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Mutations in zebrafish genes affecting the formation of the boundary between midbrain and hindbrain

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SUMMARY

Mutations in two genes affect the formation of the boundary between midbrain and hindbrain (MHB): *no isthmus* (*noi*) and *acerebellar* (*ace*). *noi* mutant embryos lack the MHB constriction, the cerebellum and optic tectum, as well as the pronephric duct. Analysis of *noi* mutant embryos with neuron-specific antibodies shows that the MHB region and the dorsal and ventral midbrain are absent or abnormal, but that the rostral hindbrain is unaffected with the exception of the cerebellum. Using markers that are expressed during its formation (*eng*, *wnt1* and *pax-b*), we find that the MHB region is already misspecified in *noi* mutant embryos during late gastrulation. The tectum is initially present and later degenerates. The defect in *ace* mutant embryos is more restricted: MHB and cerebellum are absent, but a tectum is formed. Molecular organisation of the tectum and tegmentum is disturbed, however, since *eng*, *wnt1* and *pax-b* marker gene expression is not maintained. We propose that *noi* and *ace* are required for development of the MHB region and of the adjacent mid- and hindbrain, which are thought to be patterned by the MHB region.

Presence of *pax-b* RNA, and absence of pax-b protein, together with the observation of genetic linkage and the occurrence of a point mutation, show that *noi* mutations are located in the *pax-b* gene. *pax-b* is a vertebrate orthologue of the *Drosophila* gene *paired*, which is involved in a pathway of cellular interactions at the posterior compartment boundary in *Drosophila*. Our results confirm and extend a previous report, and show that at least one member of this conserved signalling pathway is required for formation of the boundary between midbrain and hindbrain in the zebrafish.

Key words: neurogenesis, neural development, regionalization, *pax* genes, isthmus rhombencephali, midbrain, hindbrain, zebrafish, *Danio rerio*, *no isthmus*, *acerebellar*

INTRODUCTION

One of the hallmarks of the central nervous system (CNS) is the enormous number of different cell types that cooperate in its function. The great majority of these cell types derive from a common embryonic primordium, the neural plate, but the processes leading to diversification of the neural plate are only poorly understood. The neural plate forms from dorsal ectoderm as a consequence of an inductive influence from the underlying mesoderm (Spemann, 1938). Qualitative differences between the type of inducing mesoderm, as well as interactions between cells of the developing neural plate, are thought to result in the first subdivisions of the neural plate in a process termed regionalization (Nieuwkoop, 1989). As a result of regionalization, gross anatomical subdivisions become recognizable in the embryonic vertebrate CNS, such as the fore-, mid- and hindbrain, and the spinal chord. At least the fore- and hindbrain are thought to be further subdivided into segmentally arranged neuromeres, called prosomeres and rhombomeres, respectively (Puelles et al., 1987; Lumsden and Keynes, 1989; Figdor and Stern, 1993; Rubenstein et al., 1994, and references therein), which are then further subdivided into individual areas producing neurons, which contain specific and diverse cell types. The process of regionalization is therefore one of the first steps on the way to generating functional diversity in the developing vertebrate brain. Consistent with this view, position within the developing neural plate, rather than lineage history, has been shown to be an important determinant of the individual neuronal cell type in the case of zebrafish primary motoneurons (Eisen, 1994). It was therefore of interest to isolate and study mutations that affect major subdivisions of the brain, because they might define the mechanisms involved in regionalization of the neural plate.

The midbrain derives from the mesencephalic neural plate and includes as major derivatives the optic tectum and the ventral tegmentum. Additional neuromeric subdivisions within the midbrain have been tentatively suggested (Rubenstein and Puelles, 1994). Transplantation and inversion experiments in the chick embryo show that the midbrain develops in intimate association with the primordia of the fore- and hindbrain (Alvarado-Mallart, 1993; Wassef et al., 1993; Nakamura et al., 1994; Marin and Puelles, 1994). Rostral hindbrain tissue, when transplanted into caudal forebrain territory, leads to the expression of midbrain-hindbrain markers, not only in the transplanted tissue, but also in the neighbouring forebrain tissues. When such transplants are allowed to develop, the induced cells show a midbrain-like character (Gardner and Barald, 1991; Martinez et al., 1991; Bally-Cuif et al., 1992). Similar inductive interactions between fore- and hindbrain primordia are suggested by rotation experiments within the developing midbrain primordium (Marin and Puelles, 1994). If only the primordium of the midbrain is rotated, the transplanted piece heals in without any apparent reversal of polarity. If, however, a piece of rostral hindbrain tissue (the MHB primordium) is rotated along with the midbrain primordium, a duplicated midbrain with an inverted polarity results. Similarly, prospective MHB tissue grafted into hindbrain rhombomeres can induce alar rhombomeric cells to form cerebellar tissue (Martinez et al., 1995). These experiments identify the MHB region as an important organizing center with a role in midbrain induction and patterning (Marin and Puelles, 1994; Rubenstein and Puelles, 1994).

Midbrain development can be visualized with the aid of several molecular markers, such as wnt1, eng or pax-b. The corresponding genes are orthologues of the *wingless*, *engrailed* and *paired* loci in *Drosophila* that are involved in defining, through cell interactions, the posterior compartment boundary in the larval segments (reviewed by Ingham, 1991; Martinez-Arias, 1993). Mutational analysis of these genes in the mouse indicates that their vertebrate orthologues also function during development of the mid- and hindbrain (Thomas and Capecchi, 1990; McMahon et al., 1992; Wurst et al., 1994; Millen et al., 1994; Urbanek et al., 1994). In zebrafish, the function of the *paired* box-containing *pax-b* gene was studied by injecting an antibody to the pax-b protein into developing embryos (Krauss et al., 1992b). In the antibody-injected embryos, morphological malformations and a reduction in the levels of *wnt1*, *eng2* and *pax-b* RNA were observed at the midbrain-hindbrain boundary, leading to the suggestion that *pax-b* is involved in formation of this structure (Krauss et al., 1992b), consistent with misexpression experiments of *pax-b* RNA (Kelly and Moon, 1995).

We describe the isolation and initial characterization of mutations in two genes that affect the formation of the midbrain-hindbrain boundary. Our results suggest that these genes are required for successive steps in development of the isthmocerebellar primordium, and that one of these genes is the *pax-b* gene.

MATERIALS AND METHODS

Maintenance of fish, embryo collection and staging

Fish were raised and kept under standard laboratory conditions at about 27°C (Westerfield, 1994; Brand et al., 1995). Mutants were isolated as described in the accompanying paper (Haffter et al., 1996). Mutant carriers were identified by random intercrosses, and identified carriers for the mutation were then outcrossed to wild-type fish to maintain the stock. To obtain embryos showing the mutant phenotype, two heterozygous carriers for a mutation were crossed to one another. Typically, the eggs were spawned synchronously at dawn of the next morning, and embryos were collected, sorted, observed and fixed at different times of development at 28.5°C. In addition, morphological features were used to determine the age of the embryos, as described by Kimmel et al. (1995). In some cases, 0.2 mM phenylthiourea (PTU) was added to prevent melanization. For photography, live embryos were mounted in methylcellulose as described by Westerfield (1994).

Acridine orange staining

To detect degenerating cells, live embryos were dechorionated and placed for 1 hour into PBS, pH 7.1 with 2 μg/ml of acridine orange (Sigma). After two brief washes in PBS, the embryos were mounted in methylcellulose and viewed with fluourescence microscopy, using the FITC filter set.

Immunocytochemistry and antibodies

Whole-mount detection with antibodies was as described by Schulte-Merker et al. (1992). The following antibodies and concentrations were used: anti-acetylated tubulin (Sigma), 1:1000; mAb3a10 (Furley et al., 1990), 1:3; Pan-Isl (Korzh et al., 1993), 1:500; Zn12, Zn5 (Trevarrow et al., 1990), 1:1000 each; mAb 4D9 (Patel et al., 1989), 1:3. Secondary antibodies from a Vectastain elite kit were used at a dilution of 1:300 for detection.

Whole-mount in situ hybridisation

Digoxigenin-labelled RNA probes were prepared using an RNA labelling and detection kit from Boehringer. Hybridisation and detection with an anti-digoxigenin antibody coupled to alkaline phosphatase (Boehringer) was as described by Schulte-Merker et al. (1992), with modifications by C. Houart. RT-PCR isolation and sequencing of mutant *noi* alleles will be described elsewhere (K. Lun and M. Brand, unpublished).

RESULTS

Genetics

In our screen, we recovered seven recessive embryonic lethal mutations that affect formation of the boundary between mid- and hindbrain (MHB). Based on complementation tests, these mutations define two genes: *no isthmus* (*noi*) with six alleles of varying strength, and *acerebellar* (*ace*) with one allele (Table 1). In the following phenotypic analysis of *noi*, we always used one of the strong alleles, unless stated otherwise. Two other non-complementing mutations found by Schier et al. (1996) define the *spiel-ohne-grenzen (spg)* gene, which complemented both *noi* and *ace* mutations.

Development of *noi* embryos

Wild-type embryos during the pharyngula period [24-48 hours of development (h)] show a conspicuous constriction of brain tissue at the boundary between the mid- and hindbrain (Figs 1A, 7E). Embryos that are homozygous mutant for *noi* (Fig. 1B) or *ace* (Fig. 1D) lack this constriction completely, along with the cerebellum that derives from its posterior part. In addition, embryos that are homozygous for strong *noi* mutations lack the tectum, a dorsal portion of the midbrain (Fig. 1B, asterisk). For two weaker alleles of *noi*, a partially formed tectum was observed (Fig. 1C) that can still be recognized by ingrowing retinal axons (Trowe et al., 1996). Outside of the brain, *noi* mutant embryos during the late pharyngula stage lack a pronephric duct, which is normally visible as a

Table 1. Genes described in this paper

Gene	Symbol	Alleles	CNS phenotype	Other phenotype	Major description	Other references
no isthmus	*noi*	*tb21* *th44* *tm243a* *tu29a* *ty22b* *ty31a*	Absence of isthmus, tectum and cerebellum	Retinotectal projection, pronephros, circulation	This paper	a,b
acerebellar	*ace*	*ti282a*	Absence of isthmus and cerebellum, tectum enlarged	Circulation, ear	This paper	b,c
spiel-ohne-grenzen	*spg*	*m216* *m308*	Absence of isthmus and cerebellum, tectum enlarged	Circulaion, ear	d	

Seven recessive lethal mutations were isolated that affect the formation of the boundary between mid and hindbrain. In complementation tests between heterozygous carriers for these mutations, mutant embryos were observed in combinations of six of the seven mutations. These are therefore affecting the same gene, called *no isthmus* (*noi*). Two of the six *noi* alleles (*ty22b* and *ty31a*) derived from the same mutagenized founder male, but are of different phenotypic strength, and thus probably are of independent origin. All other alleles derive from independent founder males.

Two other non-complementing mutations affecting the formation of the MHB define the *spiel-ohne-grenzen* gene (*spg*) isolated by Schier et al. (1966), which complements both *noi* and *ace* mutations. We have not recovered any alleles of *spg* in our screen, and Schier et al. have not found any alleles of *noi* or *ace* in their screen (Schier et al., 1966).

References: a, Trowe et al. (1996); b, Chen et al. (1996); c, Whitfield et al. (1996); d, Schier et al. (1996).

tubular epithelium above the yolk in wild-type embryos (Fig. 1E,F, arrows). Also, whereas circulation is well established through the common cardinal vein of 36 h wild-type embryos (Kimmel et al., 1995), circulation is feeble in *noi* mutant embryos, where most blood cells accumulate in a dent on the surface of the ventral yolk sac (Fig. 1G,H), and the otic vesicle is often slightly reduced in size. The reason for the defective circulation is not known. Presumably because of this defect, embryos that are homozygous for a strong allele of *noi* show extensive edema on the third day of development, followed by severe degeneration and retardation in many tissues on the fourth day of development. Embryos homozygous for weak alleles survive to day 6 or 7, but fail to develop a swim bladder and eventually die.

Tectal cells undergo cell death in *noi* embryos

Living homozygous *noi* mutant embryos could be distinguished from their wild-type siblings from the late segmention period (about 22 h) of development onwards, based on a greater turbidity of the cells of the developing tectum. Staining of such embryos with acridine orange (AO), a dye that specifically detects apoptotic cell death in *Drosophila* (Abrams et al., 1993), revealed that a large block of predominantly dorsal cells die in the midbrain of *noi* mutant embryos for three different strong alleles (n=18), but not in their wild-type sibling embryos (n=13; Fig. 2). We observed no staining above wild-type levels at earlier stages (6-somite and 14-somite stages; n=30, wild type and mutants) or in other tissues, such as the pronephric duct. Much smaller numbers of AO-positive cells were seen in the tectum of embryos homozygous for weak alleles of *noi* (not shown). We conclude that a tectum initially forms in *noi* mutant embryos, but that it subsequently degenerates. We observed no increased cell death in homozygous mutant *ace* embryos (not shown).

Overall organisation of the CNS in *noi*

We looked at the overall neuromeric organisation and the formation of early axonal fascicles in the developing *noi* mutant brain, using an antibody to acetylated tubulin (Fig. 3; Chitnis and Kuwada, 1990; Ross et al., 1992; Wilson et al., 1990). Despite the absence of major portions of the brain, other areas are developed remarkably normally in homozygous mutant *noi* embryos at the pharygula stage. The establishment of the major comissures and longitudinal connectives in the fore- and hindbrain is not affected, like the medial longitudinal fascicle and its nucleus, which is thought to straddle the forebrain-midbrain boundary (Macdonald et al., 1994; Fig. 3A,B). Specific hindbrain neurons like the Mauthner cell and several of the reticulospinal neurons (Kimmel et al., 1985; Metcalfe et al., 1986; Hanneman et al., 1988) also seem to form normally, as revealed by staining with the 3A10 antibody (Fig. 3C,D). On the other hand, the distance between the forebrain-midbrain boundary (marked by the position of the tract of the posterior commissure) and the first hindbrain commissure (arrowheads in Fig. 3A,B) appears to be severely shortened in mutant embryos. Also, formation of the tract of the ventral tegmental commissure is severely reduced in mutant embryos (Fig. 3A,B, arrow in A). The nuclei of early differentiating cranial neurons of the mid- and hindbrain can be visualized with an antibody recognizing several Isl proteins (Pan-Isl; Korzh et al., 1993). In the tegmentum of 32 h wild-type embryos stained with this antibody, two bilateral clusters of neurons are seen, which probably are the nuclei of the oculomotor and trochlear nerves. In *noi* mutant embryos, only a small remnant of these clusters is observed, and the distance to the hindbrain nuclei is severely shortened (Fig. 3E,F; arrowheads); in addition, the trochlear nerve is absent from Zn5-stained mutant embryos (not shown). Together, these results point to a strong reduction of the occulomotor and trochlear nuclei in *noi* mutant embryos. As was seen in acetylated tubulin- and 3A10-stained embryos, organization of the fore- and hindbrain is largely normal in mutant *noi* embryos at this stage. This analysis indicates that CNS defects in *noi* mutant embryos are largely restricted to much, but not all, of the midbrain, the cerebellum and the MHB region.

Expression of markers of the MHB is affected in *noi*

Our analysis of the brain structure of *noi* mutant embryos suggested that absence of the dorsal derivatives of the midbrain (tectum) and rostral hindbrain (cerebellum) could be due to a specific deletion of tissue from the MHB region. We

therefore examined the expression of markers for both dorsal and ventral portions of the midbrain-hindbrain boundary in wild type and *noi* mutant embryos during the pharyngula stage. In wild-type embryos stained with the 4D9 antibody, which recognizes all three zebrafish eng proteins (Patel et al., 1989; Ekker et al., 1992b), expression is seen in both dorsal and ventral portions of the MHB, and the neighbouring caudal midbrain and rostral hindbrain (Fig. 4). In 30 h embryos carrying a strong allele of *noi*, the MHB constriction is not formed, and consequently no eng staining is found in this area. In addition, expression of eng protein is also not detectable in the neighbouring caudal tegmentum and rostral hindbrain, which are not overtly missing in *noi* mutant embryos (Fig. 4A,B). Expression of eng proteins is normal in other domains, such as the muscle pioneers or the clusters of hindbrain neurons (not shown). Expression of other markers, such as *wnt1* (Molven et al., 1991) and *zash1A* (Allende and Weinberg, 1994) is also affected at the MHB in mutant embryos (Fig. 4). In wild-type embryos at the 20-somite stage, *wnt1* expression is detected in an anterior cluster underneath the epiphysis, along the dorsal edge of the tectum, in the MHB, and in segmental clusters in the hindbrain (Fig. 4E). In *noi* mutant embryos, *wnt1* expression at the MHB is abolished, whereas other expression domains are not affected (Fig. 4F). The distance between the anterior cluster and the first rhombomere is decreased in *noi* mutant embryos, suggesting that the cells of the MHB area are absent at this stage. Nevertheless, a tectum is still present at this stage in *noi* mutant embryos, and its posterior edge appears to be directly joined to the first rhombomere (Fig. 4F). *zash1A* expression in wild-type embryos at the pharyngula stage occurs in two ventral clusters in the tegmentum and ventral rhombomere 1 or 2, leaving a gap at the MHB (Fig 4G, between arrowheads; Allende and Weinberg, 1994). In *noi* mutant embryos, *zash1A* expression extends across this gap (Fig. 4H, arrowhead), again indicating that the intervening tissue may be deleted or respecified. No defects are observed in the expression of markers at the forebrain-midbrain boundary (*pax-a)* or the middiencephalon (*shh*, *fkd3*; not shown). We conclude that expression of *eng*, *wnt1* and *zash1A* is specifically affected at the midbrain-hindbrain boundary in *noi* mutant embryos.

Development of the MHB primordium is affected in *noi*

In order to determine if the primordium of the MHB is already affected in *noi* mutant embryos, we looked for expression of eng proteins in *noi* embryos during somitogenesis that were doubly stained with *krx20*, a marker for rhombomeres 3 and 5 (Oxtoby and Jowett, 1993; Fig. 4C,D). In wild-type embryos at the 8-somite stage, *eng* expression is detected in a broad band across the developing MHB region of the neural keel, located half way between the rhombomere 3 stripe of *krx20* and the optic vesicle. No expression of *eng* is detected in *noi* mutant embryos, whereas expression of *krx20* is unaffected in the same embryos (Fig. 4D). The distance between the rhombomere 3 stripe and the posterior optic vesicle seems unchanged in DAPI-counter-

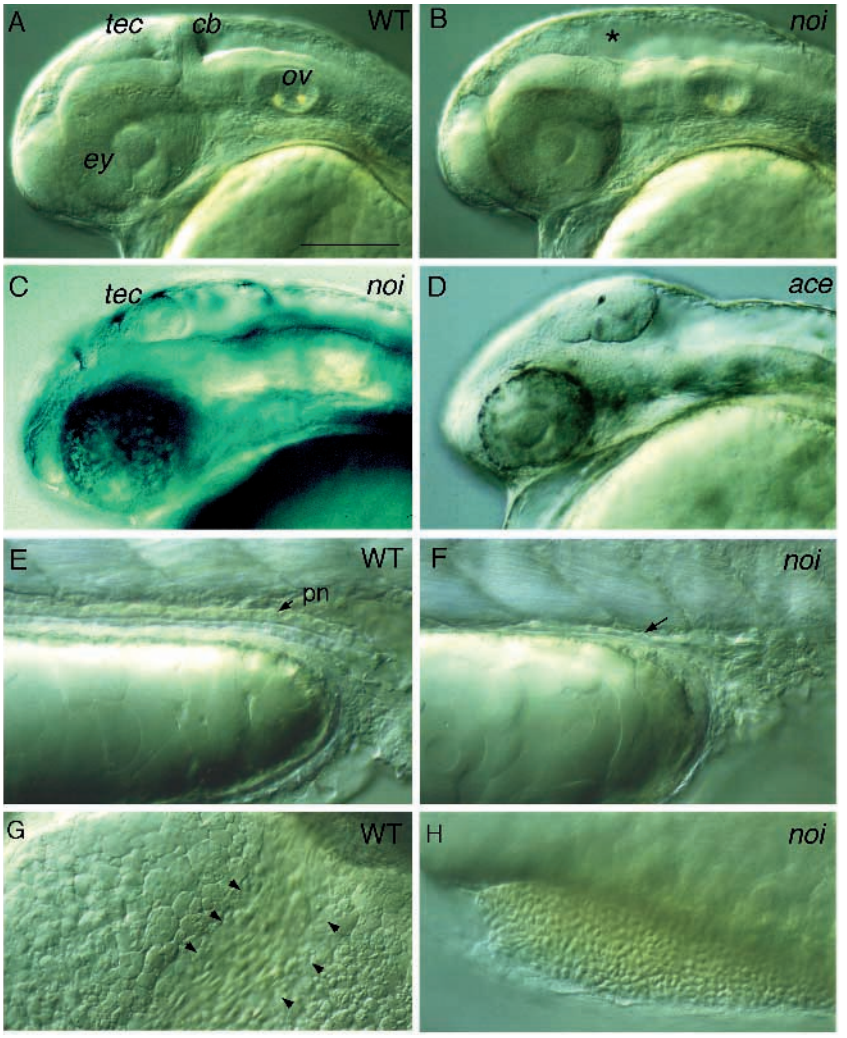

Fig. 1. Lateral views of living *noi* and *ace* mutant embryos. (A) Wild-type embryo at 36 hours of development. (B) Sibling of the embryo in A that is homozygous mutant for a strong allele of *noi* (*noi*^{th44}). Absence of the tectum, cerebellum and MHB is indicated by an asterisk. (C) Embryo homozygous mutant for a weak allele of *noi* (*noi*^{ty31a}). A partially formed tectum, but no cerebellum or MHB, is observed. (D) Embryo homozygous mutant for *acerebellar* (*ace*^{ti282a}). An enlarged tectum is present, but no cerebellum or MHB are seen (see also Fig. 7). (E) Pronephric duct (pn) in a wild type and (F) homozygous mutant embryo for *noi*^{th44}. In the mutant embryos, no pronephric duct is found; the arrow points to the few cells in the mutant that may represent partially developed pronephric duct cells; consistent with the presence of a normal duct during earlier stages of development. (G) Blood streaming through the common cardinal vein over the yolk in a 36 h wild-type embryo (bracketed by arrows). (H) Blood cells accumulate in a dent on the ventral yolk sac in a homozygous mutant embryo for *noi*^{th44}. No such accumulation is seen in wild-type embryos. Embryos in A and B and E-F were placed in PTU at about 26 h to suppress formation of melanin. cb, cerebellum; ey, eye; tec, tectum; ov, otic vesicle; pn, pronephric duct. Scale bar, 150 μm (A-D), 60 μm (E-H).

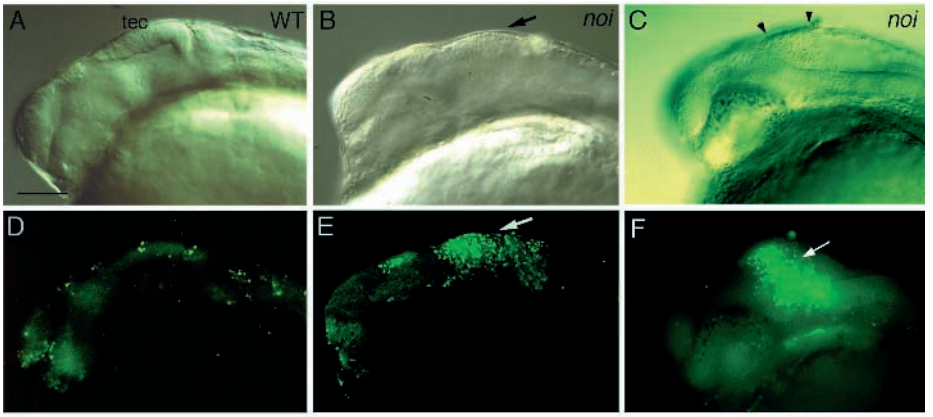

Fig. 2. Apoptosis in the tectum of *noi* mutant embryos. Shown is a comparison of Nomarski (A-C) and fluourescence (D-F) images of the same embryos, stained with acridine orange to detect apoptotic cell death. (A,D) Wild-type embryo at 22 h of development; (B,E) homozygous mutant *noi*^{tu29a} embryo at 22 h, stained with acridine orange. (C,F) Homozygous mutant *noi*^{ty22b} embryo at 26 h, stained with acridine orange. Large numbers of stained cells are observed in the tectum of mutant embryos in E and F (arrows) that are absent in the wild type. Degenerating, more refractile cells are also seen with Nomarski optics in C (between arrowheads). Scale bar, 120 μm.

stained specimens, indicating that the MHB primordium is not missing in the mutant embryos at this stage. We also do not detect any eng staining at the end of gastrulation, shortly after the onset of eng staining in wild-type embryos (*n*=16 of 67 embryos; not shown). In contrast, *pax-b* RNA expression, as another marker of the MHB primordium, is normal in late gastrula and 6-somite stage mutant *noi* embryos, but is fading to near invisibility at the 14-somite stage (Fig. 5G,H). We conclude that the MHB primordium is initially present, but defective in marker gene expression in *noi* mutant embryos.

pax-b protein, but not RNA, is eliminated in *noi*

pax-b is expressed during development of the MHB, pronephros, optic stalk and certain hindbrain and spinal chord neurons (Fig. 5A,C; Krauss et al., 1991a; Mikkola et al., 1992). In assaying the expression of pax-b protein as another marker of the MHB region, we noticed its entire absence in the mutant embryos (*n*=22 of 88; Fig. 5B,D). A survey of the different *noi* alleles using whole mount staining with a polyclonal anti-pax-b antibody showed that pax-b protein staining is eliminated in mutant embryos for three of the four strong alleles, but present for the two weak alleles (Table 2). Using whole mount in situ hybridisation, we determined that *pax-b* RNA is still present in 28 h homozygous mutant embryos for all alleles in all tissues, excepting the MHB (Fig. 5E,F). *pax-b* RNA expression is seen in the pronephric duct, optic stalk and the otic placode of mutant embryos, though the staining area in these tissues often appears slightly smaller than in the wild-type siblings (Fig. 5E,F). The optic nerve forms normally in Zn5-stained mutant embryos, and expression of *dlx3*, a marker for development of the inner ear (Ekker et al., 1992a) occurs normally in the ear up to 30 h (not shown). In addition, the overall level of *pax-b* RNA is reduced in 30 h embryos homozygous mutant for all of the strong alleles (Fig. 5F, Table 2).

The *noi* mutation is linked to the *pax-b* gene

The preceding data suggest that *noi* mutations affect the *pax-b* gene itself. To further examine this possibility, we looked for genetic linkage between the *noi* phenotype and a restriction fragment length polymorphism (RFLP), which we found in the *pax-b* gene, using the scheme depicted in Fig. 6A. The allele *noi*^{th44} was induced in the background of an inbred wild-type strain from Tübingen (Tü). Heterozygous carrier males for this allele were crossed to a female from a wild-type AB strain. In Southern blots of genomic DNA probed with a cDNA for the *pax-b* gene (Krauss et al., 1991a), these two strains yield a RFLP (Fig. 6B) that can be used to test for linkage between the *noi* mutant phenotype and the *pax-b* gene. If *pax-b* and *noi* are not linked, the *pax-b* polymorphism should assort randomly, i.e. both mutant embryos and their siblings should show both RFLP bands with equal intensity. If *noi* and *pax-b* are linked, then sorted mutant *noi* embryos should always carry only the Tü allele, which is what we observed (Fig. 6B). We then sequenced two independently amplified RT-PCR fragments containing the coding region of *pax-b* from homozygous mutant embryos for one of the strong alleles, *noi*^{th44a} (Fig. 6C). We found several ‘silent’ single base changes relative to the published wild-type sequence (Krauss et al., 1991a), but only one change leading to altered amino acid sequence, resulting in a stop codon in the middle of the coding region (Fig. 6D; K. Lun and M. Brand, unpublished data). The predicted shorter mutant protein (Fig. 6D) is presumably inactive or unstable. We conclude that *noi* mutations are located in the *pax-b* gene.

Phenotype of living *ace* embryos

Homozygous mutant *acerebellar* (*ace*) embryos can be distinguished from their wild-type siblings at the 5-somite stage, based on a slightly thicker neural keel in the area of the developing midbrain (Fig. 7A,B). Like *noi* mutant embryos, homozygous *ace* mutant embryos lack the MHB constriction at the pharyngula stage (Fig. 7C-F); they also show a similar defect in circulation (not shown), which is probably the cause

Table 2. RNA and protein in *noi* mutant embryos

noi allele	Allele strength	RNA	Protein
tb21	w	Yes	Yes
th44	s	Yes	No
tm243a	s	Yes	Yes
tu29a	s	Yes	No
ty22b	s	Yes	No
ty31a	w	Yes	Yes

Rna and protein were detected in homozygous embryos for different *noi* alleles. RNA is present in all alleles; at the pharyngula stage, RNA levels are reduced to about 50% of the wild-type level in the four strong (s) alleles. Protein and RNA presence or absence is indicated by Yes or No. The two weak (w) alleles and *noi*^{tm243a} of the strong alleles were found to still contain detectable protein.

of their death around day 7 of development. In contrast to *noi* mutant embryos, where the tectum is missing, homozygous *ace* embryos have a tectum that appears to be bigger than that of wild-type embryos (Fig. 7C-F, and below), and they have a normal pronephric duct (not shown). In addition, *ace* embryos develop a smaller otocyst that usually has only one otolith (Fig. 7E-H). On day 5 of development, overall size and the formation of semicircular canals are affected in the otocysts of *ace* mutant embryos (Fig. 7G,H; Whitfield et al., 1996).

Unlike in *noi* mutant embryos, we did not observe an increase in the amount of dying cells in acridine orange-stained *ace* mutant embryos at 24 h, compared to their wild-type siblings (not shown). Stainings with anti-acetylated tubulin and ZN12 antibodies showed an overall normal organisation of axonal tracts, and no decreased distance between the first hindbrain fascicle and more anterior comissures was observed with these antibodies (not shown). Likewise, the arrangement of Isl-stained cranial nuclei at 24 hours of development was normal (not shown); later stages were not examined. These findings indicate that the defect in *ace* mutant embryos is more restricted than in *noi* mutant embryos.

Expression of markers of the MHB region in *ace*

The absence of the MHB constriction, along with the presence of an enlarged tectum, led us to study expression of markers of the anterior portion of the constriction in *ace* mutant embryos. Does the constriction become incorporated into the tectum and/or tegmentum that is seen in *ace* mutant embryos, or is this part deleted? Does the defect in the MHB region also affect both dorsal and ventral parts of the junction between mid- and hindbrain, as seems to be the case in *noi* mutant embryos, or does only the cerebellum fail to form? We studied expression of *eng*, *pax-b*, *wnt1* and *zash1A* in homozygous mutant *ace* embryos during the pharyngula period (Fig. 8). Expression of eng proteins, as seen by 4D9 antibody staining, is not detected in the dorsal or ventral MHB region of 28 h *ace* mutant embryos (Fig. 8A,B), but is present in other parts of the body (not shown). Expression of *pax-b* RNA and protein, and of *wnt1* RNA mark the anterior wall of the constriction, which is contiguous with the tectum. In *ace* mutant embryos, expression of both markers is absent from the anterior MHB, but is not affected in other places (Fig. 8C-F). Instead, the domain of *wnt1* expression appears to extend further along the dorsal edge of the *ace* mutant tecta than in the wild-type tecta (Fig. 8E,F; the posterior end of this domain is marked by an arrow). We also observe altered expression of *eng* and *zash1A* in *ace* mutant tecta: in wild-type embryos, eng protein staining extends from the posterior midbrain border with gradually dimishing intensity into the tectum and tegmentum (Fig. 8A); no expression is observed in the tectum or tegmentum of *ace* mutant embryos at this stage (Fig. 8B). *zash1A* is weakly expressed in the ventricular zone of the tecta of wild-type embryos at the pharyngula stage (Allende and Weinberg, 1994; Fig. 8G, arrowhead). Concomitant with the loss of *eng* expression, we see a strongly increased expression of *zash1A* in the tecta of *ace* mutant embryos (Fig. 8H, arrowhead). No defect is seen in *eng* or *pax-b* expression in embryos at the end of gastrulation, but the MHB stripe of *pax-b* is thinner in mutant embryos at the 11-somite stage (not shown). In summary, the cerebellum is clearly absent in living *ace* mutant embryos. Our analysis of marker expression shows that the MHB region in general, and in particular the anterior part of the MHB constriction, are progressively lost or respecified in *ace* mutant embryos up to the pharyngula stage of development.

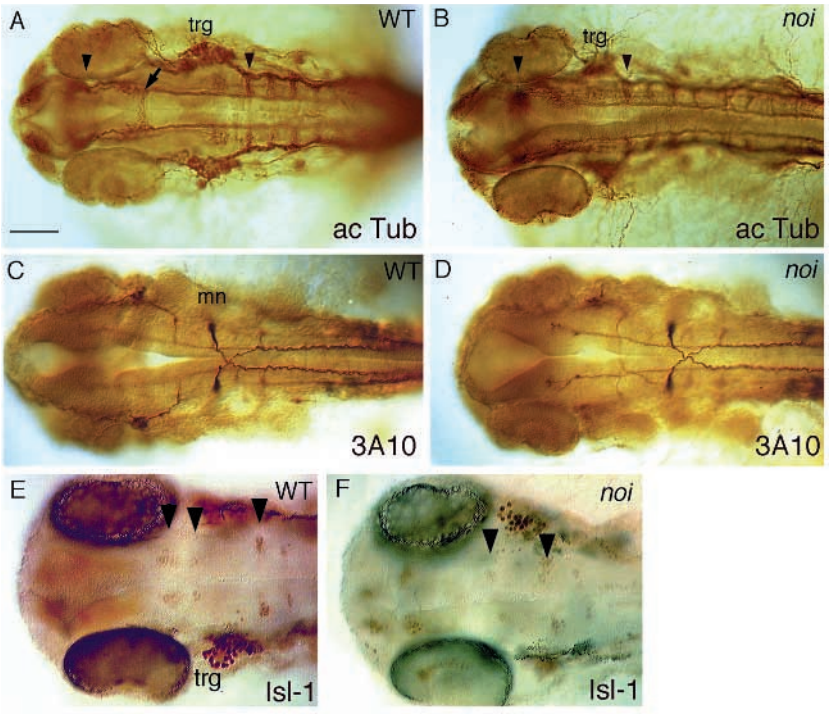

Fig. 3. Overall organisation of the CNS in *noi* mutant embryos. Shown are dorsal views of wild type and mutant embryos stained with antibodies to reveal axonal organisation and formation of neuronal nuclei. (A,B) Anti-acetylated-tubulin staining in 30 h wild-type and mutant *noi*^{ty22b} embryos. Arrowheads point to the position of the tract of the posterior commissure, and to the first hindbrain commissure. Notice the reduced distance. The arrow in A points to the ventral tegmental commissure that is absent in the mutant *noi* embryo in B. (C,D) mAb 3A10 staining in 30 h wild-type and mutant *noi*^{th44} embryos. The Mauthner neuron (mn) and the other T reticular interneurons stained by this antibody develop normally, indicating that hindbrain development is largely normal in *noi* embryos. (E,F) *Isl* staining of 32 h wild-type and mutant *noi*^{th44} embryos. Arrowheads point to bilateral pairs of clustered neuronal nuclei stained by this antibody in the midbrain and hindbrain (see text). Whereas two clusters are seen in the midbrain of the wild-type embryo in E (probably corresponding to the nucleus of the medial longitudinal fascicle), only one such pair of reduced size is seen in the mutant. Notice the reduced distance to the rhombomere 2 cluster. A group of 2-3 nuclei in rhombomere 1 is also unaffected in the mutant, but not in focus. *trg*, trigeminal ganglion. Scale bar, 100 μm (A-D), 75 μm (E, F).

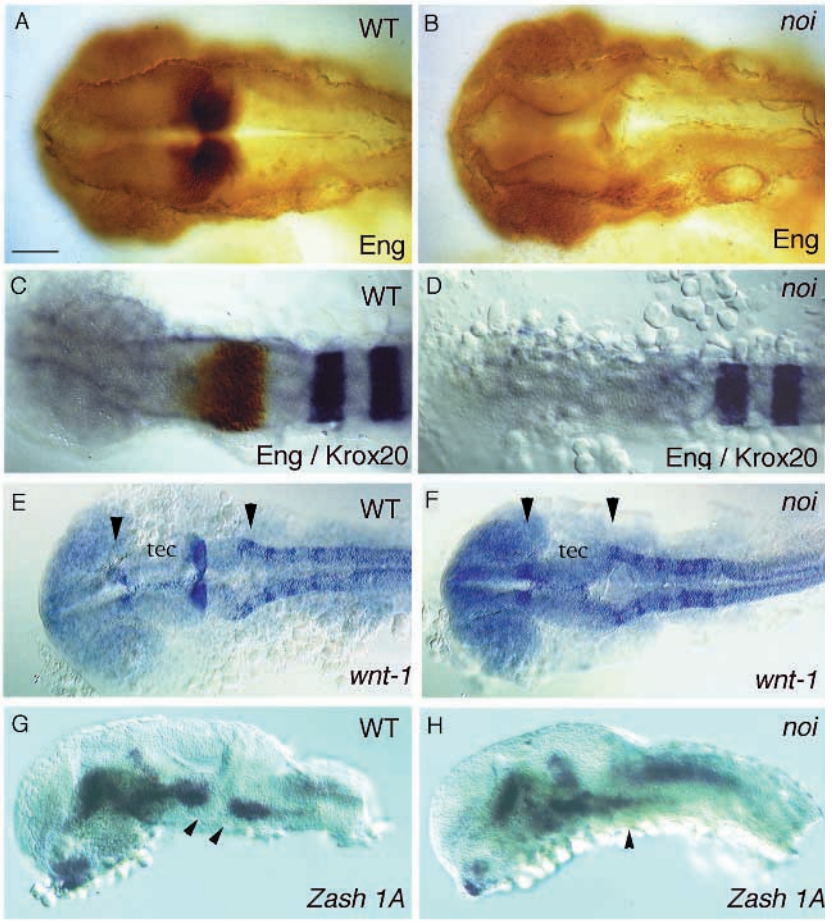

Fig. 4. Expression of markers of the MHB in *noi*. Shown are dorsal (A-F) and lateral (G,H) views of wild type and *noi*^{th44} mutant embryos, stained with markers of the MHB as indicated. (A,B) mAb4D9 staining for eng proteins at 28 h. Staining is eliminated in the MHB of mutant embryos. (C,D) Doubly stained embryos at the 8-somite stage, for *krx20* RNA in blue and eng proteins in brown. No eng proteins are detected in the primordium of the MHB of mutant embryos. (E,F) Expression of *wnt1* at the 20-somite stage posterior to the tectum (tec) is eliminated in *noi*, as is the posteriorly adjoining cerebellar fold. Notice the reduced distance between the *wnt1*-expressing domains in the dorsal forebrain and the first rhombomere (arrowheads). (G,H) *zash1A* staining at 26 h. A gap of staining (arrowheads) is observed at the MHB between the tegmental and rhombomeric expression domains, which is absent in the mutant embryo. Scale bar, 75 μm (A,B,G,H); 100 μm (C,D); 120 μm (E,F).

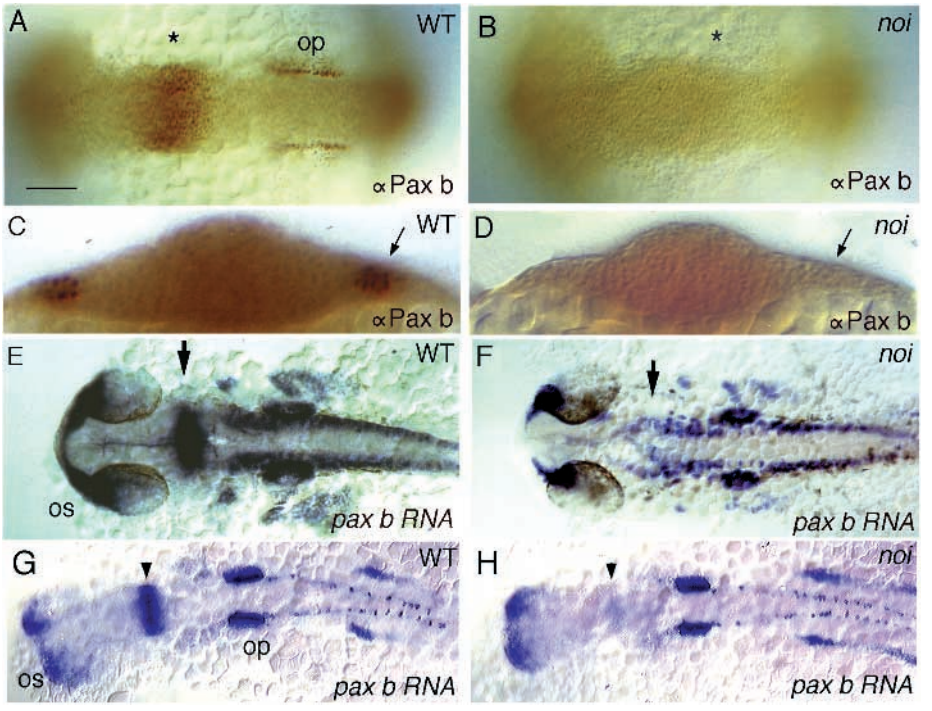

Fig. 5. Pax-b protein, but not RNA expression, is affected in *noi*. Shown are dorsal views of wild-type and *noi* mutant embryos (axial view as optical section in C and D), stained for pax-b protein and RNA, as indicated. (A-D) pax-b antibody staining is eliminated from (A,B) the MHB primordium (asterisks) and otic placode (op) and (C,D) the pronephric anlage (arrow) of mutant embryos at the 5-somite stage. (E,F) *pax-b* RNA expression in wild type and *noi*^{th44} mutant embryos. RNA expression is found in all tissues of the mutant, except for the MHB (arrow) and pronephros. Notice the overall reduction of RNA level in the mutant, which is not yet apparent at the 14-somite stage in (G,H): only a trace of RNA can be detected in the MHB of mutant embryos at this stage (arrowhead), whereas RNA expression in the optic stalk (os), otic placode, pronephric anlage and hindbrain neurons is normal. Scale bar, 100 μm (A,B); 50 μm (C,D); 140 μm (E-H).

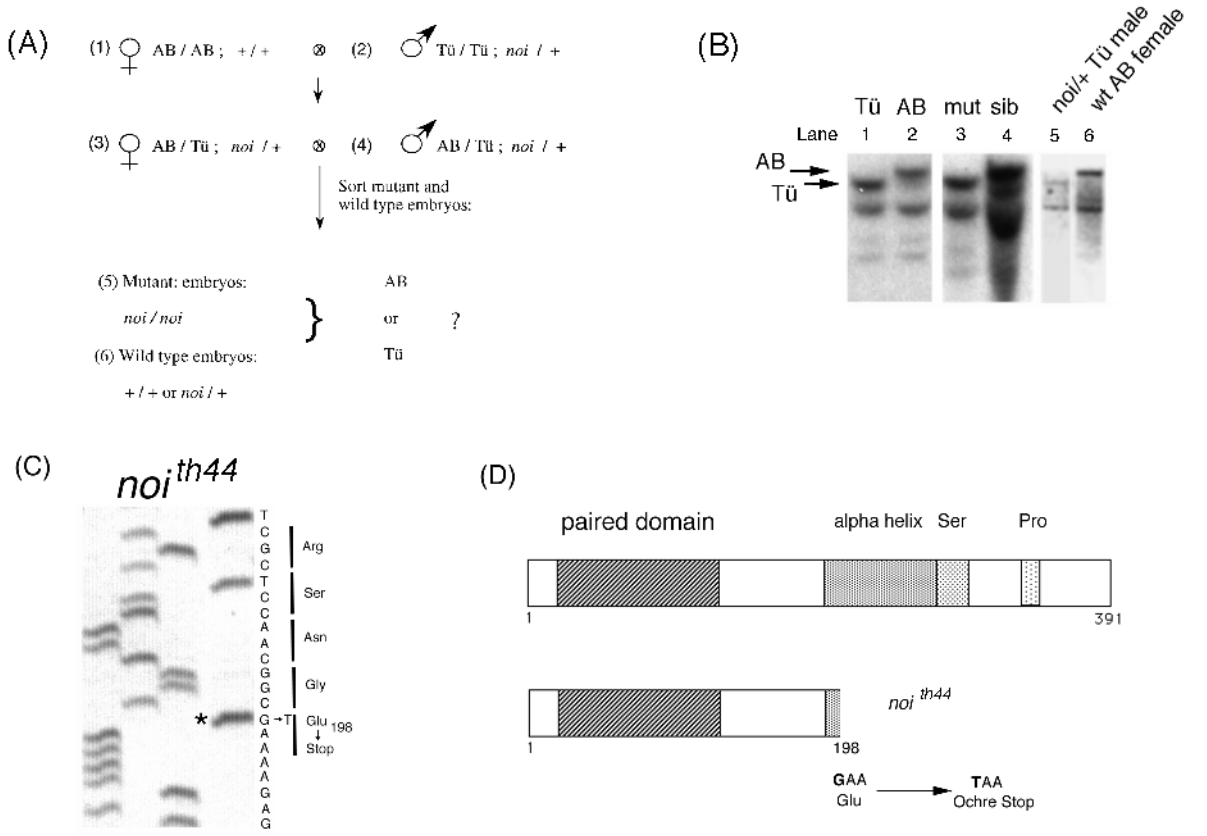

Fig. 6 A *noi* mutation maps to the *pax-b* gene. (A) Crossing scheme to determine if *pax-b* is linked to *noi*. Wild-type AB females were crossed to heterozygous *noi*/+ Tübingen males (Tü). Identified carriers in the next generation were mated to each other, and the *noi* mutant embryos were sorted from their siblings. Genomic DNA was prepared from these embryos and their parents, and probed for the presence of the Tübingen or AB band, as shown in B. (B) RFLP linkage analysis. All *noi* mutations were induced in an inbred wild-type line from Tübingen (Tü), which shows an 8 kb band, whereas the AB strain shows a band of about 9.5 kb in Southern blots of genomic DNA digested with *Pvu*II, probed with a full length cDNA for the *pax-b* gene. 10 μg DNA from approximately 40 embryos are loaded per lane (lane 4: about 20 μg). Lanes 1, 2: genomic DNA prepared from Tübingen and AB adult fish. An 8 kb band is observed in lane 1 (Tü band), and a 9.5 kb band (AB band) is seen in lane 2. A small amount of Tü band is observed in lane 2, which is due to a contamination in our AB stock (see below). Lane 3: DNA from homozygous mutant noi^{th44} embryos (genotype 5 in Fig. 6A) shows only the Tü band, indicating that the *pax-b* and *noi* genes are closely linked. Lane 4: sibling embryos of genotype 6 show both the Tübingen and AB bands. The increased signal is due to overloading. Lane 5: DNA from the parental *noi*/+ Tü male used in the mapping experiment (genotype 2). Lane 6: DNA from the parental AB female used in the mapping experiment (genotype 1). (C) Sequence of the noi^{th44} allele. The asterisk indicates the position of the altered base, sequence changes are indicated on the right. (D) Diagram of the wild-type structure of the *pax-b* transcription factor, containing the paired domain, and putative alpha-helical, serine-rich and proline-rich domains (Krauss et al., 1991a). The hypothetical truncated protein is shown underneath.

DISCUSSION

We have described mutations in two genes, *no isthmus* (*noi*) and *acerebellar* (*ace*), that are required for proper formation of the boundary between mid- and hindbrain. *noi* is required for a very early step in development of the MHB primordium during late gastrulation, and for development of the tectum and cerebellum. In addition, *noi* is also needed for maintenance of the pronephric duct. Our analysis of pax-b protein and RNA expression, and the genetic linkage between the *noi* mutant phenotype and the *pax-b* gene, strongly argue that the *paired* box gene *pax-b* is mutated in *noi* mutants. This is confirmed by the observation of a stop codon in the middle of the open reading frame in one of the strong *noi* alleles, which predicts a truncated protein of about half the size (Fig. 6; K. Lun and M. Brand, unpublished data). *acerebellar (ace)* seems to be required for a later and more restricted aspect of MHB development, possibly to prevent expansion of tectal identity into the MHB region, and for proper ear development. We do not yet know which gene is mutated in the *ace* mutant.

Detection of zebrafish mutants affecting formation of the MHB

At least three zebrafish genes (*noi* and *ace*, this paper; *spg*, Schier et al., 1996) can be mutated to a condition in which the affected embryos lack a MHB. Inactivation of the mouse genes *wnt1*, *Eng1* or *pax-5* (but not *pax-2*; M. Torres and P. Gruss, personal communication), leads to a similar phenotype (Thomas and Capecchi, 1990; McMahon et al., 1992; Millen et al., 1994; Urbanek et al., 1994), and mutations in a fourth gene, *Eng-2*, lead to more subtle defects in development of the cerebellum only (Millen et al., 1994) that we would not have detected with our morphological screening procedure. Such mutations may, however, have been kept if they affected other

structural or functional aspects of brain development (see other papers in this issue).

Extent of the defect in *noi* and *ace* embryos

In living *noi* mutant embryos, development of the MHB constriction, the tectum and the cerebellum are overtly affected. Analysis of brain structure, absence of *eng* and *wnt1* expression, and altered expression of *zash1A* in the mutant *noi* embryos, show in addition that part of the tegmentum and rostral hindbrain are affected as well. The situation is similar for *ace* mutant embryos, though the defects appear to be more confined to the MHB region itself. Overall, the observed defects in *noi* mutant embryos correlate well with the broad, early phase of *pax-b* expression throughout the midbrain and MHB primordia during gastrulation, suggesting that this could be the critically required expression phase, rather than the late expression that is restricted to the MHB. A requirement for the broader, early expression phase was also postulated for the mouse *wnt1* gene (Rowitch and McMahon, 1995). Alternatively, the progressive loss of midbrain tissue in *noi* mutant embryos may be due to loss of the organizing ability postulated for the MHB region (Marin and Puelles, 1994; see below).

Determination of the MHB region

Establishment of *pax-b* mRNA (but not protein) expression during late gastrulation stages occurs normally in *noi* mutant embryos, whereas *eng* expression is abolished. Therefore, positional information leading to formation of the MHB primordium must be generated independently of the *pax-b* gene.

pax-b most likely functions during early determination or differentiation of the MHB primordium. In contrast, early expression of *pax-b* and *eng* are not affected in *ace* mutants, indicating that *ace* affects a later or more restricted aspect of MHB development.

Are *noi* and *ace* involved in regionalization of the neural plate?

The defects of *noi* and *ace* mutant embryos in the midbrain and MHB region beg the question of how this affects development of the adjacent brain regions. We have not examined this question in detail yet. Loss of the *wnt1* and *pax-b* expression at the MHB, along with extension of the dorsal mesencephalic *wnt1* domain and the increased size of the tectum in *ace* mutant embryos, argue that the MHB region is transformed to a more anterior fate. In *noi* mutant embryos, the aberrant extension of the tegmental domain of *Zash1A* posteriorly (Fig. 4G,H) indicates that a transformation of the MHB region to a more anterior fate may have occurred. The altered marker expression we observe in *noi* and *ace* could therefore reflect disturbed interactions in regionalization of the neural plate. Since *pax-b* expression is found in the neural ectoderm, but not the underlying mesoderm, these regionalization processes must also depend on interactions in the ectoderm. Similar transformations within the forebrain anlage are observed for embryos mutant for the *masterblind* mutation found in our screen, which lack eyes and the telencephalon, and show an expanded posterior diencephalon. Transplantation chimaeras argue that *masterblind* is autonomously required in the ectoderm, again suggesting that interactions influencing regionalization of the forebrain occur in the ectoderm (Heisenberg et al., 1996).

Functional requirement of the *pax-b* gene

Previous experiments have already indicated a requirement for *pax-b* function. Injection of an antibody to pax-b protein lead to malformation of the MHB, and to reduced expression of molecular markers of this region (Krauss et al., 1992b), but it

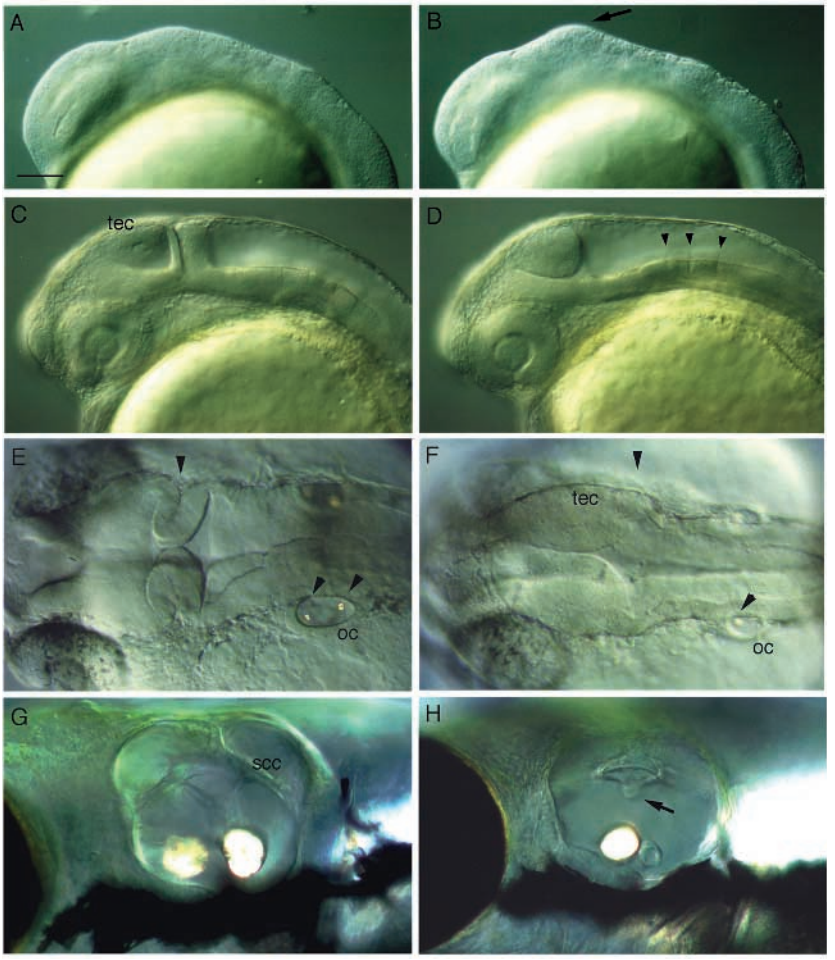

Fig. 7. Development of living *ace* embryos. Shown are lateral or dorsal (E,F) views of whole-mounted living wild type (left) and *ace* mutant siblings (right) as indicated. (A,B) At the 5-somite stage, mutant embryos show a bulge in the prospective midbrain primordium (arrow). (C,D) At the pharyngula stage, mutant embryos lack a cerebellum and the MHB fold, but show an enlarged tectum. The embryos were placed into PTU to suppress melanization. Arrowheads point to rhombomere boundaries. (E,F) In a dorsal view at 30 h, the absence of the MHB fold and the increased size of the tectum are particularly evident. Arrowheads point to the posterior edge of the tectum, coincident with the MHB fold in the wild type, and to the otoliths in the otic cyst. Notice the reduced size of the otic cyst in the mutant. (G,H) High magnification view of the otic capsule of the free swimming larva (day 6): notice the smaller size, and the truncated semicircular canal (arrow in H). Only one otolith is apparent in the mutant embryo. cb, cerebellum; oc, otic cyst; scc: semicircular canal; tec, tectum. Scale bar, 100 μm (A-D); 75 μm (E,F); 65 μm (G,H).

could not be tested whether the observed defects were solely due to inactivation of pax-b protein. Our results confirm and extend these studies. *pax-b* is expressed during development of the midbrain and the MHB, optic stalk, otic placode, certain hindbrain and spinal chord neurons and the pronephros (Krauss et al., 1991a; Mikkola et al., 1992). The requirement for *pax-b* function is most obvious for the MHB region, a large portion of the midbrain, and for the cerebellum and the pronephric duct. The common functional requirement for *pax-b* in development of the more primitive pronephros in fish, and for *Pax-2* in metanephric development of the mouse (Torres et al., 1995), points to an interesting conservation in the genetic mechanisms involved in the formation of these structures. Recent experiments also suggest a function for *pax-b* in partioning the optic vesicle into optic stalk and neural retina, in response to midline-derived signals (Macdonald et al., 1995; Ekker et al., 1995). Consistently, a mutation in the human *Pax2* gene leads, as a dominant trait, to optic nerve colobomas and kidney dysfunction (Sanyanusin et al., 1995). Our findings suggest that *pax-b* has only a minor role, if any, in development of the eyes and inner ear as a whole, since the eyes and the optic nerve appear to form normally in the mutant embryos, and the otic vesicle is only visibly affected at a time when the embryo is already rather sick from defective circulation. It remains to be determined, however, if more subtle aspects of optic stalk and inner ear development depend on the *pax-b* gene.

In the zebrafish, *pax-b* is currently the only member of the pax gene family known to be expressed at the MHB. Although only *pax-b* was isolated in two independent studies (Krauss et al., 1991a; Püschel et al., 1992), further pax genes expressed at the MHB may yet exist. In mice, *Pax-2*, *-5* and *-8* are expressed in overlapping domains at the MHB (Asano and Gruss, 1992; Stoykova and Gruss, 1994). *Pax-8* mutants have not been described yet. Inactivation of *Pax-5* leads to defective development of the posterior midbrain and cerebellum, but the defects seem to be considerably milder than in *noi* mutant embryos (Urbanek et al., 1994). Homozygous mutant *Pax-2* mice lack kidneys, ureters and genital tracts (Torres et al., 1995), but have a normal midbrain-hindbrain boundary (M. Torres and P. Gruss, personal communication). Molecularly, *pax-b* is clearly related to both *Pax-2* and *Pax-5*: whereas *Pax-5* is only expressed at the MHB and not in the developing optic stalk, ear and kidney, *pax-b* and *Pax-2* are expressed in all of these tissues (Asano and Gruss, 1992; Krauss et al., 1991a). On the other hand, the *paired* domain of *pax-b* is almost identical to the one of *Pax-5* (1 in 120 amino acids changed; Krauss et al., 1991a), consistent with the requirement for *pax-5* in MHB development. We therefore propose that the strong requirement for a single pax gene in the zebrafish may reflect the ancestral situation before the separation of mammals and teleosts in evolution. Alternatively, pax genes may have been lost in teleosts that were retained in the mammalian lineage. In either case, loss of *pax-b* function in the zebrafish is predicted to be equivalent to the knockout phenotype of all three mouse *pax* genes expressed at the MHB.

Evolutionary conservation of the *pax-b* pathway

The first vertebrate members of the pax gene family were isolated using a 'paired-box'-containing probe from the *Drosophila paired* locus (reviewed by Noll, 1993; Chalepakis et al., 1993). The *Drosophila paired* gene cooperates with *wingless*, *hedgehog* and *engrailed* in a signaling pathway controlling the formation of the posterior compartment boundary (reviewed by Bejsovec and Martinez Arias, 1991; Ingham, 1991; Martinez Arias, 1993). Several vertebrate orthologues of members of the *Drosophila* signaling cascade are now known to be expressed at the MHB in the zebrafish (Krauss et al., 1991b; Ekker et al., 1992b; Püschel et al., 1992; Krauss et al., 1992a), and targeted inactivation of the mouse orthologues *wnt-1*, *Eng*-1, *Eng*-2 and of *pax-5* have demonstrated a functional

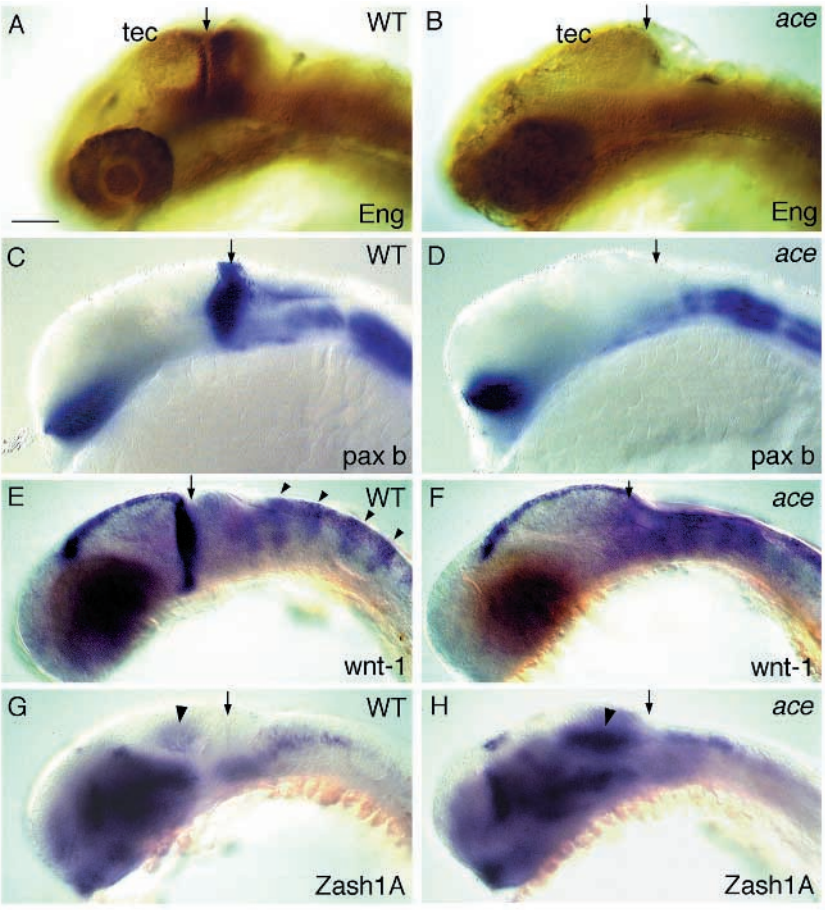

Fig. 8. Expression of MHB markers is affected in *ace* embryos. Shown are lateral views of wild-type (left) and *ace* mutant (right) embryos. Arrows point to the posterior border of the tectum. (A,B) eng protein (dark brown staining around the MHB) is not detected in the tectum, tegmentum and cerebellum of 28 h *ace* mutant embryos. (C,D) *pax-b* expression is missing in the posterior midbrain of 24 h *ace* mutant embryos, but is unaffected elsewhere. (E,F) *wnt1* expression is not found in the posterior tectum of 26 h *ace* mutant embryos. Notice the extension of the *wnt1* stripe in the dorsal tectum to more posterior levels. Expression in the rhombomeres (arrowheads) is not affected. (G,H) Expression of *Zash1A* in the tectal ventricular zone (arrowhead) is strongly increased in the mutant embryo compared to its sibling at 26 h. Stainings of the embryos in G and H were done in the same vial. Scale bar, 90 μm.

requirement for these genes in midbrain and/or cerebellum development (Thomas and Capecchi, 1990; McMahon et al., 1992; Wurst et al., 1994; Millen et al., 1994; Urbanek et al., 1994). The previous results of the antibody injection experiments by Krauss et al. (1992b) and our results show that at least one of the zebrafish orthologues of the *Drosophila* signaling pathway, *pax-b*, also functions in MHB development in the zebrafish; *ace*, and perhaps the *spg* gene (Schier et al., 1996) as well, might encode some of the missing members. Our results suggest that *noi* mutations in the *pax-b* gene affect the MHB primordium at a very early stage of its development, and that one of the functions of the *pax-b* transcription factor may be to regulate expression of the *eng* genes. Similarly, normal *eng* and *pax-b* expression in *ace* mutant embryos up to the 8-somite stage shows that *ace* is required for a later step in development of the MHB than *noi*. The expression of and phenotypic requirement for *pax-b* RNA and protein thus precedes the time when the requirement for *ace* function becomes apparent, although eventually, the phenotype of *noi* and *ace* come to resemble each other at the pharyngula stage. The *ace* gene might therefore well be one of the downstream targets of the pax-b protein, and could also be a member of the conserved signaling cascade.

noi and *ace* are required for development of an important organizing center in the brain

In *Drosophila*, the signaling events leading to the establishment of the compartment boundary take place between neighbouring cells. By inference, a similar signaling process might be necessary to establish a proper boundary between mid- and hindbrain during vertebrate neural plate development; this hypothesis can be tested in the zebrafish by performing a test for non-autonomy in transplantation experiments using the mutants we have described. In chicken, rotation experiments of the midbrain including or excluding the MHB region (Marin and Puelles, 1994), and transplantations into naive forebrain territories (Gardner and Barald, 1991; Itasaki et al., 1991; Martinez et al., 1991; Bally-Cuif et al., 1992) have shown that the MHB region can orchestrate development of the midbrain.

Our observation of cell death of tectal cells in *noi* mutant embryos, and of aberrant marker gene expression in *ace* mutant embryos, could reflect the absence of such a signal. The MHB region and the tectum seem to be affected in mutant *noi* or *ace* embryos in different ways. In mutant *noi* embryos, absence of *eng* or *wnt1* expression from the MHB is detected long before apoptotic cells are seen in the tectum. Likewise, in *ace* mutant embryos, absence of the MHB is observed when a tectum is clearly present, although abnormal in marker gene expression. Absence of *noi* and *ace* might affect the MHB and the tectum in different, but direct ways: e.g. *noi* mutations may cause a respecification in the case of the cells of the MHB, and death in the case of the tectum. Alternatively, defective development of the MHB region may lead to secondary defects in the tectum, resulting in its loss in *noi* mutant embryos, and in altered marker gene expression in *ace* mutant embryos. Such a scenario would be consistent with the proposed organizing abilities of the MHB region that have been demonstrated in chick embryos. The conserved signaling pathway that *pax-b*, and perhaps *ace* and *spg*, are involved in could be the substrate matter of this patterning influence.

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REFERENCES

Abrams, J. M., White, K., Fessler, L. I. and Steller, H. (1993). Programmed cell death during *Drosophila* embryogenesis. *Development* **117,** 29-43.

Allende, M. L. and Weinberg, E. S. (1994). The expression pattern of two zebrafish *achaete-scute* homolog (*ash*) genes is altered in the embryonic brain of the *cyclops* mutant. *Dev. Biol.* **166,** 509-530.

Alvarado-Mallart, R. M. (1993). Fate and potentialities of the avian mesencephalic/metencephalic neuroepithelium. *J. Neurobiol.* **24,** 1341-1355.

Asano, M. and Gruss, P. (1992). *Pax-5* is expressed at the midbrain-hindbrain boundary during mouse development. *Mech. Dev.* **39,** 29-39.

Bally-Cuif, L., Alvarado-Mallart, R. M., Darnell, D. K. and Wassef, M. (1992). Relationship between *Wnt-1* and *En-2* expression domains during early development of normal and ectopic met-mesencephalon. *Development* **115,** 999-1009.

Bejsovec, A. and Martinez Arias, A. (1991). Roles of *wingless* in patterning the larval epidermis of Drosophila. *Development* **113,** 471-485.

Brand, M., Beuchle, D., Endres, F., Haffter, P., Hammerschmidt, M., Mullins, M., Schulte-Merker, S., Nüsslein-Volhard, C., Lück, R., Schwarz, K. J. and Schwarz, S. (1995). Keeping and raising zebrafish (*Danio rerio*) in Tübingen. *Zebrafish Science Monitor* **3,** 2-7.

Chalepakis, G., Stoykova, A., Wijnholds, J., Tremblay, P. and Gruss, P. (1993). Pax: gene regulators in the developing nervous system. *J. Neurobiol.* **24,** 1367-1384.

Chitnis, A. B. and Kuwada, J. Y. (1990). Axonogenesis in the brain of zebrafish embryos. *J. Neurosci.* **10,** 1892-1905.

Eisen, J. S. (1994). Development of motoneuronal phenotype. *Annu. Rev. Neurosci.* **17,** 1-30.

Ekker, M., Akimenko, M. A., Bremiller, R. and Westerfield, M. (1992a). Regional expression of three homeobox transcripts in the inner ear of zebrafish embryos. *Neuron* **9,** 27-35.

Ekker, M., Wegner, J., Akimenko, M. A. and Westerfield, M. (1992b). Coordinate embryonic expression of three zebrafish *engrailed* genes. *Development* **116,** 1001-1010.

Ekker, S. C., Ungar, A. R., Greenstein, P., von Kessler, D. P., Porter, J. A., Moon, R. T. and Beachy, P. A. (1995). Patterning activities of vertebrate *hedgehog* proteins in the developing eye and brain. *Curr. Biol.* **5,** 944-955.

Figdor, M. C. and Stern, C. D. (1993). Segmental organization of embryonic diencephalon. *Nature* **363,** 630-634.

Furley, A. J., Morton, S. B., Manalo, D., Karagogeous, D., Dodd, J. and Jessel, T. M. (1990). The axonal glycoprotein TAG-1 is an immunoglobulin superfamily member with neurite outgrowth promoting activity. *Cell* **61,** 157-170.

Gardner, C. A. and Barald, K. F. (1991). The cellular environment controls the expression of engrailed-like protein in the cranial neuroepithelium of quail-chick chimeric embryos. *Development* **113,** 1037-1048.

Haffter, P., Granato, M., Brand, M., Mullins, M. C., Hammerschmidt, M., Kane, D. A., Odenthal, J., van Eeden, F. J. M., Jiang, Y.-J., Heisenberg, C.-P., Kelsh, R. N., Furutani-Seiki, M., Vogelsang, E., Beuchle, D., Schach, U., Fabian, C. and Nüsslein-Volhard, C. (1996). The identification of genes with unique and essential functions in the development of the zebrafish, *Danio rerio*. *Development* **123**, 1-36.

Hanneman, E., Trevarrow, B., Metcalfe, W. K., Kimmel, C. B. and Westerfield, M. (1988). Segmental pattern of development of the hindbrain and spinal cord of the zebrafish embryo. *Development* **103,** 49-58.

Heisenberg, C.-P., Brand, M., Jiang, Y.-J., Warga, R. M., Beuchle, D., van Eeden, F. J. M., Furutani-Seiki, M., Granato, M., Haffter, P., Hammerschmidt, M., Kane, D. A., Kelsh, R. N., Mullins, M. C., Odenthal, J. and Nüsslein-Volhard, C. (1996). Genes involved in forebrain development in the zebrafish, *Danio rerio*. *Development* **123**, 191-203.

Ingham, P. W. (1991). Segment polarity genes and cell patterning within the Drosophila body segment. *Curr. Opin. Genet. Dev.* **1,** 261-267.

Itasaki, N., Ichijo, H., Hama, C., Matsuno, T. and Nakamura, H. (1991). Establishment of rostrocaudal polarity in tectal primordium: engrailed expression and subsequent tectal polarity. *Development* **113,** 1133-1144.

Kelly, G. M. and Moon, R. T. (1995). Involvement of *wnt1* and *pax2* in the formation of the midbrain-hindbrain boundary in the zebrafish gastrula. *Dev. Genet.* **17,** 129-140.

Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B. and Schilling, T. F. (1995). Stages of embryonic development of the zebrafish. *Dev. Dyn.* **203,** 253-310.

Kimmel, C. B., Metcalfe, W. K. and Schabtach, E. (1985). T reticular interneurons: a class of serially repeating cells in the zebrafish hindbrain. *J. Comp. Neuro.* **233,** 365-376.

Korzh, V., Edlund, T. and Thor, S. (1993). Zebrafish primary neurons initiate expression of the LIM homeodomain protein Isl-1 at the end of gastrulation. *Development* **118,** 417-425.

Krauss, S., Johansen, T., Korzh, V. and Fjose, A. (1991a). Expression of the zebrafish paired box gene pax<zf-b> during early neurogenesis. *Development* **113,** 1193-1206.

Krauss, S., Johansen, T., Korzh, V. and Fjose, A. (1991b). Expression pattern of zebrafish pax genes suggests a role in early brain regionalization. *Nature* **353,** 267-270.

Krauss, S., Korzh, V., Fjose, A. and Johansen, T. (1992a). Expression of four *wnt*-related genes during embryogenesis. *Development* **116,** 249-259.

Krauss, S., Maden, M., Holder, N. and Wilson, S. W. (1992b). Zebrafish pax(b) is involved in the formation of the midbrain-hindbrain boundary. *Nature* **360,** 87-89.

Lumsden, A. and Keynes, R. (1989). Segmental patterns of neuronal development in the chick hindbrain. *Nature* **337,** 424-428.

Macdonald, R., Barth, K. A., Xu, Q., Holder, N., Mikkola, I. and Wilson, S. W. (1995). Midline signalling is required for Pax gene regulation and patterning of the eyes. *Development* **121,** 3267-3278.

Macdonald, R., Xu, Q., Barth, K. A., Mikkola, I., Holder, N., Fjose, A., Krauss, S. and Wilson, S. W. (1994). Regulatory gene expression boundaries demarcate sites of neuronal differentiation in the embryonic zebrafish forebrain. *Neuron* **13,** 1039-1053.

Marin, F. and Puelles, L. (1994). Patterning of the embryonic avian midbrain after experimental inversions: a polarizing activity from the isthmus. *Dev. Biol.* **163,** 19-37.

Martinez, S., Marin, F., Nieto, M. A. and Puelles, L. (1995). Induction of ectopic *engrailed* expression and fate change in avian rhombomeres: intersegmental boundaries as barriers. *Mech. Dev.* **51,** 289-303.

Martinez, S., Wassef, M. and Alvarado-Mallart, R. M. (1991). Induction of a mesencephalic phenotype in the 2-day-old chick prosencephalon is preceded by the early expression of the homeobox gene *en*. *Neuron* **6,** 971-981.

Martinez Arias, A. (1993). Development and Patterning of the Larval Epidermis of *Drosophila*. In *The Development of Drosophila melanogaster* (ed. M. Bate and A. Martinez Arias), pp. 517-608. Cold Spring Harbour: Cold Spring Harbour Press.

McMahon, A. P., Joyner, A. L., Bradley, A. and McMahon, J. A. (1992). The midbrain-hindbrain phenotype of *Wnt-1-/Wnt-1-* mice results from stepwise deletion of *engrailed*-expressing cells by 9. 5 days postcoitum. *Cell* **69,** 581-595.

Metcalfe, W. K., Mendelson, B. and Kimmel, C. B. (1986). Segmental homologies among reticulospinal neurons in the hindbrain of the zebrafish larva. *J. Comp. Neurol.* **251,** 147-159.

Mikkola, I., Fjose, A., Kuwada, J. Y., Wilson, S., Guddal, P. H. and Krauss, S. (1992). The paired domain-containing nuclear factor pax[b] is expressed in specific commissural interneurons in zebrafish embryos. *J. Neurobiol.* **23,** 933-946.

Millen, K. J., Wurst, W., Herrup, K. and Joyner, A. (1994). Abnormal embryonic cerebellar development and patterning of postnatal foliation in two mouse *engrailed-2* mutants. *Development* **120,** 695-706.

Molven, A., Njolstad, P. R. and Fjose, A. (1991). Genomic structure and restricted neural expression of the zebrafish *wnt-1* (*int-1*) gene. *EMBO J.* **10,** 799-807.

Nakamura, H., Itasaki, N. and Matsuno, T. (1994). Rostrocaudal polarity formation of chick optic tectum. *Int. J. Dev. Biol.* **38,** 281-286.

Nieuwkoop, P. D. (1989). The successive steps in the pattern formation of the amphibian central nervous system. *Dev. Growth Diff.* **32,** 149-154.

Noll, M. (1993). Evolution and role of Pax genes. *Curr. Opin. Genet. Dev.* **3,** 595-605.

Oxtoby, E. and Jowett, T. (1993). Cloning of the zebrafish *krox-20* gene (*krx-20*) and its expression during hindbrain development. *Nucl. Acids Res.* **21,** 1087-1095.

Patel, N. H., Martin-Blanco, E., Coleman, K. G., Poole, S. J., Ellis, M. C., Kornberg, T. B. and Goodman, C. S. (1989). Expression of *engrailed* proteins in arthropods, annelids and chordates. *Cell* **58,** 955-968.

Puelles, L., Amat, J. A. and Martinez-de-la-Torre, M. (1987). Segment-related, mosaic neurogenetic pattern in the forebrain and mesencephalon of early chick embryos: I. Topography of AChE-positive neuroblasts up to stage HH18. *J. Comp. Neurol.* **266,** 247-268.

Püschel, A. W., Westerfield, M. and Dressler, G. R. (1992). Comparative analysis of Pax-2 protein distributions during neurulation in mice and zebrafish. *Mech. Dev.* **38,** 197-208.

Ross, L. S., Parrett, T. and Easter, S. J. (1992). Axonogenesis and morphogenesis in the embryonic zebrafish brain. *J. Neurosci.* **12,** 467-482.

Rowitch, D. H. and McMahon, A. P. (1995). *Pax-2* expression in the murine neural plate precedes and encompasses the expression domains of *Wnt-1* and *En-1*. *Mech. Dev.* **52,** 3-8.

Rubenstein, J. L., Martinez, S., Shimamura, K. and Puelles, L. (1994). The embryonic vertebrate forebrain: the prosomeric model. *Science* **266,** 578-580.

Rubenstein, J. L. and Puelles, L. (1994). Homeobox gene expression during development of the vertebrate brain. *Curr. Top. Dev. Biol.* **29,** 1-63.

Sanyanusin, P., Schimmenti, L. A., Noe, L. A., Ward, T. A., Pierpont, M. E. M., Sullivan, M. J., Dobyns, W. B. and Eccles, M. R. (1995). Mutation of the Pax2 gene in a family with optic nerve colobomas, renal abnormalities and vesicouretal reflux. *Nature Genetics* **9,** 358-363.

Schier, A. F., Neuhauss, S. C. F., Harvey, M., Malicki, J., Solnica-Krezel, L., Stainier, D. Y. R., Zwartkruis, F., Abdelilah, S., Stemple, D. L., Rangini, Z., Yang, H. and Driever, W. (1996). Mutations affecting the development of the embryonic zebrafish brain. *Development* **123**, 165-178.

Schulte-Merker, S., Ho, R. K., Herrmann, B. G. and Nüsslein-Volhard, C. (1992). The protein product of the zebrafish homologue of the mouse T gene is expressed in nuclei of the germ ring and the notochord of the early embryo. *Development* **116,** 1021-1032.

Spemann, H. (1938). *Embryonic Development and Induction.* New Haven, Connecticut: Yale University Press.

Stoykova, A. and Gruss, P. (1994). Roles of Pax-genes in developing and adult brain as suggested by expression patterns. *J. Neurosci.* **14,** 1395-1412.

Thomas, K. R. and Capecchi, M. R. (1990). Targeted disruption of the murine *int-1* proto-oncogene resulting in severe abnormalities in midbrain and cerebellar development. *Nature* **346,** 847-850.

Torres, M., Gómez-Pardo, E., Dressler, G. R. and Gruss, P. (1995). Pax-2 controls multiple steps of urogenital development. *Development* **121,** 4057-4065.

Trevarrow, B., Marks, D. L. and Kimmel, C. B. (1990). Organization of hindbrain segments in the zebrafish embryo. *Neuron* **4,** 669-679.

Trowe, T., Klostermann, S., Baier, H., Granato, M., Crawford, A. D., Grunewald, B., Hoffmann, H., Karlstrom, R. O., Meyer, S. U., Müller, B., Richter, S., Nüsslein-Volhard, C. and Bonhoeffer, F. (1996). Mutations disrupting the ordering and topographic mapping of axons in the retinotectal projection of the zebrafish, *Danio rerio*. *Development* **123**, 439-450.

Urbanek, P., Wang, Z. Q., Fetka, I., Wagner, E. F. and Busslinger, M. (1994). Complete block of early B cell differentiation and altered patterning of the posterior midbrain in mice lacking *Pax5/BSAP*. *Cell* **79,** 901-912.

Wassef, M., Bally-Cuif, L. and Alvarado-Mallart, R. M. (1993). Regional specification during cerebellar development. *Perspect. Dev. Neurobiol.* **1,** 127-132.

Westerfield, M. (1994). *The zebrafish book*. Edition 2. 1, Oregon: University of Oregon Press.

Whitfield, T. T., Granato, M., van Eeden, F. J. M., Schach, U., Brand, M., Furutani-Seiki, M., Haffter, P., Hammerschmidt, M., Heisenberg, C.-P., Jiang, Y.-J., Kane, D. A., Kelsh, R. N., Mullins, M. C., Odenthal, J. and Nüsslein-Volhard, C. (1996). Mutations affecting development of the zebrafish inner ear and lateral line. *Development* **123**, 241-254.

Wilson, S. W., Ross, L. S., Parrett, T. and Easter, S. J. (1990). The development of a simple scaffold of axon tracts in the brain of the embryonic zebrafish, *Brachydanio rerio*. *Development* **108,** 121-145.

Wurst, W., Auerbach, A. B. and Joyner, A. L. (1994). Multiple developmental defects in Engrailed-1 mutant mice: an early mid-hindbrain deletion and patterning defects in forelimbs and sternum. *Development* **120,** 2065-2075.

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