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Pax2/5 and Pax6 subdivide the early neural tube into three domains

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Abstract

The nested expression patterns of the paired-box containing transcription factors Pax2/5 and Pax6 demarcate the midbrain and forebrain primordium at the neural plate stage. We demonstrate that, in Pax2/5 deficient mice, the mesencephalon/metencephalon primordium is completely missing, resulting in a fusion of the forebrain to the hindbrain. Morphologically, in the alar plate the deletion is characterized by the substitution of the tectum (dorsal midbrain) and cerebellum (dorsal metencephalon) by the caudal diencephalon and in the basal plate by the replacement of the midbrain tegmentum by the ventral metencephalon (pons). Molecularly, the loss of the tectum is demonstrated by an expanded expression of Pax6, (the molecular determinant of posterior commissure), and a rostral shift of the territory of expression of Gbx2 and Otp (markers for the pons), towards the caudal diencephalon. Our results suggest that an intact territory of expression of Pax2/5 in the neural plate, nested between the rostral and caudal territories of expression of Pax6, is necessary for defining the midbrain vesicle.

Keywords: Brain regionalization; Pax; Posterior commissure; Midbrain

1. Introduction

Around the time the neural tube closes, the primordium of the brain is divided into three vesicles termed forebrain, midbrain and hindbrain. The boundaries between the forebrain, the midbrain and the hindbrain are molecularly characterized by the nested expression patterns of the paired box containing transcription factors Pax2/5 and Pax6 (Walther and Gruss, 1991; Rowitch and McMahon, 1995; Mastick et al., 1997). While Pax6 labels the forebrain and hindbrain vesicle (Walther and Gruss, 1991), Pax2 and Pax5 demarcate the mesencephalon/metencephalon (mes/met) (Rowitch and McMahon, 1995). After the closure of the neural tube the mes/met is divided along its dorso/ventral axis into alar and basal plate. The alar plate of the mes/met gives rise to the tectum and cerebellum whereas the basal plate develops into tegmentum. The region that links the fore- and mid- brain vesicles is called the pretectum, and gives place to neuronal nuclei which serve ancillary functions related to visual perception (pupillary reflex, etc.). The caudal half of the pretectal region is covered dorsally by a characteristic group of axonal bundles that connect pretectal nuclei of both sides. This group of axonal bundles is known as the posterior commissure, and the portion of the pretectum covered by it is the commissural pretectum. The posterior commissure is an important anatomical landmark for the alar part of the forebrain/midbrain boundary (Mastick et al., 1997). From the molecular point of view, the caudal-most limit of Pax6 expression demarcates the posterior commissure at the forebrain/midbrain boundary (Stoykova and Gruss, 1994; Stoykova et al., 1996; Mastick and Easter, 1996; Mastick et al., 1997). The isthmic constriction demarcates the midbrain/hindbrain border. At the level of the basal plate, the boundary between the midbrain and the

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hindbrain is anatomically marked by a depression in the tegmentum called the fovea isthmus. Molecularly this boundary is labeled by the rostral end of expression of the homeobox containing transcription factors Gbx2 and Otp in the pons (Bulfone et al., 1993; Simeone et al., 1994). It has been reported (Millet et al., 1996), that in chicken, the border of expression of Gbx2 and Otx2 marks the midbrain/hindbrain junction; therefore, the cerebellum would be a derivative of the hindbrain. The forebrain/midbrain border in the basal plate is molecularly and morphologically less characterized.

The Pax gene family members encode a family of transcriptional regulators specifically expressed in the developing and adult central nervous system (Peter Gruss and Walther, 1992; Noll, 1993). In accordance with its expression in the commissural pretectum, mice lacking Pax6 function show a loss of commissural pretectum resulting in a disruption of the forebrain/midbrain boundary (Mastick et al., 1997). As a result the missing region is occupied by midbrain tectum and the forebrain/midbrain boundary is rostrally shifted.

As part of an ongoing project to elucidate the cooperative function of the Pax genes in the regionalization of the brain, we have created mice carrying mutations of Pax2 and Pax5 (Schwarz et al., 1997). Interestingly, and in contrast to Pax6 expression, Pax2 and Pax5 specifically label the mes/met territory and abut the Pax6 positive forebrain territory at the forebrain/midbrain boundary. Here we report on a quite unexpected result: the Pax2/5 double homozygous embryos, in contrast to Pax6 deficient mice, show a caudal expansion of the posterior commissure of the forebrain/midbrain boundary resulting in an early regionalization defect fusing the commissural pretectum of the forebrain to the choroid plexus of the hindbrain. Additionally, we demonstrate that Pax6 is necessary for the correct spatial development of the forebrain/midbrain boundary. This is shown by a rescue of the development of the posterior commissure, the morphological landmark of this junction, in Pax6 deficient mice ectopically expressing Pax6 under Pax2 enhancer sequences. Since the reported expression territories of Pax6 and Pax2/5 are early markers which show region specific expression in the neural plate, our data suggest a cooperation between Pax2/5 and Pax6 in the definition of the three vesicles of the early neural tube.

2. Results

2.1. Loss of the mes/met primordium in the Pax2/5 compound mutants

To better understand the ontogeny of the mes/met deletion, we analyzed the expression of Fgf8 and Wnt1 at e8.3 (Fig. 1) in wild-type and Pax2/5 mutant embryos. Fgf8 expression cannot be detected in the mutants, whereas two patches of Wnt1 expression can be seen in the midbrain/hindbrain junction in mutants and wild type animals. This suggests that some prospective organizer cells are present very early in the double mutants. This observation led us to investigate the integrity of the isthmic organizer in the Pax2/5 double mutants. We performed in situ hybridization for marker genes expressed after Pax2 in the midbrain/hindbrain junction and in the mes/met region. As expected on the basis of our previous results, *Fgf8*, a regulator gene of the midbrain/hindbrain organizer (Crossley and Martin, 1995; Crossley et al., 1996; Wassermann et al., 1997; Meyers et al., 1998), could not be detected in the Pax2/5 compound mutant, suggesting a loss of organizer specification at the mid/hindbrain region (Fig. 2A,B). Expression of the homeobox containing gene *En2*, which labels the metencephalon and the caudal mesencephalon (Joyner et al., 1985; Joyner and Martin, 1987; Wassef and Joyner, 1997) is lost in the Pax2/5 mutant brains (Fig. 2C,D). Expression of other marker genes for the midbrain/hindbrain junction like Wnt1 and En1 (McMahon et al., 1992; Joyner, 1996) was missing as well (data not shown). Interestingly, Pax6, a marker for the forebrain with a sharp expression boundary at the level of the forebrain/midbrain border, leaving a gap of Pax6 negative tissue between fore- and hindbrain, is expanded caudally. This results in a fusion of the Pax6 positive forebrain domain to the Pax6 positive hindbrain (Fig. 2E,F). Our gene expression data, together with previously reported data (Schwarz et al., 1997), argue for a complete loss of mes/met anlage and organizer identity in the Pax2/5 double mutant mice.

2.2. The Pax2/5 mutant lacks the tectum and the cerebellum but shows an expanded posterior commissure

As already suggested by our in situ analysis at e10.5 the double mutant embryos showed remarkable alterations in the regionalization of the midbrain tectum as well as the cerebellar primordium (Fig. 2C–E,F). In wild-type animals at later stages of development, the posterior commissure, a group of transverse axonal bundles on the dorsal side of the caudal pretectum, is the morphological landmark of the forebrain/midbrain border (Fig. 3B,C). By e13.5, in the double mutants the dorsal neural tube between the pretectum and the choroid plexus of the fourth ventricle was entirely occupied by the transverse axonal bundles of the posterior commissure (Fig. 3E,F). Each of the bundles of the commissure was of normal size and appearance, but the number of bundles was remarkably increased.

In wild-type animals, the caudal boundary of expression of Pax6 in the forebrain at the level of the posterior commissure serves as a molecular marker of the dorsal part of the forebrain/midbrain boundary (Fig. 4a,A,C,E,G) (Mastick et al., 1997). In order to confirm our whole-mount in situ hybridization studies, we investigated Pax6 transcript distribution during embryonic development using radioactive in situ hybridization. In the Pax2/5 double mutants, the caudal border of Pax6 forebrain expression

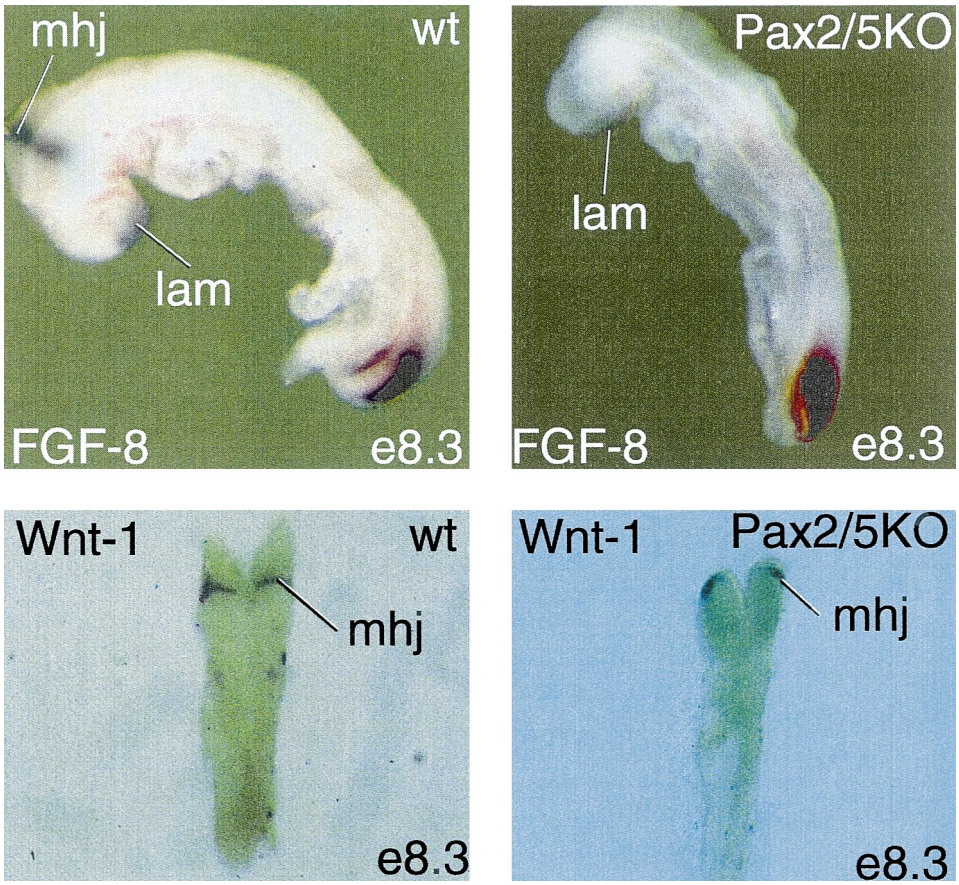

Fig. 1. Whole-mount in situ hybridization shows that Wnt1, but not Fgf8, is induced at e8.3 in the isthmic region of the Pax2/5 double mutant. Upper left panel, Fgf8 distribution in wild-type (side view); upper right, Fgf8 expression in the compound mutant (side view); lower left panel, Wnt1 distribution in wild type (dorsal view); lower right panel, Wnt1 distribution in compound mutant (dorsal view). lam, lamina terminalis; mhj, midbrain/hindbrain junction; wt, wild type.

abuts the choroid plexus of the fourth ventricle (a dorsal hindbrain structure) at e11.5 (Fig. 4a,B,D). In this way, the alar plate derivatives of the forebrain are contiguous to the alar plate derivatives of the hindbrain, demonstrating a complete absence of midbrain tectum and cerebellum (derivative of the mes/met alar plate). The enlargement of the territory of expression of Pax6 accompanied the enlarged posterior commissure at a later stage of development (Fig. 4a,F,H).

Another relevant marker for the development of this area is Otx-2 (Simeone et al., 1992; Acampora et al., 1997). The normal territory of expression of Otx-2 includes (apart from expression in the forebrain) the midbrain tectum, the midbrain tegmentum up to the fovea isthmus and the choroid plexus of the fourth ventricle (Fig. 4b,A,C); the cerebellum and the pons do not show Otx-2 expression (Otx-2 gap). In the Pax2/5 double mutant, Otx-2 expression in the alar plate was found in a continuous territory up to the choroid plexus of the fourth ventricle, completely overlapping the Pax6 expressing territory during embryonic development (data not shown and Fig. 4b,B,D). No gap of Otx2 expression between the expanded tissue and the choroid plexus of the fourth ventricle could be found in the Pax2/5 double mutant mice, suggesting that the non-Otx2-expressing tissue that would normally originate the cerebellum had disappeared completely. Since the cells which give rise to the cerebellum originate in part from the cells rostral to the midbrain/hindbrain junction, forming the midbrain tectum, this result suggests that the expanded Pax6 and Otx-2 positive tissue in the Pax2/5 mutant brains had not been specified to acquire the fate of midbrain and cerebellum.

2.3. Pax6 is responsible for the specification of the posterior commissure

It has previously been reported that in Pax6 deficient mice the posterior commissure is defective (Stoykova et al., 1996) or virtually absent (Mastick et al., 1997). Together with the observation that in Pax2/5 double mutants expansion of the Pax6 forebrain expression domain marks the expansion of the posterior commissure, these phenotypes suggest a potential role for Pax6 in the establishment of this morphological landmark. In order to assess Pax6 function in the pretectum more exactly, we generated transgenic

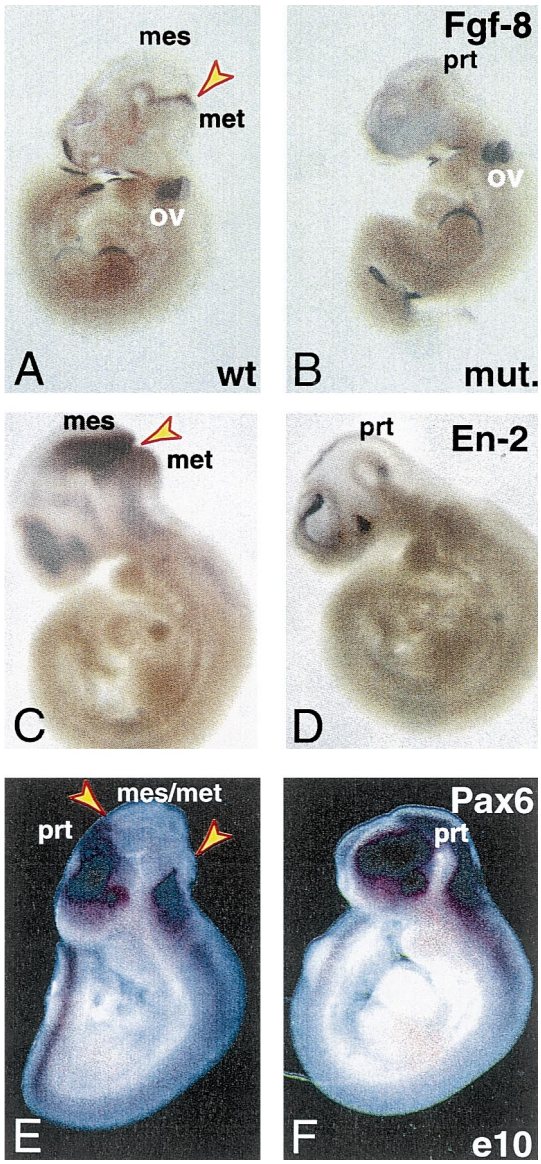

Fig. 2. Loss of the mes/met primordium in the e10.5 Pax2/5 double mutants. Whole-mount in situ hybridization of e10.5 embryos with probes for an isthmic constriction marker (Fgf8, A,B), a marker for the midbrain and cerebellum (En2, C,D) and a forebrain marker (Pax6, E,F) shows that, in the double mutants (B,D,F) the mesencephalon is lost, the organizer is not established and the forebrain is expanded and abuts the hindbrain. Arrowheads show the expression in the wild type; otic vesicle (ov).

mice expressing Pax6 cDNA and β-galactosidase cDNA under the control of Pax2 enhancer sequences (Fig. 5E–G,H); subsequently we mated these transgenic mice to Pax6 deficient mice (St-Onge et al., 1997). The phenotypes of the three different kinds of offspring were: (1) in wild type/Pax2^{Pax6} mice, the posterior commissure was an easily recognizable structure by e12.5 (Fig. 5A,B); (2) in littermates homozygous for the Pax6 mutation the posterior commissure was absent (Fig. 5C,D; Mastick et al., 1997); (3) Finally, in homozygous mice carrying the Pax6 gene under the control of Pax2 enhancer sequences (Pax6–/–/Pax2^{Pax6}), the transverse axonal bundles of the posterior commissure could be identified again (Fig. 5E,F). These results strongly suggest that Pax6 has an important function for the development of the posterior commissure. It has already been suggested that Pax6 is necessary for the development of the posterior commissure, based on data from the Pax6 loss-of-function mutant. Our results demonstrate that Pax6 can induce the formation of an ectopic (because the commissural pretectum is not present in the Pax6 mutant) posterior commissure but apparently not everywhere in the midbrain – only in the place where the diencephalon meets the mes/met. One possible explanation would be that, in the pretectal region of the neural tube, the abutment of Pax6 positive and Pax6 negative domains induces the formation of posterior commissure, irrespective of the orientation of the two domains. Alternatively, Pax6 would be able to induce the formation of posterior commissure if expressed in a hypothetical population of responsive cells sitting in the caudal diencephalon. The enlarged posterior commissure in the Pax2/5 double mutant, however, shows that the spatial and temporal expression of both Pax6 and Pax2/5 are necessary to develop a posterior commissure of normal size and position demarcating the forebrain/midbrain boundary.

2.4. Tegmental alterations in the Pax2/5 compound mutant

The whole-mount in situ gene expression studies and the morphology of the early double mutant embryos already suggested that the neural tube was altered ventrally as well as dorsally in the region between the pretectum and the fourth ventricle (which in the wild-type would be occupied by the mes/met). Anatomical and molecular analysis confirmed that the midbrain tegmentum of the double mutants is virtually absent. Comparing sagittal sections of double mutants and wild type littermates showed that the tegmentum of the double mutant embryos was heavily affected (Fig. 3E). Gbx-2 and Otp are homeobox genes whose rostral boundary of expression demarcate the isthmic constriction, thus labeling the pons (Bulfone et al., 1993; Simeone et al., 1994). In the double mutants, both genes show a rostrally shifted expression domain up to the pretectum suggesting a deletion of the tegmentum (Fig. 6A–H). Pax7 is expressed in a patch of cells in the isthmal tegmentum (Mansouri et al., 1996); in the double mutant tegmentum, expression of Pax7 could no longer be detected in the tegmentum (data not shown).

Anatomical analysis of our mutants is difficult, since they usually die before the stage in which nuclei start differentiating. However, in double mutant fetuses of e16.5, we were able to identify the principal nucleus of the trigeminal of normal size and shape (Fig. 7G,H). Since this nucleus is a major derivative of the first rhombomere (in chick embryos, (Marín and Puelles, 1994)), the pons seems to be spared. We were not able to find derivatives of the isthmic region as the locus coeruleus or the ventral- and dorsal tegmental nuclei in the double mutant fetal brain (Fig. 7C–F). The gene

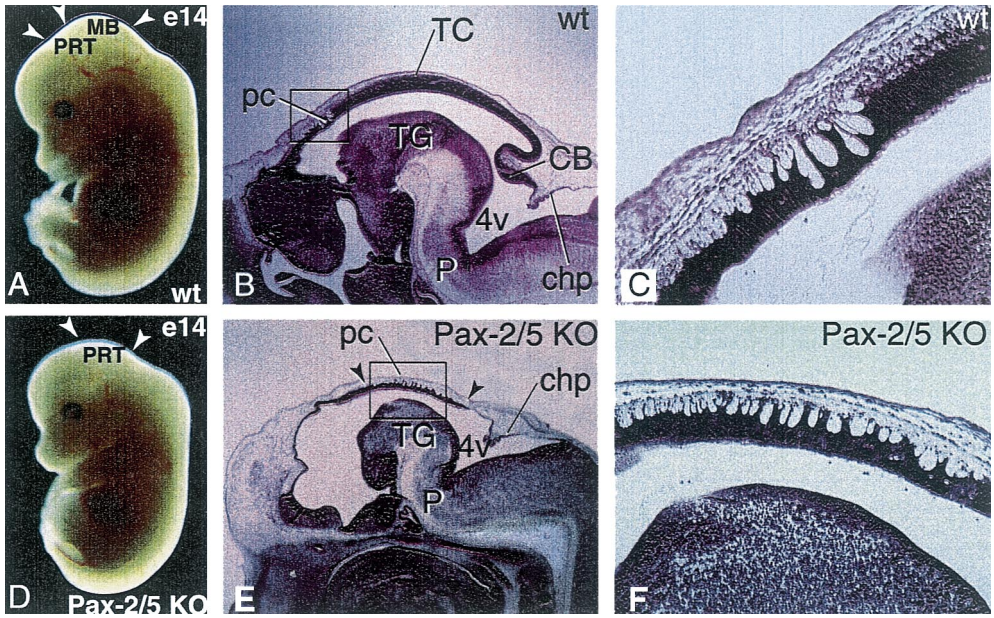

Fig. 3. The Pax2/5 mutant has an expanded posterior commissure. (A,B,C) Appearance of the midbrain and posterior commissure in the wild type mouse e14 fetus; (A) the fetal head shows a characteristic protuberance corresponding to the midbrain (arrowheads); (B) a midsagittal histological section shows the normal extent of the tectum; the framed portion encloses the posterior commissure and is shown enlarged in C (D) The fetal mutant head lacks the mesencephalic protuberance (arrowheads); (E) a midsagittal section of the mutant brain shows absence of tectum and expanded posterior commissure (arrowheads); the framed portion is shown enlarged in (F). 4V, fourth ventricle; CB, cerebellum; chp, choroid plexus; MB, midbrain; P, pons; pc, posterior commissure; PRT, pretectum; TG, tegmentum; wt, wild type.

expression studies and the morphological data suggest a complete absence of the midbrain/cerebellum in the Pax2/5 compound mutant (Fig. 7A,B).

3. Discussion

3.1. Loss of midbrain and cerebellum in the Pax2/5 mutants

Several genes (Wnt-1, En-2, Pax5, En-1, Pax2) involved in the formation of the midbrain and cerebellum have been studied, either by means of targeted mutation, or by naturally occurring mutants (McMahon and Bradley, 1990; Thomas and Capecchi, 1990; Thomas et al., 1991; McMahon et al., 1992; Wurst et al., 1994; Favor et al., 1996; Torres et al., 1996; Urbánek et al., 1997). Except for Wnt-1, none of these mutants shows a complete deletion of the tectum. This is probably due to the relative late onset of expression of these genes in the mes/met primordium. Pax2 and 5 precede the onset of the above mentioned genes and label the whole mes/met primordium at the neural plate stage (Mastick et al., 1997).

Therefore a possible explanation for the loss of mes/met in our mutants can be found in the reported early pattern of expression of Pax2 and Pax6 in the neural plate. Data from Walther and Gruss (1991), Mastick et al. (1997) and Rowitch and McMahon (1995), show that in the neural plate the domain of expression of Pax2 and Pax5 is nested inside the rostral and caudal domains of expression of Pax6. Our double mutants coincide with these results for the early neural tube (Fig. 2E,F). The phenotypical appearance (fusion of the fore- and hindbrain) of the Pax2/5 double mutant compared to the wild-type is summarized in Fig. 8. The coincidence of the forebrain/midbrain junction (rostrally) and the metencephalic/myelencephalic junction (caudally) with the rostral and caudal meeting points of the Pax2 and Pax6 territories allows us to hypothesize that a genetic interaction between both factors could contribute to setting the boundaries between forebrain and midbrain and between mes/met and myelencephalon (medulla). The data in the present study constitute genetic evidence in support of this hypothesis. The case for this interaction is particularly compelling in the formation of the forebrain/midbrain boundary and the positioning of the posterior commissure.

Since the ventral mesencephalon is not present in the double mutants (Figs. 6 and 7), the remnant En2 expression (Fig. 2) labels tissue belonging to the metencephalon (pons; hindbrain). Therefore, the development of the first rhombomere does not seem to depend on the integrity of the midbrain/hindbrain organizer.

3.2. Pax2/5 and Pax6 cooperate in the specification of the posterior commissure

This conclusion arises from the anatomical analysis of three different kinds of mutant embryos: (1) The Pax6 deficient embryo does not have a posterior commissure

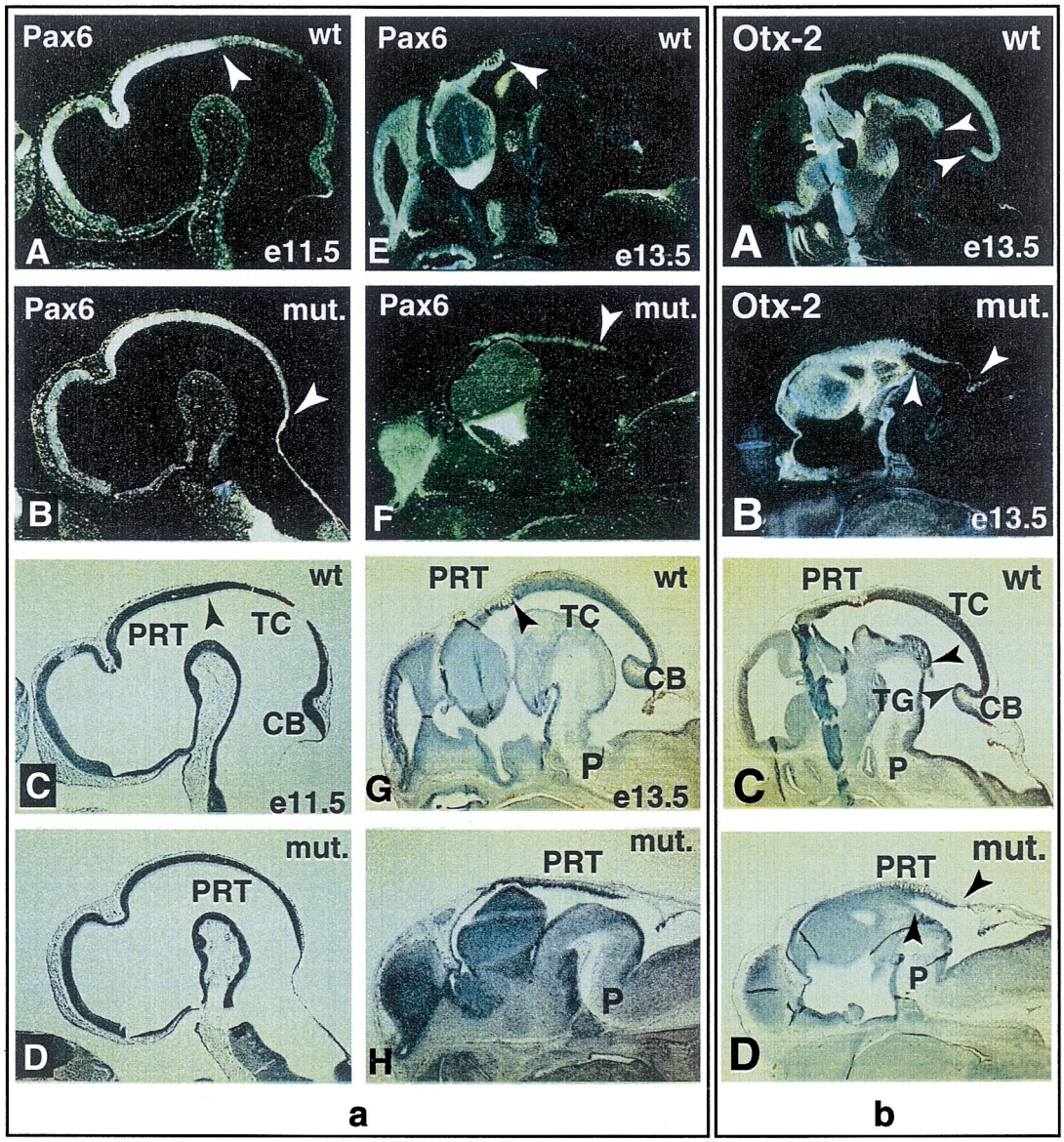

Fig. 4. The expansion of the posterior commissure is coupled to the expansion of the domain of expression of Pax6. (a) (A–D) Expression of Pax6 in the wild type embryo (e11.5, A,C) has a sharp boundary in the pretectum (arrowheads); in the mutant (B,D), Pax6 expression reaches the roof of the fourth ventricle (arrowhead in B). (E–H) Expression of Pax6 in the wild-type fetal brain (e13.5, E,G) has a sharp boundary in the posterior commissure (arrowheads in E,G); in the mutant fetal brain (F,H), Pax6 expression accompanies the expanded posterior commissure reaching the choroid plexus of the fourth ventricle (arrowhead in F). (b) Molecular markers show loss of the midbrain tectum in the Pax2/5 mutant. In the wild-type e13.5 embryo, the territory of expression of Otx2 (A,C) includes the midbrain tectum and the choroid plexus of the fourth ventricle (note that the cerebellum does not express Otx-2). In the double mutant (B,D), Otx2 expression can be found in a continuos domain in the expanded pretectal region up to the choroid plexus of the fourth ventricle; arrowheads show landmarks to facilitate comparison. CB, cerebellum; mut., Pax2/5 mutant; P, pons; PRT, pretectum; TC, tectum; TG, tegmentum; wt, wild-type.

(Stoykova et al., 1996; Mastick et al., 1997); (2) In the Pax2/5 mutant embryo the posterior commissure is not only present, but abnormally enlarged; and (3) When Pax6 is expressed in the Pax6–/– background, the posterior commissure develops normally. Together, these results suggest that Pax6 is necessary and sufficient to develop a posterior commissure, but that the concerted expression of Pax2/5 is probably needed for the proper extension and position of this structure. This notion is supported by the fact that the commissural pretectum is not affected in other reported mutant phenotypes which show alteration of the midbrain/hindbrain organizer and deletions of the cerebellum and parts of the tectum (McMahon and Bradley, 1990; Thomas and Capecchi, 1990; Thomas et al., 1991; McMahon et al., 1992; Wurst et al., 1994; Favor et al., 1996; Torres et al., 1996; Urbánek et al., 1997).

In conclusion, our results suggest that besides the formation of mesencephalon, Pax2 and Pax5 set the caudal

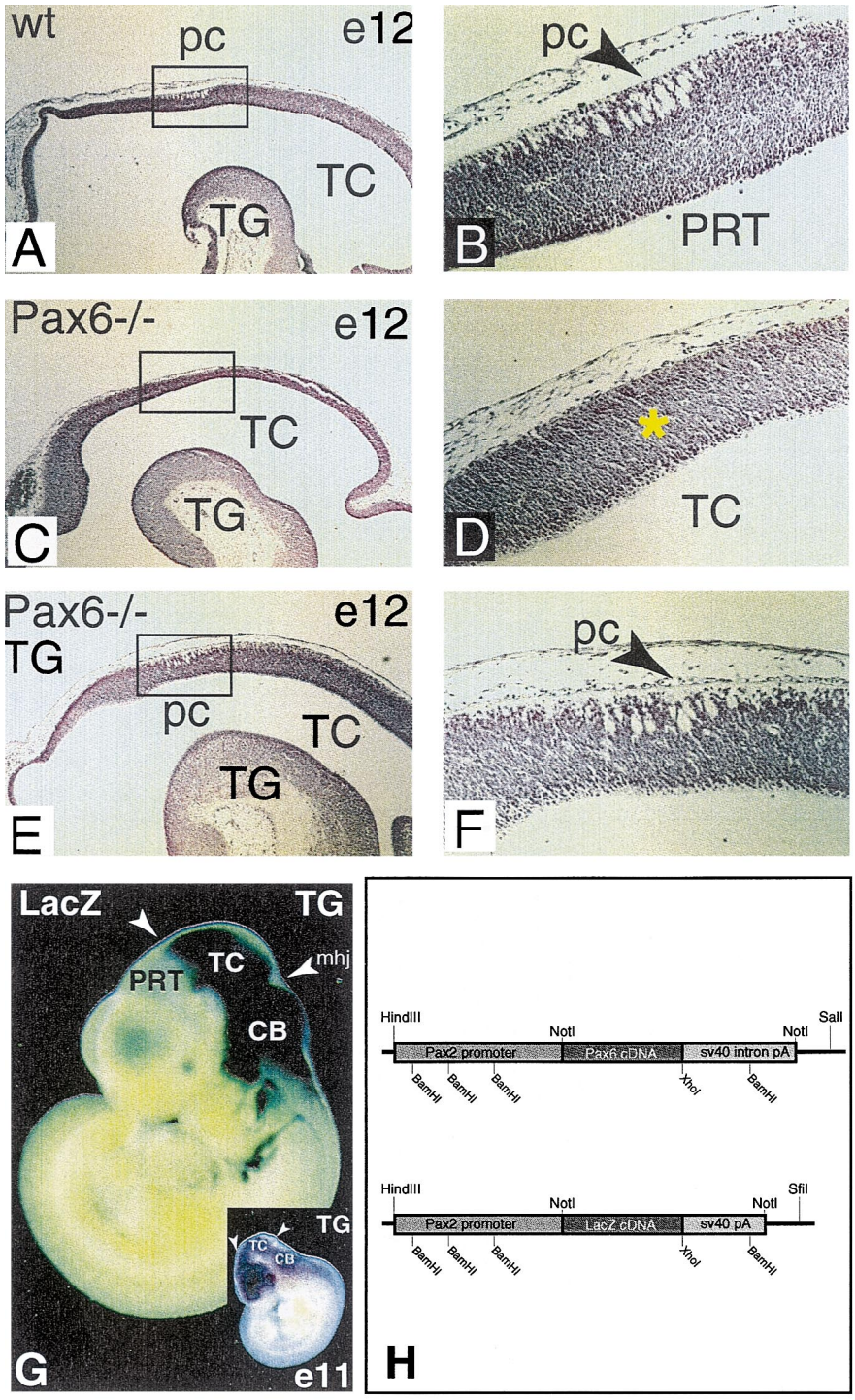

Fig. 5. Rescue of the posterior commissure in the Pax6 null mutant by transgenic expression of Pax6 under Pax2 upstream activating sequences at e12. The wild-type embryonic pretectum (A, shown in detail in B) shows the characteristic transverse bundles of the posterior commissure (pc) which are completely absent in the Pax6 null mutant (C,D). The ectopic posterior commissure is morphology evident in the Pax6 null mutant expressing Pax6 under the control of the Pax2 enhancer (E,F). (G) Expression of LacZ driven by the Pax2 enhancer shows that this enhancer is active in the region of rescue; inset shows ectopic expression of Pax6 under the control of Pax2 enhancer sequences in the tectum and cerebellum of a transgenic embryo. (H) Constructs for the transgenics used in E,F (upper) and G (lower). CB, cerebellum; pc, posterior commissure; PRT, pretectum; Pax6–/– TG, Pax6 null mutant plus transgen; TC, tectum; TG, tegmentum; wt, wild-type.

limit of the pretectum/tectum border. One possible mechanism would be the confinement of Pax6 expression to the commissural pretectum. From this point of view, our results represent an example of the cooperation between three Pax gene family members in the regionalization and boundary formation of the rostral neural tube.

3.3. The Pax2/5 mutant mes/met: a murine pair rule phenotype?

The Pax family of genes represents the mammalian counterpart of the pair-rule genes found originally in Drosophila. Mutations of these genes in the fruit fly give rise to particular kinds of abnormal phenotypes that have thrown light on the development of the segmented body of the insects. To which point is that information relevant to the interpretation of the phenotype we find in mammalian mutants? Mutations in the paired gene of Drosophila cause the appearance of a 'mixed' parasegment, that is, an abnormal parasegment with characteristics of two different ones (reviewed in Ingham and Martinez Arias, 1992). Accordingly, the phenotype of the murine Pax2/5 mutant shows an abnormal midbrain with molecular markers belonging to the forebrain (Pax6) and the forebrain and midbrain (Pax7, Otx-2). Although comparing genes, processes and phenotypes across phyla is undoubtedly attractive and intriguing, and can lead to important insights in pattern formation in living beings, caution

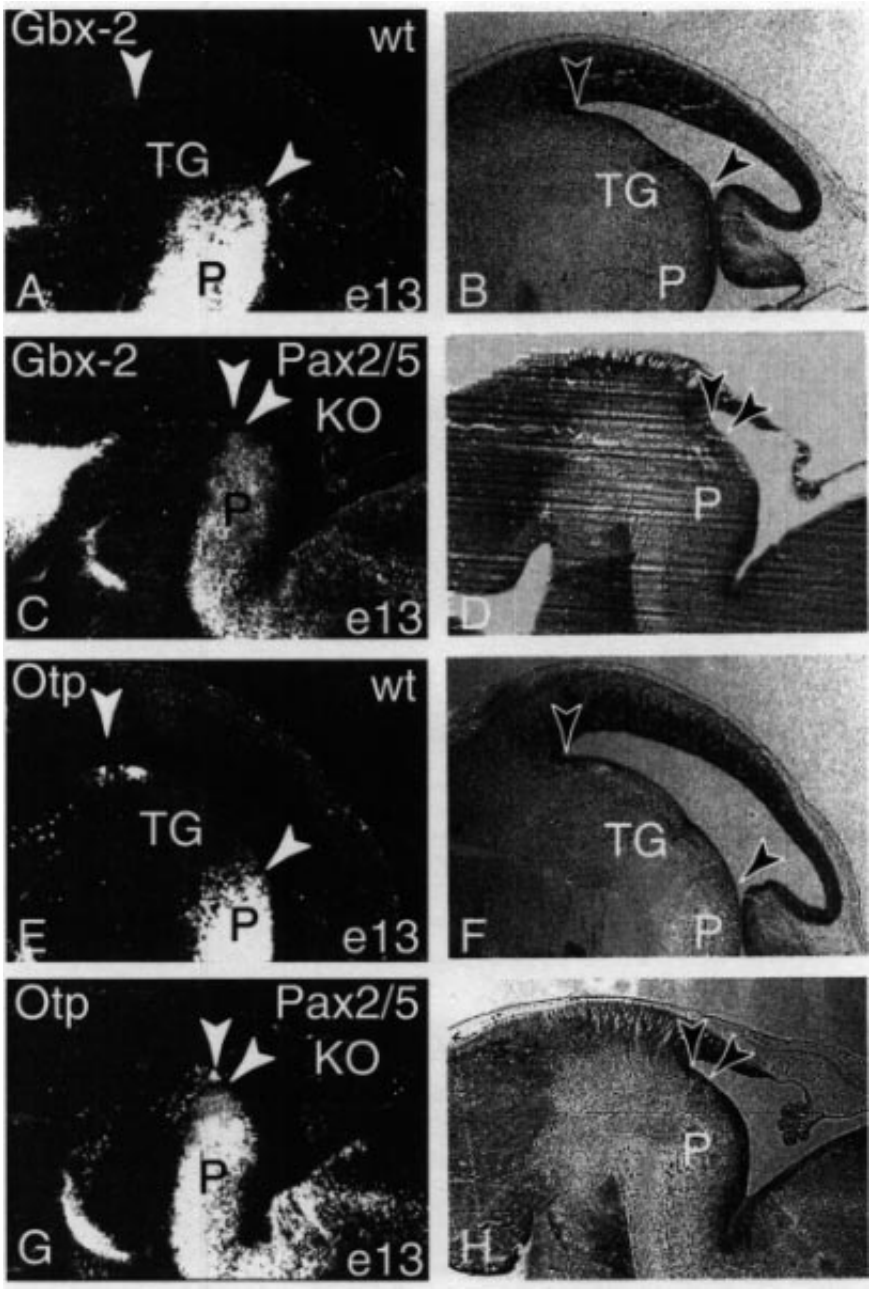

Fig. 6. Molecular markers show loss of the midbrain tegmentum in the Pax2/5 mutant. In the wild-type e13 embryos, the territory of expression of Gbx2 (A,B) has a rostral boundary in the isthmus. In the double mutant (C,D), the rostral boundary of Gbx2 expression reaches the level of the posterior commissure. The same phenomenon is evident when comparing the rostral boundary of expression of Otp in the wild type (E,F) and double mutant (G,H) e13 embryo. Arrowheads show the extent of the tegmentum in every section. P, pons; TG, tegmentum; wt, wild type.

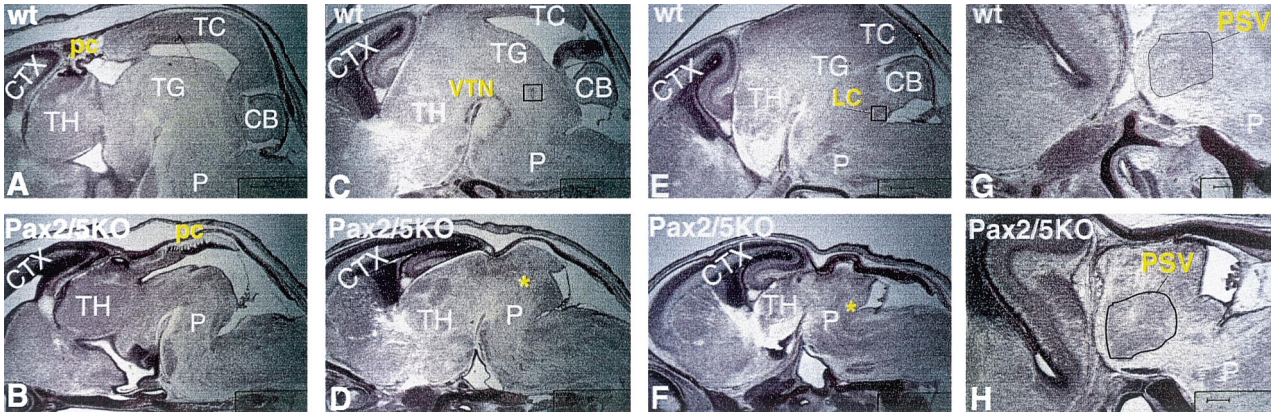

Fig. 7. In the fetal (e16.5) brain of the Pax2/5 double mutant, forebrain and hindbrain structures can be recognized, but not midbdrain tegmental structures. By e16.5, some nuclei can already be identified in the wild type mouse brain (A,C,E,G). In a midline sagittal section (A), main subdivisions of the forebrain (cortex, CTX; thalamus, TH), the midbrain (tectum, TC; tegmentum, TG) and hindbrain (cerebellum, CB; pons, P) can be recognized. At different mediolateral levels, isthmal derivatives like the ventral tegmental nucleus (VTN in C) or the locus coeruleus (LC in E) can already be identified. The principal nucleus of the trigeminal, a major derivative of the first rhombomere, is prominent in the pons (PSV in G). In the double mutant (B,D,F,H), anatomical alterations are evident. A sagittal section through the midline (B) shows a clear shortening of the distance between pons and diencephalon (compare B with A). Isthmal derivatives like the ventral tegmental nucleus (compare D to C) or the locus coeruleus (compare F to E) cannot be found in the double mutant. The principal nucleus of the trigeminal, however, seems completely normal (compare H to G).

must be exerted in the framing of these highly speculative hypotheses.

4. Materials and methods

4.1. Generation and genotyping of knockout mice

Mice and embryos were generated by crossing Pax2+/– Pax5+/– males with Pax2+/– Pax5+/– females in a BL6/129sv mixed background. DNA extraction from yolk sac or tail tip was performed as described (Torres et al., 1995; Urbánek et al., 1994). Genotypes were identified by Southern blotting using previously described DNA probes (Urbánek et al., 1994; Torres et al., 1995).

4.2. Generation and genotyping of transgenic mice

Transgenic animals were generated by microinjection of DNA into the paternal pronucleus of oocytes. The constructs contained 9.3 kb of upstream activating sequences of Pax2 (the 3′ end is at the Not I site 24 bp upstream of the ATG), followed by either the β-galactosidase or the Pax6 cDNA (Walther and Gruss, 1991) and then the SV40 intron poly A sequence. The vectors used for microinjection were linarized using a unique HindIII site at the very 5′end of the Pax2 enhancer sequence and a SalI (Pax6) or SfiI (LacZ) site at the 3′end of the construct. F0 founder mice were genotyped using the Pax6 cDNA as a probe for Southern hybridization. DNA extraction from yolk sac or tail tip was performed as described (Torres et al., 1995). Mutant mice were obtained by heterozygous intercrosses between Pax2/5+/– females with Pax2/5+/– TG (Pax2/6) males. The distribution of lacZ in the Pax2$^{\beta\text{-gal}}$ mouse embryos was performed as described (Urbánek et al., 1994).

4.3. Genetic and phenotypic analyses

All the embryos were collected from anesthetized females, fixed in 4% paraformaldehyde, embedded in paraffin blocks. The extraembryonic membranes were genotyped by Southern blotting. For the histology and the anatomy, double mutant and wt embryos were analyzed at different stages during embryonic development (see text). The embryos were dissected, photographed, sectioned and finally stained with Cresyl-Violet (Nissl staining). LacZ staining of the

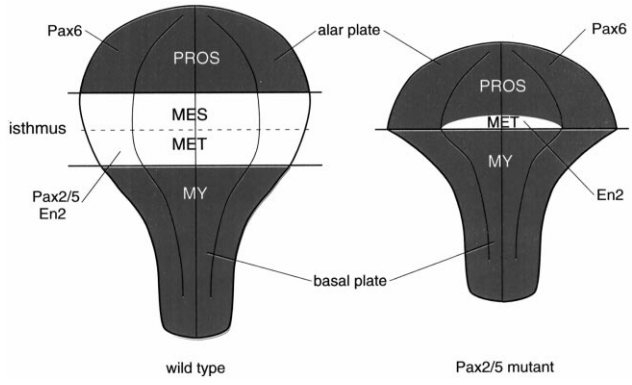

Fig. 8. Diagram summarizing the phenotypical alterations in the Pax2/5 compound mutant. In the wild-type neural plate (left side), expression of Pax6 (red) and Pax2 and 5 (white), delimit three domains (PROS, prosencephalon; MES/MET, mes-metencephalon; MY, medulla). In the Pax2/5 double mutant (right side), the middle subdivision (MES/MET) has disappeared; its only remnant is a small ventral region (white) that is En2 positive and corresponds to the basal ‘metencephalon’.

transgenic embryos was carried out essentially as described (Urbánek et al., 1994). The staining reaction was left to proceed for 12 h in order to obtain intense labeling of all structures expressing the transgene.

4.4. In situ hybridization

In situ hybridization experiments on sections were performed as previously described using ^{35}S-labeled RNA probes (Stoykova and Gruss, 1994). The Pax2, Pax6 and Otx-2 probes have been described previously (Simeone et al., 1994; Stoykova and Gruss, 1994).

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