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Expression and interactions of the two closely related homeobox genes *Phox2a* and *Phox2b* during neurogenesis

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SUMMARY

Recent evidence suggests that specific families of homeodomain transcription factors control the generation and survival of distinct neuronal types. We had previously characterized the homeobox gene *Phox2a*, which is expressed in differentiating neurons of the central and peripheral autonomic nervous system as well as in motor nuclei of the hindbrain. Targeted deletion of the *Phox2a* gene affects part of the structures in which it is expressed: the locus coeruleus, visceral sensory and parasympathetic ganglia and, as we show here, the nuclei of the IIIrd and IVth cranial nerves. We now report on the characterization of Phox2b, a close relative of Phox2a, with an identical homeodomain. *Phox2a* and *Phox2b* are co-expressed at most sites, therefore suggesting a broader role for *Phox2* genes in the specification of the autonomic nervous system and cranial motor nuclei than revealed by the *Phox2a* knock-out mice. A detailed analysis of the relative timing of *Phox2a* and *Phox2b* expression at various sites suggests positive cross-regulations, which are substantiated by the loss of *Phox2b* expression in cranial ganglia of *Phox2a*-deficient mice. In the major part of the rhombencephalon, *Phox2b* expression precedes that of *Phox2a* and starts in the proliferative neuroepithelium, in a pattern strikingly restricted on the dorsoventral axis and at rhombomeric borders. This suggests that *Phox2b* links early patterning events to the differentiation of defined neuronal populations in the hindbrain.

Key words: *Phox2a*, *Phox2b*, neurogenesis, homeodomain, mouse, nervous system

INTRODUCTION

It is still a major challenge to understand how vast numbers of different neuronal types are generated and assigned their fates in the vertebrate nervous system. Among the transcriptional regulators implicated in vertebrate neural development, homeodomain proteins expressed in differentiating neurons are good candidates to control final neuronal phenotypes. Targeted gene inactivation experiments have demonstrated that homeodomain proteins are required for the generation of various classes of neurons (Erkman et al., 1996; Gan et al., 1996; Xiang et al., 1996; McEvilly et al., 1996; Pfaff et al., 1996; Morin et al., 1997). An emerging concept from these and other studies is that structurally similar homeodomain proteins could insure the determination of subsets of related neuronal phenotypes, suggesting that duplication of transcription factor genes is causally linked to the appearance of new subclasses of neurons during evolution. For example, the three known POU-domain proteins of the Brn-3 family are expressed in overlapping but distinct patterns (Xiang et al., 1995, 1996; Turner et al., 1994). Each is uniquely expressed in some classes of neurons and is necessary for their generation as shown by the knock-out phenotypes (Erkman et al., 1996; Gan et al., 1996; McEvilly et al., 1996; Xiang et al., 1996). They are co-expressed in other neurons, most of which are apparently spared in the knock-out mice. In these cells, they could be functionally redundant with each other or, alternatively, co-operate to diversify phenotypes on a combinatorial mode. The latter model has been proposed for the closely homologous LIM-homeodomain proteins expressed in motoneurons and commissural neurons of the spinal cord (Tsuchida et al., 1994; Tanabe and Jessell, 1996).

Phox2a is a homeodomain protein specific to the nervous system (Valarché et al., 1993; Tiveron et al., 1996). Its expression pattern reveals two striking correlates. The first is with a neurotransmitter phenotype: *Phox2a* is expressed in all neurons that transiently or permanently express dopamine-β-hydroxylase (DBH), the last enzyme in the pathway of noradrenaline synthesis, suggesting that *Phox2a* is a determinant of the noradrenergic phenotype. The second correlate is with neuronal circuitry: *Phox2a* expression is largely restricted to circuits involved in medullary control of autonomic functions (Tiveron et al., 1996). Inactivation of the *Phox2a* gene leads to agenesis of the locus coeruleus and of parasympathetic ganglia, to altered morphology of the superior cervical ganglion and to massive atrophy of cranial sensory ganglia (Morin et al., 1997). In the cranial ganglia, it was possible to show the dependence of *DBH* expression on *Phox2a*, providing the first in vivo evidence that *Phox2a* may indeed regulate the noradrenergic phenotype (Morin et al., 1997). The gene coding for the GDNF (glial cell line-derived neurotrophic factor) receptor subunit Ret is also regulated, directly or indirectly, by *Phox2a* in these ganglia, sug-

gesting a mechanism by which *Phox2a* controls the survival of cranial ganglion neurons (Morin et al., 1997). Although the knock-out phenotype confirmed and extended our original hypotheses for the biological role of *Phox2a*, many cells that normally express *Phox2a* were unaffected by the mutation, either morphologically or in their expression of *DBH* and *Ret*. This raised the possibility of functional redundancy with another gene.

Here, we report the characterization of *Phox2b*, which encodes a protein with a homeodomain identical to that of Phox2a. The two genes have widely overlapping but distinct expression patterns in both the central (CNS) and peripheral (PNS) nervous systems. We re-examine the *Phox2a*$^{-/-}$ phenotype using Phox2b as a marker and demonstrate a perfect correlation between sites spared by the *Phox2a* mutation and those where *Phox2b* expression is retained, suggesting a wider role for Phox2 proteins than revealed by the *Phox2a* knock-out. We provide genetic evidence for positive cross-regulations between the two genes, which may explain at least part of their widespread co-expression. Finally, the early *Phox2b* expression pattern in the rhombencephalon suggests that this transcription factor links early patterning events with later neurogenesis in the hindbrain.

MATERIALS AND METHODS

Animals

Mice were mated overnight and females were checked the following morning for the presence of a vaginal plug; this corresponded to gestational day 0.5 (E0.5). Embryos were dissected from the embryonic annexes and fixed in 4% paraformaldehyde in PBS. Brains from neonates were dissected out and treated with the same fixative. *Phox2a*$^{-/-}$ and *Phox2a*$^{-/-}$*/DBH-lacZ* embryos were obtained as described by Morin et al. (1997).

RT-PCR cloning of the *Phox2b* homeobox and isolation of a full length cDNA clone

The *Phox2b* homeobox was amplified using two degenerate oligonucleotides, Phoxd5′ and Phoxd3′, corresponding, respectively, to N-terminal and C-terminal peptides of the Phox2a homeodomain, and containing restriction sites for subsequent cloning (Phoxd5′: 5′GGCGAATTCA(AG)(AC)GIAT(ACT)(AC)GIACIAC(ACGT)TT(CT)AC3′; Phoxd3′: 5′AATTCGCGGCCGCTTIC(GT)(AG)AA(CT)-TTIGCIC (GT)IC(GT)(AG)TT3′). Total RNA (1 μg) from E13.5 mouse embryos was subjected to reverse transcription for 45 minutes at 42°C, using an oligo(dT) primer, after an annealing step of 5 minutes at 72°C. An aliquot (7 μl) was then subjected to 35 PCR cycles consisting of 1 minute 30 seconds at 95°C, 1 minute at 55°C and 1 minute at 72°C in 100 μl of reaction buffer (Promega) supplemented with 1.5 mM $MgCl_2$ and 100 pg of each primer. PCR products of the expected size (180 bp) were extracted by phenol-chloroform, digested with *Eco*RI and *Not*I, gel-purified and subcloned into pBluescript KS II+ (Stratagene). Samples of the resulting library were ordered and analyzed by sequencing and cross-hybridization with specific oligonucleotides. An oligonucleotide specific for *Phox2b* (5′TGCTAGCTCTTCCCTGGTGT3′) was used to screen 10^6 pfu from an E13.5 mouse cDNA library constructed in λpEXlox (Novagene). One positive clone was detected and the pEXlox plasmid was recovered by the loxP-Cre auto-subcloning system following the manufacturer's instructions. The insert was isolated and subcloned into the pBluescript KS II+ vector.

Production of an anti-Phox2b antiserum

An antiserum was produced (Neosystem) against a BSA-coupled 15mer corresponding to the C terminus of the Phox2b protein with an added N-terminal tyrosine (YPNGAKAALVKSSMF). The antiserum was tested by ELISA.

The anti-Phox2a antibody has been described in Tiveron et al. (1996).

In situ hybridization and immunohistochemistry

Antisense digoxigenin(DIG)-labelled riboprobes for *Phox2b*, *Phox2a*, *DBH*, *Islet-1*, *peripherin* and *lacZ* were produced using a DIG-RNA labelling kit (Boehringer-Mannheim), following the manufacturer's instructions.

Fixed embryos from different stages (E9, E10.5, E11.5) were treated for in situ hybridization as described by Wilkinson (1992). Hindbrains from E10.5 and E11.5 embryos were dissected out, flattened and conserved in 80% glycerol in PBS.

Combined nonradioactive in situ hybridization and immunohistochemistry on cryosections was done as described (Tiveron et al., 1996). For the *Phox2b* riboprobe, an RNAse step was added to avoid non-specific signals.

Histology

Newborn mice were given an overdose of anaesthetic, skinned and fixed in Bouin's solution (Sigma) for several days, decalcified, dehydrated and embedded in paraffin wax. Coronal sections of the head were cut at 12 μm and stained with haematoxylin and Mallory's trichrome (Mark et al., 1993).

Combined BrdU staining and Phox2b immunohistochemistry

BrdU (Sigma) was injected intraperitoneally into pregnant mice (6 mg/mouse) 1 hour or 3 hours before killing. Embryos were dissected out at E10.5, fixed in 4% paraformaldehyde in PBS overnight, cryoprotected in 20% sucrose and embedded in OCT (Miles). For BrdU detection, the 10 μm sections were postfixed in 4% paraformaldehyde for 15 minutes, blocked in DMEM-10% FCS (Gibco-BRL) for 2 hours, treated with 2 N HCl for 30 minutes at 37°C and neutralized in 0.1 M sodium tetraborate. Sections were incubated with a mouse anti-BrdU monoclonal antibody (Sigma) diluted 1/200 in DMEM-10% FCS, for 1 hour and then with a rat anti-mouse IgG FITC-conjugated antibody (1/100 in DMEM-10% FCS) for 1 hour. For Phox2b detection, sections were postfixed in 4% paraformaldehyde, preincubated overnight at 70°C in PBS, blocked 30 minutes in PBS-0.05%Tween 20 (PBT)-20% FCS, incubated for 2 hours with the rabbit anti-Phox2b antiserum (1/200 in PBT-5% FCS), and then with a Cy3-conjugated donkey anti-IgG antiserum (Jackson ImmunoResearch) for 1 hour (1/300 in PBT-5% FCS).

For combined BrdU/Phox2a or BrdU/Phox2b detection, sections were postfixed, preincubated overnight at 70°C in PBS, blocked in DMEM-10% FCS, treated 30 minutes at room temperature with 2 N HCl and neutralized, incubated consecutively at room temperature with a mouse anti-BrdU antibody for 1 hour and with the anti-Phox2a or anti-Phox2b rabbit antiserum for 2 hours and then simultaneously with the two secondary antibodies for 1 hour. Photographs of the same sections were superimposed using the Photoshop 3.0 (Adobe) program.

To determine the fraction of BrdU-labelled cells that were also Phox2a/b-positive in the region where the progenitors of the facial motor nucleus are located, we counted the total number of BrdU-positive (green) nuclei, and among them, the number of nuclei that were Phox2a-positive or Phox2b-positive (red) within the ventral Phox2b-positive column, on every other section through rhombomere 4. Virtually identical results were obtained in two different embryos.

RESULTS

Isolation of Phox2b

In a search for structural relatives of the homeodomain protein

Phox2a, we constructed from E13.5 mouse RNA a library of RT-PCR fragments encoding paired-like homeodomains. Among many known or novel members of the PRX superclass of homeobox genes (Bürglin, 1994; A.P. and J-F.B., unpublished data), we identified a clone that contained a homeobox 79% identical to that of *Phox2a*. We used an oligonucleotide derived from its sequence to isolate a cDNA clone with a 1.6 kb insert. Its 933 bp-long open reading-frame encoded a novel homeodomain protein closely related to Phox2a (Valarché et al., 1993), which we named Phox2b (Fig. 1A). The homeodomain of Phox2b is identical to that of Phox2a; the N-terminal domains of Phox2a and Phox2b are 57% identical (Fig. 1B). The C-terminal domains are highly divergent and show only scattered small blocks of homology (not shown). One notable feature of the Phox2b C-terminal domain is the presence of two stretches of alanines. Such polyalanine stretches are found in the C terminus of several homeodomain proteins (Poole et al., 1985; Frasch et al., 1987; Joyner and Martin, 1987; Hérault et al., 1996; Muragaki et al., 1996) and are, in some cases, associated with transcriptional repressor domains (Han and Manley, 1993a,b).

On northern blots of RNA from the neuroblastoma N2a, a 3.3 kb band was detected with a *Phox2b* probe, implying that the clone that we isolated is not full-length (not shown). There was no evidence of a poly(A) tail in the *Phox2b* clone, consistent with the fact that it was obtained through internal priming and is missing part of the 3′-untranslated region.

We produced a rabbit polyclonal antibody directed against the C-terminal 14-mer of the Phox2b protein. Throughout this study, we observed identical *Phox2b* expression patterns using in situ hybridization with a *Phox2b* cRNA probe or immunohistochemistry with the anti-Phox2b antibody, including sites of dif-

A.

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1    AAGCAGAGCCAAGTTTATTATGAGGACTATATACTCTAGAGACCTCAGACAAGGCATCTCACAGGAGGCTTTTTCATAAA
81   ACTAGGCTCGGCTGGTAGTAAGGAGGCCAGTGTGGAGGCAGGCGTTGAGCAGTGCACATCTCCCCACTCCAGCCACCGTC
161  TCCACATCCATCTTTTATTTCATTTTTCCACTTGGCTGAGCCATCCAGAACCTTTTCAATGTATAAAATGGAATATTCTT
                                                          M  Y  K  M  E  Y  S  Y   8

241  ACCTCAATTCCTCTGCCTACGAGTCCTGTATGGCCGGGATGGATACCTCCAGCTTGGCTTCAGCATATGCAGACTTCAGT
       L  N  S  S  A  Y  E  S  C  M  A  G  M  D  T  S  S  L  A  S  A  Y  A  D  F  S   34

321  TCCTGCAGCCAGGCCAGTGGCTTCCAGTATAACCCGATAAGGACCACTTTTGGGGCCACGTCCGGTTGCCCGTCCCTCAC
     S  C  S  Q  A  S  G  F  Q  Y  N  P  I  R  T  T  F  G  A  T  S  G  C  P  S  L  T   61

401  GCCGGGATCCTGCAGCCTGGGCACCCTCAGGGACCACCAGAGCAGTCCGTACGCCGCAGTTCCATACAAACTCTTCACCG
      P  G  S  C  S  L  G  T  L  R  D  H  Q  S  S  P  Y  A  A  V  P  Y  K  L  F  T  D  88

481  ACCACGGCGGCCTCAACGAGAAACGCAAGCAGCGGCGCATCCGCACCACCTTCACAAGCGCGCAGCTCAAAGAGTTGGAG
       H  G  G  L  N  E  K  R  K  Q  R  R  I  R  T  T  F  T  S  A  Q  L  K  E  L  E   114

561  AGGGTCTTCGCTGAGACGCACTACCCTGACATCTACACCAGGGAAGAGCTAGCACTGAAGATCGACCTCACCGAGGCGAG
     R  V  F  A  E  T  H  Y  P  D  I  Y  T  R  E  E  L  A  L  K  I  D  L  T  E  A  R   141

641  AGTCCAGGTGTGGTTCCAGAACCGCCGGGCTAAGTTTCGCAAGCAGGAGCGCGCAGCCGCCGCTGCTGCCGCCGCCGCCA
       V  Q  V  W  F  Q  N  R  R  A  K  F  R  K  Q  E  R  A  A  A  A  A  A  A  A  A  K  168

721  AAAACGGCTCCTCCGGGAAGAAGTCTGACTCCTCCCGGGACGACGAGAGCAAAGAGGCCAAGAGCACTGATCCCGACAGC
       N  G  S  S  G  K  K  S  D  S  S  R  D  D  E  S  K  E  A  K  S  T  D  P  D  S   194

801  ACTGGGGGCCCGGGACCCAACCCCAACCCGACCCCCAGCTGTGGGGCAAATGGCGGCGGTGGCGGAGGGCCCAGCCCAGC
     T  G  G  P  G  P  N  P  N  P  T  P  S  C  G  A  N  G  G  G  G  G  G  P  S  P  A   221

881  CGGAGCTCCGGGGGCGGCGGGCCCGGGGGGCCCGGGAGGCGAACCCGGCAAGGGCGGTGCGGCTGCGGCTGCTGCTGCAG
       G  A  P  G  A  A  G  P  G  G  P  G  G  E  P  G  K  G  G  A  A  A  A  A  A  A  A   248

961  CTGCAGCGGCTGCAGCGGCGGCTGCGGCTGCGGCGGCGGGAGGCCTGGCTGCGGCCGGAGGCCCCGGACAAGGCTGGGCT
       A  A  A  A  A  A  A  A  A  A  A  A  A  A  G  G  L  A  A  A  G  G  P  G  Q  G  W  A   274

1041 CCTGGCCCGGGCCCCATCACCTCCATCCCAGATTCTCTTGGGGGGCCCTTCGCCAGCGTCTTATCTTCGCTCCAAAGACC
     P  G  P  G  P  I  T  S  I  P  D  S  L  G  G  P  F  A  S  V  L  S  S  L  Q  R  P   301

1121 CAACGGTGCCAAAGCCGCCTTAGTGAAGAGCAGTATGTTCTGATCTGCGATCTGCGGCGGCGGCGGGCGAGCCCGGGGCC
       N  G  A  K  A  A  L  V  K  S  S  M  F  •                                     314

1201 CGGGCTGGCGAGTGGGCGAGTGGGTAGACCCAAGGCTAGTGTCGCTGCTGTCAGTGGCTTTTTTTTTTTTTTCATCGAAG
1281 GCCTAAAATGATCGCGATTCGAAAAAACAACGAGAAAATGACGTCGCTCCATTTCAACCCCACTCCTACCCCTTTCCTCA
1361 CCCCCAAACAAAGCAAAACAAAACAAACAAAAATCTTCACCTGCCTTGGGCTGCGCATAGGACAGGGCTCCACCTGCTGC
1441 CCAAGGGATGTGAGCTTGGACTTCGGGGCACTCTCAGGGGGCTGTGTCTGAGTGACGGGTGTATGTCTGTCTCAGAGAAT
1521 GTGTGTCTGTGGCCCCAAGTAGGTGATAGGGAGAGACGGGGGCACCACAACCAACTCAGTGACTTCTTAGGAAAAAAAAA
1601 AAAA
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B.

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          10        20        30        40        50          60          70
          |         |         |         |         |           |           |
MYKMEYSYLNSSAYESCMAGMDTSSLASAYADFSSCSQASGFQYNPIRTTFGATSG--CPSLTPGS--CSLGTLRDH
   • •••••  • •• • •     •••• ••  •••  •••• • •  • •  •  •• •  ••  • •• ••••
   MDYSYLNS--YDSCVAAME----ASAYGDFGACSQPGGFQYSPLRPAFPAA-GPPCPAL--GSSNCALGALRDH
              |         |         |         |         |           |           |
              10        20        30        40        50          60

      80        90        100       110       120       130       140     150
      |         |         |         |         |         |         |       |
QSSPYAAVPYKLFTDHGGLNEKRKQRRIRTTFTSAQLKELERVFAETHYPDIYTREELALKIDLTEARVQVWFQNRR
•  •• ••••• •    •• ••••••••••••••••••••••••••••••••••••••••••••••••••••••••
QPAPYSAVPYKFFPEPSGLHEKRKQRRIRTTFTSAQLKELERVFAETHYPDIYTREELALKIDLTEARVQVWFQNRR
    |         |         |         |         |         |         |       |
    70        80        90        100       110       120       130     140

          160
          |
AKFRKQERAAA
••••••••••
AKFRKQERAAS
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Fig. 1. (A) Nucleotide sequence of *Phox2b* (1604 bp) and deduced amino acid sequence (314 aa) (GenBank accession number Y14493). The homeodomain is underlined and the two alanine stretches are shown in boldtype. (B) Comparison of the NH2-terminal domains and homeodomains of the Phox2b (top) and Phox2a (bottom) proteins. The dots correspond to conserved amino acids. The homeodomains are underlined.

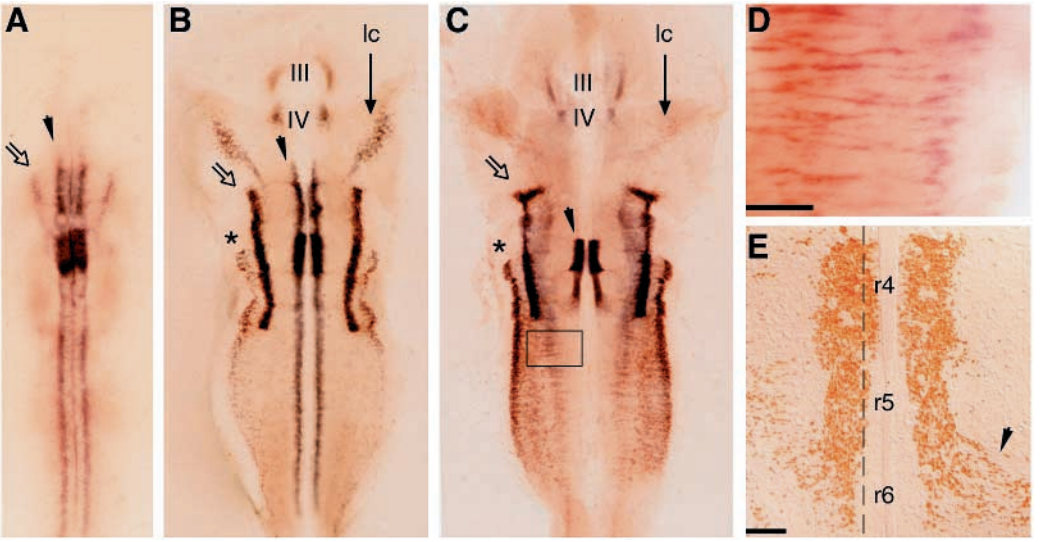

Fig. 2. Phox2b expression in the embryonic CNS. (A) Dorsal view of a whole-mount E9 embryo hybridized with a *Phox2b* probe. Expression is seen in the hindbrain, in a ventral stripe (black arrowhead) extending from the r1/r2 boundary into the cervical spinal cord, with thickenings in r2 and, most prominently, in r4. At this stage, a lateral stripe of *Phox2b*-positive cells has begun to appear at the level of r2 (open arrow). (B) Flat-mount preparation of an E10.5 CNS (midbrain + hindbrain) hybridized with a *Phox2b* probe. In the rhombencephalon, *Phox2b*-positive cells are found in a ventral stripe (black arrowhead), a lateral stripe (open arrow) and a dorsal stripe (asterisk). In the met-mesencephalic domain, *Phox2b*-positive cells are found in the oculomotor (III) and trochlear (IV) motor nuclei and in the forming locus coeruleus (lc). (C) Flat-mount of an E11.5 CNS hybridized with a *Phox2b* probe. Note that the locus coeruleus is now barely detectable. The ventral signal has disappeared, presumably by dorsal migration of the cells, except in r4 and r5. (D) Enlargement of the area marked by a window in C, showing strings of *Phox2b*-positive cells indicative of dorsal migration. (E) Anti-Phox2b immunohistochemistry on a horizontal section of a hindbrain at E11.5. Presumptive Phox2b-positive facial motoneurons seem to emerge from the neuroepithelium at the level of r4, undergo a caudal migration through r5 (Goddard et al., 1996; Auclair et al., 1996) and a lateral (i.e. dorsal) migration in r6 (black arrowhead). Note that Phox2b-positive cells are seen in the neuroepithelium of r4 but not of r5. The dotted line marks the basal limit of the neuroepithelium. Bars: 100 μm.

ference between *Phox2a* and *Phox2b* expression. This demonstrated the specificity of the antibody and the absence of discrepancy between mRNA and protein expression.

Expression pattern of *Phox2b*

In E9 brains, two ventral columns of *Phox2b*-positive cells ran on either side of the floor plate from an abrupt rostral limit just anterior to the rhombomere1/rhombomere2 (r1/r2) boundary into the cervical spinal cord (Fig. 2A). These columns were broader and more intense in r2 and, most prominently, in r4 where they most likely correspond to the anlagen of the Vth and VIIth motor nuclei, respectively (see below). One day later, the ventral column with its r2- and r4-specific thickenings was still strongly labeled (Fig. 2B). In addition, a stripe of Phox2b-positive cells had appeared laterally, sharply limited rostrally at the r1/r2 boundary and caudally at the r6/r7 boundary. Dorsally, another stripe of scattered Phox2b-positive cells extended from a sharp limit at the r3/r4 boundary into the cervical spinal cord. A group of cells in the dorsolateral aspect of r1 (Fig. 2B) could be assigned to the forming locus coeruleus by combined anti-Phox2b immunocytochemistry and *DBH* in situ

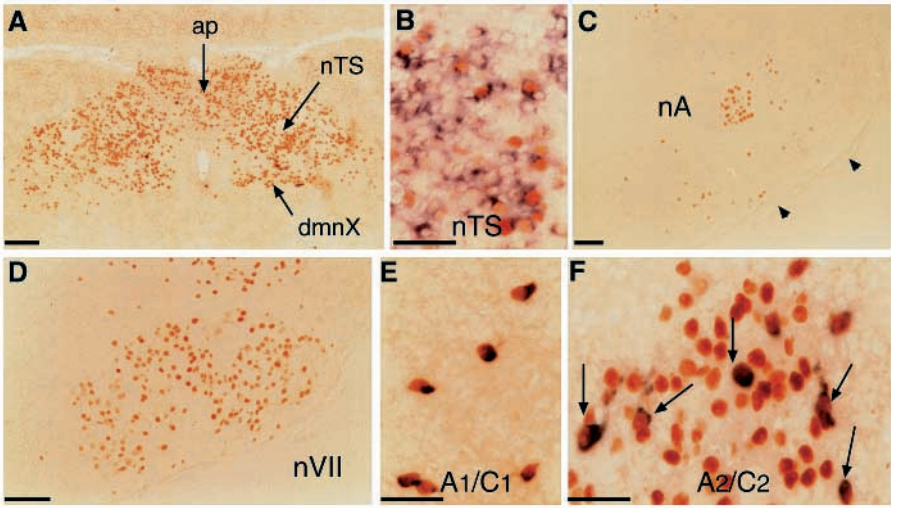

Fig. 3. Phox2b expression in the neonatal CNS. (A) Phox2b expression revealed by immunhistochemistry in the area postrema (ap), the nucleus of the solitary tract (nTS) and the dorsal motor nucleus of the vagus nerve (dmnX). (B) High magnification of a combined anti-Phox2a immunohistochemistry (orange)/*Phox2b* in situ hybridization (black) on the nTS, showing that Phox2a-positive cells represent a subset of Phox2b-positive cells. (C) Phox2b expression in the nucleus ambiguus (nA). In addition, scattered Phox2b-positive cells are visible close to the ventral surface of the medulla, marked by arrowheads. (D) Phox2b expression in the facial nucleus. (E,F) Combined Phox2b immunohistochemistry/*DBH* in situ hybridization showing the A1/C1 (nor)adrenergic groups in the ventrolateral medulla (E) and the A2/C2 groups in the dorsomedial aspect of the medulla (F). The black stain corresponds to DBH message in the cytoplasm, Phox2b protein in the nucleus is revealed in orange. Arrows point to double Phox2b/*DBH*-positive cells. In E, all Phox2b-positive cells are also *DBH*-positive and vice versa. Bars: A,C,D, 100 μm; B,E,F, 50 μm.

hybridization (not shown). Two ventral patches on both sides of the met-mesencephalic border (Fig. 2B,C) could be identified at later stages as, respectively, the forming trochlear (IV) and oculomotor (III) nuclei by combined anti-Phox2b immunohistochemistry/*choline acetyl transferase (ChAT)* in situ hybridization (data not shown).

At E11.5, the ventral columns had disappeared except in r4 and r5 (Fig. 2C), whereas the dorsal column contained many more *Phox2b*-positive cells. The scattered *Phox2b*-positive cells located outside the columns were organized in ventrodorsal strings (Fig. 2D), evocative of cell migration and suggesting that the ventral columns had been depleted and the dorsal columns populated by these migrations. Other patterns suggesting cell migrations were observed at the rostral tips of the lateral columns (Fig. 2C) and, in r6, in continuity with a ventral band of Phox2b-positive cells in r5 and r4 (Fig. 2E). These cells correspond probably to the motoneurons of the facial nucleus, which remain Phox2b-positive at later stages (see below) and have previously been reported to be born in r4 and to migrate to r5, based on the expression pattern of *Hoxb-1* (Goddard et al., 1996). Our data, in agreement with a recent study by Auclair et al. (1996), suggests that they migrate along the midline through r5 into r6, where they turn towards the lateral aspect of the tube.

In the neonatal brain, the expression pattern of *Phox2b* was similar to that of *Phox2a* (Tiveron et al., 1996) and included the nucleus of the solitary tract (nTS) and area postrema (Fig. 3A,B), the IIIrd (oculomotor), IVth (trochlear), VIIth (facial), IXth, Xth and XIth (nucleus ambiguus and dorsal motor nucleus of the vagus nerve) cranial nerve nuclei (Fig. 3A-D and not shown) and the myelencephalic noradrenergic centers (Fig. 3E,F and not shown). Although the motor nucleus of the Vth cranial nerve was *Phox2b*-negative at birth, it did express *Phox2b* (but not *Phox2a*) transiently up to midgestation (not shown). No expression was seen in the spinal cord. At many sites, *Phox2a*-positive cells represented a subset of *Phox2b*-positive cells (Fig. 3B and not shown).

In the PNS, *Phox2b*, like *Phox2a* (Tiveron et al., 1996), was found to be expressed in three cranial sensory ganglia (the distal VIIth, IXth and Xth ganglia) as soon as E9.5 (but not in other cranial or dorsal root ganglia) and in all ganglia of the autonomic nervous system as early as they form, at least up to midgestation (Fig. 4 and not shown).

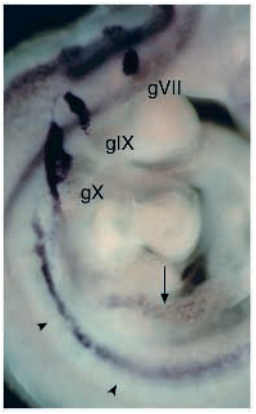

Fig. 4. *Phox2b* expression in the developing PNS. Lateral view of a whole-mount preparation of an E10.5 embryo hybridized with a *Phox2b* probe. gVII, geniculate ganglion; gIX, petrose ganglion; gX, nodose ganglion; arrow, progenitors of the enteric nervous system; arrowheads, primary sympathetic chain.

Complex spatiotemporal relationship of *Phox2a* and *Phox2b* expression

Although the list of *Phox2b* expression sites is strikingly similar to that reported for *Phox2a* (Tiveron et al., 1996), we observed systematic differences in onset, persistence and extent of expression.

In the CNS, two opposite sequences of *Phox2a* and *Phox2b* expression were observed at the met-mesencephalic junction and in most of the rhombencephalon (caudal to r1), respectively. In the forming IIIrd and IVth motor nuclei, *Phox2a* expression preceded that of *Phox2b*: it started around E9 before any *Phox2b* message was detectable (Fig. 5A,B) and, one day later, Phox2a protein was present in the neuroepithelial precursors at these sites whereas Phox2b was found only in the differentiating neurons which had moved to the mantle layer (Fig. 5E,F). Similarly, the locus coeruleus precursors were Phox2a-positive, but still Phox2b$^-$ at E9.5 (Fig. 5C,D). The situation was reversed in the rhombencephalon caudal to r1: at E10.5 and E11.5, Phox2b-positive cells were found throughout the neuroepithelium and mantle layer at the level of the ventral and lateral columns, whereas Phox2a-positive cells (which are also Phox2b-positive) were restricted to the mantle layer (Fig. 5G,H).

In the hindbrain, as in other parts of the neural tube, the proliferating neural progenitors populate the neuroepithelium or ventricular layer. Their cell bodies lie at different levels, with an endfoot at both the apical (luminar) and basal surfaces. As a general rule, cells divide close to the lumen and nuclei migrate basally as they go through S phase, in a process called interkinetic nuclear migration (Sauer, 1936; Guthrie et al., 1991). Young neurons, which have exited the cell cycle, move basally and into the mantle layer. To ascertain that *Phox2a* or *Phox2b* was indeed turned on in cycling progenitors in different parts of the hindbrain, as suggested by the topography of their expression, we combined acute (1 hour) BrdU labelling and anti-Phox2a or anti-Phox2b immunohistochemistry. Assuming a cell cycle time of at least 8 hours, as was determined in the chick hindbrain (Guthrie et al., 1991), our 1 hour-labelling period is too short to allow most cells to complete S-phase and go through mitosis. Indeed, most BrdU-positive nuclei were located in the pial aspect of the neuroepithelium as expected of S-phase nuclei. In the region of the forming oculomotor (Fig. 6A,B) and trochlear (not shown) nuclei, a large fraction of Phox2a-positive, but no Phox2b-positive cells had incorporated BrdU. The situation was reversed caudal to r1, where many Phox2b-positive /BrdU-positive but very few Phox2a-positive /BrdU-positive cells were found (Fig. 6C-F). In fact, the great majority of BrdU-positive cells expressed Phox2a in the forming oculomotor and trochlear nuclei; in r2 and r4, most Brdu-positive cells were also Phox2b-positive. Precise cell counts revealed that, in the ventral aspects of r4, at the level of the forming facial nucleus, 70% of all cells that had incorporated BrdU expressed Phox2b (720/1016 cells), whereas only 5% of the BrdU-positive cells expressed Phox2a (26/515 cells). The latter probably correspond to postmitotic cells that were labelled by BrdU in late stages of S-phase. From these data, we conclude that *Phox2a*

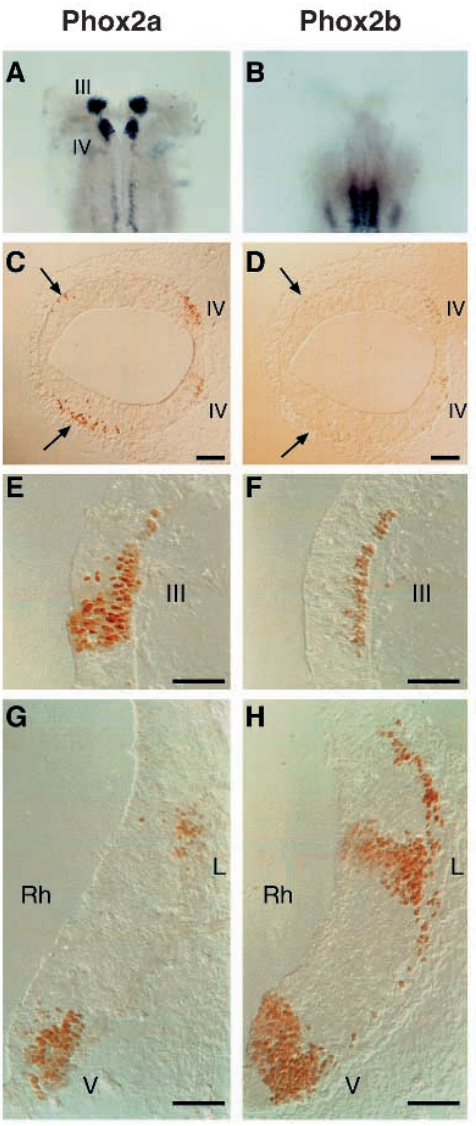

Fig. 5. Order of appearance of Phox2a (left panels) and Phox2b (right panels) in the embryonic CNS. (A,B) Anterior dorsal views of whole-mount preparations of E9.0 embryos labelled with *Phox2a* (A) and *Phox2b* (B) cRNA probes. *Phox2a* is detected in the forming oculomotor (III) and trochlear (IV) nuclei, whereas no expression of *Phox2b* can be seen. (C-H) Immunohistochemistry on sections through the hindbrain using an anti-Phox2a (C,E,G) or an anti-Phox2b (D,F,H) antibody. (C,D) Consecutive coronal sections through the metencephalon at E9.5. Ventral is to the right. In the locus coeruleus anlage (arrows), Phox2a expression (C) precedes that of Phox2b (D). The caudal aspect of the trochlear nucleus (IV) is visible ventrally, expressing Phox2a and still very low levels of Phox2b. (E,F) Consecutive sagittal sections through the isthmus at E10.5 at the level of the oculomotor nucleus. Ventral is to the right and the lumen of the isthmus to the left. Phox2a (E) is expressed in the ventricular layer and in the postmitotic neurons invading the mantle layer, whereas Phox2b (F) is only expressed in postmitotic neurons of the mantle layer. (G,H) Consecutive transverse sections through the rhombencephalon of an E10.5 embryo at the level of r4, showing a pattern opposite to that in E and F: Phox2a (G) is only expressed in the mantle layer of the ventral (V) and lateral (L) stripes whereas Phox2b (H) is detected also in the ventricular layer. The string of Phox2b-positive cells seen in the mantle layer dorsal to the lateral stripe constitutes the dorsal stripe seen in Figs. 2B and 2C. These cells are Phox2a-negative. Bars: 100 μm.

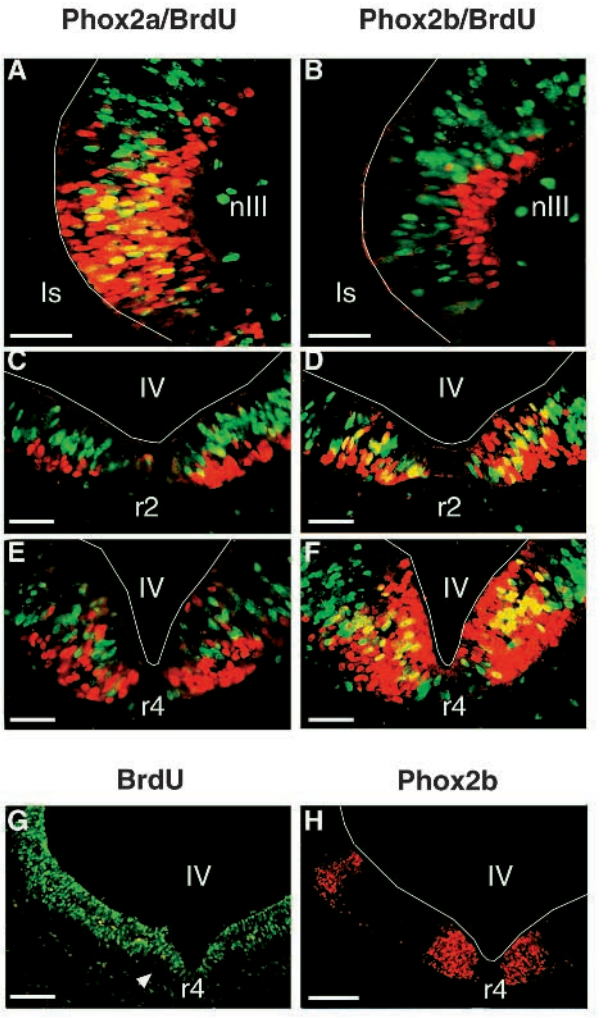

Fig. 6. Combined anti-Phox2a or anti-Phox2b and anti-BrdU immunofluorescence after pulse-labelling of S-phase nuclei on coronal sections of an E10.5 hindbrain (see Materials and Methods). (A-F) Double immunofluorescence showing in red Phox2a-positive cells (A,C,E) or Phox2b-positive cells (B,D,F) and in green BrdU-positive cells in the occulomotor nucleus (A,B) and the ventral column of Phox2-positive cells in r2 (C,D) and r4 (E,F). Phox2a-positive or Phox2b-positive cells which have incorporated BrdU after a 1-hour BrdU pulse appear yellow. S-phase nuclei express Phox2a but not Phox2b at the level of the oculomotor nucleus, and Phox2b but not Phox2a in r2 and r4. (G,H) Immunofluorescent detection of BrdU incorporation (G) and Phox2b (H) after a 3-hour BrdU pulse on adjacent sections at the level of r4. Phox2b is expressed throughout the ventricular and mantle layers in the ventral and lateral columns. BrdU incorporation is seen only thoughout the ventricular zone. The layer of BrdU-positive cells is much thinner at the level of the ventral column (white arrowhead) suggesting a depletion of the precursor pool. Is, isthmus; nIII, oculomotor nucleus; r2 and r4, second and fourth rhombomeres; IV, lumen of the IVth ventricle. Bars: A,B,C,D,F, 50 μm; G,H, 100 μm.

and *Phox2b* expression is initiated before the last mitosis in, respectively, the isthmus (containing the precursors of the oculomotor and trochlear neurons) and the rhombencephalon caudal to r1 (including the precursors of the facial nucleus). In each domain, the other *Phox2* gene is probably switched on after the last mitosis. In the ventricular layer of these domains,

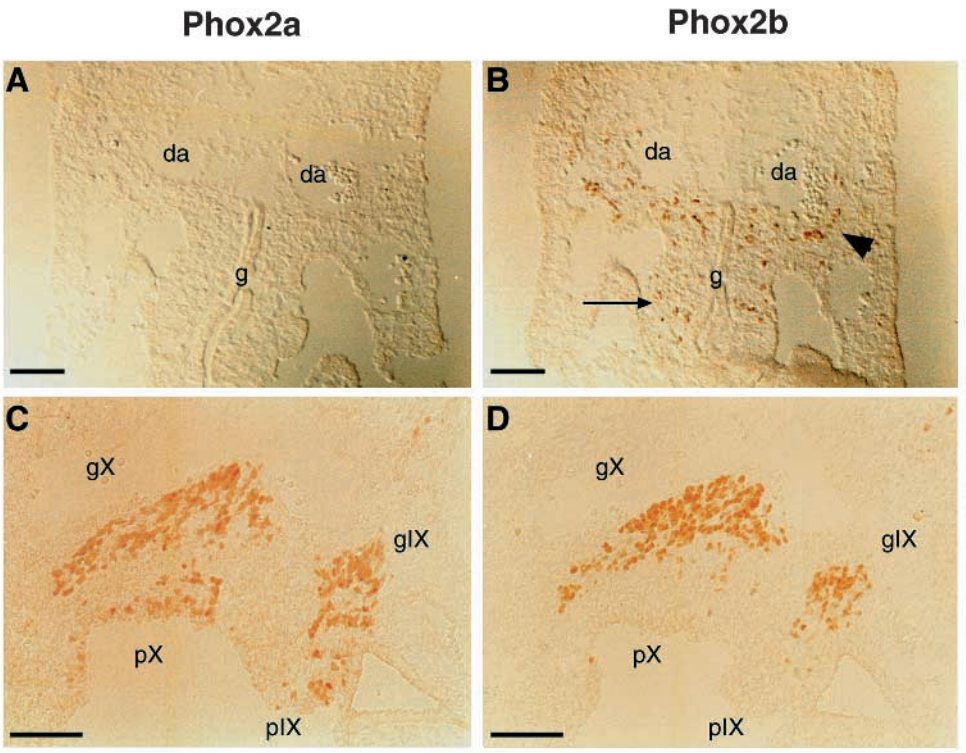

Fig. 7. Relative timing of Phox2a and Phox2b expression in the developing PNS. (A,B) Immunohistochemistry on transverse sections through the cervical region of an E9.5 embryo using anti-Phox2a (A) or anti-Phox2b (B) antibodies (ventral is down). At this stage, no expression of Phox2a is detected in the forming enteric plexus or sympathetic chain (A), whereas Phox2b (B) is expressed in migrating cells invading the gut (arrow). The arrowhead points to a cluster of cells ventral to the dorsal aorta (da) which could represent precursors of the sympathic chain that will later migrate towards the dorsal aspect of the aorta (Durbec et al., 1996). (C,D) Anti-Phox2a (C) and anti-Phox2b (D) on consecutive sagittal sections of the forming distal Xth and IXth cranial ganglia at E10.5. Phox2a is expressed in the ectodermal placodes and migrating neuroblasts, whereas Phox2b expression starts just before they aggregate to form the ganglia. da, dorsal aorta; g, gut; gX, nodose ganglion; gIX, petrose ganglion; pX and pIX, corresponding placodes. Bars: 100 μm.

we noted a reduced number of BrdU-labelled cells, particularly at the level of the ventral column in r4 (Fig. 6G,H). One interpretation is that the Phox2-positive domains in the ventricular layer correspond to hot spots of neurogenesis causing a depletion of the precursor pool.

Examples of opposite sequences of *Phox2a* and *Phox2b* expression were also found in the periphery. At E9-E9.5, we detected Phox2b, but not Phox2a protein in enteric neuroblasts invading the gut (Fig. 7A,B). We also consistently detected a few Phox2b-positive cells per section lateral to the aorta at thoracic levels (not shown), presumably corresponding to the earliest sympathetic neuroblasts, whereas no Phox2a-positive cells were found at this level. This suggests that the sympathetic ganglia anlagen express *Phox2b* before *Phox2a*. Half a day later, both genes were co-expressed in the primary sympathetic chain (Fig. 4 and Tiveron et al., 1996). In contrast, during the formation of the placodal-derived distal VIIth, IXth and Xth cranial ganglia, Phox2a protein was already detected in the epibranchial placodes before delamination of the neuroblasts, whereas *Phox2b* expression started only slightly before the migrating neuroblasts aggregate to form the ganglion anlagen (Fig. 7C,D).

The maintenance of *Phox2a* and *Phox2b* expression also differed in different cell types. Throughout embryogenesis and at postnatal stages, most neurons in the trochlear and oculomotor nuclei remained Phox2b-positive while only very few retained *Phox2a* expression. Conversely, in the locus coeruleus, Phox2a persisted until postnatal stages, whereas *Phox2b* expression was lost around E11.5.

Alteration of *Phox2b* expression in *Phox2a*$^{-/-}$ mutants

These staggered sequences of *Phox2a* and *Phox2b* expression suggested the possibility of cross-regulation between the two genes. The expression pattern of *Phox2b* in *Phox2a*$^{-/-}$ mice offered a test of this hypothesis.

Cranial ganglia lend themselves to the detection of altered gene expression in *Phox2a*$^{-/-}$ mice because they are morphologically unaffected during a 2-day window before they degenerate (Morin et al., 1997). Whole mounts of *Phox2a*$^{+/+}$ and *Phox2a*$^{-/-}$ E10.5 embryos were hybridized with a *Phox2b* cRNA probe. Whereas the anlagen of the VIIth, IXth and Xth ganglia were clearly labelled in the wild-type embryos (Fig. 8A), no cells in the VIIth and IXth and only very few cells in the Xth ganglion were *Phox2b*-positive in the mutants (Fig. 8B). The presence of an equivalent set of cells in the wild-type and mutant ganglia at that stage was demonstrated by the expression of a *lacZ* transgene (Kapur et al., 1991; Morin et al., 1997) (Fig. 8C,D). Therefore, *Phox2a* regulates *Phox2b*, directly or indirectly, in cranial ganglia.

Phox2b expression was also abolished in the locus coeruleus anlage of *Phox2a*$^{-/-}$ mice (Fig. 8E,F). Since these cells are missing in *Phox2a*$^{-/-}$ neonates and undetectable with independent markers (such as *DBH* or *tyrosine hydroxylase*) at any stage of development (Morin et al., 1997), we cannot tell whether the disappearance of the *Phox2b* signal is a consequence of the absence of *Phox2a* or of the cells themselves.

Phox2b expression reveals the loss of the oculomotor and trochlear nuclei in *Phox2a*$^{-/-}$ mice

Transient *Phox2a* expression is followed by sustained *Phox2b* expression in the oculomotor (IIIrd) and trochlear (IVth) nuclei (not shown) that were left unidentified in our previous studies of *Phox2a* expression (Valarché et al., 1993; Tiveron et al., 1996). At E13.5, a stream of Phox2b-positive cells was

seen across the midline connecting the left and right oculomotor nuclei (not shown), probably corresponding to those motoneuron precursors that migrate to the contralateral nucleus (Puelles, 1978). We noticed that *Phox2b* expression was missing in the anlagen of the IIIrd and IVth nuclei of *Phox2a*$^{-/-}$ embryos (Fig. 8E,F). To determine the fate of these structures in the mutants, we examined coronal sections through the isthmic region of neonatal *Phox2a*$^{-/-}$ brains. Both nuclei, clearly visible in wild-type brains, were undetectable in the mutants, either by histological staining (Fig. 9A,B,E,F), or in situ hybridization for *choline acetyl tranferase* (not shown) and peripherin (Fig. 9C,D,G,H). Already at E11.5, the expression of Islet-1, clearly visible in the anlage of these two nuclei on whole-mount preparations, was undetectable in the mutants (Fig. 9I,J). We conclude that *Phox2a* is necessary for the formation of the trochlear and oculomotor nuclei.

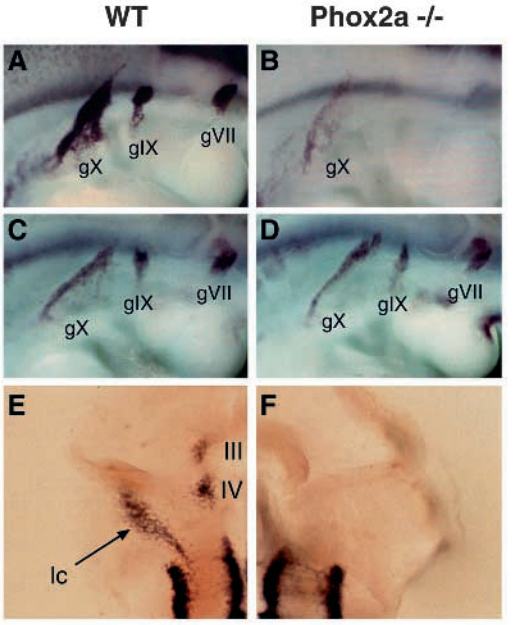

Fig. 8. *Phox2b* expression in wild-type (left) versus *Phox2a*$^{-/-}$ mutant mice (right). (A,B). Whole-mount in situ hybridizations at E10.5 with a *Phox2b* cRNA probe on wild type (A) and *Phox2a* mutant mice (B). The anlagen of the nodose (X), petrose (IX) and geniculate (VII) ganglia are clearly visible in the wild-type embryo wheras no *Phox2b* signal is detected in the petrose and geniculate ganglia of the mutant and very few positive cells are found in the nodose ganglion. (C,D) Control experiment showing that the anlagen of the VIIth, IXth and Xth ganglia are not yet morphologically affected at that stage in the mutants. Littermates carrying a *DBH-lacZ* transgene and either wild-type (C) or mutant (D) with respect to *Phox2a* were hybridized with a *lacZ* probe. The expression of the *DBH-lacZ* construct, which is normally expressed in all cells of these ganglia and, unlike the endogenous *DBH* gene, is insensitive to the *Phox2a* mutation (Morin et al., 1997), reveals that the mutant nodose, petrose and geniculate ganglia do not differ from wild-type ganglia in shape or size at E10.5. The onset of *Phox2b* expression slightly precedes that of *DBH-lacZ* (not shown), accounting for the thinner appearance of the ganglia with the latter probe. (E,F) Whole mount in situ hybridization with a *Phox2b* probe on the metmesencephalic domain of E10.5 wild-type (E) and *Phox2a* mutant (F) embryos. Phox2b expression in the forming oculomotor (III) and trochlear (IV) nuclei and in the locus coeruleus (lc) is no longer detectable in the *Phox2a*$^{-/-}$ mutants.

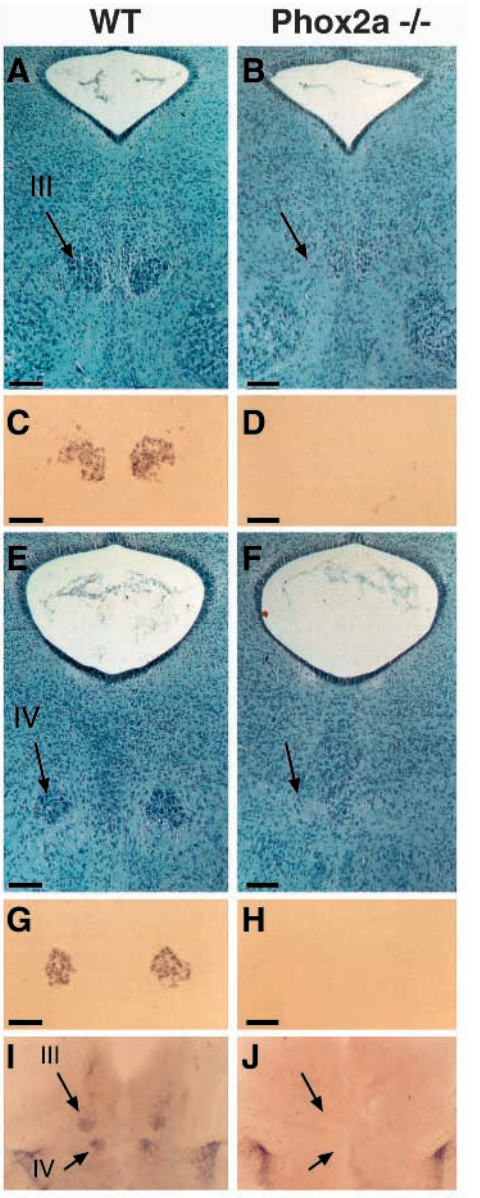

Fig. 9. Absence of oculomotor and trochlear nuclei in *Phox2a*$^{-/-}$ neonates and embryos. (A-H) Histological staining (A,B,E,F) and in situ hybridization for *peripherin* (C,D,G,H) of coronal sections through the isthmic region of wild-type (A,C,E,G) and *Phox2a*$^{-/-}$ mutant (B,D,F,H)) neonatal brains, revealing the oculomotor (A,C) and trochlear (E,G) nuclei in the wild types and their absence in mutants (B,D,F,H, arrows). (I,J) Wholemount in situ hybridization for *Islet-1* on wild type (left) and *Phox2a*$^{-/-}$ (right) E11.5 embryos showing expression in the forming oculomotor (III) and trochlear (IV) nuclei (arrow) and their absence in the mutant. Bars: 100 μm.

The expression of *Phox2b* seemed unchanged in other rhombencephalic nuclei of *Phox2a* mutants (motor nucleus of the facial nerve, nucleus ambiguus, dorsal motor nucleus of the vagus nerve, nucleus of the solitary tract, noradrenergic centers A1, A2, A5, locus subcoeruleus and area postrema), demonstrating that these structures, which also express *Phox2a* to various extents, are unaffected by the *Phox2a* mutation, at least at this level of analysis.

DISCUSSION

We have isolated and characterized Phox2b, a close homologue of Phox2a (Valarché et al., 1993). These two proteins have an identical homeodomain, an extreme case of sequence conservation between members of a structural class of homeodomain proteins. This implies that the DNA-binding properties of the homeodomain are identical between Phox2a and Phox2b. However, their C-terminal domains diverge extensively. In particular Phox2b, but not Phox2a, contains polyalanine stretches; the functional relevance of such sequences has been demonstrated by the *Hoxd-13* mutation causing Type II synpolydactyly in humans (Muragaki et al., 1996). It is therefore possible that Phox2a and Phox2b differ in their trans-regulating properties.

Several small families of homeodomain proteins with nearly identical homeodomains have now been described. Examples include En1 and En2 (Joyner and Martin, 1987) the Brn-3 family (He et al., 1989; Lillycrop et al., 1992; Turner et al., 1994; Ninkina et al., 1993), Otx1 and Otx2 (Simeone et al., 1993), Otlx1/Ptx1/p-Otx and Otlx2 (Lamonerie et al., 1996; Szeto et al., 1996; Mucchielli et al., 1996), and Islet-1 and Islet-2 (Karlsson et al., 1990; Tsuchida et al., 1994). In many cases, such highly related homeodomain proteins have overlapping but distinct expression patterns. This has been taken to suggest that they could partake in a homeobox code for neuronal or regional identity, akin to the 'Hox code' for segmental identity. The combinatorial expression of LIM homeodomain proteins in different classes of spinal motoneurons lends credence to this hypothesis (Tsuchida et al., 1994). In several nuclei of the central nervous system, such as the nTS, the facial motor nucleus or the dorsal motor nucleus of the vagus nerve, Phox2a-positive cells represent a subset of those expressing *Phox2b*. However, we do not know at this point what phenotypic heterogeneity this pattern underlies.

Evidence of cross-regulation between *Phox2a* and *Phox2b*

At all sites of *Phox2a* and *Phox2b* co-expression we analyzed, *Phox2a* expression immediately followed that of *Phox2b* or vice versa, suggesting cross-regulation between the two genes. In the placode-derived VIIth, IXth and Xth cranial ganglia, we demonstrated that *Phox2a* controls *Phox2b* expression by showing that *Phox2b* is not expressed in the mutants. An attractive possibility is that the promoters of *Phox2a* and *Phox2b* and, hence, their 'primary' expression pattern have considerably diverged while their final (or 'secondary') pattern has remained related by virtue of positive cross-regulations. In the CNS, the primary pattern of *Phox2a* would correspond to the isthmic and rostral metencephalic domains and that of *Phox2b* to the rest of the rhombencephalon, i.e. the domains in which they are expressed before the other *Phox2* gene.

There are a few precedents for cross-regulation between closely homologous homeobox genes. For example, ectopic expression of *Hoxa-1* leads to ectopic up-regulation of *Hoxb-1* in r2, suggesting direct regulation of *Hoxb-1* by *Hoxa-1* (Zhang et al., 1994). In dorsal root and trigeminal ganglia of mice mutant for the POU homeobox gene *Brn-3a*, there is down-regulation of its close homologues *Brn-3b* and *Brn-3c* (Xiang et al., 1996). There are also examples of asynchronous expression of closely related homeobox genes in the same cells compatible with cross-regulatory mechanisms: during the differentiation of spinal motoneurons, *Islet-1* is expressed in all newly formed motoneurons, soon followed by the expression of *Islet-2*, *LIM-1* or *LIM-3* and is subsequently down-regulated in all but two columns of motoneurons (Tsuchida et al., 1994). These observations, together with ours on *Phox2a* and *Phox2b*, raise the possibility that cross-regulation of homologous homeobox genes, plays a general role in the setting up of final 'codes'.

The phenotype of *Phox2a*$^{-/-}$ mice in the light of *Phox2b* expression

We previously observed that only some neurons that express *Phox2a* were affected in *Phox2a*$^{-/-}$ mutants, either morphologically or in their expression of two putative transcriptional targets of Phox2a, *Ret* and *DBH* (Morin et al., 1997). Using *Phox2b* as a marker, we have now demonstrated that the IIIrd and IVth cranial nerve nuclei are among the structures lost in the mutants. This more comprehensive picture of the *Phox2a*$^{-/-}$ phenotype reveals a striking correlation: the structures deleted are those expressing *Phox2a* before *Phox2b*. This is the case in the CNS, where the missing motor nuclei III and IV and locus coeruleus normally express *Phox2a* before *Phox2b*. In contrast, the *Phox2a*-expressing nuclei originating caudal to r1, where *Phox2b* is expressed before *Phox2a*, appear to be spared in *Phox2a*$^{-/-}$ mutants (e.g. the motor nuclei of the VIIth, IXth, Xth and XIth nerves).

This correlation seems also valid in the PNS, where the sensory cranial ganglia affected by the *Phox2a* mutation express *Phox2a* before *Phox2b* (and lose *Phox2b* expression in the mutants). In contrast, the enteric nervous system, spared by the *Phox2a* mutation, expresses *Phox2b* before *Phox2a* (and retains *Phox2b* expression in the mutants). Sympathetic ganglia, which apparently express *Phox2b* before *Phox2a*, are mostly spared by the mutation. The only structures in which we could not establish the order of *Phox2a* and *Phox2b* expression were the parasympathetic ganglia.

Altogether, these data suggest that each *Phox2* gene is required for the generation of the structures corresponding to its 'primary pattern'. This hypothesis predicts an essential role for *Phox2b* in the generation of the sympathetic chain, the enteric nervous system and the *Phox2b*-positive rhombencephalic nuclei caudal to r1.

Rhombomeric early pattern of *Phox2b* expression

The early vertebrate hindbrain is transiently subdivided into a series of eight compartments, the rhombomeres, which behave as units of lineage restriction (Birgbauer and Fraser, 1994; Wingate and Lumsden, 1996). Rhombomeres have been argued to be true metameres in the sense that some neuronal classes display a repetitive pattern of early differentiation, rhombomere-specific differences arising by variations on this same 'segmental theme' (Lumsden and Keynes, 1989; Clarke and Lumsden, 1993). These variations are thought to reflect control by distinct sets of Hox genes that are expressed by different rhombomeres (Lumsden and Krumlauf, 1996). However, the molecular circuitry that links metameric neurogenesis with regional specialization through the 'Hox code' has remained elusive.

The early expression pattern of *Phox2b* in the rhombencephalon is relatively simple. It consists of longitudinal arrays

of neuronal precursors, which form at precise locations along the dorsoventral axis. Along the anteroposterior axis, these columns respect inter-rhombomeric boundaries, which often coincide with those of *Hox* gene expression. *Phox2b* is expressed most prominently in r4 at stages when the hindbrain expression of *Hoxb1* is restricted to r4. This pattern prefigures the later expression of *Hoxb1* and *Phox2b* in presumptive migrating facial motoneurons, which are known to depend on *Hoxb-1* (Goddard et al., 1996; Studer et al., 1996) and which express Phox2 proteins up to postnatal stages. At E10.5, the lateral columns of *Phox2b* expression extend from the r1/r2 to the r6/r7 border. These borders correspond to the rostral limits of expression of the *Hox-2* and *Hox-4* paralogs, respectively. These correlations suggest that the 'primary pattern' of *Phox2b* in the CNS is under direct or indirect control by *Hox* genes, thus providing a link between *Hox*-directed patterning along the anterior/posterior axis and neurogenesis in the hindbrain.

Our BrdU-labelling study show that in their respective 'primary pattern' *Phox2b* and *Phox2a* expression occurs in proliferating precursors. This distinguishes Phox2b from other transcription factors implicated in neuronal type-determination in the CNS, such as Islet-1 (Pfaff et al., 1996; Jungbluth et al., 1997) and Brn-3.0 (Fedtsova and Turner, 1995), whose expression is initiated after the last mitosis. These data suggest that the expression of fate-determining homeobox genes is induced in defined domains in neuroepithelial precursor cells and perhaps causally linked to their exit from the cell cycle. The coordinates of these domains could be set up by the combined activity of dorsoventral patterning signals (Tanabe and Jessell, 1996) and, caudal to r1, by Hox genes. In line with the results of Lumsden et al. (1994), this early expression may fix neural phenotypic choices before cells are postmitotic, intermix with other cells and, in some cases, undergo extensive migrations.

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