Available online at www.sciencedirect.com
ScienceDirect
Developmental Biology 303 (2007) 687–702
DEVELOPMENTAL BIOLOGY
www.elsevier.com/locate/ydbio

Forced expression of Phox2 homeodomain transcription factors induces a branchio-visceromotor axonal phenotype

Marie-Rose Hirsch [a], Joel C. Glover [b], Héloïse D. Dufour [a], Jean-François Brunet [a], Christo Goridis [a,*]

[a] CNRS UMR 8542 Ecole normale supérieure, 46 Rue d'Ulm, 75005 Paris, France
[b] Department of Physiology University of Oslo, Oslo, Norway

Received for publication 3 August 2006; revised 30 November 2006; accepted 5 December 2006
Available online 15 December 2006

Abstract

What causes motor neurons to project into the periphery is not well understood. We here show that forced expression of the homeodomain protein Phox2b, shown previously to be necessary and sufficient for branchio-visceromotor neuron development, and of its paralogue Phox2a imposes a branchiomotor-like axonal phenotype in the spinal cord. Many *Phox2*-transfected neurons, whose axons would normally stay within the confines of the neural tube, now project into the periphery. Once outside the neural tube, a fraction of the ectopic axons join the spinal accessory nerve, a branchiomotor nerve which, as shown here, does not develop in the absence of *Phox2b*. Explant studies show that the axons of Phox2-transfected neurons need attractive cues to leave the neural tube and that their outgrowth is promoted by tissues, to which branchio-visceromotor fibers normally grow. Hence, *Phox2* expression is a key step in determining the peripheral axonal phenotype and thus the decision to stay within the neural tube or to project out of it.

Keywords: Phox2a; Phox2b; Axonal growth; Transcription factor; Motor neurons; Spinal accessory nerve; Chicken

Introduction

The formation of neuronal circuits depends to a large extent on the precision, with which developing neurons project their axons along predefined pathways. During the last decade, a wealth of cell surface and secreted signals have been identified that direct axons towards their targets (Charron and Tessier-Lavigne, 2005; Grunwald and Klein, 2002; Yu and Bargmann, 2001). However, much less is known about the transcriptional control of axonal pathfinding in vertebrates although a number of type-specifying transcription factors have been found to play a role in defining axonal pathway choices (Shirasaki et al., 2006; Chen et al., 2006; Dasen et al., 2005; Marion et al., 2005; Pak et al., 2004; Zhang et al., 2004; Herrera et al., 2003; Inoue et al., 2002; Kania and Jessell, 2003; Livet et al., 2002; Erkman et al., 2000; Sharma et al., 1998). One fundamental pathfinding choice made by CNS neurons is whether to project into the periphery or to extend their axons within the confines of the neural tube. In the vertebrate CNS, motor neurons are the only neuronal types, whose axons grow into the periphery. A second early choice made by motor axons is where to leave the neural tube. The somatic motor (sm) neurons generated in the caudal hindbrain and throughout the spinal cord extend axons through the ventral neural tube before emerging via a ventral exit point. The branchio- and visceromotor (bm/vm) neurons generated in the hindbrain send their axons dorsally to dorsal exit points. They maintain divergent trajectories once arrived outside of the neural tube. While sm axons avoid sensory ganglia, bm/vm axons exit close to the incoming sensory fibers and typically invade nearby cranial sensory ganglia (Jacob et al., 2000; Moody and Heaton, 1983). From there, bm axons project towards the branchial arches to innervate branchial arch-derived muscles while vm axons project to the viscera to innervate parasympathetic and enteric ganglia. Although their peripheral trajectories and the targets they innervate differ, bm and

* Corresponding author. Fax: +33 144322323.
E-mail address: goridis@biologie.ens.fr (C. Goridis).

0012-1606/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2006.12.006

hindbrain vm neurons are closely related. Both arise in the ventralmost hindbrain from the pMNv (for progenitors of visceral motor neurons) domain, share a similar transcription factor make up (there are, in fact, currently no markers available that clearly distinguish one from the other) and send their axons to dorsal exit points (Cordes, 2001; Jacob et al., 2001; Pfaff and Kintner, 1998). Finally, the oculomotor (nIII) and trochlear (nIV) motor neurons, which leave the neural tube through ventral and dorsal exit points, respectively, to innervate extraocular muscles, constitute probably a class apart (Pattyn et al., 1997).

The transcription factors Lhx3 and Lhx4 are required in early sm precursors to choose ventral instead of dorsal exit points (Sharma et al., 1998), a choice that depends in part on signaling through the CXCR4 guidance receptor (Lieberam et al., 2005). However, what makes motor axons leave the neural tube in the first place has remained a mystery as have the transcriptional regulators expressed in bm/vm and trochlear neurons that direct their choice of exiting the neural tube dorsally.

Phox2a and Phox2b are two paralogous homeodomain transcription factors, which are expressed, mostly together, in a limited number of central and peripheral neurons and their precursors (Brunet and Pattyn, 2002; Pattyn et al., 1997; Tiveron et al., 1996). *Phox2b* in particular is required for the proper development of most neuronal types, in which it is expressed. In the PNS, these include the neurons of all autonomic ganglia and the epibranchial placode-derived sensory ganglia of the VIIth, IXth and Xth nerve; in the CNS, the hindbrain bm/vm neurons, all noradrenergic neurons and those of the nucleus of the solitary tract (Dauger et al., 2003; Pattyn et al., 2000a,b, 1999). Its role in the generation of bm/vm neurons has been particularly well studied. In the absence of *Phox2b*, generic neuronal differentiation is defective in the pMNv domain and no bm/vm neurons are produced (Pattyn et al., 2000b). Conversely, ectopic expression of *Phox2b* at spinal levels promotes cell cycle exit and neuronal differentiation and at the same time induces genes characteristic for bm/vm neurons (Dubreuil et al., 2000; 2002). The consequences of *Phox2a* inactivation are more limited: it affects the three *Phox2*-expressing cranial sensory ganglia, the parasympathetic ganglia of the head, the nIII and nIV neurons and the locus coeruleus (Morin et al., 1997; Pattyn et al., 1997).

A hallmark of *Phox2b*-dependent neurons is that, in spite of their diverse origins, most of them are part of the visceral nervous system and connect synaptically to form a set of well-defined neuronal circuits, those of the medullary visceral reflexes. Since Phox2b is expressed well before these connections form, this has led to the conclusion that Phox2b must be somehow causal to their formation (Brunet and Pattyn, 2002). Another shared feature of many *Phox2b*-dependent neurons is that their axons are attracted by the viscera, vm axons to innervate parasympathetic neurons, the axons of parasympathetic and sympathetic neurons to innervate smooth and cardiac muscle and exocrine glands. Support for a role of the Phox2 factors in the specification of axonal trajectories has been obtained by loss (LOF) and gain (GOF) of function studies of the *C. elegans Phox2* orthologue (Pujol et al., 2000) and by an analysis of the *Phox2a* knockout (Jacob et al., 2000). However, complete agenesis or precocious arrest of type-specific differentiation in the knockout embryos has precluded us from further studying *Phox2* function in an axonal pathway choice by LOF experiments.

Here we have used a GOF approach to explore the potential function of the Phox2 factors in the specification of axonal trajectories. We find that forced expression of *Phox2a* or *Phox2b* in spinal regions of the neural tube entails drastic changes in axonal pathway choices. While all dorsal spinal cord neurons are interneurons and most normally generated at this stage grow commissural axons that cross to the other side, those transfected with *Phox2* have a marked tendency to project into the periphery, mostly through the dorsal roots. Upon arrival in the periphery, these axons follow other axon tracts, but they also form an ectopic nerve that runs alongside the neural tube to join the spinal accessory (XIth) nerve (SAN), made up of the axons of *Phox2b*-dependent bm neurons. In vitro studies show that the ectopic axons of *Phox2a/b*-transfected neurons need attractive cues to leave the neural tube and that their outgrowth is promoted by tissues to which bm/vm axons normally grow. The results demonstrate that *Phox2a/b* expression is sufficient to direct axons out of the neural tube and to impose an axonal phenotype that shares many features with the axons of the *Phox2*-expressing bm/vm neurons.

Materials and methods

Expression vectors and electroporation

We used *pCAGGS::Phox2a-IRES2-EGFP* (Trochet et al., 2005) and *pCAGGS::Phox2b-IRES2-EGFP* (Dubreuil et al., 2000) to express mouse *Phox2a* and *Phox2b* together with *GFP* in the chicken neural tube driven by a composite chicken beta actin-CMV promoter. As controls, we used either *pCAGGS::IRES2-EGFP*, obtained by removing the *Phox2a* coding sequence from *pCAGGS::Phox2a-IRES2-EGFP*, or *pCAGGS::Ngn2* (Dubreuil et al., 2002). The spinal region of HH 12-14 embryonic chick neural tubes was electroporated in ovo with the appropriate expression vectors (2.5 mg/ml) essentially as described (Dubreuil et al., 2000). *PCAGGS::Ngn2* was co-transfected with 0.8 mg/ml *pCAGGS::IRES2-EGFP*. Correct expression of all constructs was verified by in situ hybridization with the cognate probes. After electroporation, embryos were allowed to develop at 38 °C for 30 to 72 h.

Mutant mice

$Phox2b^{LacZ/+}$ mice and $Phox2b^{LacZ/LacZ}$ embryos were produced and genotyped as described (Pattyn et al., 1999).

Histological methods

Well-transfected chicken embryos, as assessed by GFP fluorescence, and mouse embryos were dissected out, fixed in 4% paraformaldehyde overnight, embedded in gelatin and analyzed on transverse cryosections of generally 30 μm at the brachial or thoracic level, except if stated otherwise. The extent of the transfected neural tube and the axonal trajectories of transfected neurons were visualized using rabbit (Chemicon) or monoclonal mouse (Roche) anti-GFP antibodies. Other antibodies used were: monoclonal anti-neurofilament (NF) (3A10), monoclonal anti-BEN/SC-1, monoclonal anti-Lhx1,5 (anti-LIM 1+2) (all three from Developmental Studies Hybridoma Bank), monoclonal anti-Islet1/2 (Tsuchida et al., 1994), monoclonal anti-NeuN (Chemicon), monoclonal anti-betaIII tubulin (Covance), rabbit anti-mouse Phox2a (Morin et al., 1997),

rabbit anti-mouse Phox2b (Pattyn et al., 1997), rabbit anti-TrkC (Lefcort et al., 1996), rabbit anti-peripherin (Chemicon), rabbit anti-beta-galactosidase (Cappel) and rabbit anti-laminin (Sigma L9393). Secondary antibodies were Cy3-coupled (Jackson Immunoresearch) or Alexa488-coupled (Invitrogen-Molecular Probes) goat anti-mouse or anti-rabbit IgG. In situ hybridization on cryosections using digoxigenin-labeled riboprobes for *Tbx20* (a kind gift of J. Ericson) was done as described (Dubreuil et al., 2000). For simultaneous detection of the *Sox10* (Cheng et al., 2000), *Phox2b* or *Tbx20* in situ hybridization and the anti-GFP or anti-Islet1,2 immunofluorescence signals on tissue sections, the sections were processed until the anti-digoxigenin incubation step as described (Tiveron et al., 1996). The sections were then incubated with sheep anti-digoxigenin Fab fragments coupled to HRP (1/2000) overnight at 4 °C, washed in TNT (0.1 M Tris–HCl, pH 7.5, 0.15 M NaCl, 0.1% Tween20) and overlaid with Cy3-tyramide working solution (TSA Plus fluorescence system, Perkin-Elmer) according to the manufacturer's instructions. After washing in PBS, 0.1% Tween20 and incubating with Blocking Reagent (Perkin-Elmer), sections were processed for anti-GFP or anti-Islet1,2 immunohistochemistry using rabbit-anti-GFP or monoclonal anti-Islet1,2 and Alexa488-labeled secondary antibodies. Pictures were taken either with a Leica DC 300F camera mounted on a Leica DMRXA2 microscope and Leica QFluoro software or with a Leica TCS SP2 confocal microscope and assembled using Adobe Photoshop. Whole-mount in situ hybridization using the 3A10 anti-NF antibody was done essentially as described (Maina et al., 1997) except that incubation with the first and second antibodies was done at 4 °C and for 72 h with the first antibody and that the washing step after the second antibody was overnight at 4 °C.

Retrogade labeling

Chicken embryos electroporated at HH 12 with *pCAGGS::Phox2a-IRES2-EGFP* were allowed to develop in ovo for three more days at 38 °C. Labeling with lysine fixable tetramethylrhodamine dextran 3000 (Invitrogen-Molecular Probes) (RDA) was done essentially as described (Glover, 1995). Briefly, the embryos were taken out, eviscerated, pinned to Sylgard dishes and covered with oxygenated Ringer solution. RDA crystals were then inserted into the cervical dorsal funiculus with tungsten wires and the embryos incubated for 4–6 h at room temperature in Ringer solution containing 2 g/l glucose and 1 mM HEPES buffer (pH 7.4) oxygenated with 100% O_2. At the end of the incubation period, the embryos were fixed and processed by immunohistochemistry with anti-GFP and Alexa488-labeled secondary antibodies on 30 μm sections, which were analyzed through FITC and TRITC optics.

Explant cultures and quantification of axon outgrowth

Chick embryos were electroporated at HH 12–14 with *pCAGGS::Phox2a-IRES2-EGF*, *pCAGGS::Phox2b-IRES2-EGFP* or *pCAGGS::IRES2-EGFP* and left to develop until HH 18/19 at 38 °C. Neural tube explants in collagen gels were prepared essentially as described (Tucker et al., 1997). Briefly, the embryos were dissected out in L15 medium, placed in 1 mg/ml dispase (grade 1, Roche) containing 5 μg/ml DNase for 6–10 min. Well-transfected neural tubes from the brachial or thoracic region were opened dorsally, dissected free of surrounding tissue and cut into pieces 3–5 somites in length, which were cultured in rat tail collagen (type I, Upstate) gels prepared as described (Tucker et al., 1997) in an open-book configuration in 75% Opti-MEMI, 25% Ham's F12 with GlutaMaxI and 5% fetal calf serum. The explants thus contain a central strip of floor plate surrounded on either side by basal and alar plates. After incubation for 3 days at 37 °C, 5% CO_2, the explants were fixed in 4% paraformaldehyde and processed for whole-mount immunohistochemistry using anti-GFP and anti-NF antibodies. Tissues to be co-cultured with the neural tube explants were dissected from embryos of the same stage (HH 18–19), except for the branchial arches that were from HH 12/13 and for DRG and retina that were from HH 23 embryos, and placed lateral (dorsal) to the transfected side, 100–500 μm away.

Axon outgrowth was assessed by analyzing explants stained with anti-NF and Cy3-labeled secondary antibodies. In general, all anti-NF-stained axons emanating from the transfected side were also GFP-positive. Images were captured with a Leica DC 300F camera using Leica QFluoro software and imported into ImageJ (NIH) software. After conversion into grayscale, the area lateral (dorsal) to the transfected quadrant of the neural tube explant, where axon outgrowth into the collagen gel was to be measured, was outlined using the Polygone function (see Fig. 6I). If a co-cultured tissue was also NF-positive (e.g. the cranial ganglia), care was taken to exclude axons emanating from it by comparing with the GFP image (by doing so, we also eliminate some fibers that come from the neural tube explant thus underestimating their growth). The delimited area was then thresholded so that individual fiber bundles remained well separated and the number of pixels with intensities above the threshold was counted. An equivalent area was then outlined adjacent to the control (untransfected) side. Axon outgrowth into this area was measured after applying the same threshold that was used for the transfected side, and the ratio between the values for the transfected side over that for the control side was recorded. By recording the transfected over untransfected ratios, differences in staining intensities between different experiments were corrected for. Since thresholding introduced a subjective element (although the ratios did not vary much for different thresholds), all explants were scanned by two observers, one of which was always blind to the experimental condition, and the mean values were recorded. Pixel counts were then combined for each category of co-culture and analyzed statistically using the Mann–Whitney *U*-Test (Statview).

Results

Misexpression of Phox2a or Phox2b imposes a bm/vm fate and directs ectopic axonal growth

Upon ectopic expression in the dorso-lateral neural tube of chicken embryos, *Phox2b* drives the cells to exit the cell cycle, to migrate to the mantle layer and to differentiate. At the same time, it initiates a genetic program that resembles that of bm/vm neurons (Dubreuil et al., 2000, 2002). Very similar results were obtained when misexpressing its paralogue *Phox2a* (Figs. 1A–C, 2A, B and not shown). As in the case of *Phox2b*, ectopic expression of *Phox2a* caused relocation of the cells to the mantle layer (compare Figs. 2A and B) and ectopic expression of Islet1,2 (Figs. 1B, C). Induction of *Tbx20*, which in the brain is exquisitely specific for bm/vm neurons (Dufour et al., 2006), by both *Phox2a* and *Phox2b* adds further weight to the notion that the transfected neurons have acquired a bm/vm phenotype (Figs. 1G–J). During normal development, six interneuron populations, termed dI1–dI6, can be distinguished in the early alar plate of chicken and mouse (Gross et al., 2002; Müller et al., 2002). We used Lhx1,5, expressed in dI2, dI4 and dI6 neurons, to test whether there was a complete switch to a motoneuronal phenotype or whether the cells continued to express alar plate markers. Lhx1,5 was switched off in the overwhelming majority of the *Phox2a*-transfected cells (Figs. 1D–F). This shows that the Phox2 factors not only induce bm/vm markers but also repress the expression of genes characteristic for dorsal interneurons.

The fate switch imposed by Phox2a/b entails a drastic change in axonal trajectories. Most neurons normally generated at this stage in the dorso-lateral spinal cord are commissural neurons, whose axons grow first towards the floor plate where they cross to the other side, as can be visualized after electroporation of GFP alone (Fig. 2A). As assessed by axonal GFP expression from the transfected *Phox2a::IRES-GFP* vector, neurons transfected with *Phox2a* never grow commissural axons. Instead, many of their processes, presumably axons, exit the neural tube, mostly through the dorsal roots, but also between the dorsal and ventral roots. Once in the periphery, many of them appear to join pre-existing axonal tracts. They

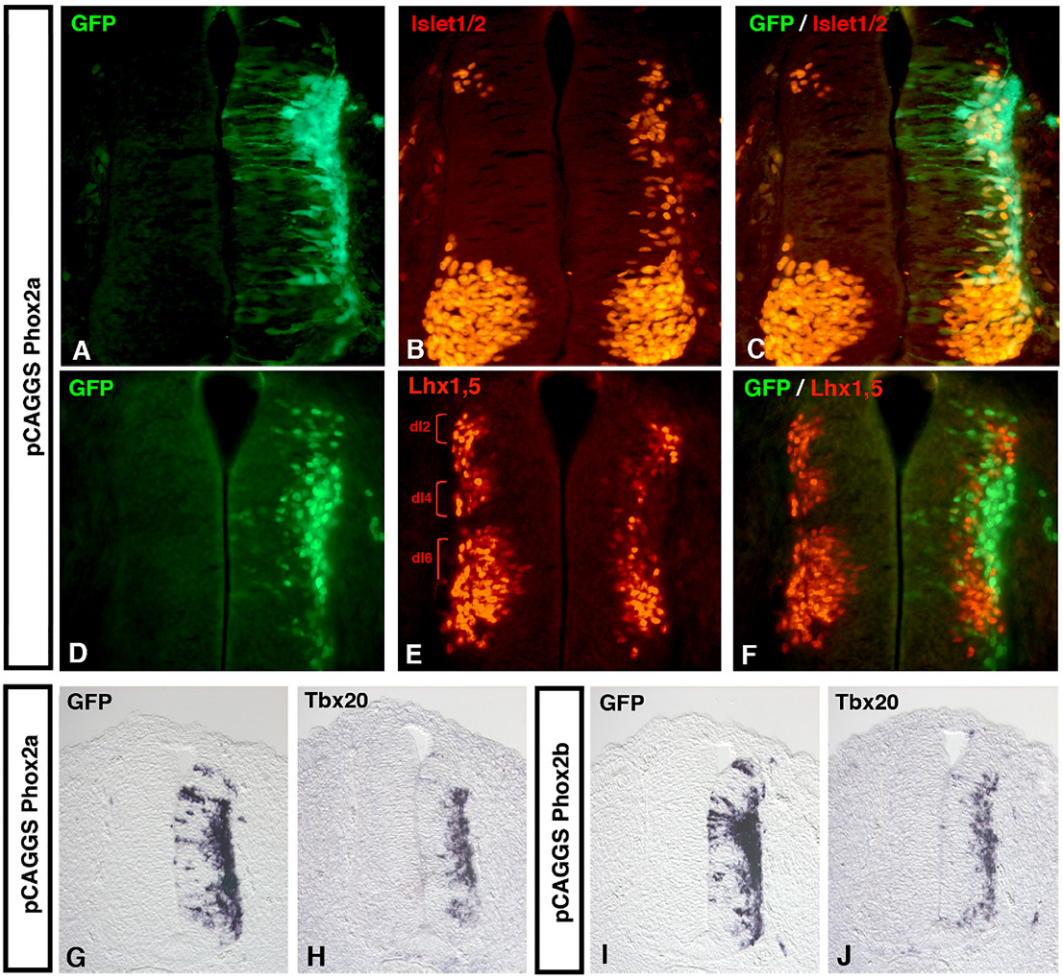


Fig. 1. Effects of *Phox2a* and *Phox2b* misexpressions in the spinal cord. Embryonic chicken neural tubes were electroporated at HH 12–13 with either *pCAGGS::Phox2a-IRES2-EGFP* (A–F, G, H) or *pCAGGS::Phox2b-IRES2-EGFP* (I, J) and analyzed 48 h after electroporation (h.a.e.) by combined anti-GFP and anti-Islet1,2 (A–C) or anti-Lhx1,5 (D–F) immunohistochemistry and by in situ hybridization with *Tbx20* (H, J) or *GFP* (G, I) probes on transverse sections of the spinal cord. *Phox2a*-transfected cells relocate to the mantle layer (A, compare with Fig. 2A) and many are induced to express Islet1,2 (B, C). *Phox2* expression induces *Tbx20*, a marker for bm/vm neurons in the hindbrain, and at the same time extinguishes expression of the dorsal interneuron marker Lhx1,5 (D–F). All pictures were captured with a Leica DC 300F camera.

pass through the dorsal root ganglia (DRG) and can be traced in the spinal nerves up to the plexus region (Figs. 2B–F, K). Others form an ectopic fiber bundle running alongside the neural tube that is absent at the non-transfected side. Easily detected in transverse sections (Figs. 2D–F), it can be followed for some length in longitudinal sections of the spinal cord (Figs. 2G–I). Still others remain inside the neural tube where they join the dorsal funiculus (DF), which normally contains the bifurcating axons of DRG neurons (Figs. 2F, L). The axonal projection phenotypes created by Phox2a versus Phox2b misexpression were indistinguishable at our level of analysis (not shown) despite the fact that other experiments have shown that the two paralogous proteins are not functionally equivalent (Coppola et al., 2005). One reason for this may be that, in our GOF experiments, *Phox2a* very efficiently induces *Phox2b* and vice versa (Dubreuil et al., 2000, 2002 and not shown).

We were concerned that the GFP-positive axons joining the dorsal roots or the DF were in fact incoming axons from occasionally transfected neural crest (NC) cells that had migrated to the DRG. To test this possibility, we used double-staining for GFP and the TrkC neurotrophin receptor, which at this stage is present in virtually all DRG neurons and their axons (Lefcort et al., 1996). We never observed fibers that could be double-labeled for GFP and TrkC, although GFP-positive fiber bundles traversed the DRG (Fig. 2J). We also traced the axonal projections of *Phox2a*-expressing cells that had joined the DF by applying the retrograde tracer RDA (Glover, 1995) specifically to the DF. As expected, we found many RDA-positive cell bodies in the DRG by analyzing sections located several segments caudal to the RDA application site, which, however, never coexpressed GFP (Figs. 2K, L). However, in contrast to the wild-type situation, cell bodies double-stained for RDA and GFP were located within the transfected neural tube, and all RDA-positive cells inside the neural tube also expressed GFP (Fig. 2L).

It may be argued that these ectopic axonal trajectories are a consequence of premature or excessive neuronal differentiation

induced by Phox2a/b. However, transfection of *Ngn2*, which has a similar propensity to induce neuronal differentiation (Dubreuil et al., 2002; Mizuguchi et al., 2001; Nguyen et al., 2006), did not change axonal trajectories. After electroporation of a *Ngn2* expression vector, many transfected cells still grew commissural axons and their axons never left the neural tube elsewhere than through the ventral roots (Figs. 2M–O).

In some cases, *Phox2*-transfected cells were also found outside the neural tube (Figs. 3A, B). Most of these seem not to be derived from the NC, which we transfect occasionally, since they did not express the NC markers *Sox10* (Figs. 3C, D) and HNK1 (not shown), yet most were neurons since they expressed the post-mitotic neuronal marker NeuN (Figs. 3E, F). Although such a scenario is difficult to exclude at present, we deem it unlikely that the GFP-positive fibers growing into the periphery emanate from neurons that have exited the neural tube. In fact, we could document many instances where GFP-positive growth cones that clearly came from *Phox2*-transfected cells located inside the neural tube were just about to leave the neural tube (Figs. 3G, H). Hence the peripheral GFP-positive cells, which often form small clusters, probably follow the axons, responding perhaps to the same guidance cues.

An intact neuroepithelium and an intact basal lamina are required to keep neurons or their precursors from exiting the neural tube (Halfter et al., 2002; Hatakeyama et al., 2004). Conceivably, *Phox2* misexpression, by promoting premature conversion into neurons, may deplete the neural tube of neuroepithelial or radial glial cells, whose integrity appears to be required for basal lamina formation, as has been observed in $Hes1^{-/-};Hes5^{-/-}$ double knockout mice or by intentional killing of radial glial cells (Hatakeyama et al., 2004). Lack of an intact basal lamina may also allow processes to inappropriately exit the tube. By staining with anti-laminin antibodies, we found places where the basal lamina surrounding the *Phox2a*-transfected neural tube was interrupted (Figs. 3I, J). However, while cell bodies spilling out of the neural tube were only found where the basal lamina was disorganized, the processes of *Phox2a*-transfected cells were able to traverse an apparently intact basal lamina (Figs. 3K, L) We thus conclude that the exit of cells we occasional observe, but not that of the axons, is due to damage to the basal lamina.

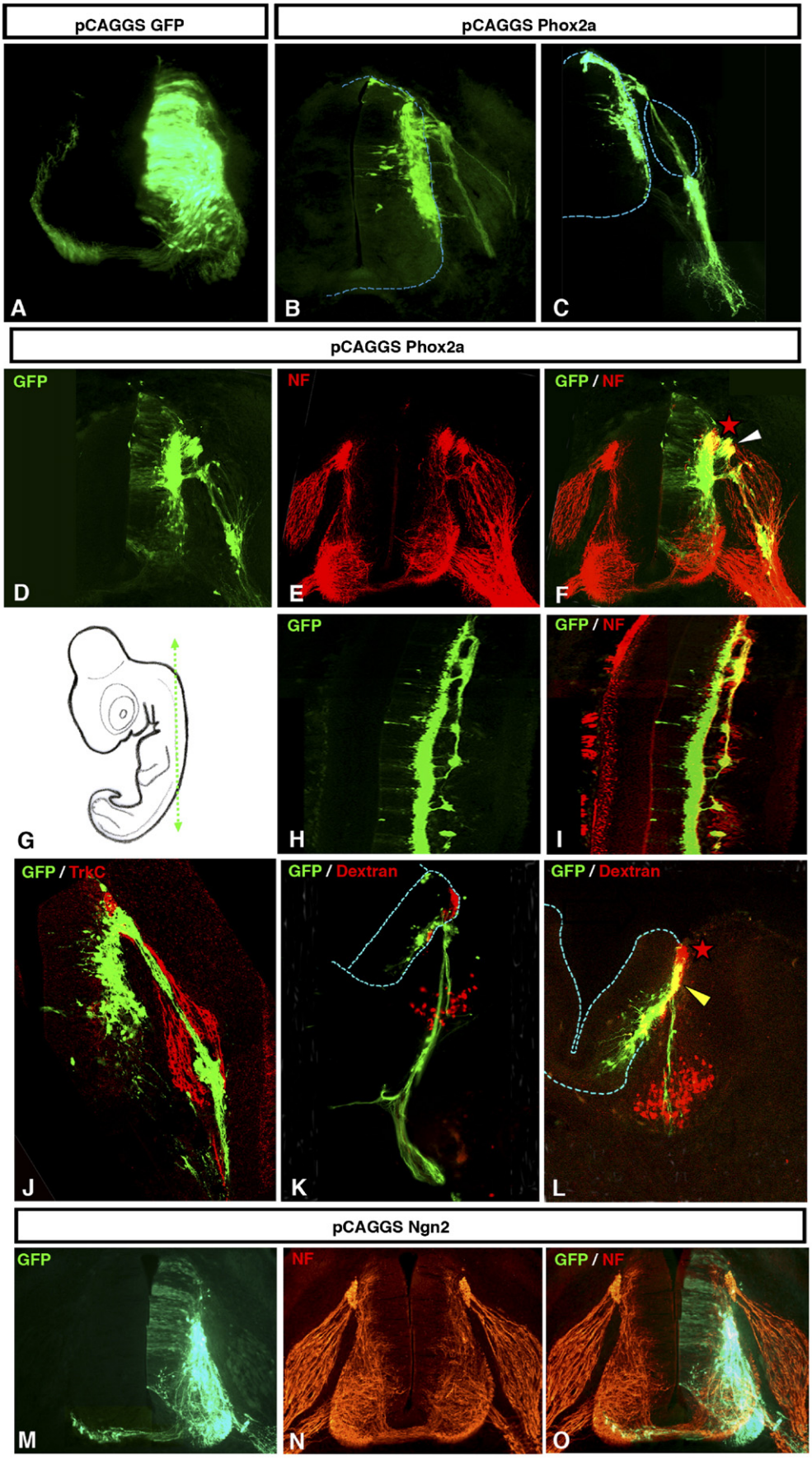
The spinal accessory nerve fails to form in Phox2b-deficient mice and is joined by axons of Phox2-transfected neurons

Many peripheral axons of the neurons that require *Phox2b* for proper development form common nerves with the axons of other *Phox2b*-expressing neurons. Examples are the cranial nerves VII, IX and X that typically contain the axons of both, the *Phox2*-expressing cranial sensory neurons and the corresponding bm/vm neurons, and the vidian nerve composed of facial vm axons and fibers from the cervical superior ganglion. The axons grown by *Phox2*-misexpressing cells often form tight fascicles (see Figs. 2B–D, H, K), but are they also attracted by the fibers of genuine *Phox2b*-expressing neurons?

At spinal levels, the spinal accessory motor (nXI) neurons located in the cervical spinal cord are considered bm neurons, although this has been debated for some time (Krammer et al., 1987). Like their hindbrain counterparts, they arise in the ventralmost progenitor domain, from which they migrate dorsally to their definite location. Their axons leave the neural tube dorsally to form the SAN that runs first alongside the neural tube and innervates the branchial arch-derived muscles of the neck and shoulder girdle (Krammer et al., 1987; Dillon et al., 2005; Lieberam et al., 2005; Pabst et al., 2003). If the nXI neurons were genuine bm neurons, they should express *Phox2a* and *Phox2b* and depend on *Phox2b* activity for proper development (Brunet and Pattyn, 2002). Indeed, we identified a ventrally located subpopulation of the Islet1,2-positive motoneurons that also expressed *Phox2b* in the cervical spinal cord of 4d-old (HH 22/23) chicken embryos (Figs. 4A, B) and were absent at brachial levels. They should thus correspond to nXI neurons, while the *Phox2b*-negative Islet1,2-positive cells are sm neurons present in the cervical spinal cord. At a slightly earlier stage, virtually all cells in the ventralmost progenitor domain expressed *Phox2b* (Fig. 4C) suggesting that, as in the ventral hindbrain (Pattyn et al., 2000b), *Phox2b* is switched on in cycling progenitors.

We then investigated whether the SAN would develop in *Phox2b* mutant mice. Using whole-mount anti-NF staining, we could visualize the SAN in embryonic day 11.5 (E11.5) wild-type mouse embryos by its characteristic trajectory alongside the rostralmost spinal cord and caudal hindbrain

Fig. 2. *Phox2a* and *Phox2b* misexpressions induce ectopic axonal trajectories. (A–J) Embryonic chicken neural tubes were electroporated at HH 12–13 with either *pCAGGS:: IRES2-EGFP* (A) or *pCAGGS::Phox2a-IRES2-EGFP* (B–F, H–J) and analyzed 3 days after electroporation (d.a.e.) by anti-GFP (A–C), combined anti-GFP and anti-NF (D–F, H, I) or combined GFP and anti-TrkC immunohistochemistry on transverse (A–F, J) or longitudinal (H, I) sections of the spinal cord. (G) Schematic of the plane of the longitudinal sections shown in panels H and I. Neurons transfected with GFP alone grow commissural axons that cross to the other side (A), and no axons are visible that leave the neural tube dorsally. *Phox2a*-transfected neurons grow axons that exit the spinal cord through the dorsal roots and between the dorsal and ventral roots (B) and that can be followed for some distance into the periphery (B–D) but that may also join the DF (red asterisk in panel F). A fraction of these axons form an ectopic fiber bundle that runs alongside the neural tube (arrowhead in panel F). Although they may traverse the DRG, the GFP-positive axons are not labeled by anti-TrkC antibodies, which mark all DRG axons at this stage (J). (K, L) At 3 d.a.e. with *pCAGGS::Phox2a-IRES2-EGFP*, RDA crystals were placed into the DF at cervical levels and the embryos were left to develop for a further 5 h in culture before analyzing transverse spinal cord sections for anti-GFP immunoreactivity and rhodamine fluorescence. Cervical spinal cord sections around 0.35 mm caudal to the crystal are shown. Note that none of the RDA-containing cell bodies in the DRG is GFP-positive, while some cells inside the neural tube are double-labeled (yellow arrowhead in panel L). The red asterisk in panel L denotes the DF. (M–O) As an additional control, chick neural tubes electroporated with *pCAGGS::Ngn2* plus *pCAGGS::IRES2-EGFP* were analyzed at 3 d.a.e. by combined anti-GFP and anti-NF immunohistochemistry. Although Ngn2 promotes migration to the mantle layer, it does not induce ectopic axonal trajectories. Note that, in contrast to *Phox2*-transfected neurons, many *Ngn2*-overexpressing cells grow commissural axons. J represents a single optical section taken with a Leica TCS SP2 confocal microscope, all other pictures were taken with a Leica DMRAX2 microscope.


pCAGGS GFP
pCAGGS Phox2a
A
B
C
pCAGGS Phox2a
GFP
NF
GFP / NF
D
E
F
G
GFP
GFP / NF
H
I
GFP / TrkC
GFP / Dextran
GFP / Dextran
J
K
L
pCAGGS Ngn2
GFP
NF
GFP / NF
M
N
O

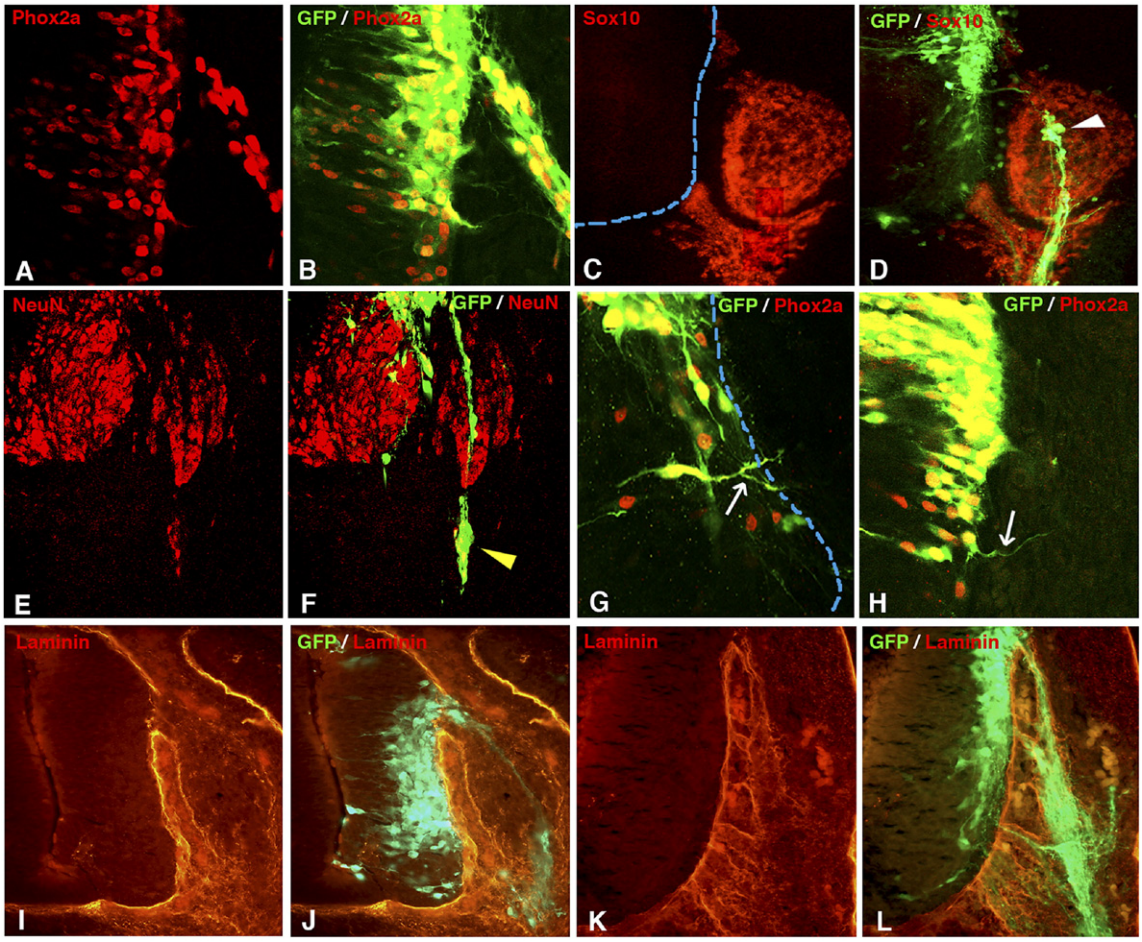
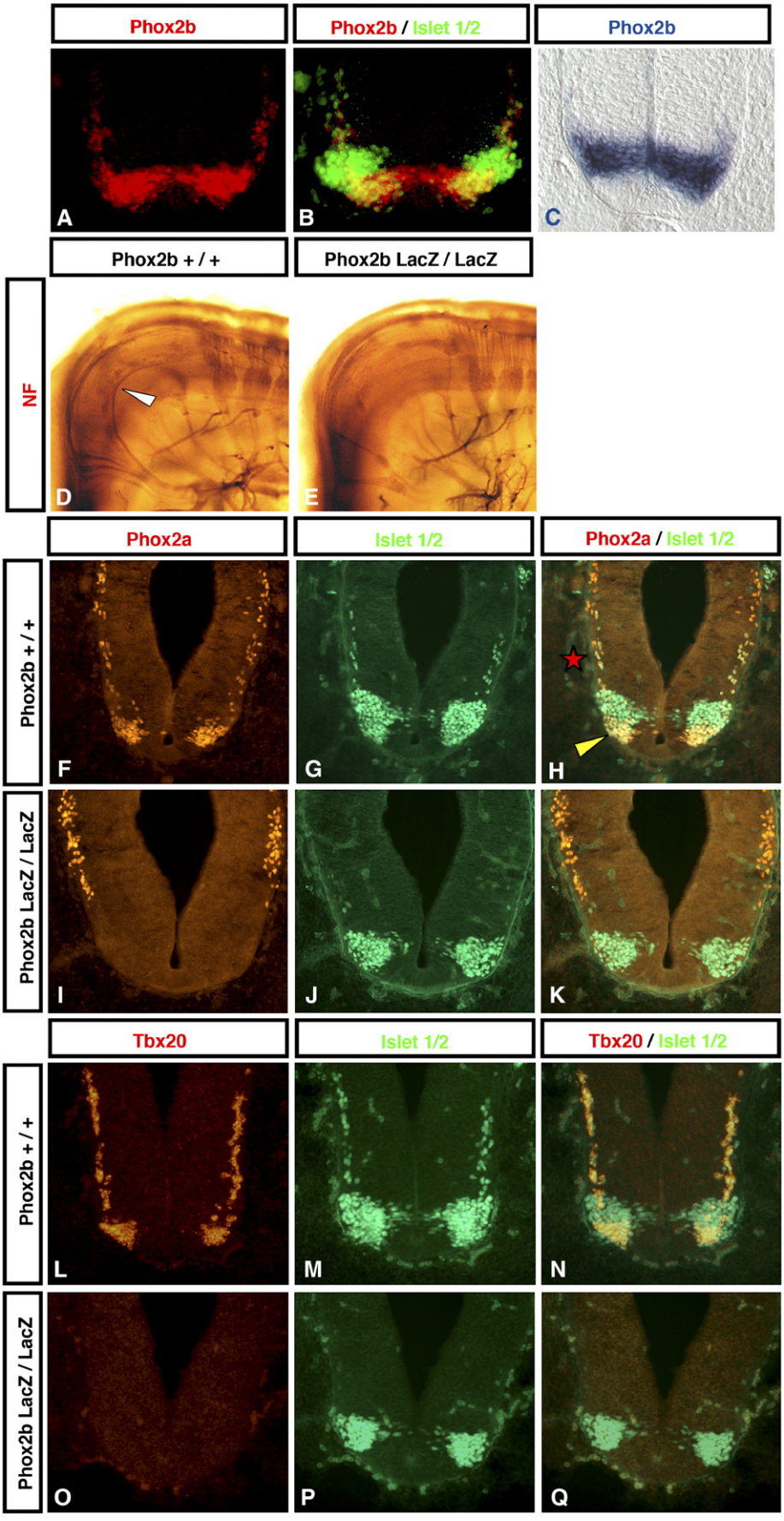


Fig. 3. Neural tube exit of *Phox2a*-transfected cell bodies and their axons. (A–L) Chicken neural tubes were analyzed at 30 h.a.e. (A, B, G, H) or 3 d.a.e. (C–F, I–L) with *pCAGGS::Phox2a-IRES2-EGFP* by combined anti-GFP and anti-Phox2a (A, B, G, H), anti-GFP and anti-NeuN (E, F) or anti-GFP and anti-laminin immunohistochemistry (I–L) or by combined anti-GFP labeling and in situ hybridization with a *Sox10* probe (C, D) on transverse sections of the spinal cord. (A, B) In some instances, transfected cell bodies, whose nuclei express mouse Phox2a, are seen outside of the spinal cord. (C, D) Although GFP-positive cells may be found within the DRG (arrowhead), they do not express *Sox10*, which at this stage marks all neural crest-derived cells. (E, F) The transfected cells that have exited the neural tube coexpress the NeuN antigen, a pan-neuronal nuclear marker (arrowhead pointing to yellow dots in panel F). (G, H) At 30 h.a.e., GFP-positive axons can be seen that are in the process of exiting the tube (arrows). In panel G, the border of the neural tube is marked by the stippled blue line. (I, J) The basal lamina is disrupted in the *Phox2a*-transfected area at places where GFP-positive cells are leaving the neural tube. (K, L) GFP-positive axons, by contrast, are seen to traverse an intact basal lamina. Panels A–H represent single optical sections taken with a Leica TCS SP2 confocal microscope, the pictures shown in panels I–L were taken with a Leica DMRAX2 microscope.

(Dillon et al., 2005; Pabst et al., 2003) (Fig. 4D). It was absent in homozygous *Phox2b* mutant littermates (Fig. 4E). We then analyzed the motoneuronal precursors on transverse sections of the cervical spinal cord of wild-type and mutant E10.5 embryos by double-staining for the generic motoneuronal marker Islet1,2 and for Phox2a or *Tbx20* that are specific for the bm/vm class of motoneurons (Pattyn et al., 2000; Dufour et al., 2006). In wild-type embryos, nXI precursors could be visualized at C1–C4 levels as a ventrally located subpopulation of Islet1,2-positive motoneurons (Dillon et al., 2005; Pabst et al., 2003) that coexpressed Phox2a (Figs. 4F–H) and *Tbx20* (Figs. 4L–N). In the homozygous *Phox2b* mutants, these Phox2a- and *Tbx20*-

Fig. 4. The spinal accessory nerve and neurons do not develop in *Phox2b* mutant embryos. (A, B) Transverse sections of the cervical spinal cord of 4 day-old (HH 23) chicken embryos were double-labeled by in situ hybridization with a *Phox2b* probe and anti-Islet1,2 antibodies. The subpopulation of Islet1,2-positive motoneurons that also express *Phox2b* and arise from the ventralmost progenitor domain corresponds to the nXI neurons. (C) In situ hybridization with a *Phox2b* probe at HH 17/18 shows that *Phox2b* is expressed throughout the progenitor domain at this stage. (D, E) Whole-mount preparations of E11.5 *wild-type* (D) and *Phox2b* mutant (E) mouse embryos were stained with anti-NF antibodies. The SAN, identified by its characteristic trajectory (white arrowhead), is absent in the mutants. (F–Q) Transverse sections of the cervical spinal cord at C2 levels of E10.5 wild-type (F–H, L–N) and *Phox2b* mutant (I–K, O–Q) mouse embryos were double-labeled by anti-Phox2a and anti-Islet1,2 antibodies (F–K) or by in situ hybridization with a *Tbx20* probe and Islet1,2 antibodies (L–Q). The double-positive cells (yellow arrowhead) are the nXI precursors, some of which are in the process of migrating dorsally (red asterisk), while the Islet1,2-positive, Phox2a- or *Tbx20*-negative cells correspond to sm neurons. No Phox2a^{+};Islet1.2^{+} or Tbx20^{+};Islet1,2^{+} double-labeled cells are present in the mutants. We counted 720±33 and 510±22 Islet1,2^{+} cells in the ventral cervical spinal cord from control and *Phox2b*$^{LacZ/LacZ}$ embryos, respectively, and 216±18 Phox2a^{+} cells in control embryos. The reduction in Islet1,2^{+} cells in the mutants (210) thus almost perfectly matches the number of Phox2a^{+} cells in the controls (216), suggesting that the nXI precursors do not transform into sm neurons.


Phox2b
Phox2b / Islet 1/2
Phox2b
A
B
C
Phox2b + / +
Phox2b LacZ / LacZ
NF
D
E
Phox2a
Islet 1/2
Phox2a / Islet 1/2
Phox2b + / +
F
G
H
Phox2b LacZ / LacZ
I
J
K
Tbx20
Islet 1/2
Tbx20 / Islet 1/2
Phox2b + / +
L
M
N
Phox2b LacZ / LacZ
O
P
Q

positive Islet1,2-expressing cells were absent (Figs. 4I–K and O–Q) while *Tbx20* expression in the heart was preserved (not shown). The Islet1,2-negative, Phox2a-positive cells at lateral positions are probably the precursors of the Phox2a-expressing subpopulation of dI5 interneurons (Ding et al., 2004). We thus conclude that nXI neurons are bona fide bm neurons that, like hindbrain bm neurons, express *Tbx20* and do not develop in *Phox2b* mutant embryos.

To test whether the axons of *Phox2*-transfected cells might have a propensity to join the SAN, we visualized the nXI neurons and their axons in the cervical spinal cord by staining for the BEN cell surface protein, which has been reported to be a specific marker for nIX neurons and their axons at corresponding stages in rodents (Dillon et al., 2005), as well as by anti-NF antibodies. In our hands, anti-BEN antibodies stained the axons of nXI neurons, of sm neurons and of the DRG, but not the axons of interneurons that remain within the neural tube (not shown). We found that the SAN contained many GFP-positive axons in *Phox2b*-transfected embryos (Figs. 5A–F). This was true even when electroporated cell bodies were found only at brachial and thoracic levels, indicating that their axons had grown rostrally to join the SAN at cervical levels (Figs. 5A–F). At thoracic levels, BEN was clearly induced by *Phox2b*, and at the transfected side, the BEN-positive fibers joined an ectopic fascicle entirely composed of GFP-positive axons that ran alongside the neural tube in the same way as the SAN does at cervical levels (Figs. 5G–I). When the transfected area extended into the cervical neural tube, GFP- and BEN-positive axons of nearby *Phox2b*-expressing neurons emerged from the dorsal spinal cord together with the endogenous BEN-expressing nXI axons, whose cell bodies are located in the basal plate (Figs. 5J–L). We consistently detected an increase in the cross-sectional area of the SAN at the transfected side (Figs. 5B, E), presumably a reflection of the additional contribution of fibers emanating from the *Phox2b*-transfected cells.

Together these results show that, by inducing a bm/vm phenotype, ectopic expression of *Phox2a* or *Phox2b* in the presumptive spinal cord profoundly affects axonal projections. Many of the axons grown by the transfected cells leave the neural tube, mostly via the dorsal roots. Once in the periphery, they may join other nerves, and some are found joining the SAN, the normal trajectory for *Phox2a/b*-expressing bm neurons located at cervical spinal levels.

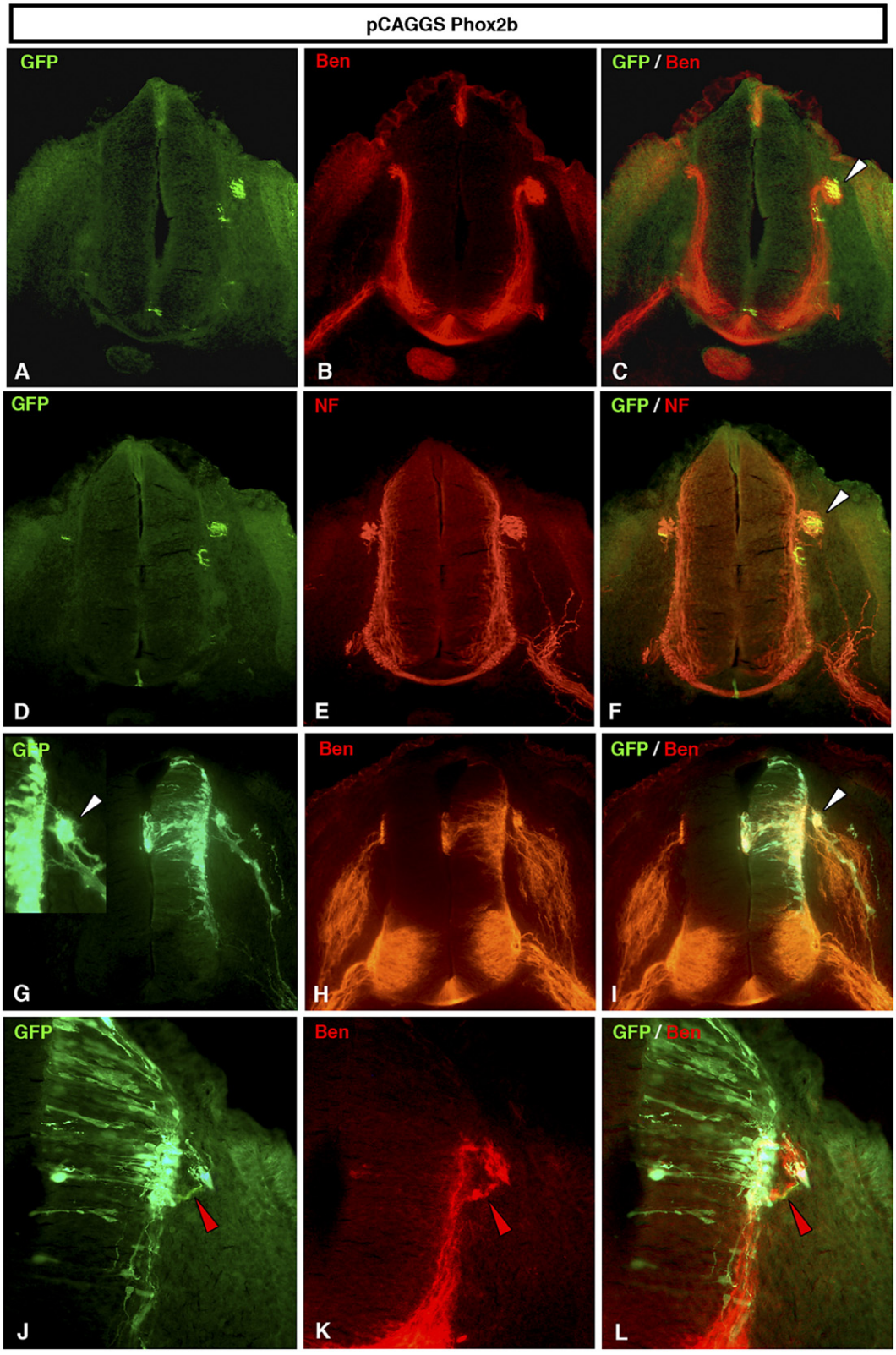
The axons of Phox2-transfected neurons are attracted by peripheral tissues

The axons grown by Phox2-misexpressing neurons may leave the spinal cord either because they have become responsive to repellent cues inside the neural tube or because they respond to attractive cues in the periphery, or both. To explore this point, we prepared explants of the spinal regions 24 h after they had been electroporated with the *Phox2a/b::IRES-GFP* vectors or with the *GFP* vector alone. After 3 days in culture, we assessed axonal growth into the surrounding collagen gel by GFP and NF staining (Figs. 6A–D) and recorded the ratio of axon outgrowth between the transfected and control sides (Figs. 6I, J). Explants transfected with *Phox2a* grew very few axons into the surrounding collagen gel, and there were no significant differences in outgrowth between *Phox2a*-transfected and control explants electroporated with *GFP* alone (Figs. 6A, B, J). The situation was more complex after electroporation of *Phox2b*; most explants (7/10) showed little outgrowth (Figs. 5C, D), but there were instances of quite diffuse growth from the transfected side in the remainder of the explants (not shown). The difference between *Phox2b*-transfected and control explants thus became marginally significant ($p=0.02$) (Fig. 6J). Hence, Phox2b but not Phox2a may also enhance responsiveness to repulsive cues or decrease that to attractants within the neural tube.

After having left the hindbrain, all bm/vm axons grow towards the cranial sensory ganglia of their respective cranial nerves that constitute their intermediate targets (Jacob et al., 2000; Moody and Heaton, 1983). From there, vm axons extend towards the viscera to innervate parasympathetic and enteric ganglia, while bm axons grow towards the branchial arches to innervate branchial arch-derived muscles. We therefore tested whether co-culture with cranial sensory ganglia or branchial arches would elicit axon outgrowth from *Phox2*-transfected explants into the surrounding collagen gel. In the following, we will use the term “attractive” in a loose sense, not denoting chemotropism, which we did not prove, but promotion of axonal growth directed towards the co-cultured tissue. In most cases, co-culture with cranial ganglia elicited profuse outgrowth of axons from *Phox2a::IRES-GFP* or *Phox2b::IRES-GFP*-expressing neurons, an effect not seen after transfecting *GFP* alone (Figs. 7A–H), and the increase in outgrowth was statistically highly significant, compared both to explants grown in isolation

Fig. 5. Axons of *Phox2b*-transfected neurons join the spinal accessory nerve. Chicken neural tubes electroporated at HH 12–13 with *pCAGGS::Phox2b-IRES2-EGFP* were analyzed at 48 h.a.e. by double-staining with either anti-GFP and anti-BEN (A–C, G–L) or with anti-GFP and anti-NF (D–F) antibodies. Anti-BEN labels the axons of the nIX neurons, and of sm and DRG neurons; anti-NF labels all axons. (A–F) Transverse sections through the rostral cervical spinal cord. The SAN is visible as a small BEN- and NF-positive cluster of fibers lying at both sides lateral and adjacent to the alar plate. The intraspinal trajectory of its axons, which arise from the nIX motor neurons in the basal plate and project dorsally towards their exit points in the alar plate, is also BEN-positive. The transfected area starts several segments caudal to the section. There are thus no GFP-positive cell bodies at this level, but GFP-positive fibers coming from more caudal levels are found within the SAN on the transfected side (arrowhead in panels C and F). Note that the SAN appears bigger on the transfected side (B, E). (G–I) Transverse section through the transfected area at brachial levels. A BEN-positive fiber bundle at a position where the SAN is located at rostral cervical levels is seen only at the transfected side (arrowheads in the insert of panel G and in panel I). The insert in panel G shows the transversally sectioned, GFP-positive fiber bundle at higher magnification. Note that *Phox2b*-expression induces BEN at the transfected side (H, I). (J–L) Transverse section through the transfected area of a cervical spinal cord. GFP-expressing neurons are visible in the alar plate, whose axons express BEN and exit the spinal cord intermingled with the BEN-expressing axons (red arrowhead in panels J–L) that come from nIX neurons in the basal plate. All pictures were taken with a Leica DMRAX2 microscope.

(Fig. 6O) and with respect to explants transfected with GFP alone (not shown). There was no obvious difference in the response to the trigeminal (Vth), geniculate (VIIth) or the petrosal (IXth) ganglia (not shown), and we thus pooled the data obtained with different ganglia. We next tested branchial arch tissue, which turned out to be highly attractive for axons derived from *Phox2a*-expressing neurons (Figs. 7I, J, O). The effect of the branchial arches appeared stage-specific since the



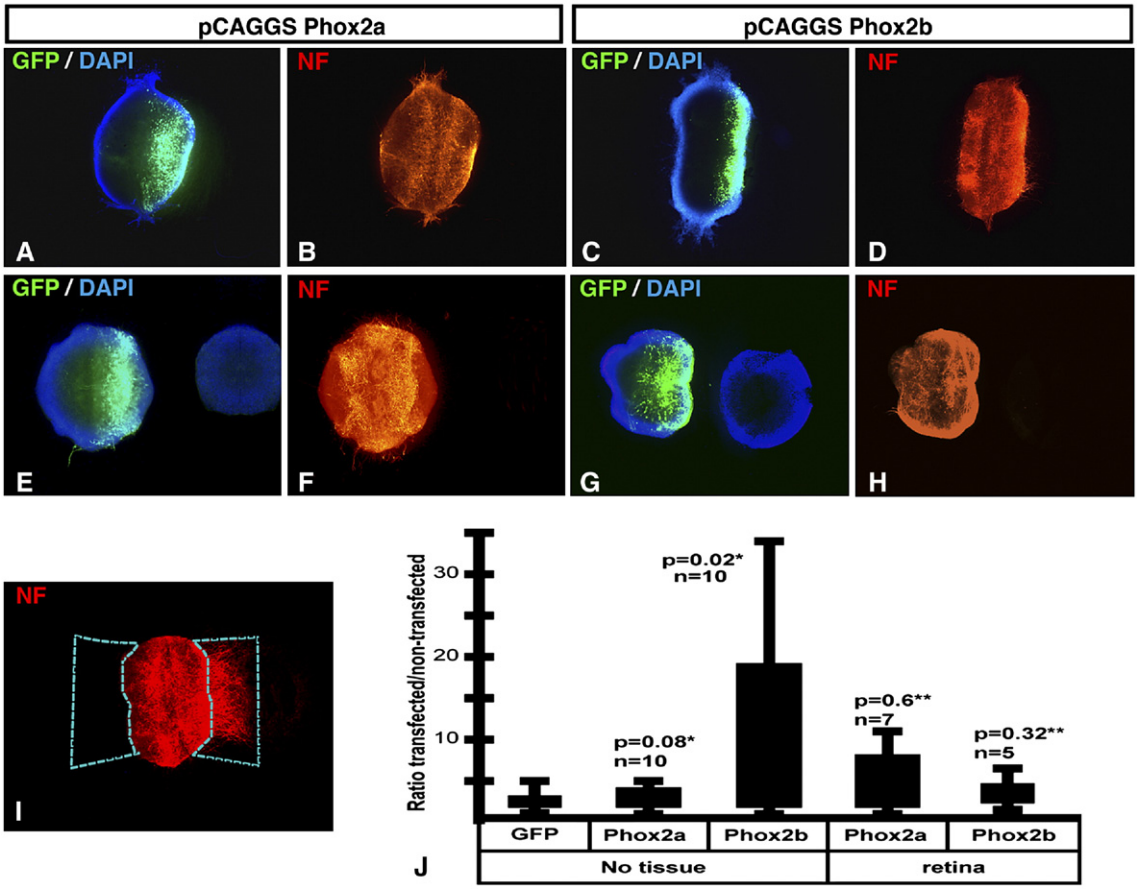


Fig. 6. *Phox2*-transfected spinal cord explants cultured in isolation or co-cultured with retina tissue show little axon outgrowth. (A–H) Explants were prepared from thoracic spinal cords 24 h.a.e. with *pCAGGS::Phox2a-IRES2-EGFP* (A, B, E, F) or *pCAGGS::Phox2b-IRES2-GFP* (C, D, G, H) and analyzed after 3 days in culture either alone (A–D) or in the presence of retinal tissue (E–H) by triple-labeling with anti-GFP and anti-NF antibodies and 4-6-diamidino-2-phenylindole (DAPI) to label nuclei. (I) Schematic representation of the areas, in which pixels were counted using ImageJ software. (J) Quantification of the results. Axon outgrowth into the surrounding collagen was estimated on NF-stained explants by counting the number of pixels in the areas lateral (dorsal) of the transfected and control sides and computing the ratio between the value for the transfected side over that for the control side. The data were analyzed using the Mann–Whitney *U*-test and are shown as box plots (Statview). The boxes represent the values lying between the 25th and 75th percentile, the bars depict the values between the 10th and the 90th percentile. **p* value computed with respect to control explants transfected with GFP alone, ***p* value with respect to explants cultured in isolation. Note that not only axon outgrowth was limited for most explants, but that also no or very few cells migrated out of the neural tube as best seen by DAPI staining. The NF-positive, GFP-negative axons leaving the explant rostrally and caudally near the floorplate are commissural axons cut during the isolation procedure.

third branchial arch taken from HH 12–13 embryos was highly attractive, but that taken from HH 19–20 embryos had no effect (not shown). Having passed through their respective sensory ganglia, vm axons project to the viscera, to innervate parasympathetic and enteric neurons. As an example of the viscera, we co-cultured spinal explants with pieces of the gut, which is invaded by the axons of the vagus (Xth) nerve on their way to the enteric plexuses. As did the cranial ganglia, co-cultured gut tissue elicited axonal growth from *Phox2*-transfected explants, which was not seen after transfecting GFP alone (Figs. 7K, L, O and not shown).

As a negative control, we used retina, which should not be a target for any normally *Phox2a/b*-expressing cells (although it may serendipitously express axonal guidance molecules shared with target tissues). Pieces of retina co-cultured with the explants did not provoke axonal growth from *Phox2*-expressing spinal explants greater than that observed for explants cultured in isolation (Figs. 6E–H, J).

Which tissue provides attractive cues to the axons from *Phox2a/b*-expressing neurons in the spinal cord that make them leave the neural tube? The DRG are an obvious possibility since they share some characteristics with cranial sensory ganglia and are frequently invaded by the misdirected fibers. Indeed, the DRG proved highly attractive for axons growing out of *Phox2a*-transfected explants, even more so than cranial sensory ganglia (Figs. 7M–O).

Together, these data suggest that ectopic expression of *Phox2a/b* makes the cells respond to attractive cues to which bm/vm axons normally respond, cues that may be provided by the DRG in spinal regions of the neural tube.

Discussion

Over the last 10 years, a wealth of cell surface and secreted signals that control growth and guidance of axons along defined pathways have been identified (Charron and Tessier-Lavigne,

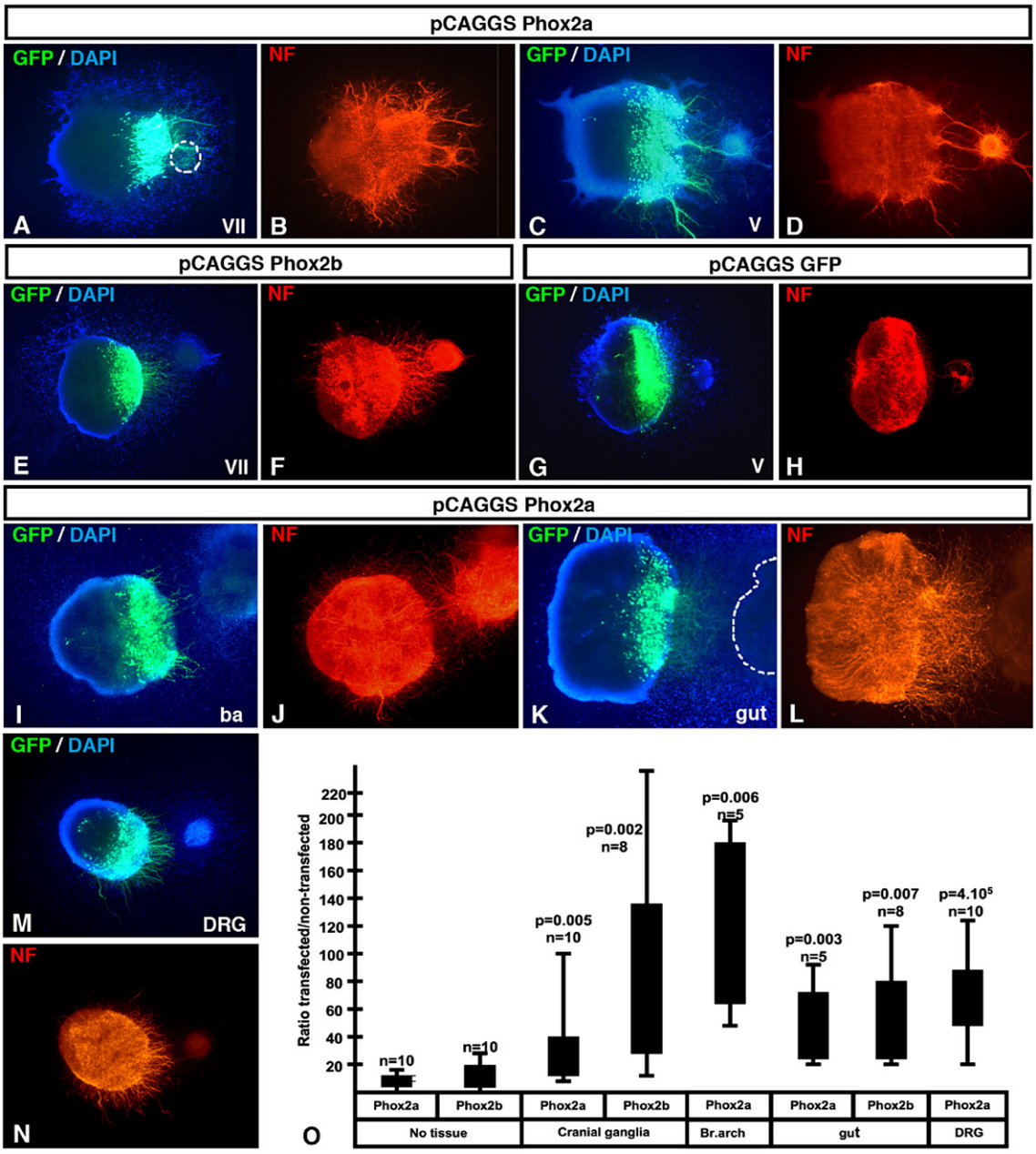


Fig. 7. Response of axons of *Phox2*-transfected spinal neurons to cranial ganglia, branchial arches, gut and DRG. (A–N) Explants were prepared from thoracic spinal cords 24 h.a.e. with *pCAGGS::Phox2a-IRES2-EGFP* (A–D, I–N), *pCAGGS::Phox2b-IRES2-GFP* (E, F) or *pCAGGS::IRES2-GFP* as control (G, H) and analyzed after 3 days of co-culture with cranial ganglia V (V) or VII (VII) (A–H), the 3rd branchial arch (ba) (I, J), gut tissue (K, L) or DRG (M, N) by triple-labeling with anti-GFP, anti-NF antibodies and DAPI. (O) Box plots of the data giving the 10%, 25%, 75% and 90% percentiles as in Fig. 6. All *p* values are computed with respect to explants cultured in isolation. Qualitatively similar results were obtained for explants prepared from brachial level spinal cords. Axon outgrowth is stimulated by the cranial ganglia that are intermediate targets for hindbrain bm/vm axons, by the 3rd branchial arch, a target for bm axons, and the gut, a target for vm axons. The DRG also stimulate axonal growth and may be responsible for attracting axons of the neurons that express *Phox2* ectopically at spinal levels. The results for cranial ganglia were most variable, perhaps due to small differences in developmental stage. Qualitative similar results were obtained for co-cultures with the IXth ganglion or the 2nd branchial arch (not shown). Cranial ganglia and gut were also co-cultured with explants transfected with GFP alone as control; very little or no fiber outgrowth was observed, and the results were not statistically significant from GFP-transfected explants cultured in isolation (not shown).

2005; Grunwald and Klein, 2002; Yu and Bargmann, 2001). Obviously, the expression of these molecules is somehow linked to the earlier deployment of fate-specifying transcription factors, but the role of transcriptional regulators in directing axonal growth and connectivity has begun to be explored only recently. Here we show that the paralogous Phox2a and Phox2b transcription factors, when ectopically expressed at spinal levels of the neural tube, impose a striking axonal phenotype. Neuronal precursors, which normally would grow mainly commissural axons that remain within the confines of the

neural tube, are now reprogrammed to grow axons, which may exit the neural tube or inappropriately join incoming sensory fibers or both.

The Phox2 transcription factors implement a branchio-visceral motoneuronal fate

Previous GOF studies have shown that forced expression of *Phox2b* imposes a gene expression program that resembles that of bm/vm neurons. The *Phox2b*-misexpressing cells resemble motor neurons in that they express Islet1 and choline acetyltransferase; they resemble the bm/vm subclass in that they express Islet1 (but not Islet2) and *Phox2a* (Dubreuil et al., 2000). Our finding that *Phox2b* also induces *Tbx20*, which in the brain is exquisitely specific for bm/vm neurons (Dufour et al., 2006), provides further evidence that *Phox2b* implements a genuine bm/vm phenotype. Here we show that its paralog *Phox2a* produces the same fate switch. This contrasts with other results showing that the Phox2a and Phox2b proteins are not functionally equivalent (Coppola et al., 2005). However, in GOF paradigms, the two paralogs have been invariably found to produce very similar if not identical effects (Adachi et al., 2000; Seo et al., 2002; Stanke et al., 1999, 2004). One likely explanation is that expression of either one induces expression of the other (Dubreuil et al., 2000, 2002; Stanke et al., 1999), but it is also possible that biochemical differences are masked by the high-level expression achieved in GOF experiments. In LOF experiments, *Phox2b*, but not *Phox2a* is essential for all aspects of bm/vm differentiation (Morin et al., 1997; Pattyn et al., 2000b), and *Phox2a* can only partially replace *Phox2b* (Coppola et al., 2005). This makes it likely that, in our experiments, Phox2a acts in part via Phox2b. The main difference we found was that *Phox2b*-transfected explants cultured in isolation were slightly more prone to extend axons out of the neural tube than *Phox2a*-transfected ones.

Phox2a/b expression induces an axonal phenotype resembling that of branchio-visceromotor neurons

In vertebrates, motor neurons are the only CNS neurons that project into the periphery. Hence, the neural tube exit of many axons derived from *Phox2*-transfected neurons may reflect the fact that the Phox2 transcription factors impose a generic motoneuronal fate. According to their axonal trajectories, motor neurons fall into two main classes: sm and spinal vm neurons, whose axons have ventral exit points, and the bm/vm neurons found in the hindbrain and cervical spinal cord, whose axons leave the neural tube dorsally. The axonal phenotype driven by *Phox2* expression clearly is of the bm/vm type. First, the misdirected axons mostly leave the neural tube dorsally as hindbrain bm/vm axons do. Second, in striking contrast to sm neurons, the axons of hindbrain bm/vm neurons typically invade the nearby sensory ganglia of their respective cranial nerves and form mixed nerves with sensory fibers. Similarly, the axons derived from the *Phox2*-expressing cells often project out of the neural tube along incoming DRG axons, grow across the DRG or join the DF made up of incoming sensory fibers, and sensory ganglia strongly promote axonal outgrowth from *Phox2*-transfected explants. A similar propensity for attraction by the DRG has also been seen for CXCR4-deficient sm axons, some of which appear to adopt bm axonal trajectories (Lieberam et al., 2005). Third, tissues, to which bm/vm axons normally grow, stimulate axonal growth from the transfected explants, an effect not seen with retinal tissue, which is never contacted by axons from normally *Phox2*-expressing cells. Clearly, the axons of *Phox2a/b*-misexpressing spinal neurons adopt two cardinal characteristics of bm/vm axons: they have a tendency to leave the spinal cord via dorsal exit points and are attracted by sensory neurons and their processes.

To which extent do the axons of the ectopically *Phox2a/b*-expressing neurons acquire an authentic bm/vm phenotype? The only endogenous bm neurons located at spinal levels are the nXI neurons in the rostral cervical spinal cord, whose axons assemble into the SAN that projects longitudinally alongside the cervical neural tube. They are considered bm neurons since, like bm neurons, they arise from progenitors adjacent to the floor plate, send their axons to dorsal exit points and innervate branchial arch-derived muscles (Dillon et al., 2005; Krammer et al., 1987; Pabst et al., 2003; Snider and Palavali, 1990). Our present results, showing that like all bm neurons they also express the Phox2 factors and depend on *Phox2b*, corroborate the notion that nXI neurons are of the bm class. In transfected chicken embryos, the *Phox2*-misexpressing cells located at cervical levels grow axons, which emerge from the dorsal spinal cord along with the nXI axons, thus behaving like authentic bm axons. Perhaps our most striking finding was the observation that, once in the periphery, a fraction of the axons of *Phox2*-transfected cells at thoracic levels form an ectopic nerve, which, similar to the SAN at cervical levels, runs alongside the neural tube and appears to join the SAN further rostrally. The fact that the axons of *Phox2*-transfected cells frequently leave the neural tube between the ventral and dorsal roots may similarly reflect their SAN-like character since normally, the rootlets of the SAN typically leave the spinal cord caudal to the dorsal roots (Snider and Palavali, 1990). The axons of *Phox2*-expressing neurons thus not only select aberrant pathways, but join the SAN, the only bm nerve found at spinal levels. Hence, *Phox2* expression appears sufficient to confer an authentic bm axonal trajectory.

The axonal phenotype imposed by the Phox2 factors may be a far downstream consequence of their capacity to implement a full-fledged bm/vm fate, but they may also control the expression of axonal guidance molecules more directly. Phox2a and Phox2b are typically switched on in the cycling progenitors or just around the time they become post-mitotic, yet remain expressed during later steps of neuronal differentiation, in some neuronal populations till after birth (Brunet and Pattyn, 2002; Tiveron et al., 1996). They are thus in a position to directly control later events such as axonal pathfinding and connectivity, but precocious arrest of differentiation when they are absent has precluded us from demonstrating a role in axonal guidance by simple LOF experiments.

Phox2a/b-expressing neurons respond to attractive cues in the periphery

Explants of *Phox2a/b*-transfected neurons cultured in isolation only rarely extend axons out of the neural tube, but they do so if confronted with tissues that are normally invaded by bm/vm nerves such as cranial sensory ganglia, branchial arches or gut. These findings suggest that repulsive cues within the neural tube are not sufficient to force the axons out of the neural tube and that attractive cues in the periphery are required. In contrast, another study found that attenuation of Slit-mediated repulsive signals from the floor plate prevented bm/vm axons from exiting the neural tube (Hammond et al., 2005). However, overexpressing *Slit* did not squeeze axons out of the hindbrain. Hence, Slit-Robo-signaling may be more involved in directing initial axonal trajectories within than in causing exit out of the neural tube. Cranial sensory ganglia and the branchial arches have been shown previously to promote axon outgrowth of bm/vm neurons in vitro (Caton et al., 2000; Tucker et al., 1997). These studies did not reveal selective responses of different types of bm/vm neurons to appropriate and inappropriate targets (that is from the same or a different rostro-caudal level, respectively). In our experiments, we also did not find obvious differences in the response to different cranial ganglia, and the DRG, which are not normally contacted by bm/vm nerves, promoted axonal growth equally well or better. It looks as if forced expression of *Phox2* results in the acquisition of a generic bm/vm phenotype, which causes the axons to be attracted by sensory neurons in general and by the viscera.

The axonal projection patterns of the neurons that require *Phox2b* for proper development typically share three main characteristics: most of their axons project to the viscera, many join fibers originating from other *Phox2*-expressing neurons and many synapse with other *Phox2b*-dependent neurons to form circuits (Brunet and Pattyn, 2002; Jacob et al., 2000; Tiveron et al., 1996). The GOF experiments presented here confirm the first two aspects. As an example of the viscera, gut tissue promoted axonal growth in vitro, and a fraction of the ectopic axons joined the SAN, derived from *Phox2b*-expressing neurons.

Which are the molecular changes that underlie the axonal phenotype elicited by Phox2 misexpression?

Previous work has implicated two types of molecules normally expressed by sm neurons in their choice of a ventral exit point: Lhx3/4 and CXCR4. In the absence of the *Lhx3* and *Lhx4* transcription factors, many sm motor axons that normally leave the spinal cord through ventral exit points now exit dorsally (Sharma et al., 1998). As does *Phox2* misexpression, loss of *Lhx3/4* causes the formation of a SAN-like nerve at levels where it does not exist normally. Conversely, forced expression of *Lhx3* in the hindbrain produces aberrant ventral roots, consistent with the possibility that fibers that normally exit dorsally are induced to leave the neural tube ventrally. In contrast to Phox2a/b, however, Lhx3/4 affects the choice where to leave the neural tube, but not the decision whether to project into the periphery or to remain within the CNS. Lhx3 and 4 are specific for sm neurons and thus not in a position to play a role in the axonal phenotype imposed by the Phox2 factors. In the light of the Lhx3/4 data, one may ask whether misexpression of *Phox2* in sm neuron precursors would also force them to grow axons that exit the neural tube dorsally. However, the main effect we saw after misexpressing *Phox2a/b* in sm progenitors was a marked reduction in the production of differentiated neurons (MRH and CG, unpublished results). Most likely, at the stage at which we transfect the neural tube, sm progenitors are already partially specified and *Phox2* expression becomes incompatible with further differentiation.

At the effector level, loss of CXCR4-signaling has been found to cause spinal sm neurons to adopt trajectories reminiscent of bm/vm neurons and neurons misexpressing *Phox2* (Lieberam et al., 2005). A fraction of sm axons, instead of exiting the spinal cord through the ventral roots, now project dorsally within the neural tube and some of them appear to join the DF at thoracic and lumbar levels. At cervical levels, loss of CXR4-signaling causes sm neurons to extend axons that exit dorsally and join the SAN, but at more caudal levels, the CXCR4-deficient axons did not project out of the neural tube. Since *CXCR4*-deficient sm neurons select axonal trajectories pathways reminiscent of those taken by bm/vm axons, we considered that downregulation of CXCR4 may play a role in the response to *Phox2a/b*. We found that *CXCR4* expression in the ventricular zone was indeed downregulated by *Phox2a/b*. This, however, concerned only neuroepithelial cells, and we did not detect *CXCR4* expression in any post-mitotic neurons in the dorso-lateral spinal cord (MRH and CG, unpublished results).

Future work will be directed towards elucidating the downstream events, in particular the changes in the expression of guidance receptors, which cause the axonal phenotype we observe. So far, the molecular nature of the cues that direct bm/vm axons to dorsal exit points and from there to cranial ganglia and the branchial arches have remained largely unknown. Slit-Robo-signaling may be involved in steering bm/vm axons within the neural tube (Hammond et al., 2005). HGF has been found to participate in the attractive response to branchial arch tissue. However, outgrowth was only reduced, not abolished by anti-HGF antibodies, and in vivo, lack of HGF-Met-signaling affected the development of the XIIth nerve, which is of sm type, while bm/vm nerves were normal (Caton et al., 2000). Finally, the cues that cause motor axons to extend into the periphery have remained enigmatic. The GOF paradigm we describe lends itself to searches for the downstream effectors of the Phox2 transcription factors, which should comprise those that steer bm/vm axons out of the neural tube along characteristic pathways.

Acknowledgments

We thank Patrick Pla for providing mouse cranial ganglia used in initial experiments, Frances Lefcort and Tom Jessell for anti-TrkC and anti-Islet1/2 antibodies, respectively, and Johan Ericson and Paul Scotting for the *Tbx20* and *Sox10* probes, respectively. The monoclonal anti-BEN/SC1, anti-

neurofilament and anti Lhx1/5 antibodies developed by O. Pourquié, J. Dodd and T.M. Jessell, respectively, were obtained from Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences. This work was supported by grants from Agence Nationale de la Recherche (to JFB), Association Française contre les Myopathies (to CG) and the Fondation Franco-Norvegienne pour la Recherche Scientifique et Technique (to CG and JCG).

References

Adachi, M., Browne, D., Lewis, E.J., 2000. Paired-like homeodomain proteins Phox2a/Arix and Phox2b/NBPhox have similar genetic organization and independently regulate dopamine beta-hydroxylase gene transcription. DNA Cell Biol. 19, 539–554.

Brunet, J.-F., Pattyn, A., 2002. Phox2 genes—From patterning to connectivity. Curr. Opin. Genet. Dev. 12, 435–440.

Caton, A., Hacker, A., Naeem, A., Livet, J., Maina, F., Bladt, F., Klein, R., Birchmeier, C., Guthrie, S., 2000. The branchial arches and HGF are growth-promoting and chemoattractant for cranial motor axons. Development 127, 1751–1766.

Charron, F., Tessier-Lavigne, M., 2005. Novel brain wiring functions for classical morphogens: a role as graded positional cues in axon guidance. Development 132, 2251–2262.

Chen, A.I., de Nooij, J.C., Jessell, T.M., 2006. Graded activity of transcription factor Runx3 specifies the laminar termination pattern of sensory axons in the developing spinal cord. Neuron 49, 395–408.

Cheng, Y., Cheung, M., Abu-Elmagd, M.M., Orme, A., Scotting, P.J., 2000. Chick sox10, a transcription factor expressed in both early neural crest cells and central nervous system. Brain Res. Dev. Brain Res. 121, 233–241.

Coppola, E., Pattyn, A., Guthrie, S.C., Goridis, C., Studer, M., 2005. Reciprocal gene replacements reveal unique functions for Phox2 genes during neural differentiation. EMBO J. 24, 4392–4403.

Cordes, S.P., 2001. Molecular genetics of cranial nerve development in mouse. Nat. Rev., Neurosci. 2, 611–623.

Dasen, J.S., Tice, B.C., Brenner-Morton, S., Jessell, T.M., 2005. A Hox regulatory network establishes motor neuron pool identity and target-muscle connectivity. Cell 123, 477–491.

Dauger, S., Pattyn, A., Lofaso, F., Gaultier, C., Goridis, C., Gallego, J., Brunet, J.-F., 2003. Phox2b controls the development of peripheral chemoreceptors and afferent visceral pathways. Development 130, 6635–6642.

Dillon, A.K., Fujita, S.C., Matise, M.P., Jarjour, A.A., Kennedy, T.E., Kollmus, H., Arnold, H.H., Weiner, J.A., Sanes, J.R., Kaprielian, Z., 2005. Molecular control of spinal accessory motor neuron/axon development in the mouse spinal cord. J. Neurosci. 25, 10119–10130.

Ding, Y.Q., Yin, J., Kania, A., Zhao, Z.Q., Johnson, R.L., Chen, Z.F., 2004. Lmx1b controls the differentiation and migration of the superficial dorsal horn neurons of the spinal cord. Development 131, 3693–3703.

Dubreuil, V., Hirsch, M.-R., Pattyn, A., Brunet, J.-F., Goridis, C., 2000. The Phox2b transcription factor coordinately regulates neuronal cell cycle exit and identity. Development 127, 5191–5201.

Dubreuil, V., Hirsch, M.R., Jouve, C., Brunet, J.F., Goridis, C., 2002. The role of Phox2b in synchronizing pan-neuronal and type-specific aspects of neurogenesis. Development 129, 5241–5253.

Dufour, H.D., Chettouh, Z., Deyts, C., de Rosa, R., Goridis, C., Joly, J.-S., Brunet, J.-F., 2006. Pre-craniate origin of cranial motoneurons. Proc. Natl. Acad. Sci. U. S. A. 103, 8727–8732.

Erkman, L., Yates, P.A., McLaughlin, T., McEvilly, R.J., Whisenhunt, T., O'Connell, S.M., Krones, A.I., Kirby, M.A., Rapaport, D.H., Bermingham, J.R., O'Leary, D.D., Rosenfeld, M.G., 2000. A POU domain transcription factor-dependent program regulates axon pathfinding in the vertebrate visual system. Neuron 28, 779–792.

Glover, J.C., 1995. Retrograde and anterograde axonal tracing with fluorescent dextrans in the embryonic nervous system. Neurosci. Protocols 30, 1–13.

Gross, M.K., Dottori, M., Goulding, M., 2002. Lbx1 specifies somatosensory association interneurons in the dorsal spinal cord. Neuron 34, 535–549.

Grunwald, I.C., Klein, R., 2002. Axon guidance: receptor complexes and signaling mechanisms. Curr. Opin. Neurobiol. 12, 250–259.

Halfter, W., Dong, S., Yip, Y.P., Willem, M., Mayer, U., 2002. A critical function of the pial basement membrane in cortical histogenesis. J. Neurosci. 22, 6029–6040.

Hammond, R., Vivancos, V., Naeem, A., Chilton, J., Mambetisaeva, E., Andrews, W., Sundaresan, V., Guthrie, S., 2005. Slit-mediated repulsion is a key regulator of motor axon pathfinding in the hindbrain. Development 132, 4483–4495.

Hatakeyama, J., Bessho, Y., Katoh, K., Ookawara, S., Fujioka, M., Guillemot, F., Kageyama, R., 2004. Hes genes regulate size, shape and histogenesis of the nervous system by control of the timing of neural stem cell differentiation. Development 131, 5539–5550.

Herrera, E., Brown, L., Aruga, J., Rachel, R.A., Dolen, G., Mikoshiba, K., Brown, S., Mason, C.A., 2003. Zic2 patterns binocular vision by specifying the uncrossed retinal projection. Cell 114, 545–557.

Inoue, K., Ozaki, S., Shiga, T., Ito, K., Masuda, T., Okado, N., Iseda, T., Kawaguchi, S., Ogawa, M., Bae, S.C., Yamashita, N., Itohara, S., Kudo, N., Ito, Y., 2002. Runx3 controls the axonal projection of proprioceptive dorsal root ganglion neurons. Nat. Neurosci. 5, 946–954.

Jacob, J., Tiveron, M.-C., Brunet, J.-F., Guthrie, S., 2000. Role of the target in the pathfinding of facial visceral motor axons. Mol. Cell. Neurosci. 16, 14–26.

Jacob, J., Hacker, A., Guthrie, S., 2001. Mechanisms and molecules in motor neuron specification and axon pathfinding. BioEssays 23, 582–595.

Kania, A., Jessell, T.M., 2003. Topographic motor projections in the limb imposed by LIM homeodomain protein regulation of ephrin-A:EphA interactions. Neuron 38, 581–596.

Krammer, E.B., Lischka, M.F., Egger, T.P., Riedl, M., Gruber, H., 1987. The motoneuronal organization of the spinal accessory nuclear complex. Adv. Anat. Embryol. Cell Biol. 103, 1–62.

Lefcort, F., Clary, D.O., Rusoff, A.C., Reichardt, L.F., 1996. Inhibition of the NT-3 receptor TrkC, early in chick embryogenesis, results in severe reductions in multiple neuronal subpopulations in the dorsal root ganglia. J. Neurosci. 16, 3704–3713.

Lieberam, I., Agalliu, D., Nagasawa, T., Ericson, J., Jessell, T.M., 2005. A Cxcl12-CXCR4 chemokine signaling pathway defines the initial trajectory of mammalian motor axons. Neuron 47, 667–679.

Livet, J., Sigrist, M., Stroebel, S., De Paola, V., Price, S.R., Henderson, C.E., Jessell, T.M., Arber, S., 2002. ETS gene Pea3 controls the central position and terminal arborization of specific motor neuron pools. Neuron 35, 877–892.

Maina, F., Hilton, M.C., Ponzetto, C., Davies, A.M., Klein, R., 1997. Met receptor signaling is required for sensory nerve development and HGF promotes axonal growth and survival of sensory neurons. Genes Dev. 11, 3341–3350.

Marion, J.F., Yang, C., Caqueret, A., Boucher, F., Michaud, J.L., 2005. Sim1 and Sim2 are required for the correct targeting of mammillary body axons. Development 132, 5527–5537.

Mizuguchi, R., Sugimori, M., Takebayashi, H., Kosako, H., Nagao, M., Yoshida, S., Nabeshima, Y., Shimamura, K., Nakafuku, M., 2001. Combinatorial roles of olig2 and neurogenin2 in the coordinated induction of pan-neuronal and subtype-specific properties of motoneurons. Neuron 31, 757–771.

Moody, S.A., Heaton, M.B., 1983. Developmental relationships between trigeminal ganglia and trigeminal motoneurons in chick embryos: I. Ganglion development is necessary for motoneuron migration. J. Comp. Neurol. 213, 327–343.

Morin, X., Cremer, H., Hirsch, M.-R., Kapur, R.P., Goridis, C., Brunet, J.-F., 1997. Defects in sensory and autonomic ganglia and absence of locus coeruleus in mice deficient for the homeobox gene Phox2a. Neuron 18, 411–423.

Müller, T., Brohmann, H., Pierani, A., Heppenstall, P.A., Lewin, G.R., Jessell, T.M., Birchmeier, C., 2002. The homeodomain factor Lbx1 distinguishes two major programs of neuronal differentiation in the dorsal spinal cord. Neuron 43, 551–562.

Nguyen, L., Besson, A., Heng, J.I., Schuurmans, C., Teboul, L., Parras, C., Philpott, A., Roberts, J.M., Guillemot, F., 2006. p27kip1 independently

promotes neuronal differentiation and migration in the cerebral cortex. Genes Dev. 20, 1511–1524.

Pabst, O., Rummelies, J., Winter, B., Arnold, H.H., 2003. Targeted disruption of the homeobox gene Nkx2.9 reveals a role in development of the spinal accessory nerve. Development 130, 1193–1202.

Pak, W., Hindges, R., Lim, Y.S., Pfaff, S.L., O'Leary, D.D., 2004. Magnitude of binocular vision controlled by islet-2 repression of a genetic program that specifies laterality of retinal axon pathfinding. Cell 119, 567–578.

Pattyn, A., Morin, X., Cremer, H., Goridis, C., Brunet, J.-F., 1997. Expression and interactions of the two closely related homeobox genes Phox2a and Phox2b during neurogenesis. Development 124, 4065–4075.

Pattyn, A., Morin, X., Cremer, H., Goridis, C., Brunet, J.-F., 1999. The homeobox gene Phox2b is essential for the development of autonomic neural crest derivatives. Nature 399, 366–370.

Pattyn, A., Goridis, C., Brunet, J.-F., 2000a. Specification of the central noradrenergic phenotype by the homeobox gene Phox2b. Mol. Cell. Neurosci. 15, 235–243.

Pattyn, A., Hirsch, M.-R., Goridis, C., Brunet, J.-F., 2000b. Control of hindbrain motor neuron differentiation by the homeobox gene Phox2b. Development 127, 1349–1358.

Pfaff, S., Kintner, C., 1998. Neuronal diversification: development of motor neuron subtypes. Curr. Opin. Neurobiol. 8, 27–36.

Pujol, N., Torregrossa, P., Ewbank, J.J., Brunet, J.-F., 2000. The homeodomain protein CePHOX2/CEH-17 controls antero-posterior axonal growth in *C. elegans*. Development 127, 3361–3371.

Seo, H., Hong, S.J., Guo, S., Kim, H.S., Kim, C.H., Hwang, D.Y., Isacson, O., Rosenthal, A., Kim, K.S., 2002. A direct role of the homeodomain proteins Phox2a/2b in noradrenaline neurotransmitter identity determination. J. Neurochem. 80, 905–916.

Sharma, K., Sheng, H.Z., Lettieri, K., Li, H., Karavanov, A., Potter, S., Westphal, H., Pfaff, S.L., 1998. LIM homeodomain factors Lhx3 and Lhx4 assign subtype identities for motor neurons. Cell 95, 817–828.

Shirasaki, R., Lewcock, J.W., Lettieri, K., Pfaff, S.L., 2006. FGF as a target-derived chemoattractant for developing motor axons genetically programmed by the LIM code. Neuron 50, 841–853.

Snider, W.D., Palavali, V., 1990. Early axon and dendritic outgrowth of spinal accessory motor neurons studied with DiI in fixed tissues. J. Comp. Neurol. 297, 227–238.

Stanke, M., Junghans, D., Geissen, M., Goridis, C., Ernsberger, U., Rohrer, H., 1999. The Phox2 homeodomain proteins are sufficient to promote the development of sympathetic neurons. Development 126, 4087–4494.

Stanke, M., Stubbusch, J., Rohrer, H., 2004. Interaction of Mash1 and Phox2b in sympathetic neuron development. Mol. Cell. Neurosci. 25, 374–382.

Tiveron, M.-C., Hirsch, M.-R., Brunet, J.-F., 1996. The expression pattern of the transcription factor Phox2 delineates synaptic pathways of the autonomic nervous system. J. Neurosci. 16, 7649–7660.

Trochet, D., Hong, S.J., Lim, J.K., Brunet, J.F., Munnich, A., Kim, K.S., Lyonnet, S., Goridis, C., Amiel, J., 2005. Molecular consequences of PHOX2B missense, frameshift and alanine expansion mutations leading to autonomic dysfunction. Hum. Mol. Genet. 14, 3697–3708.

Tsuchida, T., Ensini, M., Morton, S.B., Baldassare, M., Edlund, T., Jessell, T.M., Pfaff, S.L., 1994. Topographic organization of embryonic motor neurons defined by expression of LIM homeobox genes. Cell 79, 957–970.

Tucker, A., Lumsden, A., Guthrie, S., 1997. Cranial motor axons respond differently to the floor plate and sensory ganglia in collagen gel co-cultures. Eur. J. Neurosci. 8, 906–916.

Yu, T.W., Bargmann, C.I., 2001. Dynamic regulation of axon guidance. Nat. Neurosci. 4, 1169–1176.