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The Phox2b transcription factor coordinately regulates neuronal cell cycle exit and identity

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SUMMARY

In the vertebrate neural tube, cell cycle exit of neuronal progenitors is accompanied by the expression of transcription factors that define their generic and sub-type specific properties, but how the regulation of cell cycle withdrawal intersects with that of cell fate determination is poorly understood. Here we show by both loss- and gain-of-function experiments that the neuronal-subtype-specific homeodomain transcription factor Phox2b drives progenitor cells to become post-mitotic. In the absence of *Phox2b*, post-mitotic neuronal precursors are not generated in proper numbers. Conversely, forced expression of *Phox2b* in the embryonic chick spinal cord drives ventricular zone progenitors to become post-mitotic neurons and to relocate to the mantle layer. In the neurons thus generated, ectopic expression of *Phox2b* is sufficient to initiate a programme of motor neuronal differentiation characterised by expression of *Islet1* and of the cholinergic transmitter phenotype, in line with our previous results showing that *Phox2b* is an essential determinant of cranial motor neurons. These results suggest that Phox2b coordinates quantitative and qualitative aspects of neurogenesis, thus ensuring that neurons of the correct phenotype are generated in proper numbers at the appropriate times and locations.

Key words: Phox2b, Neuronal cell cycle, Vertebrate, Motor neuron, Spinal cord

INTRODUCTION

All neurons of the vertebrate CNS are derived from the neuroepithelium, which forms the wall of the embryonic neural tube and generates a vast array of neuronal phenotypes. Early in CNS development, neuroepithelial cells generate more neuroepithelial cells by symmetrical divisions. At a given stage, which varies according to rostrocaudal and dorsoventral position, the dividing neuroepithelial progenitors start generating neuronal precursors, which leave the neuroepithelium or ventricular zone (VZ), generally after completion of their last mitosis, and begin to differentiate. Hence, exit from the cell cycle and the decision to become a neuron are tightly linked (Altman and Bayer, 1984; Rakic, 1988; McConnell, 1995; Huttner and Brand, 1997). Lateral inhibition mediated by the Delta-Notch intercellular signalling pathway is thought to negatively regulate cell cycle withdrawal, thus limiting the number of neurons produced (Chitnis et al., 1995; de la Pompa et al., 1997; Henrique et al., 1997; see Lewis, 1998, for review). In addition, some of the growth factor requirements for neuroepithelial proliferation have been identified (Cameron et al., 1998). However, the cell-intrinsic determinants that positively regulate cell cycle exit in the vertebrate neural tube remain largely unknown.

In *Drosophila*, differentiation along a neural pathway requires the activity of the proneural genes that code for basic-helix-loop-helix (bHLH) transcription factors (Jan and Jan, 1993). In vertebrates, a number of related genes have proneural activity in that they induce ectopic or supernumerary neurons when misexpressed in *Xenopus* or zebrafish embryos (for reviews, see Lee, 1997; Anderson, 1999; Brunet and Ghysen, 1999). The ectopic neurons generated express genes characteristic of post-mitotic neurons (Ma et al., 1996; Blader et al., 1997). This implies that the vertebrate proneural genes promote cell cycle withdrawal in addition to conferring neuronal properties, but positive regulation of cell cycle exit by these genes has not been demonstrated in the neural tube. Rather, lack of *Mash1*, a gene of the bHLH class homologous to *Drosophila* proneural genes, has been found to cause a decrease in progenitor proliferation in the VZ (Casarosa et al., 1999; Horton et al., 1999). Recently, the winged helix transcription factor XBF-1 has been found to promote neuronal differentiation and to inhibit cell proliferation when misexpressed at low doses in the *Xenopus* neural plate (Hardcastle and Papalopulu, 2000). Paradoxically, however, inactivation of its mouse orthologue caused reduced proliferation and premature differentiation of neuroepithelial cells (Xuan et al., 1995).

The period of acquisition of neuronal identity starts before and extends beyond completion of the final mitosis. This has been studied most intensively during motor neuron development (Ericson et al., 1997; Sharma et al., 1998; Tanabe

et al., 1998; Briscoe et al., 1999; Briscoe et al., 2000). Three general classes of motor neurons can be distinguished: the somatic motor (sm) neurons that innervate most skeletal muscles in the body, the branchiomotor (bm) neurons that innervate the branchial arch-derived muscles of the face and jaw, and visceral motor (vm) neurons that innervate sympathetic and parasympathetic ganglia. The dividing progenitors of most sm and of the spinal vm neurones, i.e. of the motor neurones that send axons ventrally from the neural tube, express the LIM homeobox genes *Lhx3* (*Lim3*) and *Lhx4* (Varela-Echevarría et al., 1996; Ericson et al., 1997). In embryos lacking both genes, post-mitotic spinal motor neurons are still generated in proper numbers, but acquire identities reminiscent of cranial bm or vm neurons (Sharma et al., 1998; Tanabe et al., 1998). Conversely, ectopic expression of *Lhx3* in the hindbrain leads to the generation of ventrally projecting neurons at the expense of a cranial vm phenotype, but does not seem to influence the number of neurons generated (Sharma et al., 1998). Likewise, the homeobox gene *MNR2* is switched on by chick motor neuron progenitors just before their final division. When misexpressed in the spinal cord region of the chick neural tube, ectopic *MNR2* is able to induce an sm differentiation programme (Tanabe et al., 1998), characterised by expression of the Islet1 and HB9 transcriptional regulators, which are required for post-mitotic sm differentiation (Pfaff et al., 1996; Arber et al., 1999; Thaler et al., 1999). However, timing and extent of neurogenesis appear to be controlled by an *MNR2*-independent process (Tanabe et al., 1998). In the mouse, an *MNR2* homologue has not been found, and the closely related *HB9* gene may have subsumed *MNR2* function in this species. In mice lacking *HB9*, motor neurons transiently express interneuron markers and acquire inappropriate subtype identities, but they are still generated in correct numbers (Arber et al., 1999; Thaler et al., 1999). Upstream of these transcription factor genes, another set of homeodomain proteins subdivides the VZ into different progenitor domains and is responsible for conferring distinct progenitor identities (Briscoe et al., 2000). Nkx6.1 in particular is expressed in the motor neuron progenitors and induces a sm phenotype when misexpressed in the dorsal spinal cord. However, no effects of Nkx6.1 on neurogenesis per se have been reported. Thus, although exit from the cell cycle and acquisition of subtype-specific (as well as generic) neuronal identity appear tightly coordinated in time, how this is achieved has not been elucidated.

In the embryonic mouse (Pattyn et al., 1997) and chick (J. Gay and J. F. B., unpublished results) hindbrain, the closely related paired-type homeobox genes *Phox2a* and *Phox2b* are expressed by all bm and vm, but not by sm neurons. *Phox2b*, but not *Phox2a*, is already expressed in the dividing progenitors and expression of both genes persists in the differentiating mantle layer neurons (Pattyn et al., 1997; Pattyn et al., 2000). The analysis of the *Phox2b* knockout phenotype has uncovered two successive functions of this transcription factor in hindbrain motor neurons. In the progenitors, *Phox2b* activity is required for high level expression of the bHLH family member *Mash1*. In the post-mitotic precursors, it is necessary for all further differentiation, both generic and type-specific (Pattyn et al., 2000). Here, we demonstrate that in the absence of *Phox2b*, post-mitotic motor neurons are not generated in proper numbers in the VZ. Conversely, forced expression of *Phox2b* in the presumptive spinal cord of chick embryos, where it is normally not expressed, promotes expression of early post-mitotic markers and migration into the mantle layer. In addition, *Phox2b* induces ectopic expression of motor neuron markers in the dorsal spinal cord. Together, these results suggest that Phox2b coordinately regulates the decisions to exit the cell cycle and to become a particular type of neuron.

MATERIALS AND METHODS

Mice

The generation of the *Phox2b*$^{LacZ/LacZ}$ mice used in this study has been described (Pattyn et al., 2000). In this line, targeted insertion of the *LacZ* gene allows for visualisation of the mutant cells that would normally express the inactivated gene.

Expression vectors

The coding regions of mouse *Phox2b* (Pattyn et al., 1997) and mouse *Otlx2* (*Pitx2*) (Mucchielli et al., 1996) were cloned between the *Eco*RI and *Xho*I sites of the pCAGGS vector, which drives expression by a CMV/actin hybrid promoter (Koshiba-Takeuchi et al., 2000). *GFP* was expressed from the *pCAGGS-AFP* vector (Momose et al., 1999).

Electroporation

Chick embryos 44-52 hours old (HH 12-14) were electroporated in ovo essentially as described (Funahashi et al., 1999). The *mPhox2b* (3 mg/ml) or *Pitx2* (5 mg/ml) expression vectors plus *pCAGGS-AFP* (0.8 mg/ml) or the empty vector (3 mg/ml) plus *pCAGGS-AFP* (0.8 mg/ml) were injected into the lumen of the neural tube at spinal cord levels. A pair of electrodes flanking the neural tube delivered six pulses (25 V, 30 mseconds each) from a BTX square electroporator. Embryos were allowed to develop at 38°C before processing at 24 or 48 hours after electroporation (24 h.a.e. or 48 h.a.e., respectively). GFP expression was used to monitor efficiency and extent of electroporation.

Histological methods

Antisense RNA probes for *ChAT* (Tanabe et al., 1998), *EGFP* (Clontech), *Dll1* (Bettenhausen et al., 1995), *Delta1* (Henrique et al., 1997), *E. coli LacZ* (Pharmacia), *Hes5* (Takebayashi et al., 1995), *Islet2* (Tsuchida et al., 1994), *NeuroM* (Roztocil et al., 1997), *cPhox2a* (Ernsberger et al., 1995), *mPhox2b* (Pattyn et al., 1997), *Pitx2* (Mucchielli et al., 1996) and *class III β-tubulin* (Lee et al., 1990) were labelled using a DIG-RNA labelling kit (Roche). In situ hybridization and combined in situ hybridization and immunohistochemistry on cryosections were done as described (Hirsch et al., 1998), except that for chick embryos post-hybridization washes were in 50% formamide, 2×SSC, 0.1% Tween 20. For immunohistochemistry, the following antibodies were used: monoclonal anti-BrdU (Sigma), monoclonal anti-GFP (Roche), monoclonal anti-Islet1/2 (Tsuchida et al., 1994) and rabbit anti-mouse Phox2b (Pattyn et al., 1997). TUNEL analysis was done as described (Pattyn et al., 2000). BrdU incorporation and detection in mouse (Pattyn et al., 2000) and chick (Sechrist and Marcelle, 1996) embryos were done as described. Pictures were taken with a Kappa DX30 CCD camera using Kappa software and assembled using Adobe photoshop.

Quantitative analyses

The number of *Dll1*$^{+}$ cells in the VZ from *Phox2b* knockout embryos was assessed on *Dll1*/BrdU double-stained sections through the hindbrain of embryonic day (E)10.5 embryos. The *LacZ*-expressing area was delimited by β-galactosidase staining of adjacent sections. A minimum of 680 *Dll1*$^{+}$ cells were counted for each genotype on every third section, and 16 sections from four embryos were analysed for each genotype.

The effects of *Phox2b* misexpression were quantified at 24 h.a.e. by counting the number of BrdU$^+$, *Delta1*$^+$ and *NeuroM*$^+$ cells in the electroporated region on transverse spinal cord sections from *mPhox2b*-transfected and control embryos. Positive cells were counted on every fifth section throughout the optimally transfected region both within the transgene-expressing area, as determined by GFP expression on adjacent sections, and in the equivalent area of the nontransfected side. To normalize for slight differences in developmental stage and rostrocaudal position, the results were expressed as the difference in positive cell numbers between the transfected and the nontransfected side (number of positive cells in the transfected minus the number of positive cells in the nontransfected area). A minimum of five sections was analyzed per embryo, and the results given as the difference in the number of positive cells per section of at least four embryos analyzed.

The distribution of *GFP* fluorescence along the mediolateral axis of the neural tube was analysed at 24 and 48 h.a.e. on transverse spinal cord sections. On each section, the transfected area of the neural tube was divided into three bins of equal dimensions, from the luminal to the pial surface: a luminal bin, an intermediate bin and a pial bin. The mean fluorescence intensities in each bin and in the total areas were measured using a Zeiss confocal microscope and NIH imaging software for 4-10 sections of four (control transfections) or six (*mPhox2b* transfections) embryos. Results were expressed in relative fluorescence intensities by dividing the value for each bin by the mean fluorescence of the total transfected area to obtain a measure of the relative distribution of the transfected cells across the mediolateral extent of the neural tube wall.

RESULTS

The generation of post-mitotic neuronal precursors in the ventricular zone is defective in *Phox2b* knockout mice

In the vertebrate neural tube, the dividing progenitors form a single layer of neuroepithelial cells, in which the nuclei migrate in a cell cycle-dependent manner. The neuronal precursors that have exited the cell cycle migrate radially out of the VZ towards the mantle layer and start their post-mitotic differentiation programme (Sauer, 1936; Guthrie et al., 1991). In the following, we operationally define the VZ as the compartment delimited internally by the lumen of the tube, and externally by the outermost nuclei in S phase as determined by a BrdU pulse and the mantle layer as the BrdU-negative area lying basal to it.

Our previous analysis of bm neuron generation has shown that in *Phox2b* knockout embryos, mantle layer cells do not switch on generic or type-specific differentiation genes and maintain expression of markers normally expressed only in the VZ, although the partition of the wall of the neural tube into an apical VZ and a basal mantle layer is preserved. Moreover, the mantle layer is reduced in size (Pattyn et al., 2000). As shown before, the number of cells in the lateral aspects of the mutant neural tube that do not incorporate BrdU – i.e. post-mitotic precursors – is massively reduced in the ventral region of rhombomere 4 (r4) from E10.5 *Phox2b*$^{LacZ/LacZ}$ hindbrain, which normally gives rise to the facial bm neurons (Pattyn et al., 2000; Fig. 1A,B). A cluster of *LacZ*$^+$ cells that had not incorporated BrdU after a 3 hour pulse was still found lateral to the BrdU$^+$ cells in the homozygous mutants. This shows that after completion of their last mitosis, they have followed the usual pattern of radial migration from the VZ to the mantle layer. Although the results show that fewer post-mitotic precursors accumulate in the mutants, they leave open several possibilities as to how this could come about, such as (1) a reduced rate of progenitor proliferation, (2) the generation of fewer post-mitotic cells in the VZ, (3) a reduced rate of exit from the VZ or (4) preferential death of post-mitotic mantle layer cells. We observed increased cell death in the mutant territory, but the apoptotic cells were distributed throughout the wall of the neural tube and there was no sign of preferential death of cells in the mantle layer (Pattyn et al., 2000). We then counted the number of cells in the mutant territory that had incorporated BrdU after a 1 hour pulse. If anything, this value was slightly higher for *Phox2b*$^{-/-}$ than for *Phox2b*$^{+/-}$ embryos (+15%, results not shown).

To test whether fewer post-mitotic neuronal precursors are generated within the mutant VZ, we examined the expression of *Dll1*, a mouse homologue of *Drosophila Delta*, which is the earliest known marker expressed after the last S phase (Bettenhausen et al., 1995; Myat et al., 1996; Henrique et al., 1997). *Dll1* expression displayed a salt-and-pepper pattern throughout the VZ of heterozygotes (Fig. 1C), presumably corresponding to cells that have just completed their last mitosis close to the lumen and are on their way out of the VZ (Myat et al., 1996; de la Pompa et al., 1997; Dunwoodie et al., 1997; Henrique et al., 1997). In the *LacZ*$^+$ region from mutant embryos, *Dll1* was still expressed (Fig. 1D). However the *Dll1* expression pattern was altered in two ways. First, mutant precursors fail to downregulate *Dll1* upon exit from the VZ, as they normally do (compare Fig. 1C and D). The persistence of VZ markers on the post-mitotic mantle layer cells in the mutants had also been observed for other genes (Pattyn et al., 2000). Since the mutant cells that have become post-mitotic still migrate to the mantle layer, this results in the accumulation of *Dll1*$^+$ cells in the lateral aspects of the neural tube. Second and more relevant to the question under study, *Dll1* expression in the VZ was reduced by 50% (Fig. 1C-E). To test whether this decrease was due to the selective death of *Dll1*$^+$ cells, we performed *Dll1*/TUNEL double-labelling experiments. Virtually none of the TUNEL$^+$ cells in the VZ were also *Dll1*$^+$ (Fig. 1F), arguing that *Dll1* is not activated at its normal rate. The failure of detecting doubly labelled TUNEL$^+$/*Dll1*$^+$ cells cannot be ascribed to mRNA degradation in dying cells, since virtually all TUNEL$^+$ cells could be labelled with a *LacZ* probe (not shown). These data thus indicate that, at any point in time, a smaller proportion of the mutant progenitors become post-mitotic, switch on *Dll1* and migrate out of the VZ.

We reasoned that the reduced numbers of *Dll1*-expressing cells should result in a lesser degree of Notch-mediated lateral inhibition in the VZ. A major output of Notch signalling is the induction of the *Enhancer of split* and related genes (Weinmaster, 1997; Wettstein et al., 1997; Ohtsuka et al., 1999). We therefore examined the *Enhancer of split* homologue *Hes5*, whose normal expression throughout the VZ of the embryonic neural tube is reduced or abrogated in mouse mutants with disrupted Notch signalling (de la Pompa et al., 1997). *Hes5* has also been implicated in lateral inhibition in another neurogenic region, the olfactory placode (Cau et al., 2000). In the VZ of ventral r4 from wild-type or heterozygous embryos, *Hes5* transcripts were not uniformly expressed, clusters of cells with high expression levels juxtaposing cells where *Hes5* expression was barely detectable (Fig. 1G). A

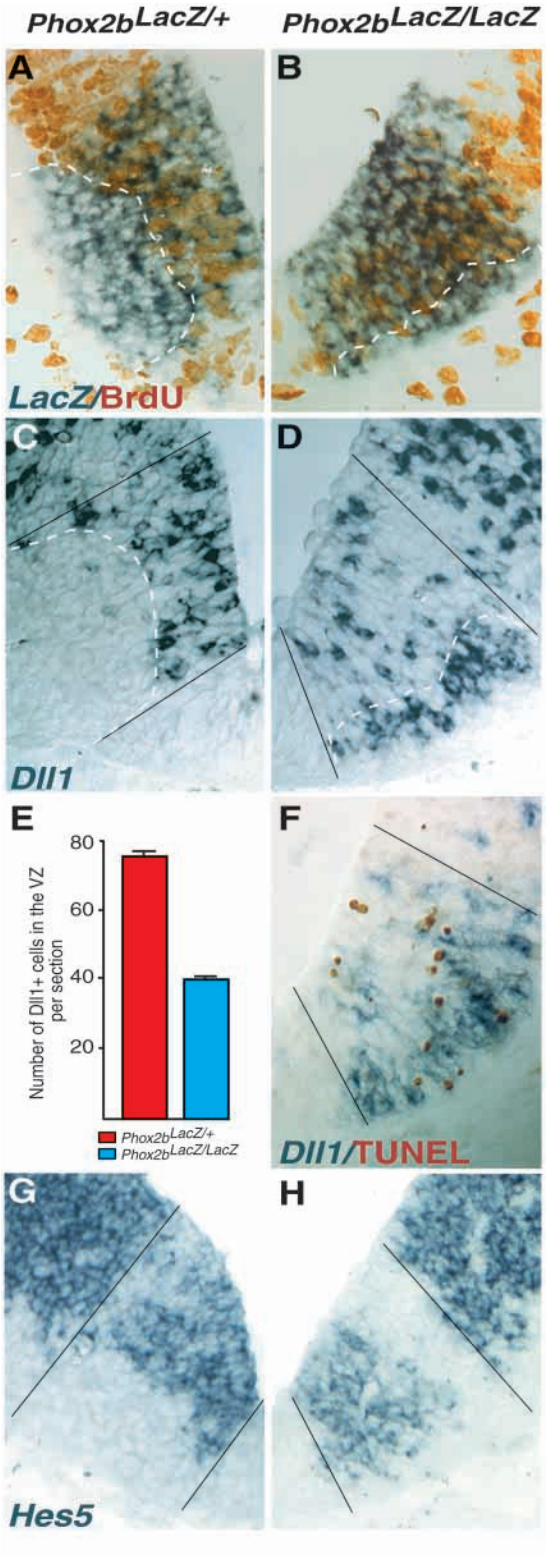

Fig. 1. Defective generation of post-mitotic motor neurons in the ventricular zone of *Phox2b*$^{LacZ/LacZ}$ mouse embryos. In situ hybridization with *LacZ* (A,B), *Dll1* (C,D,F) or *Hes5* (G,H) probes on transverse sections through the r4 region of the hindbrain from heterozygous or homozygous E10.5 mutant embryos. Shown is the ventral region of the neural tube where the facial motor neuron precursors are born at this stage. (A,B) Combined in situ hybridization with a *LacZ* probe (blue) and BrdU labelling (brown) after a 3 hour BrdU pulse shows accumulation of BrdU^{-} cells laterally in both *Phox2b*$^{LacZ/+}$ and *Phox2b*$^{LacZ/LacZ}$ embryos, but their number is massively reduced in the homozygotes. The broken white line delimits the VZ as determined by the outermost (basal) BdU^{+} nuclei. (C,D) In the homozygous mutants, *Dll1* is still expressed, but the number of *Dll1*$^{+}$ cells is reduced in the VZ and it fails to be downregulated in the mantle layer. The limit of the VZ, defined as the outermost limit of BrdU^{+} nuclei, was determined by BrdU-labelling of an adjacent section. After superimposition of the two pictures in Adobe Photoshop, the VZ border was drawn on the *Dll1*-labelled section (broken white line). The solid black lines delimit the *LacZ*$^{+}$ region, as determined in the same way by β-galactosidase staining on an adjacent section. (E) Cell counts of *Dll1*$^{+}$ cells in the LacZ^{+} neuroepithelium of ventral r4. The *Dll1*$^{+}$ cells were counted in the area delimited laterally by the outermost BrdU^{+} cells, dorsally and ventrally by the limits of *LacZ* expression as determined on an adjacent section. Values are given as the number of cells per section (mean ± s.e.m., 16 sections). (F) Double labelling for *Dll1* and TUNEL stain shows no sign of preferential cell death of *Dll1*$^{+}$ cells. (G,H) *Hes5* expression in the VZ is reduced in ventral r4 from homozygous mutants. The black line delimits the *LacZ*$^{+}$ region. Note that in both heterozygous and homozygous mutants, *Hes5* expression is confined to the VZ.

similar pattern has been described in the olfactory placode (Cau et al., 2000). In *Phox2b*-deficient embryos, *Hes5* expression was reduced in the normally *Phox2b*$^{+}$ area (Fig. 1H). *Hes5* transcripts in the homozygous mutants were only detectable in the regions where expression was highest in the heterozygotes while the areas of weak expression appeared negative.

Phox2b misexpression drives cells to become post-mitotic and to migrate to the mantle layer

The foregoing data suggested to us that in *Phox2b* mutants, exit from the cell cycle is defective. Alternatively, *Phox2b* could be required for the survival of committed precursors at a stage that precedes *Dll1* expression. To test whether *Phox2b* activity is sufficient to drive progenitors to leave the cell cycle and to trigger their post-mitotic differentiation, we turned to gain-of-function experiments in chick embryos. We used misexpression in chick embryos since in ovo electroporation offers a convenient means for temporally and spatially controlled expression in one side of the neural tube (Funahashi et al., 1999; Itasaki et al., 1999). We electroporated a mouse *Phox2b* (*mPhox2b*) expression vector into the presumptive spinal cord region at Hamburger and Hamilton (1951) stage (HH) 12-14 and examined the embryos 24 hours (HH 18-19) or 48 hours (HH 22-23) later. At these stages, the chick spinal cord normally expresses neither *Phox2b* nor *Phox2a*. As in mouse (Tiveron et al., 1996; Hirsch et al., 1998), a small population of interneurons just dorsal to the sulcus limitans expresses Phox2a, but not Phox2b, at slightly later stages (>HH24) (results not shown). A *GFP* expression plasmid was coelectroporated alongside to visualise the transfected cells. As a control, we expressed either *GFP* alone or the homeobox gene *Pitx2* (formerly called *Otlx2*), which is also a *paired*-class homeodomain protein (Mucchielli et al., 1996). Virtually all GFP-expressing cells coexpressed *Phox2b* or *Pitx2* (Fig. 2A,B), which allowed us to use either the homeobox transgenes or the *GFP* reporter as markers of the transfected cells.

We first tested whether *mPhox2b* induced expression of two

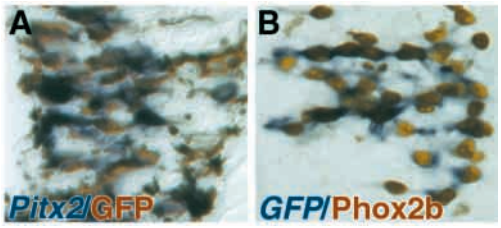

Fig. 2. Reporter and transgene expression are coextensive. In situ hybridization (blue) with *Pitx2* (A) or *GFP* (B) probes followed by immunohistochemistry (brown) with antibodies directed against GFP protein (A) or mPhox2b (B) on spinal cord sections taken at 24 h.a.e. of *Pitx2* (A) or *mPhox2b* (B) expression vectors. The lumen is to the left. Coexpression is maintained at least for up to 72 h.a.e. (not shown).

early markers of post-mitotic VZ cells, the chicken *Dll1* orthologue *Delta1* and *NeuroM*, which codes for a bHLH transcription factor and, like its mouse orthologue *Math3* (Takebayashi et al., 1997), is transiently expressed by post-mitotic VZ cells en route to the mantle layer (Roztocil et al., 1997). 24 h.a.e. there was a marked increase in *Delta1*- and *NeuroM*-expressing cells in the *mPhox2b*-transfected VZ (Fig. 3E-H). The dorsalmost *Phox2b*-transfected cells consistently failed to respond with *Delta1* or *NeuroM* induction, possibly because they constitute neural crest-derived cells. An increase in *Delta1* or *NeuroM* expression was never observed when *Pitx2* (Fig. 3A-D) or *GFP* alone (not shown) were electroporated. Cell counts revealed that the differences in the numbers of positive cells between the *mPhox2b*-transfected and the nontransfected sides of the embryos were highly significant. In absolute terms, we counted approximately 16 *Delta1*$^+$ or *NeuroM*$^+$ cells/section on the *mPhox2b*-transfected compared to approximately 6 *Delta*$^+$ or *NeuroM*$^+$ cells on the nontransfected side, a >2.6 fold increase. *Pitx2* expression produced a decrease in positive cells in the transfected area, whereas *GFP* expression alone was without effect (Fig. 3J). *Phox2b* misexpression thus produces a mirror image of the phenotype observed in *Phox2b*-deficient embryos, where both *Dll1* (Fig. 1) and *Math3* (Pattyn et al., 2000) are downregulated in the VZ. Most *Delta1*$^+$ cells in the transfected region coexpressed mPhox2b, suggesting that *Phox2b* acts cell-autonomously (Fig. 3I). In line with the normally observed expression pattern of *Dll1/Delta1* on VZ cells en route to the mantle layer, the *mPhox2b*-induced increase in *Delta1* expression was transient and confined to VZ cells. At 32 h.a.e., an increase in the number of *Delta1* cells was not observed any more, and at 48 h.a.e., when the great majority of the transfected cells has shifted to the mantle layer and expresses generic neuronal differentiation markers (see below), virtually none of the *mPhox2b*$^+$ cells also express *Delta1* (not shown). This finding shows that the normal temporal relationship between expression of *Delta1* and markers of later stages of differentiation is preserved in ectopic *Phox2b*-induced neurons.

As expected from the increase in post-mitotic *Delta1*$^+$,

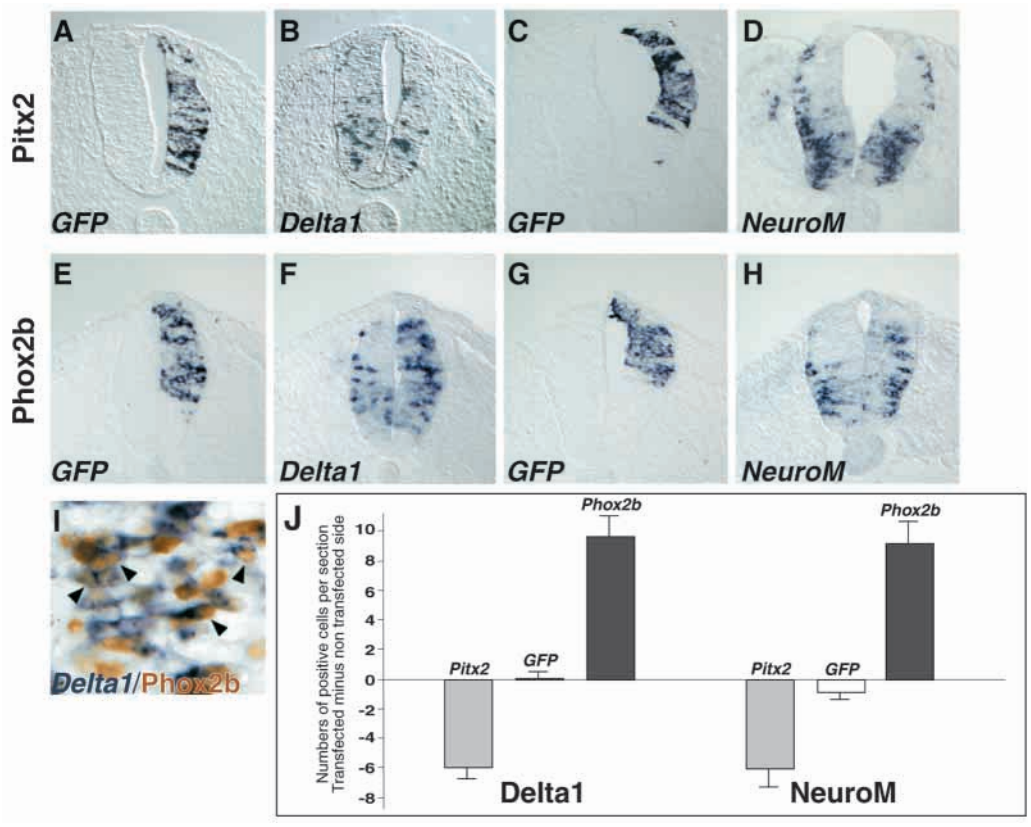

Fig. 3. Forced expression of *mPhox2b* induces expression of the early post-mitotic markers *Delta1* and *NeuroM*. In situ hybridization with *Delta1* (B,F) or *NeuroM* (D,H) probes or combined *Delta1* in situ hybridization and mPhox2b immunohistochemistry (I) on transverse sections through the chick embryo spinal cord at 24 h.a.e. of either *Pitx2* (A-D) or *mPhox2b* (E-H) expression vectors. *GFP* was transfected alongside. (A-H) Adjacent sections of *Pitx2*- or *mPhox2b*-transfected spinal cords were hybridized with either a *GFP* probe to show the transfected cells or with *Delta1* or *NeuroM* probes as indicated. There is a marked increase in *Delta1*$^+$ and *NeuroM*$^+$ cells in the transfected area of the spinal cords after electroporation with the *mPhox2b*, but not after electroporation with the *Pitx2* expression vector. (I) Combined *Delta1* in situ hybridization (blue) and mPhox2b immunohistochemistry (brown) shows that most *Delta1*$^+$ cells also express *mPhox2b* (arrowheads). (J) Quantitative analysis of *Delta1* and *NeuroM* expression in the *mPhox2b*- or control-transfected areas of the spinal cord. Results are expressed as the numbers of positive cells per section in the transfected area minus the numbers in the equivalent area on the nontransfected side (means ± s.e.m.) for 4-6 embryos. At least 400 cells on a minimum of 21 sections from each embryo were analysed; P=0.0001 for *mPhox2b* and *Pitx2* transfections; P=0.88 (*Delta1*)and P=0.09 (*NeuroM*) for the *GFP* control (two-tailed paired *t*-test).

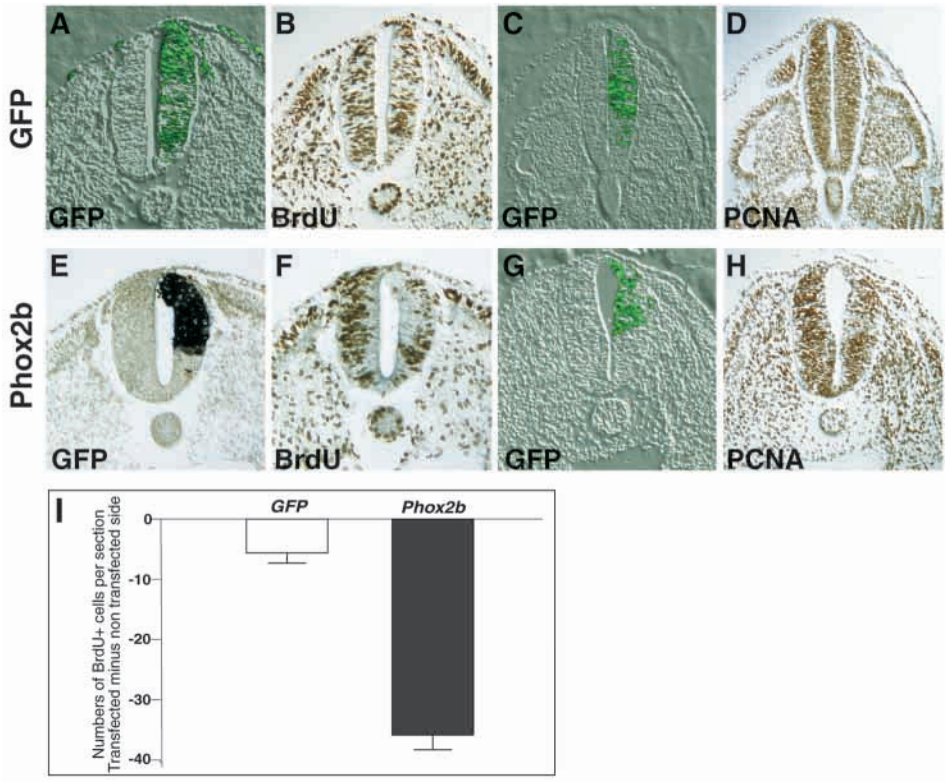

Fig. 4. Forced expression of *mPhox2b* reduces the number of cycling progenitors. GFP fluorescence (A,C,G), GFP immunohistochemistry (E), BrdU immunohistochemistry (B,F) and PCNA immunohistochemistry (D,H) on transverse sections through the chick embryo spinal cord at 24 h.a.e. with either the *GFP* expression vector alone (A-D) or with both *mPhox2b* and *GFP* (E-H) expression vectors. At 24 h.a.e., the number of BrdU-incorporating cells after a 3 hour BrdU pulse is markedly decreased in the *mPhox2b*-transfected area (F), as revealed by *GFP* in situ hybridization on an adjacent section (E), but only marginally in the area transfected with *GFP* alone (A,B). Expression of PCNA is likewise reduced in the *mPhox2b*-transfected area (G,H), but not after electroporation of *GFP* alone (C,D). GFP fluorescence was converted to green pseudocolour and overlaid onto a view of the same area through Nomarski optics. (I) Quantitative analysis of the BrdU incorporation experiments. Results are expressed as the numbers of positive cells per section in the transfected area minus the numbers in the equivalent area on the nontransfected side (means ± s.e.m.) for 4-6 embryos. At least 3000 cells in a minimum of 28 sections from each embryo were analysed; P=0.0001 for *mPhox2b* transfections; P=0.0024 for the *GFP* control (two-tailed paired t-test).

NeuroM$^+$ neurons, there was a marked reduction in the number of BrdU-incorporating cycling progenitors in the *mPhox2b*-transfected area at 24 h.a.e. (Fig. 4E,F) compared to control transfections (Fig. 4A,B). We counted around half as many BrdU$^+$ cells on the *mPhox2b*-transfected than on the nontransfected side compared to an 11% decrease after electroporation of *GFP* alone (Fig. 4I). We tested expression of proliferating-cell nuclear antigen (PCNA) as an additional marker of cycling cells. PCNA is a nuclear protein that is part of the elongation apparatus and essential for DNA replication (Tsurimoto, 1998). In the wall of the neural tube, DNA-bound PCNA has been found in late G_1 to mid-S phase nuclei (Takahashi and Caviness Jr, 1993). We found a clear reduction in the number of PCNA$^+$ nuclei after transfection with *mPhox2b* (Fig. 4G,H), but not after transfection with *GFP* alone (Fig. 4C,D), again suggesting that Phox2b promotes cell cycle exit.

Does *mPhox2b* also drive the cells to migrate to the mantle layer? At 24 h.a.e., the vast majority of the transfected cells were found in the VZ and had the typical morphology of neuroepithelial cells, in both *mPhox2b*-transfected and control neural tubes. One day later, however, the great majority of *mPhox2b*-transfected cells were found in the lateral region of the neural tube while in the controls, most electroporated cells were still in the neuroepithelium and looked like neuroepithelial cells (Fig. 5A-H). Quantification of the GFP fluorescence showed that at 48 h.a.e., 55% of the *mPhox2b*-transfected cells were found in the lateral third of the neural tube compared to 33% in the *GFP* control (Fig. 5I-K). We confirmed that the *Phox2b*-transfected cells that had translocated laterally were indeed post-mitotic differentiating mantle layer neurons using BrdU incorporation and class III *β-tubulin* (Lee et al., 1990) as markers (Fig. 6). At 48 h.a.e., few, if any, of the *mPhox2b*$^+$ cells incorporated BrdU after a 3 hour pulse. Most were located external to the BrdU$^+$ VZ progenitors, showing that they had shifted to the mantle layer, and many of them expressed *β-tubulin*. Together, these data demonstrate that Phox2b positively regulates the decisions to become post-mitotic, to migrate to the mantle layer and to initiate generic neuronal differentiation.

Phox2b induces branchio- or visceromotor neuronal markers

In the mouse (Pattyn et al., 1997) and chick (J. Gay and J. F. B., unpublished results) hindbrain, *Phox2b* is expressed in the bm and vm, but not in the sm neurons, and is required for their formation as shown by targeted gene inactivation in the mouse (Pattyn et al., 2000). We therefore tested whether the ectopically *mPhox2b*-expressing neurons acquire a bm or vm identity. In the ventral hindbrain, where the cranial motor neurons are being born, *Phox2b* is already expressed in the cycling progenitors and persists on the post-mitotic neurons. The LIM homeobox gene *Islet1* is expressed most prominently in all post-mitotic motor neuron precursors, but also in a small population of dorsal interneurons (Ericson et al., 1992; Liem et al., 1997), while *Islet2* is specific for sm neurons (Varela-Echevarría et al., 1996; Ericson et al., 1997). Finally, all motor neurons and no other neurons in the spinal cord express *choline*

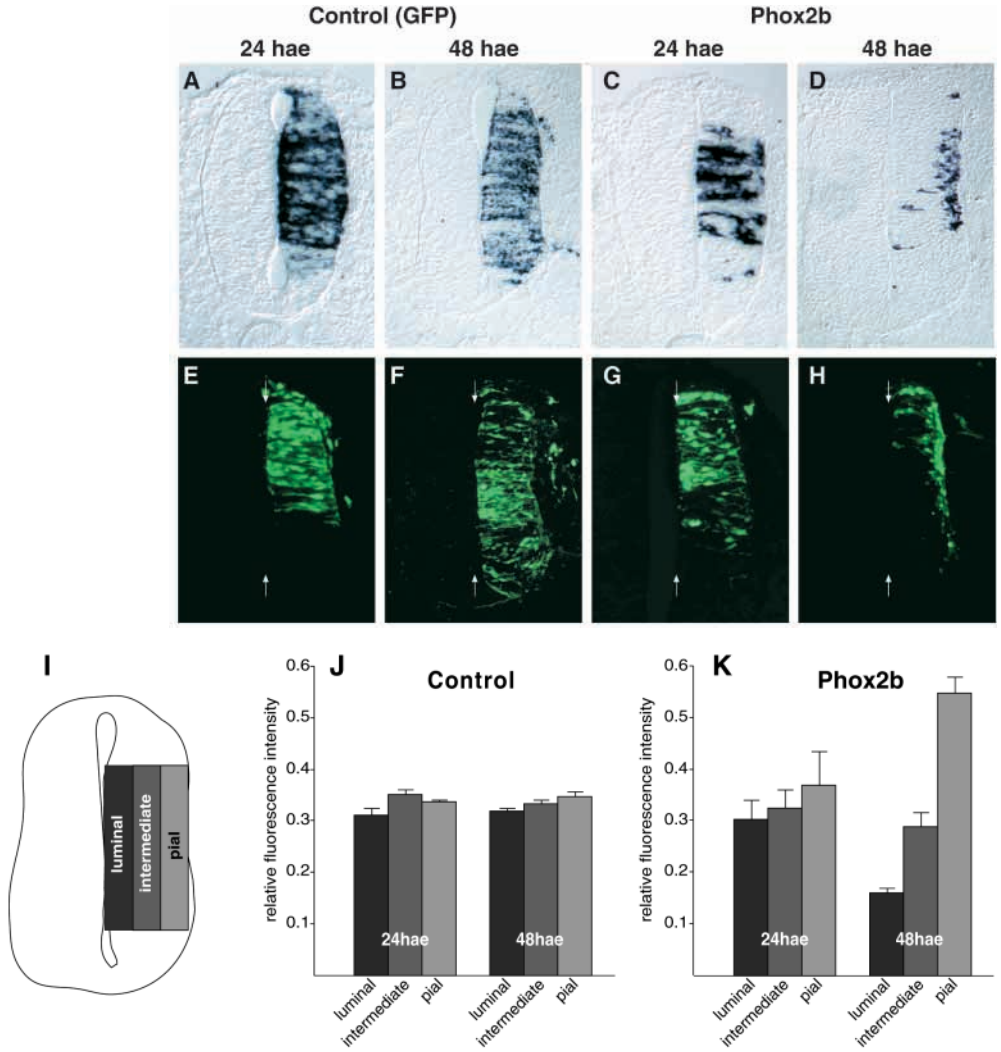

Fig. 5. Relocation of *mPhox2b*-transfected cells to the lateral aspects of the neural tube. Distribution of cells transfected with *GFP* alone (A,B,E,F) or with *mPhox2b* and *GFP* (C,D,G,H) at 24 (A,E,C,G) or 48 h.a.e. (B,F,D,H) as visualized by *GFP* in situ hybridization (A-D) or GFP fluorescence (E-H) on transverse sections through the spinal cord. The arrows point to the luminal side of the tube wall. (I) Schematic representation of the way the transfected area was sub-divided into three bins to measure fluorescence intensities. (J,K) Quantification of the GFP fluorescence shows that at 24 h.a.e., the transfected cells are evenly distributed across the mediolateral extent of the neural tube wall in both *mPhox2b* and control transfections. At 48 h.a.e., more than half of the *mPhox2b*-transfected cells have relocated to the lateral aspects of the neural tube, while the control-transfected cells are still evenly distributed. Values are presented as relative fluorescence intensities in each bin. Values are means ± s.e.m. for four (control) or six (*mPhox2b*-transfected) embryos ($P<0.001$ for pairwise comparisons between pial, intermediate and luminal bins at 48 hours after *mPhox2b* electroporation; $P>0.5$ for the other comparisons).

acetyltransferase (*ChAT*) at the stages studied (see Fig. 7D). At 48 h.a.e., many mantle layer cells that expressed *mPhox2b* could be labelled by an antibody recognising both Islet1 and Islet2. There was thus a clear increase in Islet1/2$^+$ cells in the area that expressed the transgene (Fig. 7E-H), an effect not seen in *GFP* (Fig. 7A,B) or *Pitx2* (not shown) control electroporations. *Islet2* was only expressed ventrally in differentiating spinal motor neurons, showing that Islet1/2 immunoreactivity is due to *Islet1*, but not *Islet2* expression (Fig. 7I,J). While *ChAT* expression was confined to the ventrally differentiating motor neurons in control transfections and on the nontransfected side, it was detected dorsolaterally throughout the *mPhox2b*-transfected area (Fig. 7C,D,K,L). Hence, forced expression of *Phox2b* generates ectopic neurons that, like cranial bm or vm neurons, are *Islet1*$^+$, *ChAT*$^+$ and *Islet2*$^-$.

We used chicken *Phox2a* (cPhox2a) as an additional marker to confirm the bm or vm phenotype of the ectopically *Phox2b*-expressing neurons. In both mouse (Pattyn et al., 1997) and chick (J. Gay and J. F. B., unpublished results) embryos, *Phox2a*, whose expression follows that of *Phox2b*, is also expressed by cranial bm and vm but not by sm neurons. Unlike *Phox2b*, *Phox2a* is downregulated on cranial motor neurons beyond E13 in the mouse (Tiveron et al., 1996; Jacob et al., 2000). In *Phox2b*-transfected embryos examined at 20 h.a.e. (HH 18), many of the transfected cells also expressed *cPhox2a*

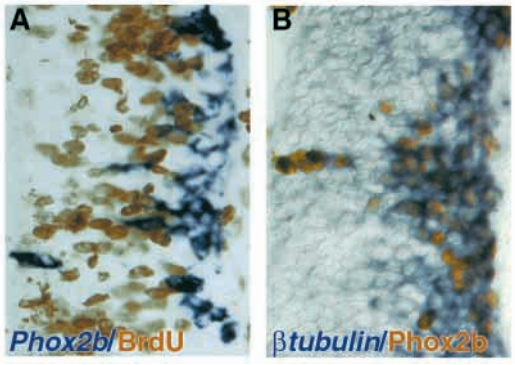

Fig. 6. The *mPhox2b*-expressing cells in the lateral aspects of the spinal cord are post-mitotic and express neuron-specific *β-tubulin*. (A) Combined *mPhox2b* in situ hybridization (blue) and BrdU labelling (brown) after a 3 hour BrdU pulse on sections taken at 48 h.a.e. shows that the *mPhox2b*-expressing cells do not incorporate BrdU. (B) Virtually all mPhox2b-expressing cells also express *β-tubulin* at 48 h.a.e as shown by combined *β-tubulin* in situ hybridization (blue) and anti-mPhox2b immunohistochemistry (brown).

(Fig. 7M), an effect not seen after electroporation of GFP alone (not shown). Ectopic *cPhox2a* expression was transient; its expression had become weaker by 32 h.a.e. (HH 20) (Fig. 7N) and had declined to barely detectable levels by 48 h.a.e. (not shown). At 32 h.a.e., many of the *cPhox2a*$^+$ cells had shifted to the mantle layer and were thus most likely differentiating post-mitotic neurons. Hence, *Phox2b*-expression also induces *Phox2a*, which is never expressed by sm neurons, further suggesting that *Phox2b* activates at least some aspects of a bm or vm differentiation programme. The transitory character of this expression may be related to the downregulation of Phox2a that is normally observed in cranial motor neurons at later stages.

DISCUSSION

Neurogenesis in the vertebrate neural tube is marked by a rapid succession of events in precursor cells. Before the first post-mitotic neurons are generated, different homeodomain transcription factors already subdivide the VZ into distinct progenitor domains (Briscoe et al., 2000). Once neurogenesis is under way, the progenitors committed to become a neuron complete their last mitosis close to the lumen in most regions of the neural tube. They then migrate away from the VZ while transiently activating genes such as *Math3*/*NeuroM* or the *Ebf* family members (Roztocil et al., 1997; Garel et al., 1997; Pattyn et al., 2000), which mark the earliest steps of generic neuronal differentiation. This is accompanied or soon followed by expression of downstream transcription factor genes that specify neuronal sub-type identity (see for reviews, Goulding, 1998; Edlund and Jessell, 1999). These decisions must be regulated coordinately, but how this is achieved is poorly understood. The evidence presented in this study shows that the homeodomain transcription factor Phox2b promotes neural tube progenitors to exit the cell cycle and to start a generic neuronal differentiation programme, and that its expression is sufficient to initiate differentiation towards a motor neuronal phenotype. Thus, Phox2b appears to be a critical determinant that successively regulates commitment to become a neuron and execution of a sub-type-specific differentiation programme.

Phox2b expression promotes cell cycle exit and migration to the mantle layer

In mice lacking *Phox2b*, there is a massive reduction in post-mitotic mantle layer cells in the region where the bm neurons of the facial nerve normally develop (Pattyn et al., 2000). Our previous results showed that the depletion of the mantle layer is not due to an early death of mantle layer cells, as observed for example in *Islet1* knockout embryos (Pfaff et al., 1996). However, they did not allow us to conclude that Phox2b is required for proper exit from the cell cycle since other possibilities remained, such as a reduced rate of proliferation of the mutant progenitors or a reduced rate of exit from the VZ. We here show that expression of *Delta1/Dll1*, the earliest known marker expressed by VZ cells after completion of their last S phase (Myat et al., 1996; de la Pompa et al., 1997; Dunwoodie et al., 1997; Henrique et al., 1997), is defective in the VZ of mutant embryos. Since this defect is not due to a requirement of *Phox2b* for the survival of *Dll1*$^+$ cells, this suggests that *Phox2b* positively regulates cell cycle exit of the progenitors in which it is expressed. Our gain-of-function experiments in the chick neural tube fully confirm the role of Phox2b as a positive regulator of cell cycle exit. Forced expression of *Phox2b* reduces the numbers of BrdU-incorporating and PCNA-expressing cycling cells and upregulates *Delta1/Dll1* and *NeuroM/Math3*, which are transiently expressed by early post-mitotic precursors on their way out of the VZ (Myat et al., 1996; de la Pompa et al., 1997; Henrique et al., 1997; Roztocil et al., 1997). Hence Phox2b clearly acts on progenitor cells in the VZ. Lack of Phox2b has the opposite effect: the number of *Delta1/Dll1*$^+$ cells in the VZ is reduced by half (this study) and *NeuroM/Math3* expression is extinguished (Pattyn et al., 2000). It could be argued that Phox2b, rather than driving cells to exit the cell cycle, increases the survival of progenitors committed to a neuronal fate, in particular since spontaneous cell death has been observed in the dorsal chick spinal cord at the stages studied (Homma et al., 1994). However by TUNEL analysis, we found spontaneous cell death to occur only in the dorsalmost part of the VZ and its extent not to be reduced by *Phox2b* transfection (results not shown). This makes it very unlikely that the effects of *Phox2b* misexpression can be explained by a positive effect on survival.

2 days after transfection, most cells ectopically expressing *Phox2b* have relocated to the mantle layer and express the generic neuronal marker *class III β-tubulin*, an effect not seen in control transfections. Hence, *Phox2b* expression is sufficient to drive cells to become post-mitotic, to start a neuronal differentiation programme and to migrate to the mantle layer. In the absence of *Phox2b*, neuroepithelial cells are still able to become post-mitotic, albeit at reduced rates. This argues that rather than being absolutely required for cell cycle withdrawal, *Phox2b* positively regulates the rate at which it occurs.

At what step in the pathway does *Phox2b* act? The bHLH transcription factors with proneural activity are another class of transcriptional regulators that appear to control timing and extent of neuronal differentiation (Fode et al., 1998; Ma et al., 1998). In *Phox2b* knockout embryos, *Mash1*, a homologue of *Drosophila* proneural genes, is expressed at strikingly reduced levels in the bm progenitors and is downregulated prematurely in the sympathetic ganglion primordia (Pattyn et al., 1999; Pattyn et al., 2000). One possibility thus is that Phox2b acts by maintaining or boosting expression of proneural genes, a possibility corroborated by our finding that *Phox2b* upregulates expression of *Delta1/Dll1*, which is thought to be a target of transcription factors with proneural activity (Chitnis et al., 1995; Casarosa et al., 1999). Alternatively, Phox2b may function downstream of, or in parallel with, proneural gene products, by promoting expression of bHLH transcription factors that lie further downstream in the regulatory cascade (Fode et al., 1998; Ma et al., 1998; Cau et al., 2000) or by cooperating with bHLH factors in the control of cell cycle regulators. Finally, Phox2b acts in the context of the Delta-Notch lateral inhibition system. As judged by the reduction in *Hes5* expression (de la Pompa et al., 1997; Casarosa et al., 1999; Ohtsuka et al., 1999; Cau et al., 2000), Notch signalling is activated to a lesser degree in the *Phox2b* mutant territory, probably as a consequence of reduced expression of the Notch ligand *Dll1*. This should initiate a feedback loop (de la Pompa et al., 1997; Wettstein et al., 1997; Lewis, 1998), counteracting the negative effect of *Phox2b* deficiency on *Dll1* expression

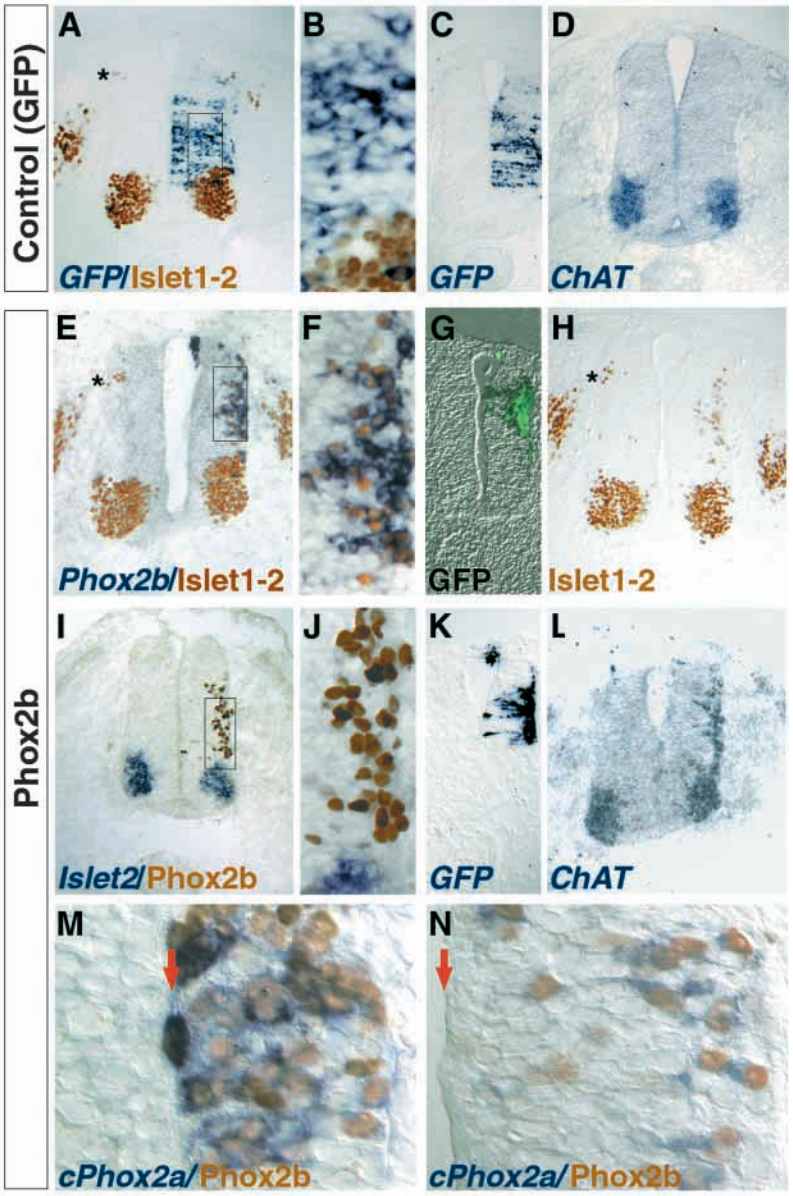

Fig. 7. Forced expression of *mPhox2b* induces a motor neuron-like phenotype. *GFP* (A,B,C,K), *mPhox2b* (E,F), *Islet2* (I,J), *ChAT* (D,L) or *cPhox2a* (M,N) in situ hybridizations combined with Islet1/2 (A,B,E,F) or mPhox2b (I,J,M,N) immunohistochemistry or GFP fluorescence (G) or Islet1/2 immunohistochemistry alone (H) on transverse sections through the chick embryo spinal cord at 20 (M), 32 (N) or 48 (all others) h.a.e. with *GFP* alone (A-D) or with *GFP* plus *mPhox2b* (E-N). (A-L) Islet1/2 immunohistochemistry (H) and combined *GFP* (A,B) or *mPhox2b* (E,F) in situ hybridizations (blue) and anti-Islet1/2 immunohistochemistry (brown) show supernumerary Islet1/2-expressing cells in the transfected area in *mPhox2b*-, but not in control-transfected embryos. (G) The transfected area in H is visualized by GFP fluorescence on the same section. Equivalent results were obtained in 11 out of 12 embryos electroporated with the *mPhox2b* expression vector. At the nontransfected side and in control embryos, Islet1/2 is expressed in the ventrally located motor neurons as well as in a small population of dorsal interneurons (asteriks in A,E,H), but not in the lateral spinal cord. (I) Combined *Islet2* in situ hybridization (blue) and mPhox2b immunohistochemistry (brown) show that, in contrast to the ventrally located sm neurons, the *mPhox2b*$^{+}$ cells do not express *Islet2* and are thus *Islet1*$^{+}$, *Islet2*$^{-}$. (B,F,J) Higher magnifications of the fields drawn in A,E,I, respectively, to show that many cells coexpress *mPhox2b* and Islet1/2 after electroporation of the *mPhox2b* expression vector, but not *GFP* and Islet1/2 in control embryos nor *Islet2* after *mPhox2b* transfection. (C,D,K,L). Forced expression of *mPhox2b*, but not expression of *GFP* alone, induces *ChAT* in the lateral spinal cord as shown by in situ hybridization with a *ChAT* probe (D,L) and labelling of adjacent sections with a *GFP* probe (C,K) to visualize the transfected area. (M,N) Combined *cPhox2a* in situ hybridization (blue) and mPhox2b immunohistochemistry (brown) show that at 20 h.a.e. many mPhox2b-expressing cells strongly express *cPhox2a* (M). There is no *cPhox2a* expression at the nontransfected side. At 32 h.a.e., *cPhox2a* expression has decreased (N); it is bareley detectable at 48 h.a.e. (not shown). The red arrow marks the lumen of the neural tube.

and cell cycle exit. Conversely, increased *Delta1* expression in the gain-of-function experiments should initiate a feedback loop that opposes the effect of *Phox2b* misexpression. It is thus easy to understand that in *Phox2b* mutants not all cells remain cycling progenitors, and that not all cells misexpressing *Phox2b* can be driven to express *Delta1* or *NeuroM* at any one time.

Phox2b initiates motor neuronal differentiation

Our analysis of the loss-of-function phenotype has shown that *Phox2b* is absolutely required for the generation of the bm and vm neurons in which it is expressed (Pattyn et al., 2000). Here we show that *Phox2b* is also sufficient to induce characteristics of a motor neuronal phenotype in the lateral spinal cord, where motor neurons normally never develop. The ectopic *Phox2b*-expressing cells switch on the motor neuronal markers Islet1/2 and *ChAT*, but not *Islet2*, which is specific for sm neurons. Forced expression of *Phox2b* also transiently activates *Phox2a*, whose expression in motor neurons is confined to bm and cranial vm neurons. Hence, the expression of *Phox2b* in neural progenitor cells appears sufficient to activate a differentiation programme resembling that of bm or hindbrain vm neurons, but in the absence of specific markers we cannot distinguish between these two fates.

Motor neurons are normally generated only in the ventral neural tube in response to sonic hedgehog signalling from the notochord and floor plate (Briscoe et al., 1999; Briscoe et al., 2000; for a review, see Edlund and Jessell, 1999). Generation of alar plate neurons depends on BMPs and related molecules that are expressed in the dorsalmost regions of the neural tube (Liem et al., 1995; Lee et al., 2000), and exposure of progenitors to BMPs inhibits motor neuron generation (Basler et al., 1993; Liem et al., 1995). Our results show that like *MNR2* and *Hb9* (Tanabe et al., 1998), *Phox2b* is able to override such negative control of motor neuronal differentiation in the dorsal spinal cord. In the hindbrain, however, the dorsal *Phox2b*-expressing precursors do not give rise to motor neurons, but to sensory relay neurons, to neurons

of the reticular formation and to noradrenergic neurons (Pattyn et al., 1997, A. P. and J. F. B., unpublished). Other studies have shown that forced expression of *Phox2b* or *Phox2a* in the chick neural crest pathway (Stanke et al., 1999) or of *Phox2a* in the zebrafish embryo (Guo et al., 1999) generates ectopic noradrenergic neurons. One possibility thus is that Phox2b must cooperate with other factors to specify dorsal sub-type identities in the hindbrain, and that these factors lack in the spinal cord.

Taken together, our studies show that a single transcription factor is able both to increase cell cycle exit of neuronal progenitors and to initiate at least some aspects of sub-type-specific differentiation of the neurons thus generated. Transcriptional regulators, which assume this role in neuronal types other than bm and vm neurons, remain to be identified.

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