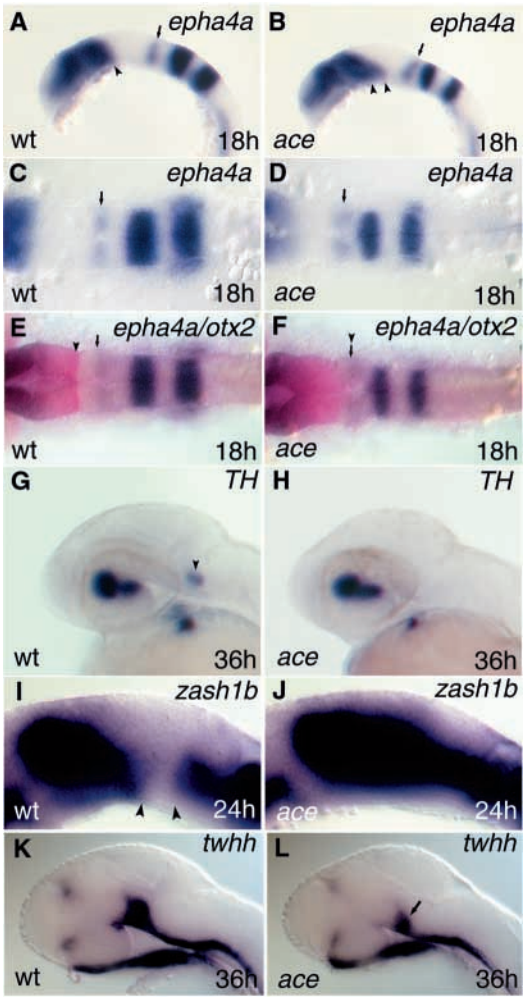

Fig. 4. Analysing rhombomere 1 (r1) and the ventral mesencephalon in *ace* mutants. The rostralization stops at caudal r1 in *ace* mutants (A-H). The ventral mesencephalon displays altered gene expression profiles in *ace* mutants (A,B,I-L). All views are rostral to the left. A,B,G-L are lateral views; C-F are dorsal views. (A-D) Expression of *epha4a*, labeling the caudal part of r1, is still detectable in *ace* mutants. However, this domain in *ace* embryos (B) shows a distorted, slanted orientation in comparison with wild-type siblings (A), where it is approximately perpendicular to the axis of the hindbrain. Small arrows (A-D) point to the *epha4a*-expressing caudal r1 compartment. Note that the expression of *epha4a* at the fore-midbrain junction is expanded toward the ventral mesencephalon in *ace* mutants. Arrow (A) points to the caudal limit of *epha4a* expression at the fore-midbrain region. Two arrowheads (B) point to the expanded *epha4a* expression domain in the mutant embryo. (E,F) In the hindbrain, a new interface is detectable between the *otx2* (red) and *epha4a (*purple) expression domains in *ace* embryos. The arrowhead (E) points to the caudal limit of *otx2*; the small arrow labels the rostral end of *epha4a* in the caudal r1 of wild-type embryos. The arrowhead above the small arrow (F) marks the new *otx2*/*epha4a* interface in the *ace* mutant. (G,H) The *Locus ceruleus* (LC; arrowhead in G) is missing from r1 in *ace* mutants. (I,J) In *ace* mutant embryos, the diencephalic expression domain of *zash1b* expands toward the mesencephalic tegmentum and fuses to the hindbrain expression domain (J). Arrowheads (I) mark the gap between the rostral and caudal expression domains of *zash1b*. (K,L) Expression of *twhh* is severely compromised in the ventral mesencephalic region of pharyngula-stage mutant embryos in comparison with wild-type embryos. The arrow (L) points to the reduced ventral mesencephalic *twhh* expression domain.

diencephalic domain can be observed (Fig. 4B,D). These findings are consistent with the recently reported caudal expansion of *fgfr3* expression along the basal plate of the midbrain, and with the consequent fusion of the diencephalic and hindbrain expression domains in *ace* embryos (Sleptsova-Friedrich et al., 2002). Expression of *twhh*, instead of being expanded, shows a dramatic reduction in the ventral mesencephalon in *ace* mutants (Fig. 4K,L). The alteration in expression of *twhh* may reflect the expansion of more rostral, caudal diencephalic or rostral mesencephalic fates toward caudal co-ordinates of the mesencephalon. Taken together, these changes in gene expression are compatible with the observed rostralization taking place at the dorsal metencephalic alar plate, and are indicative of a caudal-to-rostral transformation of the basal plate of the MHB. However, the degree of rostralization along the mesencephalic/metencephalic basal and alar plates may be different.

Caudal enlargement of the tectum in *ace* mutants is not a consequence of enhanced cell proliferation or decreased cell death

Various mechanisms could account for the successive rostralization and loss of isthmic indentation in *ace* mutants, such as regionally distinct cell proliferation or apoptosis, or transformation of cell fate in the affected tissue. We therefore analyzed the mitotic behaviour of the dorsal mesencephalic (tectal) and MHB primordial cells using an antibody raised against the proliferation marker phospho histone H3 (PH3). H3 phosphorylation has previously been described to correlate with mitosis in mammalian cells, *Xenopus* and *Tetrahymena*. Before the actual formation of the anatomical isthmic constriction, marked differences cannot be detected in cell proliferation between wild-type and mutant embryos (Fig. 5A-D). However, at later stages [36, 57 and 72 hours postfertilization (hpf)], the immunoreactivity of PH3 is markedly reduced at the caudal-most edges of *ace* tecta, and the cerebellar proliferative zone typical for wild-type embryos is absent in *ace* (Fig. 5E-G; data not shown). Increased proliferation, therefore, is very likely not the cause of the enlarged tectum. However, the decreased proliferation observed in young *ace* mutant larvae, may account for the overall smaller than wild-type size of *ace* mutants at later stages, at day 5, for example (Fig. 3I,J) (Picker et al., 1999).

Next, we analyzed whether a decreased rate of apoptotic cell death could contribute to the altered MHB development in *ace* mutants. Although pronounced rostralization is evident in gene expression during early somitogenesis stages in *ace* embryos, marked deviation from the wild-type cell death pattern cannot be detected by TUNEL staining (Fig. 5H,I). However, from the 12-somite stage on, a higher number of dead cells can be detected in *ace* embryos than in wild-type siblings, as demonstrated by DIC images of wild type and *ace* mutants (Fig. 5J,K). In accordance with this, an increased nick-end labeling can be observed at the 15-somite stage in *ace* embryos, indicating a higher number of apoptotic events in mutant tecta in comparison with wild-type structures (Fig. 5L-O). As marked differences in cell death cannot be detected at early segmentation stages, when pronounced differences in gene expression are observed, cell death is therefore likely to be a secondary consequence of the earlier, abnormal mis-patterning of the MHB area in *ace* mutants.

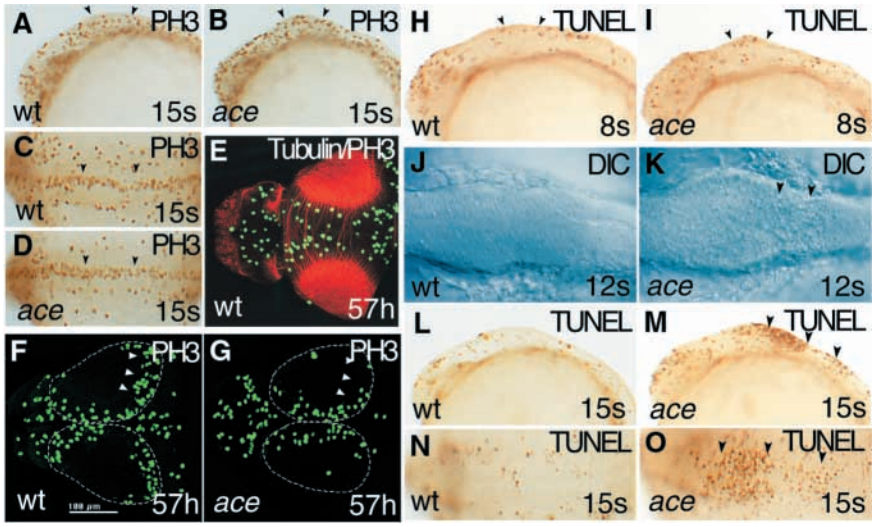

Fig. 5. Comparison of cell proliferation and cell death patterns in wild-type embryos and *ace* mutants. (A,B,H,I,L,M) lateral views; (C-G,J,K,N,O) dorsal views. (A-G) Comparison of the cell proliferation characteristics of wild-type and mutant embryos using anti-phospho histone H3 (anti-PH3) whole-mount in situ hybridization staining. (A-D) No differences can be detected, during the mid-somitogenesis period, in the cell proliferation patterns of wild-type and *ace* mutant embryos after anti-PH3 immunostaining (brown reaction product). Arrowheads mark the range between the mesencephalon and rostral hindbrain under investigation. (E) Double immunostaining depicting the anatomical relationship of proliferation zones (anti-phospho histone; green) and axonal trajectories (anti-acetylated tubulin; red) of the mesencephalic tectal region during normal development, at 57 hpf. (F,G) Comparing the anti-PH3 staining pattern in 57 hpf wild-type and *ace* mutant larvae reveals a dramatic reduction of cell proliferation. The mutants lose the typical caudal tectal and cerebellar proliferation zones seen in wild-type larvae. The white arrowheads (F,G) delineate the caudal proliferation zones. (H-O) Analysis of cell death in wild-type and mutant embryos. (H,I) TUNEL staining (brown) fails to detect marked differences between wild-type and *ace* mutant embryos at early somitogenesis as demonstrated at the 8-somite stage. The arrowheads (H,I) label the region of interest for comparison. (J,K) At later stages of the segmentation period the amount of cell death is increasing in the mutant embryos in comparison with wild-type siblings, as revealed by Nomarski (DIC) optic. In the mutants, dead cells (small, round, excluded superficial structures) can be seen all over the midbrain and rostral hindbrain, but are more concentrated above the rostral hindbrain region. The arrowheads (K) indicate the area where a higher number of dead cells is visible. (L-O) Detecting apoptotic cell death (brown reaction product) at the 15-somite stage reveals an increased number of dead cells above the rostral hindbrain and r4 in *ace* mutant embryos (M,O; arrowheads).

Cells at the location of the wild-type MHB give rise to caudal tectum in *ace* embryos

The rostralized gene expression and the lack of cerebellar cell types in *ace* mutants, along with the results of the proliferation and the cell-death studies, all suggest that the isthmic and cerebellar primordium might have already adopted a new tectal identity by early- to mid-somitogenesis stages. To address this issue directly, we performed cell lineage tracing experiments to reveal the fate of the MHB primordial cells. We investigated whether MHB primordial cells in *ace* mutants are retained in a position that corresponds to the MHB compartment of wild types, or whether they end up elsewhere, acquiring a new fate. We labeled groups of cells at the level of the prospective MHB with the lipophilic dye DiI during early somitogenesis (5-somite stage) in wild-type and *ace* embryos. The morphological MHB has not yet formed at this stage, but MHB primordial cells can be targeted by their position along the rostrocaudal axis relative to the posterior edge of the optic vesicle. By comparing the position of DiI-labeled cells at the 10-somite stage (Fig. 6A,D), and at 24 hpf (Fig. 6B,C,E,F), in wild-type and *ace* mutant embryos, we were able to compare the fate of these marked cells. Table 1 summarizes the results of our labeling experiments. In all wild-type cases, the labeled cells ended up in either the posterior tectum or in the cerebellum/r1, i.e. in the MHB region (Fig. 6B,C). In all *ace* cases, cells ended up in the posterior part of the enlarged tectum (Fig. 6E,F). Moreover, we did not observe a loss of the labeled cell population in *ace* embryos, when compared with wild-type siblings. These results suggest that cells that are normally fated to become MHB tissue in the wild-type are transformed into tectal cells in the *ace* mutants.

Transformation of the MHB region is a reversible process in *ace* mutants

In *ace* mutants, the molecular and anatomical identity of the isthmic and cerebellar primordial cells is not maintained, and they acquire a more rostral fate as we have visualized by various markers. At later stages, lack of tectum polarization in *ace* mutants leads to a somewhat variable, but always severely distorted, retino-tectal map formation, including altered expression of *ephrin A2* and *ephrin A5b* (Picker et al., 1999). To test the reversibility of the alterations in the affected brain structures, functional Fgf8 protein was applied by implanting Fgf8-coated beads into wild-type and mutant embryos, and their MHB/tectal morphology was then evaluated. Implantation of Fgf8-coated beads between the 5- and 20-somite stage, into the prospective MHB of *ace* embryos, rescues the MHB phenotype (*n*=9/11), reconstituting the MHB fold and the upper rhombic lip (data not shown). Moreover, the coated beads induce an ectopic MHB fold when implanted in the posterior diencephalon of wild-type embryos (*n*=5/7). Although the

Table 1. Statistics of DiI labeling

	Wild type*	*ace*†
Average	155	201
Minimum	134	153
Maximum	182	225
Standard deviation	21	23

Table shows distance (in μm) of DiI-labeled cells from the posterior tip of the eye field in wild-type and *ace* embryos at the 10-somite stage.
*n=7 embryos.
†n=8 embryos.

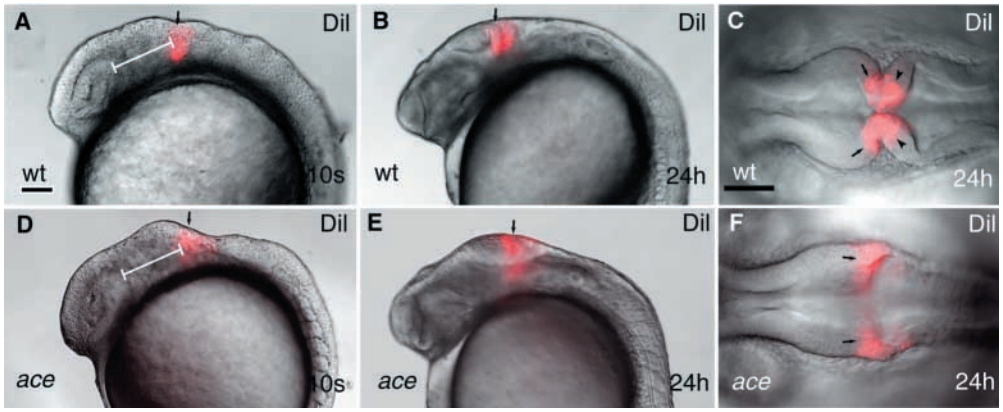

Fig. 6. Dil lineage-tracing reveals fate alteration of MHB primordial cell in *ace* mutants. All views are rostral to the left. (A,B,D,E) Lateral views; (C,F) dorsal views. (A-F) Labeling (red) wild-type and *ace* mutant embryos at equivalent rostrocaudal positions along the neuraxis reveals that the labeled cells in the mutants are not retained in the MHB compartment. The labeled mutant cells always end up at the caudal enlargement of the tectum (E,F). Arrows (A,B,D,E) point to the Dil-labeled group of cells. Arrows (C,F) point to the mesencephalic side of the labeled compartment; arrowheads (C) point to the hindbrain side of the Dil-labeled cell population. The white bar (A,F) shows the distance between the caudal edge of the otic vesicle and the Dil injection.

induced phenotypes are stronger after early bead implantation, reversion of the *ace* phenotype and induction of an ectopic MHB in wild-type embryos are possible even when implantation is performed very late, at the 20-somite stage, the latest stage we tested (data not shown). To assay the restorative and inductive abilities of Fgf8 on polarized marker expression, Fgf8 beads were implanted at the 15-somite stage into wild-type and *ace* mutant embryos. As a read-out, expression of ephrin A proteins was assessed at 30 hpf, detected using an Epha3-AP-fusion protein recognizing all known ephrin A proteins (Fig. 7A) (Brennan et al., 1997; Picker et al., 1999). In wild-type embryos, distribution of ephrin A proteins shows an increasing rostral-to-caudal gradient at this stage (Fig. 7A). However, this gradient is completely absent in *ace* embryos (Fig. 7B). Upon implantation of Fgf8-coated beads, graded ephrin A expression is restored in *ace* embryos on the operated side (n=5/6; Fig. 7D). Moreover, by implanting Fgf8 beads at various ectopic anteroposterior locations within the diencephalon and tectum of wild-type individuals, we find that Fgf8 is able to induce an ectopic MHB fold, and is sufficient to establish a mirror gradient of ephrin A proteins (n=4/4; Fig. 7C) by polarizing the diencephalon and tectum. Apparently, Fgf8 induces ephrin A protein expression in locations experiencing low or no Fgf8 concentration, corresponding to wild-type diencephala and *ace* tecta, respectively. From these experiments, we conclude that the expanded tectal tissue retains its ability to respond to Fgf8, and that the transformation of the MHB and cerebellar primordium is a reversible process, as scored by morphological and molecular criteria.

Discussion

We have analysed the mechanisms underlying cerebellar agenesis in the zebrafish *fgf8* mutant *acerebellar*. Our analysis revealed that in *ace* mutants: (1) there is a marked rostralization in gene expression profiles of the midbrain, MHB and cerebellar primordium; (2) the cerebellar developmental program is not initiated because the cells of the MHB and cerebellar primordium are transformed, acquiring a more rostral, tectal identity; and (3) isthmic folding and tectal polarization can be elicited in ectopic locations, and can be restored in *ace* mutants, upon local application of an Fgf8 protein source, suggesting that the absence of the inductive organizer signal Fgf8 is responsible for the observed fate alteration in these mutants.

The lack of isthmic indentation is preceded by marked changes in molecular identity

The examination of MHB region specific marker gene expression enabled us to investigate the processes underlying the special anatomical features of *ace* embryos (Brand et al., 1996; Reifers et al., 1998). In *ace* mutants, expression of specific genes that prefigure the future anatomical constriction (*pax2a*, *her5*, *wnt1*, *eng2*, *eng3*, *erm*, *pea3*, *spry4*, *spa1a* and *pax8*) is perturbed (Brand et al., 1996; Reifers et al., 1998; Raible and Brand, 2001; Fürthauer et al., 2001) (present study). Consequently, in *ace* embryos a caudal shift of the expression of forebrain and mesencephalic markers (*otx2*, *dmbx1*, *wnt4*) is evident in positions where the isthmic

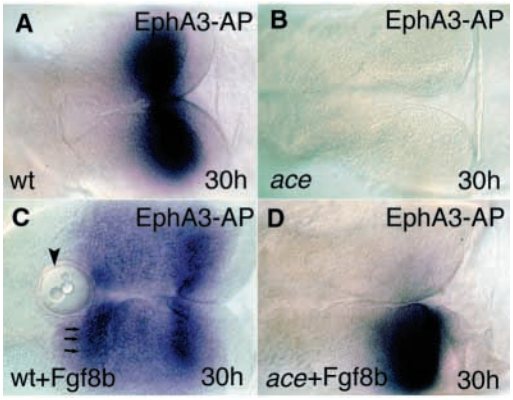

Fig. 7. Fgf8-bead implantation restores the molecular and anatomical identity of the MHB territory. All views are rostral to the left, and are dorsal aspects. (A) The Epha3-AP fusion protein reveals the distribution of ephrin ligands (blue) in the mesencephalic tectum. (B) The fusion protein fails to detect the typical ephrin gradient expression in the mutant tecta. (C) After implanting Fgf8-coated beads into the wild-type diencephalon, a second, mirror gradient of ephrin A proteins can be detected. Arrowhead indicates the implanted beads; arrows indicate the second, ectopic ephrin A domain. (D) Unilateral implantation of the Fgf8-coated beads into *ace* embryos is able to restore the graded ephrin A expression on the operated side.

primordium and upper rhombic lips should differentiate. From the expression of *wnt4*, it can also be estimated how rostral the molecular identity of the structures in *ace* embryos is. This gene normally starts to be expressed at the junction of the caudal diencephalon and the rostral tectum (Ungar et al., 1995). Its appearance at more caudal positions indicates that *ace* mutants show gene expression profiles characteristic for the rostral tectum of wild-type embryos. Pharmacological inhibition of Fgf signaling in wild-type embryos faithfully mimics the expansion of *otx2* expression seen in *ace* mutants. The rostralized gene expression profiles of the mutants, along with the lack of the rhombic lip/early cerebellar marker *zath1* and other markers of the developing cerebellum (*gap43*, *tag1/cntn2*, *neurod*, *zebrin II*), suggest a possible fate transformation of the isthmic and cerebellar regions preceding the agenesis of the isthmic fold and the lack of a distinct cerebellar domain at later stages. Our present results, combined with earlier observations (Reifers et al., 1998; Raible and Brand, 2001), show that a molecular MHB is initially formed but is then lost during the early- to mid-somitogenesis period in the mutants. The significant structural reorganization taking place at the embryonic neuraxis of the mutant is preceded by a marked alteration in the molecular identity of the cells of the isthmic and cerebellar primordia. The identity of the MHB domain is not maintained and, as a consequence, both the isthmic and cerebellar primordia acquire a more rostral, mesencephalic character in *ace* mutants. In fact, in *ace* mutants the dorsocaudal compartment (upper rhombic lip/dorsal r1), which normally gives rise to the cerebellum, develops as an enlargement of the midbrain tectum. Taken together, these results show that the agenesis of the isthmic constriction in *ace* embryos is not only a simple dysmorphology. The scheme in Fig. 8 summarizes the most important features of *ace* mutants in comparison with wild-type siblings.

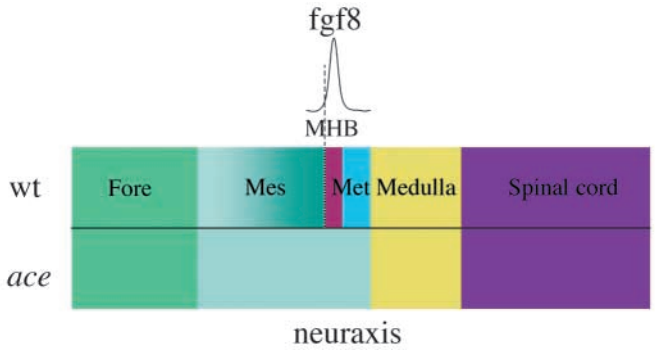

Fig. 8. Organization of the MHB region in the presence (wt) and absence (*ace*) of functional Fgf8 signaling. In the presence of the functional organizer signal Fgf8 (red block) the MHB region is correctly patterned, and the mid- and hindbrain regions are separated by the isthmic constriction (dotted line). The mesencephalic tectum (Mes) is polarized, as revealed by the graded expression of various markers (gradient green). In the *ace* mutant, where functional Fgf8 is absent, the isthmic indentation fails to form, the tectum expands caudally at the expense of the metencephalon (Met), and it loses its polarized character, showing gene expression profiles characteristic of the anterior tectum in wild-type embryos (gray block).

Qualitative rather than quantitative alterations explain the lack of isthmus and the expansion of the tectum in *ace*

The severe rostralization in gene expression and lack of cerebellar development suggests that the isthmic and cerebellar primordia in *ace* mutants acquire a new identity. Mechanistically, however, the enlargement of a particular tissue domain could also be the result of excessive proliferation or a decrease in developmental cell death. Upon monitoring the cell proliferation characteristics of the mutants, a significant decrease in cell proliferation can be detected. *ace* mutants lose their caudal (upper rhombic lip) proliferation zone typical of wild-type brain, as revealed by anti-phospho histone H3 staining. However, this difference in the cell proliferation pattern is a relatively late phenomenon, typically seen from pharyngula stages onwards. During the segmentation period, the differences in cell proliferation are not evident. Therefore, we think that an increased proliferation is highly unlikely to contribute to the loss of the isthmic structures in *ace* mutants. Furthermore, the results obtained by analyzing the TUNEL and DIC images of *ace* mutant embryos show that a decrease in the rate of ontogenetic cell death cannot account for the expansion of the tectum and the lack of the isthmus. In *ace* mutants, apoptotic cell death is increased when compared with wild-type siblings. However, an increased number of dead cells can only be detected from mid-somitogenesis stages, when the rostralized gene expression profiles, at the relative positions where the isthmic and cerebellar primordia should form, are already evident in the mutant embryos. The decreased cell proliferation and increased apoptosis rate at later stages of development are consistent with the role of Fgfs, including Fgf8, as mitogen and survival factors for various neuronal and non-neuronal cell types (Lee et al., 1997; Hajihosseini et al., 1999; Trumpp et al., 1999; Chi et al., 2003). The sequence of mis-patterning and cell death seen in *ace* embryos is somewhat reminiscent of the kreisler phenotype in mice, where the cells in the developing hindbrain that would normally become specified as r5 and r6 adopt an r4 character instead, producing an excess of r4 cells that is disposed subsequently by apoptosis (McKay et al., 1994).

Our analysis suggests that quantitative processes, such as increased proliferation or a decreased apoptotic rate, are unlikely to play a major role in the restructuring process occuring within the mesencephalic-metencephalic region in *ace* mutants. By contrast, our cell lineage tracing experiments clearly indicate that the isthmic and cerebellar primordial cells become part of the tectum in *ace* embryos. Cells at the location of the wild-type MHB give rise to caudal tectum in *ace* embryos. Therefore, we think that the cells of the putative isthmic primordium in the mutants are qualitatively different to those of the wild type. The marked rostralization in gene expression is connected to the alteration of positional information and caudal-to-rostral transformation in the absence of the functional organizer signal Fgf8. Our results suggest that MHB and cerebellar primordial cells are transformed to tectal ones, or show a default tectal fate in *ace*.

Possible molecular mechanisms underlying the isthmus-to-midbrain transformation

In our bead implantation experiments the molecular and morphological features of the MHB could be restored in *ace*

mutant embryos, indicating that Fgf8 is either directly or indirectly necessary to execute the proper morphogenetic program at the mesencephalic and hindbrain alar plate. Moreover, when Fgf8 is provided ectopically in wild-type embryos it is able to restructure even the caudal parts of the forebrain, as was known from previous work in chick (Martinez et al., 1999; Shamim et al., 1999). Fgf8, besides being necessary and sufficient to reprogram and restructure the surrounding tissues, seems to act in a dose-dependent manner. Several pieces of evidence support the idea that different doses of Fgf8 may be responsible for the specification of distinct structures of the MHB region, and that the activity of Fgf8 is directly coupled to the dose of Fgf8 protein acting on the target tissue (Xu et al., 2000; Sato et al., 2001). Gain-of-function experiments performed with two different Fgf8 isoforms (MacArthur et al., 1995; Liu et al., 1999; Sato et al., 2001) show that the type-difference in the activity of the a and b isoforms can be attributed to the signal intensity, as electroporation of 100-fold less Fgf8b expression vector exerts similar effects to Fgf8a in chick embryos (Sato et al., 2001). To achieve cerebellar differentiation a high Fgf8 concentration is required, as reflected by the transformation of the tectal structures into cerebellum upon ectopic overexpression of the stronger Fgf8b isoform. During normal development, the stronger Fgf8b is the predominant isoform of Fgf8 in the isthmic region (Sato et al., 2001). The region that is exposed to strong Fgf8 signal (e.g. to the more abundant and physiologically more active Fgf8b), which expresses *Gbx2* and *Irx2*, may then acquire r1 characteristics, from which the dorsal rhombic lip and, later, the cerebellum will differentiate (Sato et al., 2001). Regions in which the tectal differentiation program will be executed receive only weak Fgf8 signaling, as inferred from the results of diencephalic overexpression of the weakly active Fgf8a isoform (Sato et al., 2001). In addition, different doses of Fgf8 might influence the fine patterning of midbrain regions (Lee et al., 1997; Picker et al., 1999). Our bead implantation experiments support dose dependency of Fgf8 action, as reflected by the graded induction of tectal ephrin A expression at ectopic locations in wild-type embryos, or in *ace* embryos at positions where the isthmic organizer normally forms. Close to the Fgf8 source, this protein is normally expressed at high levels, and its expression gradually decreases as a function of the distance from the source. In *ace* embryos the gradient is absent, and the mutants show no or only low levels of expression, characteristic of the anterior tectum in wild types (Picker et al., 1999) (present study).

The next question is how Fgf8 triggers and orchestrates the reorganization of the isthmic region when applied in the form of coated beads. We have suggested previously that the implantation of Fgf8 beads into the mesencephalic alar plate of the mutants may re-activate a set of regulatory genes that are acting during an earlier phase of the normal MHB development (Reifers et al., 1998). During normal development, these genes are initially independent of Fgf8, but at the time the bead implantation experiments were performed many of these key regulators (*her5*, *wnt1*, *pax2a*, *pax8*, *spa1a*, *spry4*) are directly or indirectly dependent on Fgf8 function, as is evident from the fact that they disappear in the absence of functional Fgf8 in *ace* mutants. Indeed, bead implantation experiments done on chick and fish embryos support this notion. Fgf8- or Fgf4-soaked beads implanted into the posterior forebrain or hindbrain alar plate are capable of inducing isthmic, cerebellar or midbrain structures, and the key regulatory genes (*Wnt1*, *En2* and *Fgf8* itself) are induced around the bead in the implanted embryos (Crossley et al., 1996; Martinez et al., 1999; Shamim et al., 1999). Similarly in zebrafish, we found that implanted Fgf8-coated beads in wild-type embryos are able to induce *fgf17*, *en2*, *sprouty4* and other targets of the MAPK pathway (Reifers et al., 2000b; Fürthauer et al., 2001; Raible and Brand, 2001). Similar to the results of the above-mentioned in vivo bead implantation experiments, Fgf8b-coated beads are able to trigger expression of *En1*, *En2*, *Pax5*, *Wnt1* and *Gbx2* in mouse embryonic midbrain and diencephalic explants, and repress *Otx2* in mesencephalic explants (Liu et al., 1999). This proposed action of Fgf8 triggering key regulators of MHB development, which then create ectopic MHB identity, is presumably different from the normal steps of MHB development because early expression of these markers is independent of Fgf8. These events can be considered as a shortcut in the developmental program, leading to recapitulation of the MHB cascade. Upon re-initiation of key regulators of the MHB cascade, it is likely that the mutual effects of regulatory genes create secondary genetic events that then stabilize the identity of the tissue, as is presumed to happen during the maintenance phase of MHB development.

During normal development, repressive genetic interactions between Otx2 and Gbx2 (Gbx1 in zebrafish) seem to determine the caudal limit of the tectum and the position of the molecular MHB (Broccoli et al., 1999; Millet et al., 1999; Katahira et al., 2000; Martinez-Barbera et al., 2001; Rhinn et al., 2003) (M. Rhinn and M.B., unpublished). In vertebrates, both *Fgf8* and *Gbx2* have a repressive activity on *Otx2* expression in the metencephalic alar plate (Liu and Joyner, 1999; Martinez et al., 1999; Millet et al., 1999; Tour et al., 2002). In addition, zebrafish *gbx2* is fully dependent on Fgf8 during the maintenance period (Rhinn et al., 2003). In *ace* mutants, one of the key determinants of the MHB position, *otx2*, expands at the expense of the *fgf8* and *gbx1* (*Gbx2* in amniotes) domain. It is therefore conceivable that upon displaying functional Fgf8 to *otx2*-expressing *ace* mutant tectal cells, the repressive genetic interactions (Martinez-Barbera et al., 2001) playing a role during normal MHB maintenance are re-established, accounting for the reversal of the tectal morphology and identity on the operated side in *ace* embryos. Conversely, in *ace* mutants where functional Fgf8 signaling is absent (Reifers et al., 1998; Araki and Brand, 2001), *otx2* transcripts are not repressed, causing a strong similarity to rostral mesencephalic regions experiencing putatively low or no Fgf8 signal as a function of the distance from the emitting source. Consequently, the mesencephalic/tectal differentiation program is executed in place of the metencephalic alar plate in *ace* mutants. Our observations are in good agreement with the results of *Otx2* misexpression studies, where the ectopic appearance of *Otx2* changes the fate of the metencephalic alar plate to a more rostral, tectal fate (Broccoli et al., 1999; Katahira et al., 2000). Taken together, the transformation process that takes place at the expense of the isthmic and cerebellar primordia can be viewed as an imbalance between mutual repressive effects of the MHB cascade genes, caused by lack of the orchestrating Fgf8 signal. As a consequence of the lack of Fgf8 function,

the self-maintenance of isthmic and cerebellar primordial cells is impaired resulting in a rostralization of gene expression profiles, and transformation of the isthmic and cerebellar primordium in *ace* mutants. In agreement with recent observations (Tallafuß and Bally-Cuif, 2003), our results suggest a role for Fgf8 in protecting the isthmic and metencephalic precursors from acquiring more rostral identities, and in self-maintaining the MHB identity.

Modularity in the developing upper brainstem structures

The embryonic brain seems to be composed of sequential modules that represent histogenetic fields specified by position-dependent expression of patterning genes (reviewed by Redies and Puelles, 2001). This early, embryonic modularity of the nervous system is later on translated into functional modularity. Furthermore, the suggested modular organization of the early brain allows for adaptive modification during evolution (Redies and Puelles, 2001). The modularity of the developing brainstem structures allows for spatial and temporal changes at early stages of development, for example, transformation of one particular structure to another one. During early stages of ontogenesis, such plasticity of the developing CNS makes escape from an immediate developmental arrest possible in CNS mutant embryos. Cases of transformation and their mechanisms are well documented during insect development (Sato and Denell, 1985; Klingensmith et al., 1989), and during development of the vertebrate caudal hindbrain, where the modular organization is evident owing to the rhombomeres (McKay et al., 1994; Gavalas et al., 1998; Popperl et al., 2000; Wiellette and Sive, 2003) (reviewed by Lumsden and Krumlauf, 1996). The rostralization, transformation and reversal of the abnormalities by bead implantation in *ace* mutants reveal the plasticity of the developing upper brainstem. An interesting question is therefore whether the observed transformation of the isthmic region hints at a modular organization of this region as well. Anatomically, a modular organization of the midbrain and the isthmic region has not yet been revealed (Redies and Puelles, 2001). Experiments performed in chick reveal the unique developmental fate of r1, being the only rhombomere in which no Hox genes are expressed as consequence of Fgf8 action in the anterior hindbrain. Fgf8 acts to set aside the territory from which the cerebellum will eventually develop through restriction of *Hoxa2* expression in r1 (Irving and Mason, 2000). Visualizing the formation of a new *otx2*/*epha4a* interface in *ace* mutants reveals a bipartite organization of r1, where the rostral part of r1 acquires a new, tectal identity. The modular plasticity also applies to more anterior parts of the mesencephalon and the diencephalon as seen, supported by bead implantation and electroporation experiments, both in chick (Martinez et al., 1999; Sato et al., 2001) and fish (present study). We demonstrated that functional Fgf8 is able to both revert the transformation of the isthmic and cerebellar primordia in mutant embryos and induce the diencephalon and mesencephalon to undergo transformation in wild-type embryos. Taken together, this reveals an extensive plasticity of the isthmic and r1 region, suggesting that transformation of cell fates seems to be the most economic way of restructuring the MHB territory in the absence of functional organizer activity in *ace* mutants.

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