

Control of hindbrain motor neuron differentiation by the homeobox gene

Phox2b

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SUMMARY

Motor neurons are a widely studied model of vertebrate neurogenesis. They can be subdivided in somatic, branchial and visceral motor neurons. Recent studies on the dorsoventral patterning of the rhombencephalon have implicated the homeobox genes *Pax6* and *Nkx2.2* in the early divergence of the transcriptional programme of hindbrain somatic and visceral motor neuronal differentiation. We provide genetic evidence that the paired-like homeodomain protein *Phox2b* is required for the formation of all branchial and visceral, but not somatic, motor neurons in the hindbrain. In mice lacking *Phox2b*,

both the generic and subtype-specific programs of motoneuronal differentiation are disrupted at an early stage. Most motor neuron precursors die inside the neuroepithelium while those that emigrate to the mantle layer fail to switch on early postmitotic markers and to downregulate neuroepithelial markers. Thus, the loss of function of *Phox2b* in hindbrain motor neurons exemplifies a novel control point in the generation of CNS neurons.

Key words: Neurogenesis, Motor neuron, Homeobox gene, Rhombencephalon, *Phox2b*, Mouse

INTRODUCTION

The specification of neuronal subtype identity in the developing central nervous system (CNS) appears to be controlled by the interplay between inductive signals secreted by embryonic signaling centers and the transcription factors that transmit this information onto the transcriptional apparatus (Lumsden and Krumlauf, 1996; Edlund and Jessell, 1999). A large number of different transcription factors have been found to be expressed in the developing CNS in discrete temporal and region- or cell-type-specific patterns. They are thought to control the generation of neuronal cell type diversity, in complex combinations and sequences. A major challenge is to assemble an integrated picture of this transcriptional logic of neuronal type-specific differentiation.

In vertebrates, motor neurons represent one of the better-understood model systems for exploring the molecular mechanisms that specify neuronal fate. Three general classes of motor neurons are found in the spinal cord and hindbrain: the somatic motor (sm) neurons that innervate most skeletal muscles in the body, the branchiomotor (bm) neurons that innervate muscles derived from the branchial arches, and visceral motor (vm) neurons that innervate sympathetic and parasympathetic ganglia. Sm neurons differentiate from progenitors in the ventral half of the neural tube characterized by the expression of the homeobox gene *Pax6*; bm and vm neurons in the hindbrain arise ventral to the former from progenitors that express the homeobox genes *Nkx2.2* and *Nkx2.9* (Ericson et al., 1997; Briscoe et al., 1999). Generation

of sm neurons has been studied most extensively and some of the transcriptional cascades controlling their differentiation are beginning to be understood. *Pax6* activity is required for sm differentiation, but appears to act mainly by suppressing expression of *Nkx2.2*. In the hindbrain, lack of *Pax6* diverts sm neurons towards a vm fate (Ericson et al., 1997; Osumi et al., 1997). Studies in chick embryos have implicated the homeobox genes *MNR2* and its close homologue *HB9* in sm differentiation (Tanabe et al., 1998). *MNR2* is switched on by sm progenitors just before their final division, whereas *HB9* is expressed during further differentiation. When misexpressed in neural tube cells, both *MNR2* and *HB9* are able to instruct the sm phenotype.

A separate line of research has concentrated on the acquisition of generic neuronal properties. In vertebrates, many genes that are related to the *Drosophila* proneural genes and encode bHLH transcription factors, have been found to be expressed in the developing nervous system; a number of them have been shown to have proneural activity, in that they induce formation of ectopic or supernumerary neurons when overexpressed in *Xenopus* embryos (see Anderson and Jan, 1997; Brunet and Ghysen, 1999 for review). Using this type of analysis and loss-of-function experiments in the mouse (Fode et al., 1998; Ma et al., 1998), it has been possible to place them in epistatic cascades, those expressed in dividing progenitors (such as the *Neurogenins* and *Mash1*) driving the expression of downstream genes activated at later stages of differentiation (such as *NeuroD* and *Math3*). These factors have thus been studied mostly for their capacity to induce a generic neuronal

phenotype, although there is evidence both in the fly and in vertebrates that they also participate in specifying the type of neuron that is generated (Hirsch et al., 1998; Lo et al., 1998). It is thus not known how the transcriptional cascades initiated by the transcription factors with proneural activity intersect with the pathways specifying neuronal subtype identities.

We have been studying the function of the closely related paired-like homeobox genes *Phox2a* and *Phox2b* by loss-of-function experiments in the mouse. Expression of the two genes is restricted to certain classes of developing neurons and neuronal progenitors in both the peripheral (PNS) and central nervous systems (Tiveron et al., 1996; Pattyn et al., 1997). In the PNS, they are co-expressed in all neurons of the visceral nervous system and are essential for their generation (Morin et al., 1997; Pattyn et al., 1999). In the CNS, expression of the *Phox2* genes is mostly confined to the hindbrain, where they are expressed in all bm and vm neurons, in the oculomotor (nIII) and trochlear (nIV) nuclei and in the (nor)adrenergic centers. *Phox2a* is essential for the generation of the rostral sites of *Phox2* gene expression in the CNS, i.e. nIII and nIV and the locus coeruleus (Morin et al., 1997; Pattyn et al., 1997). However, it is dispensable for the formation of the bm and vm neurons. Here, we demonstrate that *Phox2b* is required for the formation of all bm and cranial vm neurons. In the anlage of the facial (bm) motor nuclei, motoneuronal precursors die in large numbers inside the neuroepithelium. In those that exit from the neuroepithelium and reach the mantle layer, differentiation is disrupted before the downregulation of neuroepithelial markers and the concomitant upregulation of postmitotic markers. Therefore, *Phox2b* exemplifies a novel control point in the generation of CNS neurons.

MATERIALS AND METHODS

Generation of *Phox2b^{lacZ/lacZ}* mice

Mice heterozygous for the *Phox2b^{lacZ}* allele (Pattyn et al., 1999) were interbred; the day of detection of a vaginal plug was designated E0.5. Embryos were genotyped by PCR analysis on DNA of extraembryonic membranes using two pairs of primers: one pair (antisense CCAGACCAACTGGTAATGGTAGCG, sense TCGAGC-TGGGTAATAAGCGTTGGC) located in the *lacZ* gene, reveals a 800 bp product in heterozygotes and homozygotes; the other one, located in the deleted region of the *Phox2b* locus (antisense primer AGTGCTAGCTCTCCCTGGT, sense primer AGTGGCCCTTCATCCTGA) reveals a 220 bp product in heterozygous and wild-type mice.

Histology, β -galactosidase staining, in situ hybridization and immunohistochemistry

Nissl staining (Hirsch et al., 1998) and β -gal histochemistry (Knittel et al., 1995) were done as described. Antisense RNA probes for *peripherin* (Escurat et al., 1990), *Islet1* (Karlsson et al., 1990), *Phox2a* (Tiveron et al., 1996), *classIII β -tubulin* (Grove et al., 1998), *Ebf1*, *Ebf2* and *Ebf3* (Garel et al., 1997), *Math3* (Takebayashi et al., 1997), *Nkx2.2* (Shimamura et al., 1995), *Mash1* (Guillemot et al., 1993) and *E. coli lacZ* (Pharmacia) were labelled using a DIG-RNA labelling kit (Roche). In situ hybridization on cryosections or whole-mount preparations of embryos and combined in situ hybridization and immunohistochemistry were done as described (Tiveron et al., 1996; Hirsch et al., 1998).

For double-immunofluorescence, cryosections were blocked for 30 minutes in PBS/0.05% Tween-20/20% FCS and incubated overnight

at 4°C with the two primary antibodies: rabbit anti- β -gal (Rockland) (diluted 1/5000) and mouse anti-class III β -tubulin (Sigma) (diluted 1/400). They were then incubated for 1 hour with the two fluorescent antibodies: a rat anti-rabbit fluorescein-conjugated antibody (diluted 1/100), and a goat anti-mouse Texas red-conjugated antibody (Rockland) (diluted 1/200). Analyses were performed using a Zeiss confocal microscope.

BrdU labelling

BrdU (Sigma) was injected intraperitoneally into pregnant mice (6 mg/mouse), 1 hour or 3 hours before dissecting the embryos. Cryosections were incubated in 0.5% H₂O₂ in ethanol for 30 minutes, blocked for 2 hours in DMEM-10% FCS, treated with 2 N HCl for 30 minutes at 37°C and neutralized in 0.1 M sodium tetraborate. Sections were then incubated with a mouse anti-BrdU antibody (Sigma) (diluted 1/500), which was revealed using the peroxidase-ABC Vectastain kit (Vector) and diaminobenzidine substrate (Sigma).

For combined in situ hybridization and BrdU immunohistochemistry, sections were first treated for in situ hybridization and then for BrdU detection as described above.

Detection of apoptotic cell death

Cells undergoing apoptotic cell death were detected on sections by TUNEL analysis using the Apop-Tag Kit (Oncor).

Cell counts

The fraction of *lacZ*-expressing cells located in the mantle layer of heterozygotes and homozygotes was determined on β -gal/BrdU double-stained transverse sections through the hindbrain of E10.5 embryos after a 1 hour BrdU pulse. To count the cells in the mantle layer, defined as the region of the neural tube lateral to the outermost BrdU-positive cells, the total β -gal-positive cells and those in the mantle layer were counted in the ventral β -gal-positive column on every third section through the entire extent of r4. A minimum of 4,000 cells on 11 sections from two embryos were counted for each genotype.

RESULTS

Absence of visceral and branchial motor neurons in the hindbrain of late-gestational *Phox2b^{lacZ/lacZ}* embryos

The bm and vm nuclei of the mammalian hindbrain constitute the bm nuclei of the trigeminal (nV) and facial (nVII) nerve, the vm neurons of the facial nerve, the nucleus ambiguus (nA), which contains both bm and vm neurons, and the vm dorsal motor nucleus of the vagal nerve (dmnX), all of which express *Phox2b* (Pattyn et al., 1997). We examined *Phox2b^{lacZ/lacZ}* mutant embryos for the presence of these nuclei at late gestational stages, when most of them can be identified as discrete histological structures in wild-type mice. *Phox2b^{lacZ/lacZ}* mice die around embryonic day 14 (E14), but they can be rescued up to birth by supplying them with noradrenergic agonists (Thomas and Palmiter, 1997), demonstrating that their embryonic lethality is due to the lack of noradrenaline (Pattyn et al., 1999, 2000). In rescued E18.5 homozygous mutants, nV, nVII, dmnX and nA were conspicuously absent on the grounds of either Nissl stain (Fig. 1A,A',C,C' and not shown) or *peripherin* (Escurat et al., 1990) expression (Fig. 1B,B',D-F'). By contrast, the sm nuclei of the hypoglossal (nXII) and abducens (nVI) nerves appeared intact (Fig. 1F-F'), consistent with the fact that they never express *Phox2b*. The nIII (Fig. 1H,H') and nIV (not shown), which do

express *Phox2b*, also looked grossly normal, presumably because of compensation by *Phox2a* whose expression precedes that of *Phox2b* in these nuclei and is required for their formation (Pattyn et al., 1997). The vm neurons of the facial nerve could not be examined at this stage, since they do not form a recognizable nucleus. Their absence can be inferred, however, from observations made at earlier stages.

Therefore, all bm and vm motor nuclei fail to form or degenerate in the hindbrain of *Phox2b^{lacZ/lacZ}* embryos. To analyze more precisely the effects of the *Phox2b* mutation on neuronal differentiation, we examined the fate of the cranial motor neuron precursors at earlier stages of embryogenesis.

Lack of cell-type-specific differentiation of branchial and visceral motor neuron precursors in *Phox2b^{lacZ/lacZ}* embryos

To explore the differentiation status of motor neuron precursors, we made use of the *lacZ* gene which is integrated in the *Phox2b* locus of the mutants and whose expression in heterozygotes faithfully reproduces the expression pattern of the wild-type allele (data not shown). Therefore, β -galactosidase (β -gal) expression is a reliable marker of mutant cells, with the possible exception of stages and sites where *Phox2b* promoter activity depends on the Phox2b protein itself.

In E10.5 heterozygotes, *lacZ* expression in the hindbrain is organized in three longitudinal columns, in line with our previous data on Phox2b mRNA and protein expression (Pattyn et al., 1997; Fig. 2A). Bm and vm neurons are generated in the ventral column (arrowhead in Fig. 2A,A'; Ericson et al., 1997), starting at around E9.5 (Taber Pierce, 1972). The thickenings of the ventral column in rhombomere 2 (r2) and r4 correspond to the *Phox2b*-expressing precursors of the trigeminal (nV) and facial (nVII) bm neurons, respectively (Pattyn et al., 1997). Two hallmarks of early motor neuronal differentiation were revealed by the β -gal stain in the hindbrains of E10.5 *Phox2b^{+/lacZ}* embryos: first, axonal outgrowth was already well under way in r2 and r4, where the axons could be seen converging towards the future exit points of the Vth and VIIth cranial nerves, respectively (asterisks in Fig. 2A). *lacZ* expression also revealed the canonical patterns of vm and bm neuronal migrations: at E10.5, dorsal migration of motor neurons resulted in mediolateral streaks of β -gal staining, most conspicuously caudal to r6, where the dmnX neurons are known to arise at this stage (Ericson et al., 1997). By E11.5, this migration had entirely depleted the ventral columns of β -gal-positive cells except in r4 (Fig. 2B). At this stage, the nVII bm precursors born in r4 were seen migrating caudally through r5 and into r6, where they turn dorsally (Pattyn et al., 1997). In homozygous mutants, the *lacZ*-expressing precursors were readily visualized by β -gal staining, although there were already fewer cells at E10.5 except in r4 (Fig. 2A'). This may reflect the fact that the neurons of nV, nA and dmnX are born slightly earlier than those of nVII (Taber Pierce, 1972). Conspicuously, the mutant cells showed neither signs of axonal outgrowth nor signs of lateral or caudal cell migration (Fig. 2A'). This was especially obvious for the nVII precursors, which remained confined to r4 in E11.5 mutants (Fig. 2B'). Hence, precursors of bm and vm neurons do arise on schedule in *Phox2b^{lacZ/lacZ}* embryos, but they fail to put out axons and to emigrate from the region where they are born.

The inference that the mutant precursors are unable to

differentiate was further substantiated by using two early markers of cranial bm and vm differentiation, *Phox2a* and *Islet1*. In the rhombencephalon, *Phox2a* expression in motor neurons is restricted to vm and bm neurons and is initiated after that of *Phox2b*, once the cells have become postmitotic (Tiveron et al., 1996; Pattyn et al., 1997; Fig. 2C). *Islet1* is expressed by all motor neuron precursors and is similarly restricted to postmitotic cells (Pfaff et al., 1996; Fig. 2D). There was a complete lack of *Phox2a* expression in the ventral column of *Phox2b^{lacZ/lacZ}* embryos (Fig. 2C').

Islet1 expression was drastically reduced and restricted to r5 and to the caudal rhombencephalon and cervical spinal cord (Fig. 2D'). This residual expression corresponds to precursors of sm neurons, which do not express either *Phox2a* or *Phox2b*. Their identity as sm neurons was confirmed by *Islet2* expression, which is absent from vm and bm neurons (Varela-Echevarría et al., 1996; Ericson et al., 1997; Osumi et al., 1997; data not shown). Therefore, no sign of bm or vm neuronal differentiation is detectable in the hindbrain of *Phox2b^{lacZ/lacZ}* embryos.

Early differentiation block of branchial motoneuronal precursors in *Phox2b^{lacZ/lacZ}* embryos

To analyze in detail the abnormalities of neuronal differentiation in *Phox2b^{lacZ/lacZ}* mice, we focused on the ventral region of r4 at E10.5. At this stage, the ventral aspect of r4 is the site of an intense generation of *Phox2b*-positive cells, which are destined to become nVII bm neurons (Pattyn et al., 1997; McKay et al., 1997). In this region, as elsewhere in the ventral *Phox2b*-positive column, *Phox2b* expression starts in the dividing progenitors of the neuroepithelium and persists in their postmitotic descendants, which migrate basally (i.e. away from the lumen) and into the mantle layer (Pattyn et al., 1997). *lacZ* expression faithfully reproduced this pattern (see Fig. 3B). For the sake of clarity, we will hereafter reserve the term "progenitor" for the cycling neuroblasts in the neuroepithelium and will use the term "precursor" for, collectively, progenitors and neurons that are not yet fully differentiated, even though they are postmitotic.

At E10.5, many cells expressing class III β -tubulin, a general marker of early postmitotic neurons, accumulate in the lateral aspects (or mantle layer) of ventral r4 in wild-type and *Phox2b^{+/lacZ}* animals (Fig. 3A). By contrast, in the corresponding region of the mutants, very few β -tubulin-positive cells are found (Fig. 3A') that, in double-labelling experiments, do not express *lacZ* (Fig. 3B') and therefore arise from *Phox2b*-negative progenitors. The *Ebf1* gene, a member of the Ebf/Olf-1/collier family of transcription factors, whose expression is similarly restricted to early postmitotic neurons (Garel et al., 1997; Wang et al., 1997), showed an expression pattern very similar to β -tubulin in heterozygotes in this region (Fig. 3C) and was also absent in homozygotes (Fig. 3C').

Next, we examined genes that are transiently expressed in postmitotic precursors as they emerge from the neuroepithelium, but are downregulated when the neurons settle in the mantle layer. These genes thus define an intermediate stage of differentiation and are mainly expressed in cells located at the internal margin of the mantle layer. *Math3* is a bHLH gene related to *Drosophila atonal* (Takebayashi et al., 1997) that fits in this category. Its avian homologue, *NeuroM*, is expressed in postmitotic cells just

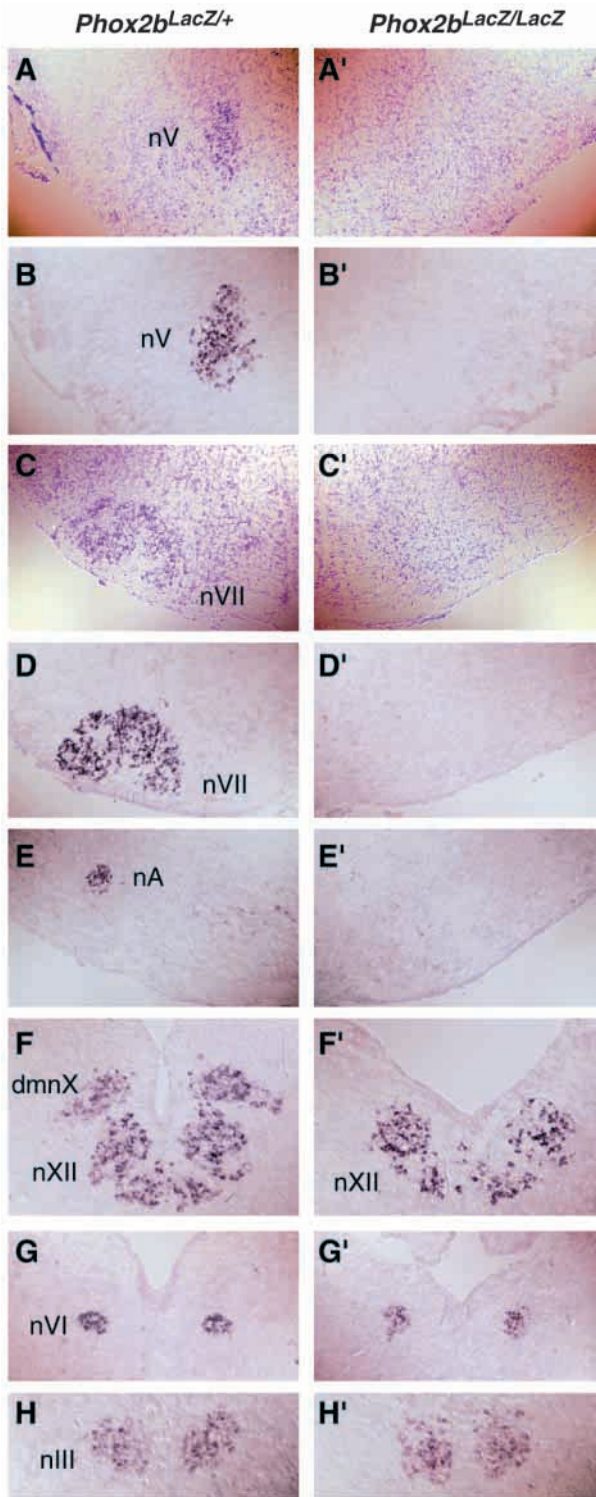
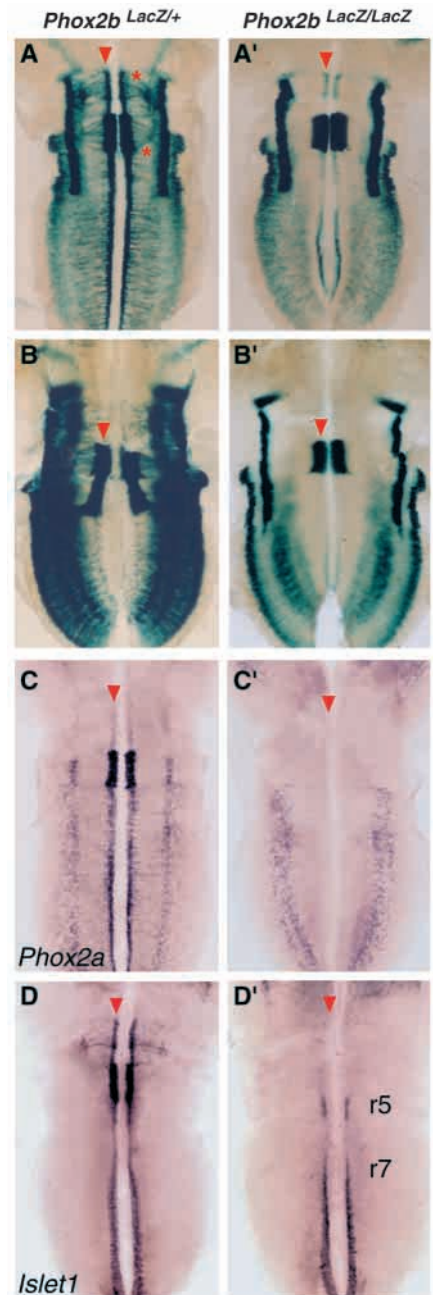


Fig. 1. Absence of visceral and branchial motor nuclei in the hindbrain of E18.5 *Phox2b^{lacZ/lacZ}* mice. Nissl stain (A,A',C,C') and in situ hybridization with a *peripherin* probe (B,B',D-H') on transverse sections through the brainstem of E18.5 heterozygous (A-H) or homozygous mutant embryos (A'-H'). The bm and vm nuclei – the trigeminal (nV, A-B') and facial (nVII, C-D') nuclei, the nucleus ambiguus (nA, E,E') and the dorsal motor nucleus of the vagus nerve (dmnX, F,F') – are missing in *Phox2b^{lacZ/lacZ}* mice. By contrast, the sm nuclei of the hypoglossal (nXII, F,F') and abducens nerve (nVI, G,G') are spared by the mutation, as is the oculomotor (nIII, H,H') nucleus.

Fig. 2. Absence of early differentiation of branchial and visceral motor neuron precursors in *Phox2b^{lacZ/lacZ}* mice. (A-B') β -gal stain on flat-mounted E10.5 (A,A') and E11.5 (B,B') hindbrains of heterozygous (A,B) and homozygous (A',B') mice. At E10.5, the *Phox2b* locus is normally expressed in three longitudinal columns on either side (A), of which the most ventral corresponds to the precursors of the vm and bm neurons (arrowhead). β -gal staining also reveals the dorsal migration of bm and vm precursors, most conspicuously caudal to r6, and axon outgrowth in r2 and r4 where the nV and nVII precursors are differentiating (asterisk). In homozygous embryos (A'), this ventral column (arrowhead) is well preserved only in r4 where, however, no signs of axon outgrowth are visible. (B) At E11.5, the ventral column has been depleted of β -gal-positive cells by their dorsal migration, except for the facial bm precursors, which are now migrating caudally through r5 and r6 (arrowhead). In homozygous embryos (B'), β -gal-positive precursors are still present in r4 (arrowhead), but the caudal migration of the facial precursors does not occur. (C-D') Whole-mount in situ hybridization on E10.5 flat-mounted hindbrains of heterozygous (C,D) and homozygous (C',D') mutants with *Phox2a* (C,C') and *Islet1* (D,D') probes. In heterozygotes (C), *Phox2a* is expressed by the precursors of the vm and bm neurons, but is absent in homozygotes (C') (arrowhead). The residual *Phox2a* staining probably corresponds to precursors of neurons of the reticular formation, which do not depend on *Phox2b*. In the hindbrain, *Islet1* is normally expressed in all vm, bm and sm neurons (D). In homozygous embryos (D'), *Islet1* expression is absent in the bm and vm precursors, but is still observed in precursors of sm neurons: nVI neurons, which derive from r5, nXII neurons which derive from r7-r8, and sm neurons of the cervical spinal cord further caudally.



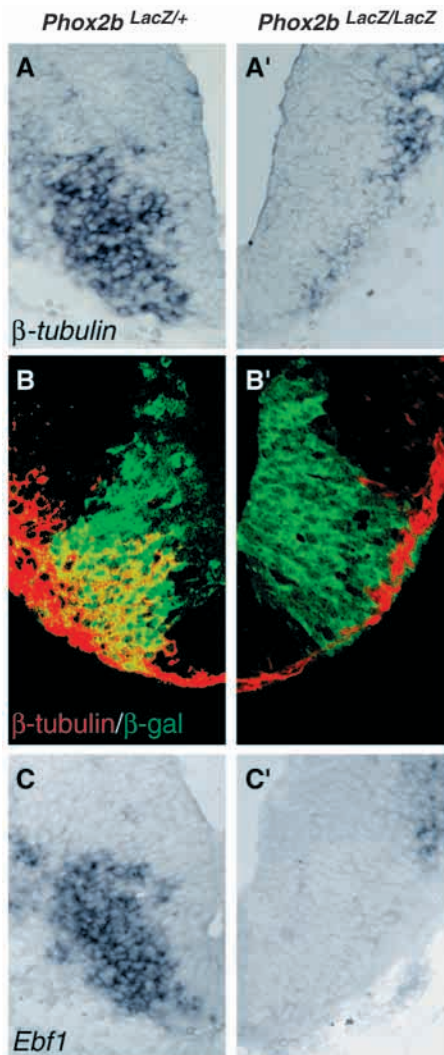


Fig. 3. Lack of generic neuronal differentiation in facial motor neuron precursors from *Phox2b^{lacZ/lacZ}* mice. (A,A',C,C') In situ hybridization with *class III β-tubulin* (A,A') and *Ebf1* (C,C') probes on transverse sections through r4 of heterozygous (A,C) and homozygous (A',C') E10.5 embryos. In ventral r4 of heterozygotes, *β-tubulin* and *Ebf1* are expressed in postmitotic neurons, which accumulate in the mantle layer (A,C). In the homozygotes, *Ebf1* expression is abolished in ventral r4 and *β-tubulin* is weakly expressed in very few cells. (B,B') Confocal views of double-immunofluorescence stains with anti-*β-gal* (green) and anti-*β-tubulin* (red, double-labeled cells in yellow) on transverse sections of r4 from E10.5 heterozygous (B) and homozygous mutant (B') embryos, showing that the *β-tubulin*-positive cells remaining in ventral r4 are *lacZ*-negative and thus presumably correspond to neurons that have immigrated from more dorsal regions.

outside the neuroepithelium (Roztocil et al., 1997). We found a similar pattern of expression for *Math3* in the mouse hindbrain, except that, in addition, we could detect scattered *Math3*-positive cells in the ventricular zone, which probably correspond to postmitotic precursors en route to the mantle layer (Fig. 4A). A very similar expression pattern has been described for *Ebf2* and *Ebf3*, members of the same gene family as *Ebf1* (Garel et al., 1997). In ventral r4, *Ebf2*- and *Ebf3*-positive cells were essentially confined to the internal margin

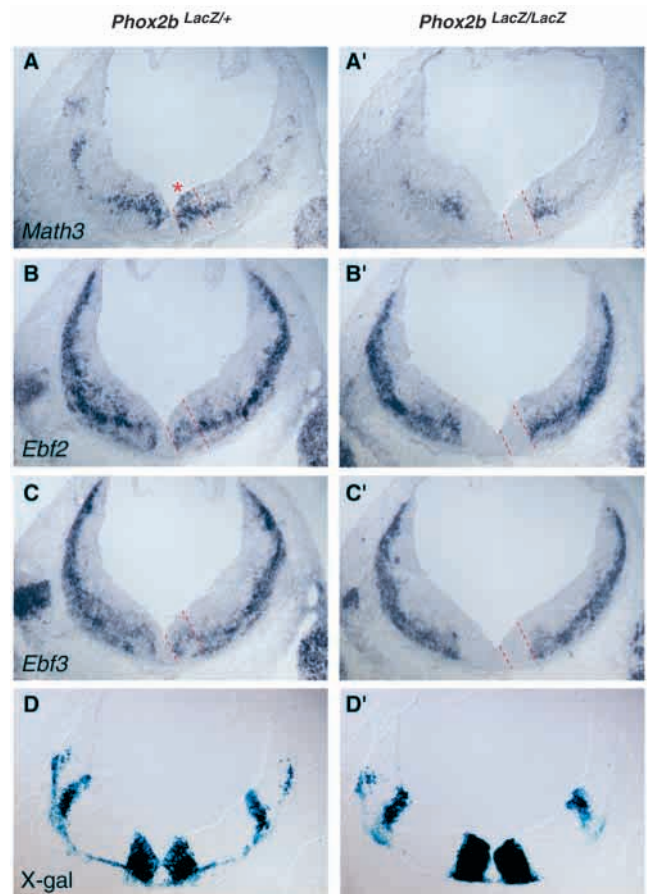


Fig. 4. Lack of early postmitotic markers in the ventral region of rhombomere 4 in *Phox2b^{lacZ/lacZ}* mice. In situ hybridization with *Math3* (A,A'), *Ebf2* (B,B') and *Ebf3* (C,C') probes, and *β-gal* stain (D,D') on serial consecutive sections through r4 of E10.5 heterozygous (A-D) and homozygous mutant (A'-D') mice. In ventral r4 of heterozygotes, *Math3*, *Ebf2* and *Ebf3* are transiently expressed on cells in the process of emigrating from the neuroepithelium and accumulate at the internal margin of the mantle layer. Note that scattered *Math3*- (but not *Ebf2*- or *Ebf3*-) positive cells are present close to the lumen (asterisk). In homozygotes, expression of these genes is abrogated in the ventral region of r4 that corresponds to the intensely *β-gal*-stained region on adjacent sections (D' and dotted lines in A',B',C').

of the mantle layer (Fig. 4B,C). In *Phox2b^{lacZ/lacZ}* embryos, *Math3*, *Ebf2* and *Ebf3* failed to be expressed in the r4 *β-gal*-positive ventral region (Fig. 4A'-D').

Finally, we examined genes whose expression is normally restricted to the ventricular zone and can therefore be assumed to coincide with or precede that of *Phox2b*. The expression of the homeobox gene *Nkx2.2* (Shimamura et al., 1995) precisely delimits the neuroepithelial region where the *Phox2b*-positive cells are born (Fig. 5A) and is sharply downregulated in the mantle layer where *β-tubulin*-expressing cells accumulate in heterozygotes (Fig. 5B,C). In the *Phox2b^{lacZ/lacZ}* embryos, *Nkx2.2* expression was preserved (Fig. 5C'). However, in contrast to the heterozygotes, where a large complement of *Phox2b*-positive, *Nkx2.2*-negative cells accumulates in the lateral aspect of the tube (Fig. 5A,C), in the homozygotes, no downregulation was

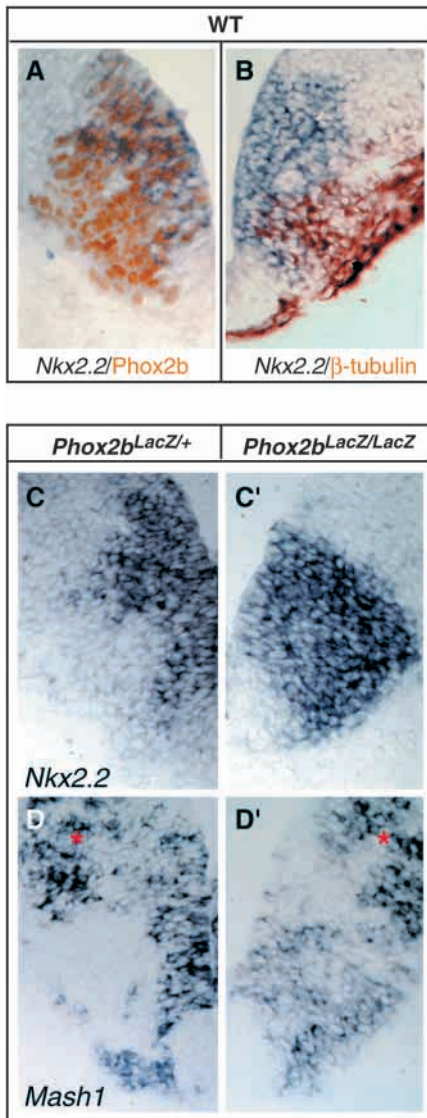


Fig. 5. *Nkx2.2* and *Mash1* expression is preserved in the neuroepithelium of *Phox2b^{lacZ/lacZ}* mice, but fails to be downregulated in the lateral aspects of the neural tube. (A,B) Combined in situ hybridization with a *Nkx2.2* probe and immunohistochemistry using anti-Phox2b (A) or anti-classIII β -tubulin (B) antibodies on transverse sections of E10.5 wild-type embryos. *Nkx2.2* is expressed in the ventral region of r4, which matches precisely the expression domain of Phox2b (A). In this region, *Nkx2.2* is restricted to the neuroepithelium and is downregulated in the mantle layer where β -tubulin is expressed (B). (C-D'). In situ hybridization with *Nkx2.2* (C,C') or *Mash1* (D,D') probes on transverse sections through r4 of E10.5 heterozygous (C,D) and homozygous mutant (C',D') embryos. In the heterozygotes (C), *Nkx2.2* is expressed in the neuroepithelium of ventral r4, excluding the mantle layer, while in the homozygotes (C'), its expression extends up to the external surface of the neural tube. In ventral r4 of heterozygous embryos (D), *Mash1* expression is also restricted to the neuroepithelium, while in homozygotes (D') it is expressed throughout the whole width of the neural tube. *Mash1* expression in the homozygous mutants is drastically reduced, compared to heterozygous or wild-type animals. This is best seen by comparing the signal intensity in the more dorsal regions (asterisk) to that in the ventral region where Phox2b is normally expressed. Note that the neuroepithelium is compressed in ventral r4, by the massive accumulation of facial motor neuron precursors in the mantle layer.

observed and *Nkx2.2* expression extended up to the basal surface of the neural tube (Fig. 5C').

We also found that the bHLH gene *Mash1*, a homologue of *Drosophila achaete-scute* (Johnson et al., 1990), was expressed in the neuroepithelium of ventral r4 in the salt-and-pepper pattern typical for vertebrate proneural genes (Ma et al., 1997). Like *Nkx2.2*, *Mash1* is downregulated in the wild-type mantle layer (Fig. 5D). *Mash1* expression was preserved in the neuroepithelium of homozygous embryos, albeit at a drastically reduced level (Fig. 5D'). As in the case of *Nkx2.2*, downregulation in the lateral aspects of the tube was not observed in the mutants.

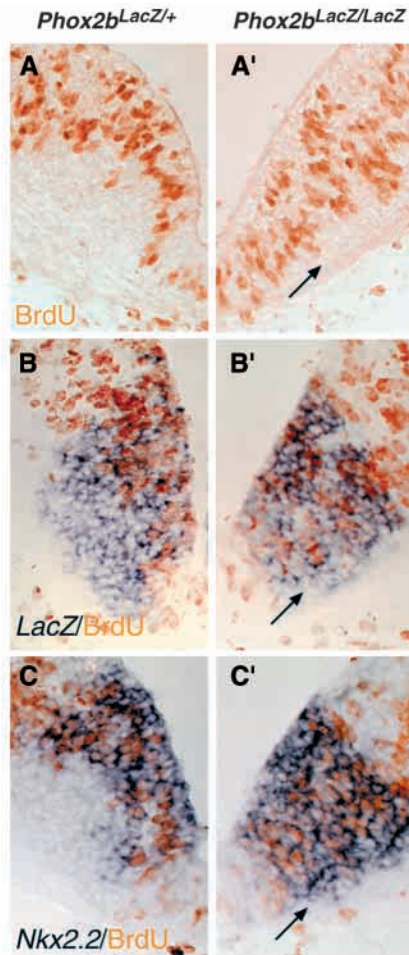
Therefore, differentiation events subsequent to the onset of *Phox2b* expression, including the induction of *Math3*, *Ebf1-3* and β -tubulin, as well as the downregulation of *Nkx2.2* and *Mash1*, fail to occur in the nVII anlage of *Phox2b^{lacZ/lacZ}* embryos. Furthermore, the decrease in *Mash1* expression suggests that Phox2b acts already in dividing progenitors.

Atrophic mantle layer and early cell death of motor neuronal precursors in *Phox2^{lacZ/lacZ}* embryos

As described in the previous section, the region where nVII bm precursors form shows neither upregulation of postmitotic markers nor downregulation of neuroepithelial markers in the mutants. The question thus arose whether this region would entirely fail to form a mantle layer. We investigated this possibility by bromodeoxyuridine (BrdU) labelling of mutant and heterozygous embryos. BrdU is incorporated into nuclei in S-phase, which are located in the basal (i.e. outer) part of the neuroepithelium (Guthrie et al., 1991) before migrating apically, where the cells divide close to the lumen. In the following, we operationally define the neuroepithelium, where the progenitors are found, as the compartment delimited internally by the lumen of the tube and externally by the outermost nuclei in S-phase, as detected by a 1- to 3-hour pulse of BrdU, and the mantle layer as the compartment external to those nuclei (see Fig. 6A,B).

In the heterozygotes, BrdU-positive nuclei labelled by a 1 hour pulse accumulated, as expected, in the outer two thirds of the neuroepithelium. In ventral r4, this compartment was compressed towards the midline by the ventrolateral accumulation of postmitotic cells in the mantle layer (Fig. 6A). In homozygous mutants, this compression was not seen and the pattern in ventral r4 resembled that found more dorsally (Fig. 6A'). However, a cluster of cells that had not incorporated BrdU was found lateral to the BrdU-positive cells, i.e. in the mantle layer (arrow in Fig. 6A',B'), suggesting that some mutant cells are capable of migrating and settling outside the neuroepithelium. These cells expressed *lacZ* (Fig. 6B') and remained unlabelled even after a 3-hour BrdU pulse, which labels the whole width of the neuroepithelium (Fig. 6B,B'), further suggesting that they have followed the usual pattern of emigration from the neuroepithelium after completion of their last mitosis. Cell counts revealed that the β -gal-positive cells in the mantle layer represented only $14 \pm 0.7\%$ (mean \pm s.e.m.) of the total β -gal-positive population in ventral r4 of *Phox2b^{lacZ/lacZ}* embryos, compared to $41 \pm 0.8\%$ (mean \pm s.e.m.) in the heterozygotes. The proportion of β -gal-positive cells located in the mutant mantle layer was thus decreased by 65%, demonstrating a substantial depletion of the pool of postmitotic bm precursors in *Phox2b^{lacZ/lacZ}* embryos. Double-labelling

Fig. 6. Depletion of the mantle layer in the ventral r4 region of *Phox2b^{lacZ/lacZ}* mice. (A-B') BrdU labelling after a 1-hour BrdU pulse (A,A') or combined in situ hybridization with a *lacZ* probe and BrdU labelling after a 3-hour BrdU pulse (B,B') on transverse sections through r4 of E10.5 heterozygous (A,B) and homozygous mutant (A',B') mice. In heterozygotes, BrdU-positive nuclei are located in the basal two thirds of the neuroepithelium after a 1-hour pulse (A). After a 3-hour pulse, BrdU is detected throughout the width of the neuroepithelium, but is still excluded from the mantle layer (B). In this region, BrdU-positive cells are compressed towards the midline probably because of the massive accumulation of postmitotic cells laterally. In the homozygotes (A',B'), the neuroepithelium is not compressed towards the lumen. The lateral aspects of the neural tube in the *lacZ*-expressing region remain BrdU-negative even after a 3-hour pulse, suggesting that they are composed of postmitotic cells which have emigrated from the neuroepithelium (B'). The BrdU-negative mantle layer is massively decreased in size (arrow in A',B'). (C,C') Double labelling for *Nkx2.2* and BrdU incorporation shows that the population of BrdU-negative cells in the lateral aspects of the tube express *Nkx2.2* (arrow).



experiments confirmed that this same group of cells had failed to downregulate *Nkx2.2* and *Mash1* (Fig. 6C,C' and not shown).

One possible cause for the depletion of the mantle layer is the early death of motor neuron precursors. We monitored apoptotic cell death in the ventral column of the rhombencephalon by TUNEL analysis. Flat mounts of hindbrains from heterozygous animals showed a very low incidence of cell death in the ventral β -gal-positive region (Fig. 7A). There was a marked increase in TUNEL-positive cells in the same region from homozygous mutants (Fig. 7A'). On transverse sections, cell death appeared to occur throughout the wall of the neural tube, including the neuroepithelium (Fig. 7B,7B'). Therefore, many motor neuron precursors die before their exit from the neuroepithelium. Those that do reach the mantle layer fail to upregulate postmitotic markers and to downregulate neuroepithelial markers. Fig. 8 summarizes the

Fig. 7. Massive apoptosis of motor neuron precursors in homozygous *Phox2b^{lacZ/lacZ}* embryos. (A,A') flat mounts of the hindbrain of E10.5 heterozygous (A) and homozygous (A') mutants stained by the TUNEL method. (B,B') Transverse sections through r4 of E10.5 heterozygous (B) and homozygous mutant (B') mice shows that the TUNEL-positive cells are distributed throughout the wall of the neural tube.

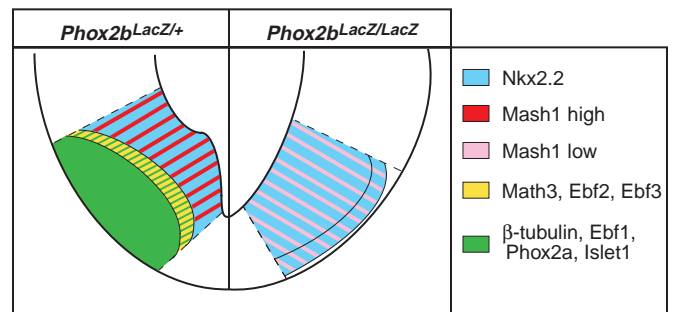
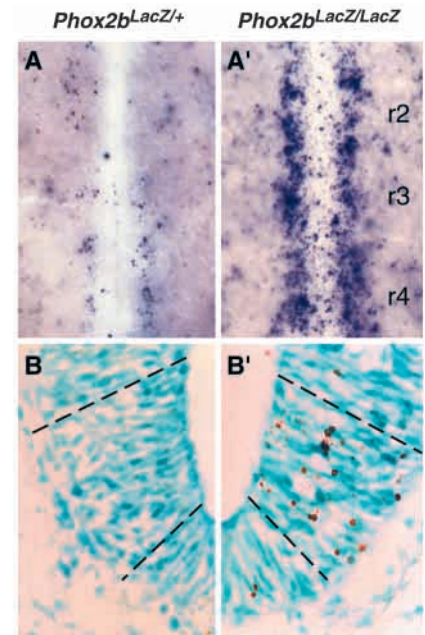


Fig. 8. Schematic representation of gene expression by facial branchial motor neuron precursors from E10.5 wild-type and mutant embryos. (Left) The cycling facial branchiomotor progenitors in the neuroepithelium express *Nkx2.2* and *Mash1* (blue and red stripes). Following their last mitosis, the cells, which are emigrating to the mantle layer, switch on *Math3* (not shown). As these cells leave the neuroepithelium, they accumulate at the internal margin of the mantle layer, downregulate *Nkx2.2* and *Mash1*, maintain *Math3*, switch on *Ebf2* and *Ebf3* (yellow) and slightly later *Ebf1*, *class III β -tubulin*, *Phox2a* and *Islet1* (green stripes). Once cells have settled in the mantle layer, they downregulate *Math3*, *Ebf2* and *Ebf3* but maintain expression of *Ebf1*, β -tubulin, *Phox2a* and *Islet1* (green). (Right) In homozygous *Phox2b* mutants, the cycling cells in the neuroepithelium still express *Nkx2.2* and *Mash1* (albeit reduced levels of *Mash1*, see Fig. 6) (blue and pink stripes). Some cells do become postmitotic as defined by the absence of BrdU incorporation and accumulate in the mantle layer (demarcated by a line), but fail to downregulate *Nkx2.2* and *Mash1* (blue and pink stripes) such that *Nkx2.2* and *Mash1* are now expressed throughout the whole width of the neural tube. *Math3*, the *Ebf* genes, *class III β -tubulin*, *Phox2a* and *Islet1* are not expressed in the mutants. Dashed lines demarcate the *lacZ*-expressing region where *Phox2b* is expressed normally. Note that the compression of the β -gal-positive ventricular zone in the wild types compared to mutants (see for example Fig. 6C,C') is compensated for by its broadening on the dorsoventral axis, such that the size of the neuroepithelium is in fact virtually unchanged between mutant and wild-type.

alterations in gene expression patterns in the facial bm precursors that occurs in *Phox2b*^{lacZ/lacZ} embryos.

DISCUSSION

Phox2b controls the generation of branchial and visceral motor neurons of the hindbrain

Our results establish that *Phox2b* is necessary for the formation of all bm and vm neurons of the hindbrain, whereas it is dispensable for the formation of sm neurons. This ontogenetic partition parallels earlier observations that the bm and vm neurons of the hindbrain share features that set them apart from sm neurons. They are generated from *Nkx2.2*-positive progenitors ventral to sm neurons (Ericson et al., 1997; Briscoe et al., 1999; this study). Bm and vm neuron precursors then migrate dorsally (Ericson et al., 1997) and their axons exit laterodorsally from the hindbrain while sm neurons remain ventral and have ventral axonal exit points. The pattern of LIM-homeobox gene expression (Varela-Echevarría et al., 1996; Sharma et al., 1998) shows that, unlike sm neurons, neither bm nor vm neurons express *Islet-2* or *Lim3/Lhx3*. Finally, a comparative analysis of cranial nerves in lamprey and spinal nerves in amphioxus has led to the proposal that bm and vm motor neurons of vertebrates could both be homologues of the so-called visceral motor neurons of cephalochordates (Fritzsch and Northcutt, 1993a).

Phox2b is dispensable for the formation of oculomotor (nIII) and trochlear (nIV) nuclei, in superficial agreement with the fact that they are usually classified as somatic. However, we reported earlier (Pattyn et al., 1997) that they depend for their formation on *Phox2a*, the paralogue of *Phox2b*. This, in turn, echoes earlier suggestions that nIII and nIV differ from bona fide sm nuclei in several features that they share with bm and vm nuclei. First, their LIM code in chicken resembles that of bm and vm nuclei in that they never express *Lim3* (Varela-Echevarría et al., 1996; Ericson et al., 1997) (although they express *Islet2*, like sm neurons). Second, the axons of nIV neurons resemble those of vm and bm neurons in that they exit dorsally from the neural tube. Intriguingly, in lampreys, nIV is located dorsally, that is, in a position topologically equivalent to bm nuclei, and there is evidence that nIV neurons, like bm/vm neurons are generated ventrally before they migrate to their adult dorsal position (Fritzsch and Northcutt, 1993b). Third, they do not express the homeobox gene *HB9* unlike all other hindbrain and spinal somatic motor neurons (Thaler et al., 1999). Finally, a notable feature by which nIII and nIV differ from all other motor nuclei, is that they are the only ones to arise outside the domain of *Hox* gene expression. These features, together with their dependency on a *Phox2* gene, argue that nIII and nIV motoneurons are more related ontogenetically, and maybe phylogenetically, to bm and vm than to sm motoneurons and should probably be placed in a class of their own.

Phox2b controls two early steps in the differentiation of branchiomotor neurons

The mantle layer of the ventral rhombencephalon is markedly depleted in *Phox2b* knock-out embryos. Massive cell death occurs throughout the wall of the neural tube, showing that differentiation is disrupted inside the proliferative

neuroepithelium and that most precursors die before they emigrate to the mantle layer. Further support for a role of *Phox2b* in neuroepithelial cells is that the bHLH gene *Mash1* is expressed at strikingly reduced levels. This parallels our observation of *Mash1* early downregulation in the sympathetic ganglion primordia of *Phox2b* mutants (Pattyn et al., 1999b). Therefore, as in sympathetic ganglia, *Phox2b* maintains and/or boosts *Mash1* expression in motor neuron progenitors. This, in turn could increase the probability that a progenitor will reach a threshold for commitment to a neuronal fate. However, there is no defect in the facial nucleus of *Mash1*^{-/-} newborn mice (Hirsch et al., 1998) arguing that, in that region, *Mash1* function can be taken over by another, still unknown bHLH gene whose dependency on *Phox2b* is yet to be documented.

Some mutant neuroepithelial cells escape this early differentiation block, become postmitotic and exit the neuroepithelium, allowing the detection of a second, later requirement for *Phox2b* in early postmitotic precursors of the mantle layer. Indeed, in *Phox2b* mutants, the colonization of the mantle layer is uncoupled from all gene regulatory events that normally accompany it: there is neither upregulation of the earliest known markers for postmitotic differentiation, *Ebf2*, *Ebf3* and *Math3*, nor of later markers such as *Phox2a*, *Islet-1* and β -tubulin, nor downregulation of the neuroepithelial markers *Nkx2.2* and *Mash1*. As a consequence, mantle layer cells maintain a neuroepithelial identity. The lack of any sign of further generic differentiation (such as β -tubulin expression and axon outgrowth) makes it unlikely that the mutant cells undergo a fate switch but argue that whatever neurogenesis is preserved in the ventral r4 region of *Phox2b* mutants is abortive.

Generic versus subtype-specific control of neuronal differentiation by Phox2b

One of the main conceptual divides in the neuronal differentiation program is between general and specific aspects of neuronal differentiation. Clear cases of neuronal subtype-specific programs of differentiation are provided by transcription factors whose inactivation or ectopic expression results in fate switches. Among the few vertebrate transcription factors in this category are the homeobox genes *Pax6* (Ericson et al., 1997), *Nkx2.2* (Briscoe et al., 1999), *Lhx3* and *Lhx4* (Sharma et al., 1998). In contrast, the case for a generic subprogram of neuronal differentiation, which would be controlled independently of neuronal-subtype-specific subprograms, is weaker. For example, bHLH transcription factors of the proneural class have often been studied for their capacity to promote generic aspects of neuronal differentiation. However, it has been known for some time in the fly, and it has recently become clear in vertebrates as well, that they in fact couple specific and generic aspects of neuronal differentiation (reviewed in Brunet and Ghysen, 1999).

The exquisite specificity of *Phox2* gene expression in both the PNS and CNS makes them good candidates for controlling neuronal-subtype-specific differentiation programs. Such a role has been demonstrated in the PNS by gain-of-function experiments, both in vivo and in vitro (Stanke et al., 1999; Lo et al., 1999; Guo et al., 1999), thus confirming the role of *Phox2* genes in the induction of noradrenaline biosynthetic enzymes and the tyrosine kinase *Ret*, suggested first by loss-of-function experiments (Morin et al., 1997; Pattyn et al.,

1999b). Here we show that, in the CNS, *Phox2b* functions upstream of both genes that define a bm/vm neuronal phenotype (*Phox2a* and *Islet1*) and genes that are more general markers of early postmitotic precursors (*Ebf2*, *Ebf3*, *Math3*) and whose expression precedes or coincides with subtype-specific differentiation. Moreover, in the absence of *Phox2b* motor neuron progenitors express lower levels of *Mash1* and die in large numbers in the neuroepithelium, arguing for a role of *Phox2b* in the progression of cycling progenitors towards a neuronal fate. Thus, *Phox2b* exemplifies a type of transcriptional regulator that directly or indirectly controls both very general and sub-type-specific aspects of neuronal differentiation.

A transcription factor that plays an analogous role in the sm neuronal lineage has not been found to date. *Isl1* appears to be required for differentiation of all motor neurons, but its expression is restricted to postmitotic neurons and it can thus not act at an early commitment step (Pfaff et al., 1996). In embryos deficient in both *Lhx3* and *Lhx4*, motor neurons are generated initially on schedule and in normal numbers, but they acquire an identity resembling that of bm neurons (Sharma et al., 1998). Similarly, loss of *Hb9*, which is required for proper sm motor neuron specification, has no discernable effect on the number of motor neurons generated and their initial differentiation (Arber et al., 1999; Thaler et al., 1999). Clearly, further work is required to decide whether the level of control exerted by *Phox2b* on bm neuron differentiation operates in other neuronal lineages as well.

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