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Pax3 and *Pax7* are expressed in commissural neurons and restrict ventral neuronal identity in the spinal cord

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Abstract

Pax3 and *Pax7* are transcription factors sharing high sequence identity and overlapping patterns of expression in particular in the dorsal spinal cord. Analysis of *Pax3* and *Pax7* double mutant mice demonstrates that both genes share redundant functions to restrict ventral neuronal identity in the spinal cord. In their absence, the *En1* expression domain is expanded dorsally but that of *Evx1* is not affected. In addition, *Pax3* and *Pax7* are expressed in commissural neurons and double mutant embryos exhibit highly reduced ventral commissure. Our findings reveal two distinct regulatory pathways for spinal cord neurogenesis, only one of which is dependent on *Pax3/7* and *6*.

Keywords: Pax; Spinal cord; Patterning; Neuronal identity; Mouse

1. Introduction

The fate of cells within the neural tube depends on ventral and dorsal signals from the notochord and epidermal ectoderm, respectively (Placzek, 1995; Dickinson et al., 1995; Tanabe and Jessel, 1996). The *Pax3* and *Pax7* genes, which encode two members of the *Pax* family of transcription factors, are expressed in the dorsal neural tube (Goulding et al., 1991; Jostes et al., 1991). Transplantation experiments in the chick have shown that they are regulated both ventrally by sonic hedgehog (Shh) and dorsally by bone morphogenetic proteins (BMPS) 4 and 7 (Goulding et al., 1993; Liem et al., 1995, 1997; Ericson et al., 1996). These observations led to the hypothesis that these *Pax* genes, together with *Pax6*, are involved in the dorso-ventral patterning of the spinal cord (Goulding et al., 1993; Liem et al., 1995, 1997; Ericson et al., 1996). Loss of *Pax3* function results in the *Splotch* (*Sp*) phenotype which has defects in neural tube closure and neural crest emigration and/or differentiation (for review see Mansouri et al., 1994). Ectopic expression of *Pax3* in the spinal cord of the mouse, using the Hoxb4 enhancer, leads to the agenesis of the floor plate in the affected area (Tremblay et al., 1996). Recently it has also been shown that in ascidians, the overexpression of *HrPax-37* (a homologue of *Pax3* and *Pax7*) causes ectopic expression of dorsal neural markers (Wada et al., 1997). The analysis of $Pax7^{-/-}$ mice, generated by homologous recombination in embryonic stem cells, revealed defects in some cephalic neural crest derivatives only and indicated that *Pax3* and *Pax7* may share redundant functions due to coactivity in several tissues (Mansouri et al., 1996).

In order to specifically address the role of both genes in the spinal cord, *Pax3/Pax7* double mutant embryos (*Pax3/* $Pax7^{-/-}$) were generated. Our analysis revealed that *Pax3* and *Pax7* are expressed in commissural neurons and are necessary to restrict ventral neuronal identity in the spinal cord. In the absence of both genes, some cells located dorsally have acquired a ventral cell fate of interneuron type. This is documented by a dorsal expansion of *En1* which has been shown to define V1 interneurons and corroborated by a distinct change in the *Pax2* and *Lim1* pattern. Our results provide evidence that there may be two distinct pathways for neurogenesis in the spinal cord, one dependent on *Pax3/7* and *6*, shown by *En1* cells, and one pathway independent of *Pax* genes, exemplified by *Evx1* cells.

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2. Results

Pax3/Pax7$^{-/-}$ embryos die at E11.0 and exhibit extensive exencephaly and spina bifida. Histological analysis revealed no obvious alterations in the spinal cord. Due to early lethality it was impossible to study the consequence of the mutations in more mature spinal cords. Therefore, the expression of several early ventral and dorsal molecular markers have been examined, in order to analyse if the dorso/ventral patterning, i.e. the dorsal cell fate has been changed.

2.1. Wnt4 is not expressed in the dorsal neural tube of Pax3/Pax7$^{-/-}$ *embryos*

The expression of several markers remained unchanged. Specifically, the expression of dorsal markers like the homeobox genes *msx1* and *msx2* and the signalling molecules *Wnt1*, *Wnt3* and *Wnt3a* is not modified, indicating that the formation of the roof plate is not affected (data not shown). At E9.5 *Wnt4* normally starts to be expressed in the dorsal spinal cord (Parr et al., 1993). In *Pax3/Pax7*$^{-/-}$ embryos *Wnt4* transcripts are not detectable at this stage (data not shown) or at E10.5 (Fig. 1F), while the expression in the ventral spinal cord is only reduced (floor plate, see white arrow in Fig. 1F). Thus, *Pax3* and *Pax7* are necessary for the initiation and/or maintenance of *Wnt4* expression in the dorsal spinal cord.

In the spinal cord of the mouse, the first neurons are born at E9.5 of gestation (Nornes and Carry, 1978). The expression of *Pax* and *Wnt* genes is detected prior to or coincident with the generation of these first neurons (Parr et al., 1993; Mansouri et al., 1994). Like *Pax* genes, the *Wnt* genes expression patterns also clearly display dorso-ventral restrictions in the spinal cord and have been therefore suggested to play an important role in dorso-ventral variations of neuronal precursor cells (Parr et al., 1993). Accordingly, *Pax3* and *Pax7* may specify different neuronal precursor cells by acting directly or indirectly on the *Wnt4* gene.

For this reason we studied the expression of several genes

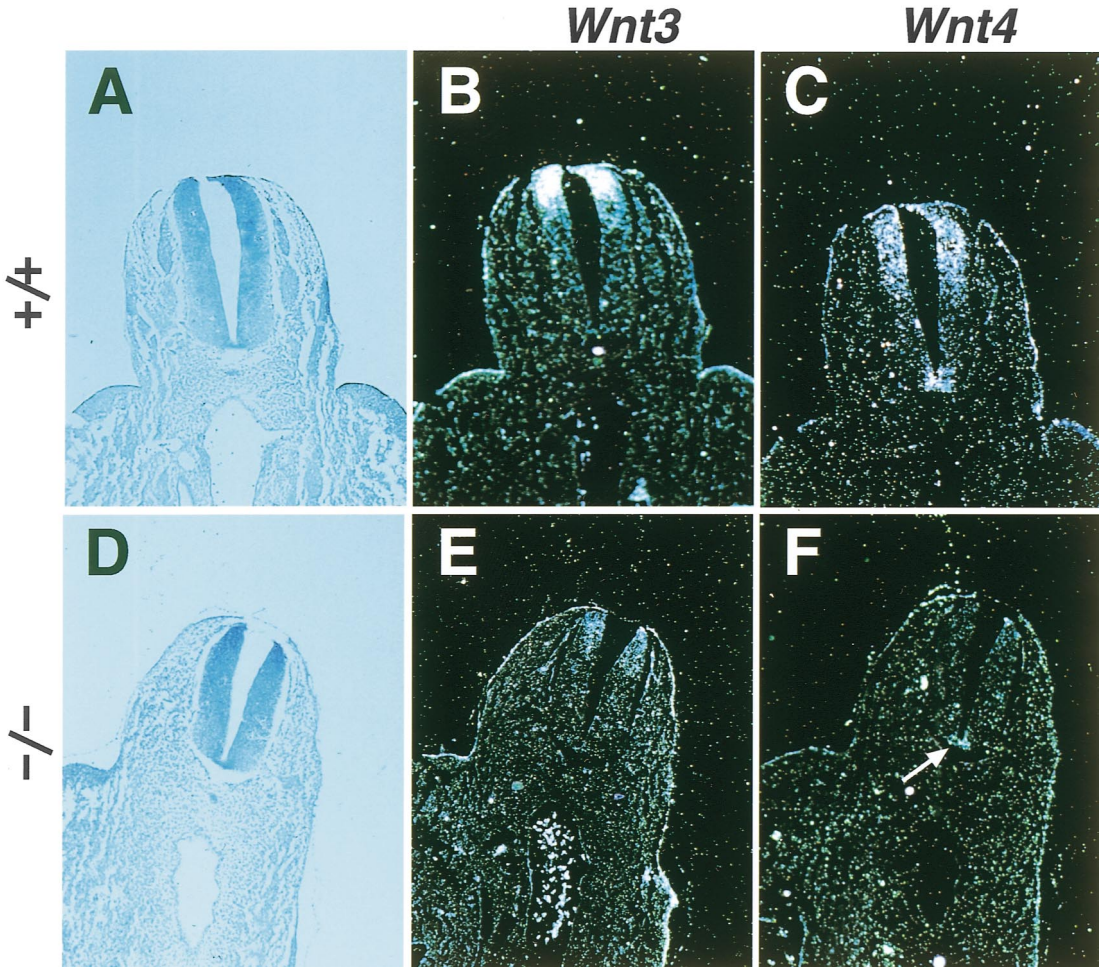

Fig. 1. Expression of *Wnt3* and *Wnt4* in the spinal cord of wild-type and *Pax3/Pax7*$^{-/-}$ embryos at E10.5. In situ hybridization analysis of *Wnt3* (B,E) and *Wnt4* (C,F) in the spinal cord of E10.5 embryos is shown in adjacent transverse sections. While the expression of *Wnt3* is still present in the mutant embryos (E), the *Wnt4* expression is missing in the dorsal spinal cord of the mutant (F). The expression in the floor plate is only reduced (white arrow). The expression of *Wnt3* is also reduced in the dorsal spinal cord of the double mutant. A,D: light-field; B,C,E,F: dark-field. +/+, wild-type; –/–, double mutant embryo.

associated with a function for neuronal subtypes in the spinal cord (including *Pax6*, *En1*, *Isl1* and *Lim1*).

2.2. Pax3 and Pax7 restrict ventral neuronal identity in the spinal cord

En1 has been shown to define V1 interneurons generated ventral to *Pax7* expressing cells (Ericson et al., 1997). In *Pax3/Pax7*$^{-/-}$ embryos the domain of *En1* expression is extended into the dorsal spinal cord, where *Pax3* and *Pax7* are normally expressed (Fig. 2E). In contrast, the *Evx1* expression domain which abuts the ventral limit of *Pax7* expression (Burill et al., 1997) is not affected (Fig. 2F). To further demonstrate that the expression of *En1* is expanded dorsally into the *Pax7* domain, we analysed *Pax3/Pax7*$^{-/-}$ embryos which contain the LacZ gene inserted into the *Pax7* locus (Mansouri et al., 1996). As illustrated in Fig. 2J,K, at E10.5, the expression of *En1* is extended dorsally

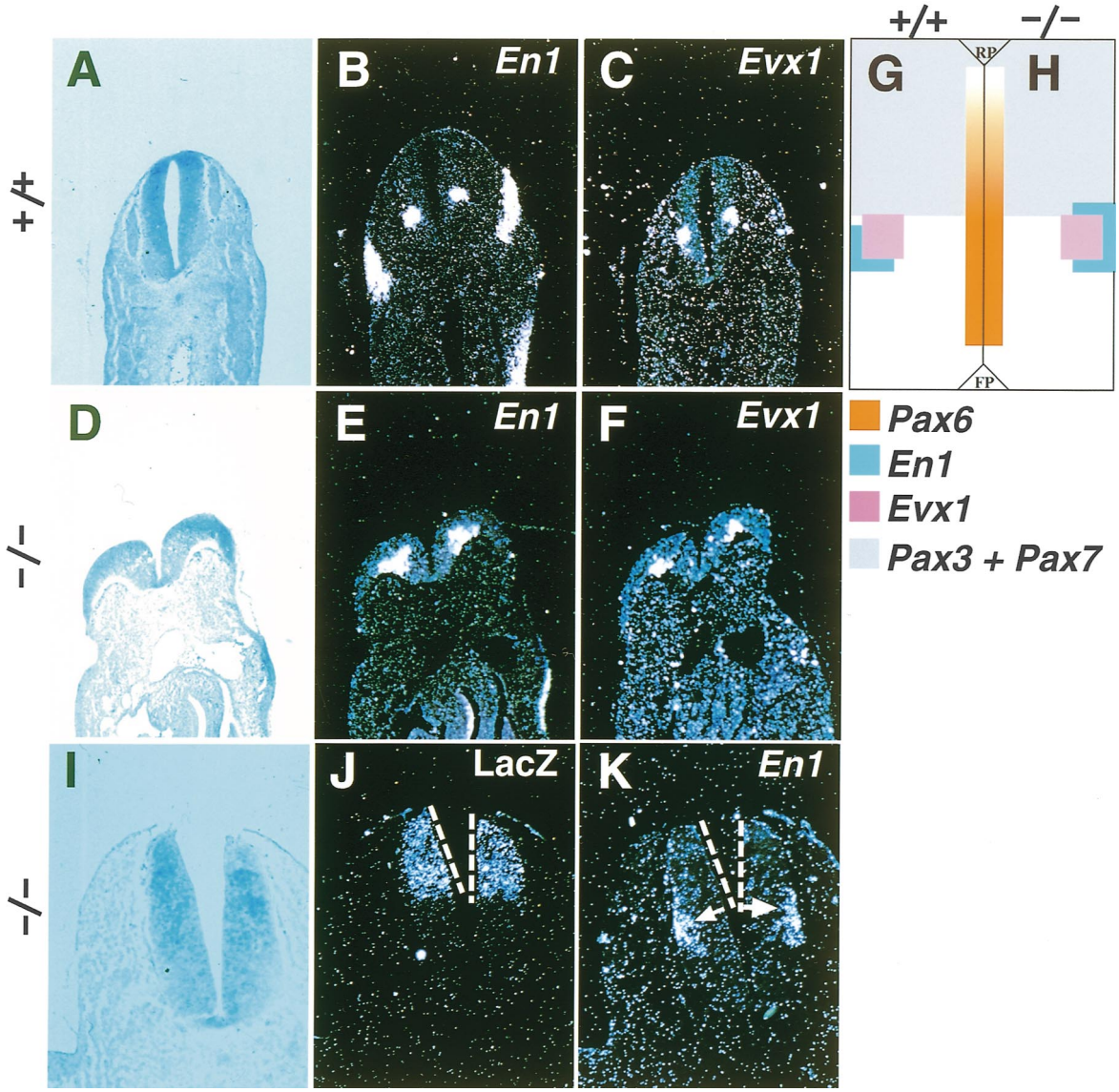

Fig. 2. Expression of *En1* and *Evx1* in the spinal cord of wild-type and *Pax3/Pax7*$^{-/-}$ embryos, and comparison of *En1* to LacZ expression in mutant embryos at E10.5. In situ hybridization analysis of *En1* (B,E) and *Evx1* (C,F) in the spinal cord of E10.5 embryos is shown on transverse adjacent sections. In the mutant embryo (E), the expression of *En1* is shifted dorsally as compared to the *Evx1* expression domain shown in the adjacent section (F). This is shown schematically in G and H. Also, the expression (not changed) of *Pax6* is shown in the scheme. Comparison of the expression of *En1* and LacZ in the double mutant embryo at E10.5 is shown in one part of the spinal cord without spina bifida. The LacZ gene has been inserted in the *Pax7* locus by homologous recombination and reflects its expression pattern (Mansouri et al., 1996). As shown in J and K, the expression of *En1* extends dorsally into the LacZ domain. To highlight the extension of the *En1* expression into the LacZ area, the ventral limit of LacZ expression is indicated by white arrows. Thus, the dorsal area where the expression of *En1* extends acquires ventral identity of interneurons. A,D,I: light-field; B,C,E,F,J,K: dark-field. G and H indicate schematically the expression of *En1*, *Evx1* and *Pax6* in control (G) and mutant (H) embryos as compared to *Pax3* and *Pax7* expression domain in the dorsal spinal cord (grey). FP, floor plate; LacZ, β-galactosidase; RP, roof plate; + / + , wild-type; − / − , double mutant embryo.

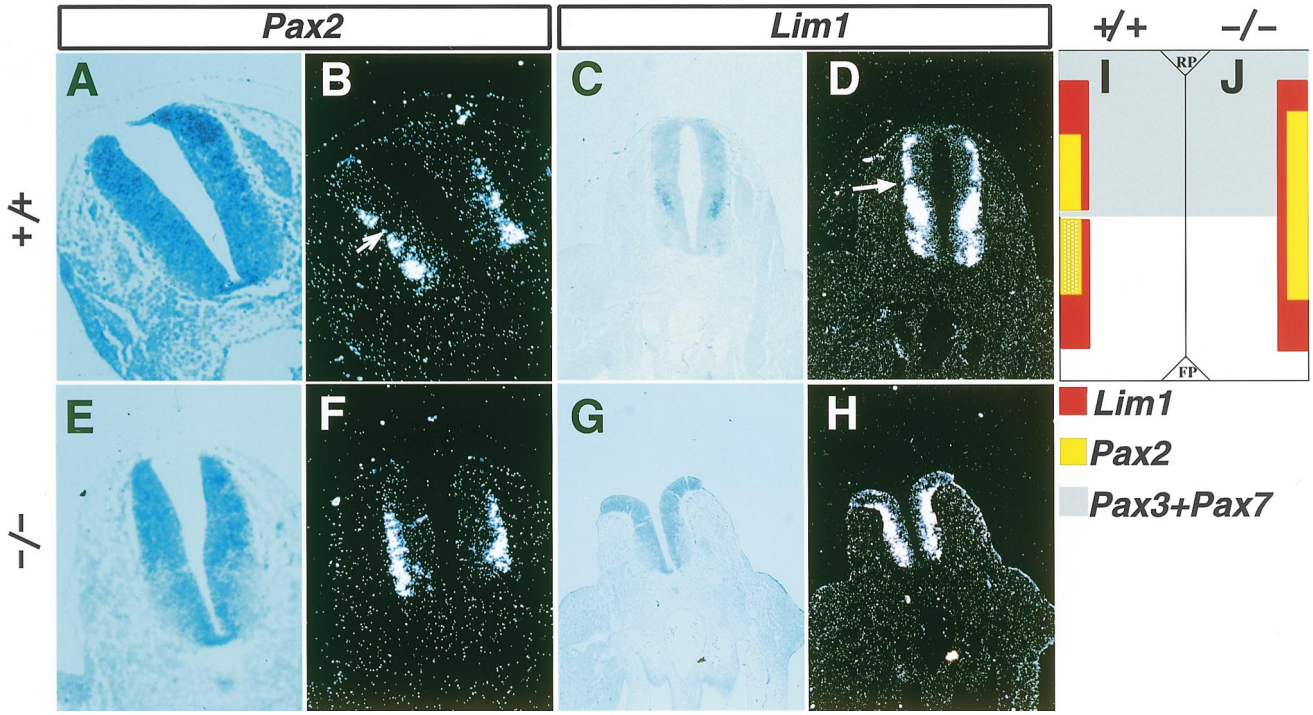

Fig. 3. Expression of *Pax2* and *Lim1* in the spinal cord of wild-type and *Pax3/Pax7*$^{-/-}$ embryos at E10.5. In situ hybridization analysis of the expression of *Pax2* and *Lim1* on transverse sections in the spinal cord of E10.5 control (A–D) and mutant (E–H) embryos, respectively. In the control embryos there is a gap between the ventral and dorsal expression domains of *Pax2* and also *Lim1*, indicated in both cases by white arrow in B and D. These gaps are not present in the mutants. For Pax2 it is shown in a part of the spinal cord without spina bifida (E,F). The results are schematically shown in I for control and J for the mutant embryo in comparison to the expression of *Pax3* and *Pax7* (grey). A,C,E,G: light-field; B,D,F,H: dark-field. +/+, wild-type; –/–, double mutant embryo; FP, floor plate; RP, roof plate.

within the LacZ-positive domain. This suggest some dorsally located cells might have acquired ventral cell fate of the V1 interneuron type. Therefore, one possible role of *Pax3* and *Pax7* might be to restrict ventral neuronal identity.

Since *Pax3* and *Pax7* inhibit the dorsal extension of some ventral cell fate determining gene in the spinal cord, we studied whether the expression of additional neuronal markers is also affected. The *Lim* family of transcription factors defines different cell progenitors in the spinal cord (Tsuchida et al., 1994; Lumsden, 1995). In the *Pax3/Pax7*$^{-/-}$ embryos, *Lim1* and *Isl1* expression domains exhibit subtle modifications, while that of *Lim3* remains unchanged. The overall amount of *Isl1* mRNA is reduced in the medial but not in the longitudinal dorsal spinal cord (data not shown). The *Lim1* expression domain is usually discontinuous and exhibits a gap at the limit of ventral and dorsal cell types (Tanabe and Jessel, 1996). This gap is not detectable in *Pax3/Pax7*$^{-/-}$ embryos (Fig. 3D,H). A similar modification is also observed for the *Pax2* expression domain. Normally, *Pax2* is expressed in longitudinal columns on both sides of the sulcus limitans (Nornes et al., 1990). In the *Pax3/Pax7*$^{-/-}$ embryos the ventral and dorsal columns are fused (Fig. 3B,F; see also Fig. 3I,J). The *En1*-positive interneurons coexpress *Lim1* and *Pax2* (Burill et al., 1997; Ericson et al., 1997). Accordingly, the observed modifications of the *Lim1* and *Pax2* expression domains are consistent with the expansion of ventral cell fate (interneurons) into the dorsal part of the spinal cord in the mutant embryo (see scheme of Fig. 3I,J). From these observations we conclude that *Pax3* and *Pax7* appear to restrict ventral neuronal identity in the spinal cord.

2.3. Pax3 and Pax7 are expressed in commissural neurons

Recently *Pax6* has been shown to be responsible for the establishment of different ventral cell populations. It mediates *Shh* signals to control the identity of motor neurons and ventral interneurons in the ventral spinal cord (Ericson et al., 1997). By analogy, *Pax3* and *Pax7* may control the identity of some dorsal cell populations by mediating dorsally located *BMP* signals (Liem et al., 1997). We generated a new *Splotch* mouse mutant by introducing the LacZ gene via homologous recombination in the *Pax3* locus (data not shown). The comparison of the LacZ pattern with the previously described expression of *Pax3* revealed a new expression domain in the spinal cord. As shown in Fig. 4A,B, *Pax3* LacZ staining is observed in fibres originating in *Pax3/7*-positive alar plate, elongating ventrally, and crossing the ventral midline. Contralateral axonal projections of this type are considered as dorsal commissural neurons (Altman and Bayer, 1984). Thus *Pax3* is expressed in commissural neurons. In adjacent sections the staining is shown for β-galactosidase and N-CAM protein, which labels commissural neurons (Dodd et al., 1988) (Fig.

5A,B). This prompted us to look at the *Pax7* knock-out embryos also carrying the LacZ gene (Mansouri et al., 1996). It revealed that also *Pax7* is expressed in commissural neurons (Fig. 4C). The analysis of *Pax3/Pax7*$^{-/-}$ embryos using LacZ staining for *Pax3* showed no staining in commissural neurons (Fig. 4D). As compared to *Splotch* embryos, *Pax3/Pax7*$^{-/-}$ embryos have greatly reduced ventral commissure underlying the floor plate of the spinal cord. Also, little LacZ staining is observed there and the N-CAM and neurofilament expression are highly reduced (Fig. 5C,D, and data not shown). This phenotype has been already observed in Netrin-1-deficient mice (Serafini et al., 1996). However, the Netrin-1 gene is expressed in *Pax3/Pax7*$^{-/-}$ mice as revealed by whole-mount in situ hybridization (data not shown).

3. Discussion

To elucidate the function of *Pax3* and *Pax7* in the spinal cord, we generated double mutant embryos. The results of our analysis provide evidence that *Pax3/7* restricts ventral neuronal identity. This is documented by a dorsal expansion of *En1* expression domain into the *Pax3* and *Pax7* expression area, and corroborated by a distinct change in the *Pax2* and *Lim1* pattern. However, *Evx1* which also labels a subpopulation of ventral interneurons (Burill et al., 1997; Ericson et al., 1997) and abuts the ventral limit of *Pax7* expression, is not modified. Interestingly, also in *Pax6*$^{-/-}$ mice the *En1* cells and *Evx1* cells behave differently. While the *En1* expression disappears, that of *Evx1* is not affected (Burill et al., 1997; Ericson et al., 1997). Taken together, these results suggest that there are two distinct pathways for neurogenesis in the spinal cord. One pathway is dependent on *Pax3/7* and *6* as shown for *En1* cells, and the other, independent of *Pax* genes, as exemplified by *Evx1* cells.

In the brain, the absence of *Pax6* function affects the border between two adjacent domains, the cortex and the ganglionic eminence (Stoykova et al., 1996). In addition, a failure in cell adhesion properties of cortical but not striatal cells has been established (Stoykova et al., 1997). Our results demonstrate that *Pax3* and *Pax7* are necessary for the expression of *Wnt4* in the dorsal spinal cord. The *Wnt* family of signalling molecules have been implicated in cell–cell adhesion mechanisms (Papkoff et al., 1996). Therefore, *Pax3* and *Pax7* may also mediate cell–cell adhesion properties to restrict ventral neuronal identity. Also *Pax6* is involved in the specification of hindbrain motor neuron subtypes through the regulation of *Wnt7b* expression (Osumi et al., 1997). Such a mechanism may be common to the function of *Pax* genes in the CNS. However, recently the analysis of mice lacking both *Wnt1* and *Wnt3a* revealed that these genes are involved in the proliferation of dorsal neural precursors in the hindbrain (Ikeya et al., 1997). Mice lacking *Wnt4* have no phenotype in the spinal cord (Stark et al., 1994). Furthermore, *Wnt4* (also *Wnt1* and *Wnt3*) has been

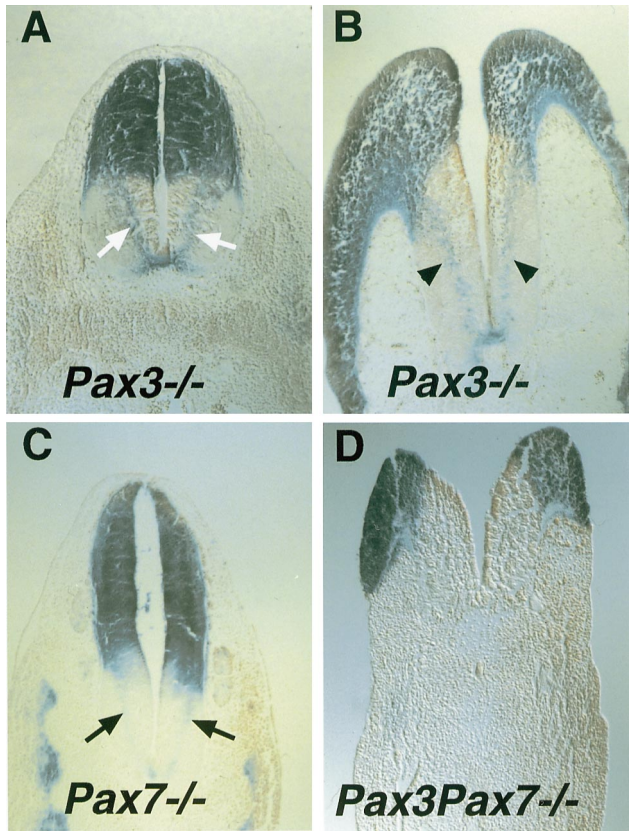

Fig. 4. Expression of *Pax3* and *Pax7* in commissural neurons. Transverse sections of E10.5 embryos showing LacZ staining for *Pax3*$^{-/-}$ embryos (A,B), at two different levels, *Pax7*$^{-/-}$ embryo (C) (Mansouri et al., 1996) and *Pax3/Pax7*$^{-/-}$ (D). LacZ staining is observed in fibres originating in *Pax3/7*-positive alar plate, elongating ventrally, and crossing the ventral midline. Contralateral axonal projections of this type are considered as dorsal commissural neurons (Altman and Bayer, 1984). Thus *Pax3* and *Pax7* are expressed in commissural neurons. A: *Pax3*$^{-/-}$; white arrows indicate the LacZ-positive fibres; B: *Pax3*$^{-/-}$ at the level of the spina bifida; black arrowheads indicate the LacZ-positive fibres; C: *Pax7* – / – ; black arrows indicate the LacZ-positive fibres; D: *Pax3/Pax7*$^{-/-}$, LacZ staining is not observed, indicating that the fibres are missing; the LacZ staining is shown for the *Pax3* allele.

proposed to be involved in signals from the dorsal neural tube on somite development (Münsterberg et al., 1995). Therefore, the absence of *Wnt4* in the neural tube of *Pax3/Pax7*$^{-/-}$ mutant mice may not be related to the phenotype described here but rather may be responsible for the phenotype observed in the somites of *Pax3/Pax7*$^{-/-}$ embryos (Mansouri and Gruss, in preparation).

The regulation of *En* by *Pax* genes have been also observed for *Pax2,5* and *8*. Two binding sites for *Pax2,5* and *8* proteins were identified in *En2* regulatory sequences. Mutation of the binding sites disrupts initiation and maintenance of *En2* expression in the brain of transgenic mice (Song et al., 1996). Therefore by analogy, *Pax3/7* might directly regulate transcription of the *En1* gene.

Using the LacZ knock-in we show that *Pax3* and *Pax7* are expressed in commissural neurons. The observation that ventral commissure underlying the floor plate are highly reduced in the double mutant suggests that both genes are

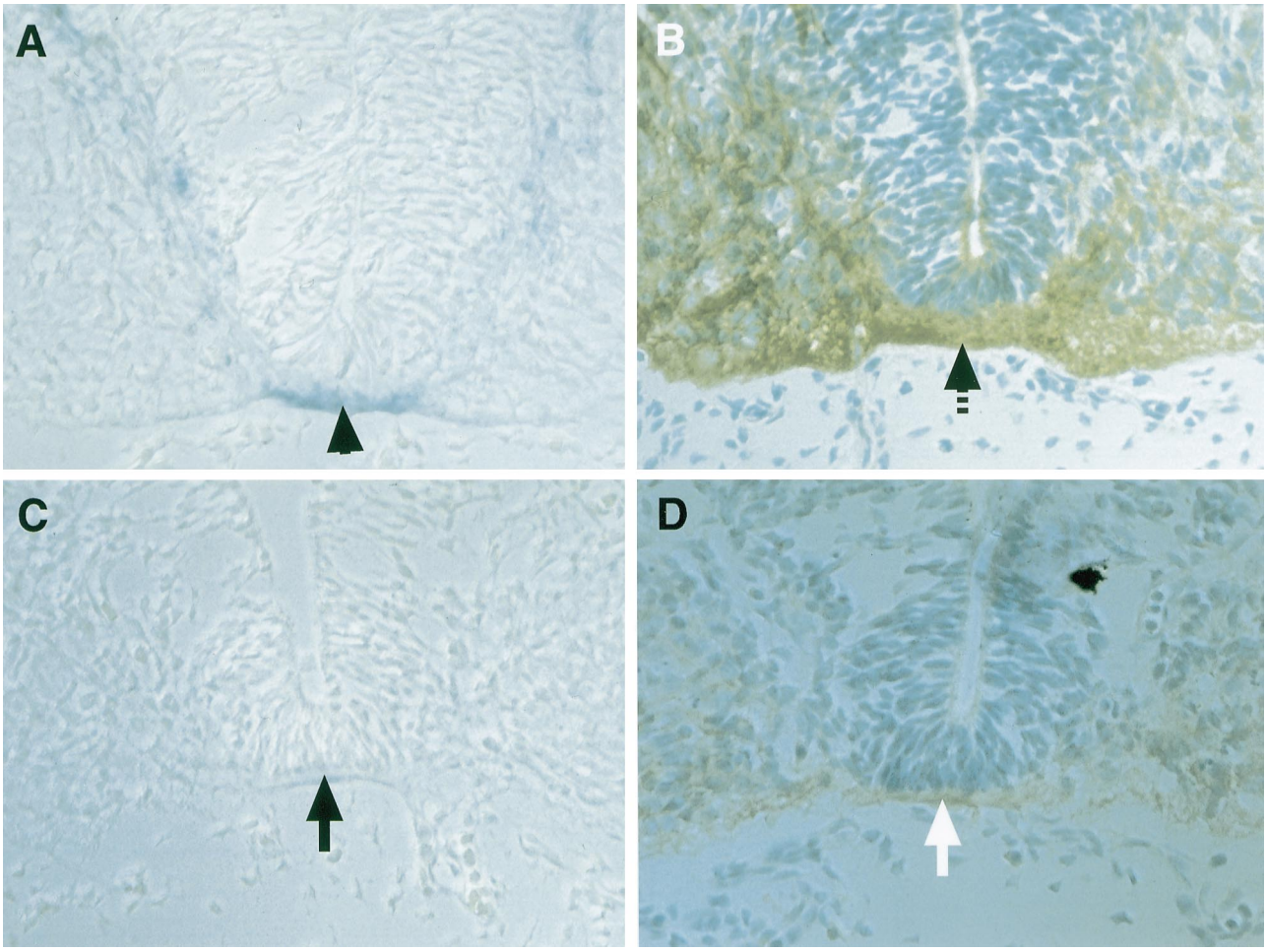

Fig. 5. The ventral commissure underlying the floor plate of the spinal cord is greatly reduced in *Pax3/Pax7*$^{-/-}$. Transverse sections of *Pax3*$^{-/-}$ and *Pax3/Pax7*$^{-/-}$ embryos at E10.5 showing the ventral commissure. No LacZ-staining is observed in *Pax3/Pax7*$^{-/-}$ (C). Also, N-CAM staining is highly reduced in *Pax3/Pax7*$^{-/-}$ (D). Arrows and arrowheads indicate the ventral commissure in the respective section. A: *Pax3*$^{-/-}$ stained for LacZ (*Pax3*); B: adjacent section to A, stained with N-CAM antibody; C, *Pax3/Pax7*$^{-/-}$ stained with LacZ (*Pax3*); D: adjacent section to C stained with N-CAM antibody. In B and D the sections were counterstained with methyl green.

expressed in a subpopulation of commissural neurons. Alternatively, the differentiation of these neurons is affected so that their axons are not projected to the floor plate. The fact that Netrin-1 expression is not modified in *Pax3/Pax7*$^{-/-}$ embryos indicate that they act in parallel pathways, and it is worth speculating what happens to the expression of *Evx1* in the spinal cord of Netrin-1-deficient mice. Alternatively, Netrin-1 acts upstream of *Pax3,7* genes. In *Drosophila*, two structurally related genes, *gooseberry-proximal* and *gooseberry-distal*, which are homologous to the *Pax3* and *Pax7* genes, specify row 5 neuroblasts (Noll, 1993; Zhang et al., 1994; Skeath et al., 1995; Duman-Scheel et al., 1997). In zebrafish, it has been reported that the *Pax2* homologue is expressed in a subpopulation of commissural neurons (Mikkola et al., 1992).

In summary, *Pax3* and *Pax7* may specify a subpopulation of commissural neurons. They restrict ventral neuronal cell fate in the spinal cord. Our results provide evidence that there may be two distinct pathways of spinal cord neurogenesis, only one of which is dependent on *Pax* genes.

4. Experimental procedures

4.1. Animals

To generate double mutant embryos, *Pax3/Pax7* compound heterozygotes were generated by mating *Splotch* 2H mice (Epstein et al., 1991) to *Pax7* knock-out mice (Mansouri et al., 1996). Two different alleles of *Pax7* were used (one with β-galactosidase insertion and the second with neomycin insertion) (Mansouri et al., 1996). Mice are kept in a mixed genetic background of 129sV and C57Bl/6. Embryos were staged on the assumption that the day of vaginal plug is considered as E0.5.

4.2. In situ hybridization and immunohistochemistry

Embryos were fixed in 4% paraformaldehyde overnight at 4°C and further processed for whole-mount hybridization or in situ hybridization on sections using ^{35}S-labelled riboprobes as described (Wilkinson, 1992). Immunohistochemis-

try was performed on cryostat sections as described (Dodd et al., 1988). After fixation embryos were passed through 30% sucrose overnight and embedded in OCT compound (Tissue Tek). Embryonic membranes were used to genotype the embryos for *Pax3* and *Pax7* by PCR or Southern blot as described (Epstein et al., 1991; Mansouri et al., 1996).

4.3. Antibodies

Primary antibody was monoclonal anti-N-CAM (Developmental Studies Hybridoma Bank). Anti-mouse antibody coupled to HRP (Capell) was detected with a colour-development substrate solution containing diaminobenzidine. Antibodies were diluted according to the companies' recommendations.

4.4. LacZ staining

Embryos were dissected from the uterus in cold PBS and fixed in a solution of PBS containing formaldehyde, glutaraldehyde and NP-40, and stained overnight for β-galactosidase as described (Allen et al., 1988). For sectioning, β-galactosidase stained embryos were fixed for 1 h in 4% paraformaldehyde at 4°C. After dehydration in ethanol, embryos were washed in isopropanol, cleared in xylene, and embedded in paraffin. Ten-μm sections were deparaffinized and counterstained with neutral red.

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