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**Phox2b Influences the Development of a Caudal Dopaminergic Subset**

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**Abstract**

The developing mesodiencephalic dopaminergic (mdDA) neuronal field can be subdivided into several molecularly distinct domains that arise due to spatiotemporally distinct origins of the neurons and distinct transcriptional pathways controlling these neuronal subsets. Two large anatomically and functionally different subdomains are formed that eventually give rise to the SNc and VTA, but more subsets exist which require detailed characterization in order to better understand the development of the functionally different mdDA subsets, and subset-specific vulnerability. In this study, we aimed to characterize the role of transcription factor Phox2b in the development of mdDA neurons. We provide evidence that Phox2b is co-expressed with TH in a dorsal-caudal subset of neurons in the mdDA neuronal field during embryonic development. Moreover, Phox2b transcripts were identified in FAC-sorted Pitx3 positive neurons. Subsequent analysis of Phox2b mutant embryos revealed that in the absence of Phox2b, a decrease of TH expression occurred specifically in the midbrain neuronal subset that normally co-expresses Phox2b with TH. Our data suggest that Phox2b is, next to the known role in the development of the oculomotor complex, involved in the development of a specific caudal mdDA neuronal subset.

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**Introduction**

In Parkinson’s disease (PD) the onset is highlighted by a specific degeneration of mesodiencephalic dopaminergic (mdDA) neurons of the Substantia nigra pars compacta (SNc). To understand this neuron specific vulnerability, a thorough understanding of the development of mdDA neurons is essential. Based on a study in Lmx1b null mutants, it was suggested that Lmx1b is required for the generation of properly differentiated mdDA neurons. This study demonstrated a remarkable loss of developing mdDA neurons co-expressing Pitx3 and Th, in E12.5 Lmx1b−/− tissue [1]. Furthermore, Lmx1b plays an important role in the correct specification of the mid-hindbrain boundary (MHB), where it regulates expression of Fgf8, Wnt1 and several isthmus-related transcription factors, and it is required for the inductive activity of the isthmic organizer (Iso) itself [2,3]. Notably, the clear reduction of mdDA neurons in Lmx1b−/− embryos is likely due to an early loss of a large part of the midbrain, due to affected MHB patterning [2].

Previously, we identified Lmx1b as upstream activator of Phox2a (unpublished data). These data were recently confirmed as they showed the dependence of Phox2a expression in oculomotor neurons on the activity of Lmx1b [4]. Phox2a is expressed in the midbrain oculomotor complex (OMC), that partially overlaps with the mdDA neuronal field during development. Over the years, Phox2a has been identified as an important regulator of midbrain motorneuron development, in mice and humans, and in Phox2a−/− mice, midbrain oculomotor neurons are absent [5–7]. A recent study in chick suggested that exogenous Phox2a can induce a complete OMC molecular program, and can act as a primary developmental determinant for the oculomotor complex [8]. The functional parologue of Phox2a, Phox2b, is expressed in the hindbrain where it plays an essential role in the specification of cranial motor neurons [6,9–11]. Importantly, Phox2b is expressed in the OMC as well, and recently some degree of cooperation between Phox2a and Phox2b was discovered in motorneuron development [12]. In addition, molecular evidence was provided that Phox2b can regulate the expression of Phox2a by specifically interacting with the 5’-regulatory region of Phox2a [13].

The collective data on regulation of Phox2a by Lmx1b and Phox2b, aimed our interest towards a putative role of Phox2b in caudal subset specification of mdDA neurons. In this study, we show that Phox2b is co-expressed with TH and importantly, with Pitx3 in mdDA neurons during development. Interestingly, subsequent analysis of Phox2b null mutants revealed decreased expression of TH in the exact subset that normally expresses Phox2b, indicating that Phox2b plays a role in the development of this specific caudal subset of mdDA neurons.

**Materials and Methods**

**Ethics statement**

Mice were bred in our laboratory under standard conditions and all procedures were fully approved by the Dutch Ethical Committee (DEC) for animal experimentation of the University Medical Center Utrecht in the Netherlands (DEC-UMC-U) and international guidelines.
Animals

Experiments were carried out in C57Bl/6J wild-type mice (Charles River). Pregnant mice were decapitated or euthanized by CO2 asphyxiation and embryos were collected at E12.5, E13.5 and E14.5 (the morning of detection of a copulatory plug was considered E0.5). Pups were euthanized by CO2 asphyxiation and brains were isolated at postnatal (P) day 0, P7 and P14. Phox2b-LacZ mutant mice [14] were maintained under the same conditions. Embryos were collected at E12.5, E14.5, E16.5 and E18.5. Since homozygous mutants rarely survive after E13.5, due to a noradrenalin deficit [10], we treated drinking water of pregnant Phox2b-LacZ/+ mice by supplementing with 100 ug/mL of L-phenylephrine (Merck), 100 ug/mL isoproterenol (Sigma) and 2 mg/mL ascorbic acid (Sigma), from E8.5 onwards. Animals were genotyped by means of PCR, using a forward primer located in intron 1 of the Phox2b coding sequence (5'-GTTCTGGTTCAGTGGCCCTTC) and a reverse primer in the LacZ inserted sequence (5'-AGGCTGCGCAACTGTTGG) resulting in a product of 260 bp in mutants, or no product, in wild-type animals. To discriminate between heterozygous and homozygous LacZ mutants, the same forward primer was used, with a reverse primer in the wild-type sequence directly after the LacZ insertion (5'-GCAAAAGCATTCAAGCATTAG). Pitx3-GFP mice were described previously [15]. RNA from Pitx3-GFP/+ embryos was used for fluorescence-activated cell sorting (FACS).

In situ hybridization

Embryos were collected in ice-cold buffer and immediately frozen on dry ice. Sagittal and coronal sections (14 or 16 um) were cut and collected on SuperFrost plus slides (Menzel-Glaser). In situ hybridization (ISH) with digoxigenin (DIG)-labeled RNA probes was performed as described previously [16,17]. The following DIG-labeled probes were used: Th, a 1142 bp fragment of rat cDNA [18]; Lmx1b, a 1.3 kbp fragment containing full Lmx1b mouse coding sequence; Phox2b, a 1.6 kbp fragment containing full length coding sequence (a kind gift of J.F. Brunet).

Combined ISH-immunohistochemistry

ISH on fresh frozen sections was performed as described [16,17]. After termination of the alkaline phosphatase coloring reaction of the ISH, slides were washed in 1x TBS, incubated in 0.3% H2O2 in 1x TBS for 30 min, washed again, blocked with 4% FCS in 1x TBS for 30 min, washed again and incubated overnight at 4°C with rabbit anti-TH (Pel-Freez, Arkansas, 1:1000) in TBST (0.05 M Tris-HCl pH 7.4, 0.9% NaCl, 0.5% Triton. Next day, the sections were washed in 1x TBS, incubated for 1h with avidin-biotin-peroxidase reagent mix (ABC Elite kit, Vector Laboratories) in TBST. After this, slides were washed again, and stained with 3,3’-diamino-benzidine (DAB) until a maximum of 10 minutes. Color reaction was stopped by washing with water, slides were dehydrated with ethanol and mounted with Entellan (Merck).

Immunohistochemistry

Embryos were directly after isolation incubated in 4% paraformaldehyde (PFA) in 1x PBS at 4°C for at least 3 h or overnight, followed by cryoprotection in 30% sucrose solution in 1x PBS. After this, embryos were frozen on dry ice. For immunohistochemistry (IHC), sections were washed twice for 5 min in 1x TBS, blocked in 4% fetal calf serum (FCS) in 1x TBS for 30 min, and

Figure 1. Expression of Phox2b and Lmx1b in the midbrain of wild-type mouse embryos. Sagittal sections of E12.5, E13.5 and E14.5 wild-type mouse brains, from lateral to medial to lateral. ISH staining is shown for Phox2b, and for Lmx1b as a reference. Dashed lines represent the mid-hindbrain boundary. (a–l) Lmx1b and Phox2b mRNA expression at E12.5. Lmx1b is broadly expressed throughout the midbrain, in P1, P2 and P3, and in the hindbrain. Except for the most medial part, Phox2b is expressed in the posterior midbrain and anterior hindbrain. (m–x) Lmx1b and Phox2b mRNA expression at E13.5, and (y–jj) at E14.5. (kk) Schematic overview of a sagittal mouse brain at E14.5, depicting several neuronal fields in the midbrain area (red box). C, caudal; R, rostral; FB, forebrain; MB, midbrain; HB, hindbrain; RN, red nucleus; OM, oculomotor complex.

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**Figure 2. Phox2b expression overlaps with Th during development.** (a-f) Th and Phox2b mRNA expression at E12.5 wild-type mouse brains from medial to lateral, (g-l) at E13.5 and (m-r) at E14.5. Th marks the mdDA neuronal field. Dashed lines represent the mid-hindbrain boundary. Phox2b is expressed in the posterior midbrain and anterior hindbrain. At E13.5 and E14.5, Phox2b expression overlaps with the dorsal-caudal Th domain. (s-w) In coronal E14.5 sections, Phox2b expression overlaps with the caudal mdDA neuronal field. (u’-w’) Phox2b positive cells in the caudal midbrain co-express TH protein. C, caudal; R, rostral; for a schematic picture of the embryonic midbrain, see figure 1kk. doi:10.1371/journal.pone.0052118.g002

were washed again. When incubating with sheep anti-TH, blocking was performed in 5% normal donkey serum in 1x TBS. Slides were incubated with primary antibody in THZT (50 mM Tris-HCl pH 7.6, 0.5 M NaCl, 0.5% Triton) at 4°C overnight, washed 3x with 1x TBS for 5 min and incubated for 1 h with secondary antibody in THZT at room temperature. Slides were washed three times in 1x PBS for 10 min and mounted using FluorSave (Calbiochem, Darmstadt). Antibodies used: rabbit anti-TH (Pel-Freeze, Arkansas, 1:1000), sheep anti-Th (Millipore, 1:500), mouse anti-b-GAL (Promega, 1:300), rabbit anti-b-GAL (Cappel, 1:1000). Secondary antibodies: goat anti-rabbit Alexa-Fluor-488, donkey anti-sheep Alexa-Fluor-488, goat anti-mouse Alexa-Fluor-555, goat anti-rabbit Alexa-Fluor-555, all 1:1000 (Invitrogen).

**Fluorescence-activated cell sorting (FACS)**

The micro-dissected mdDA region of several Pitx3-GFP/+ embryos was dissociated using a Papain dissociation system (Worthington) and cells were sorted on a Cytopeia Influx Cell sorter. Sort gates were set on forward scatter versus side scatter (live cell gate), on forward scatter versus pulse width (elimination of clumps) and on forward scatter versus fluorescence channel 1 (528/38 filter; GFP fluorescence). Cells were sorted (90% purity) using a 100 um nozzle at a pressure of 25 PSI with an average speed of 7000 cells/second and collected in Trizol reagent (Invitrogen). [13].

**One-step RT PCR**

Total RNA was purified from MN9D cells [19] or Pitx3-GFP/+ FAC-sorted neurons, using Trizol according to manufacturer’s protocol (Invitrogen). Gene expression levels were determined using a One-step RT-PCR kit (Qiagen). We used 1 ng total RNA from MN9D per 20 uL PCR reaction. Samples were separated on 1.5–2.0% agarose gels, and gels were scanned using a FLA-5000 imaging system (Fuji). Primers used: Phox2a Forward primer 5’-GCTTTCTTAGGAACAGGGATC, Phox2a Reverse primer 5’-GGCTCTTCCCCTCTAGATGC; Phox2b Forward primer 5’-GAAAGTGAGCCGAGGCATC, Phox2b Reverse primer 5’-CTTTTGCTCTCGTGCTGCC (product size 228 bp); Slc18a2 Forward primer 5’-ATATCTGCTGCTGCTGCC (product size 226 bp); Lmx1b Forward primer 5’-CAAAAGGATTTGAGAGGTTC, Lmx1b Reverse primer 5’-GCTATGCCTTCCTGCTGATC, Vmat2 reverse primer 5’-9GCTCTTCCCCTCTAGATGC; Vmat2 forward primer 5’-9GCTCTTCCCCTCTAGATGC, Vmat2 reverse primer 5’-AGCTGAATAGCTCCAATCCAAG (product size 259 bp).

**Results**

**Phox2b is expressed in developing mdDA neurons**

Since Phox2a and Phox2b are paralogues and it is suggested that Phox2a and Lmx1b can regulate Phox2a [4,12,13], we were interested whether Phox2b is expressed in a similar, specific pattern in the dorsal-caudal mdDA neuronal field as we previously found for Phox2a (unpublished data). Therefore, we analyzed the expression pattern of Phox2b by means of in situ hybridization (ISH) on E12.5, E13.5 and E14.5 sagittal sections of wild-type (C57Bl/6J) embryos, and compared this to Lmx1b expression (Fig. 1).

At E12.5, Phox2b was clearly expressed in the posterior midbrain, and anterior hindbrain (Fig. 1G–L), except for the most medial sections, where no expression was detected (Fig. 1i). In the lateral areas, where Phox2b was highly expressed, this expression overlapped with the caudal domain of Lmx1b. At E13.5 and E14.5, Phox2b was also expressed in the most medial sections (Fig. 1S–X, EE–JJ). When comparing with Lmx1b expression, in both developmental stages a clear overlap of both Phox2b and Lmx1b expression fields was observed. In order to assess the possibility that Phox2b is involved in developing mdDA neurons, we compared Phox2b expression to Th. At E12.5, Phox2b only partially overlapped with Th (Fig. 2A,D); whereas more laterally, Phox2b was expressed more caudally and outside the Th domain (Fig. 2B,C,E,F). At E13.5, major overlap of Phox2b with the dorsal-caudal Th domain was observed, a pattern that was confirmed at E14.5 (Fig. 2g–r). In addition, by using Phox2b ISH analysis
domains. However, close to the expression domains of Lmx1b has been analyzed before [26,27], and we confirmed

In adjacent sections, no nuclei (PB) and principal sensory trigeminal nucleus (PSV) (Fig. 3a).

expression in the (dorsal) raphe nucleus (DR), the parabrachial nucleus (PB) and the principal sensory trigeminal nerve (V), which is in the same domain as the PB and PSV but represents a different set of neurons. In the anterior hindbrain and posterior midbrain, Phox2b is expressed in neurons of the trochlear and oculomotor nuclei (OM). Phox2b is not expressed in neurons of the VTA or SNc. (c–q) Th is expressed in the periaqueductal gray (F), the retrorubral field (I) and the VTA and SNc. Some Th positive cells are located near the OM nuclei, but not in the same domain (U, L, C, caudal, R, rostral).

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together with TH protein staining, the observed dorsal-caudal overlap was further validated in coronal brain sections, where many TH positive neurons overlapped with Phox2b expression, mainly in the dorsal-caudal mdDA domain (Fig. 2s–w').

In summary, Phox2b is expressed in the caudal midbrain, at high levels during early and late developmental stages. At E13.5 and E14.5 it largely overlaps with dorsal-caudal Lmx1b and Th midbrain expression domains, suggesting a possible role during the development of this mdDA neuronal subset.

Phox2b expression is restricted to the developing mdDA neuronal field

The expression of Phox2b in the developing midbrain may relate to the known role of Phox2b in the development of (midbrain) oculomotor neurons (OMNs). However, it might also suggest an additional involvement in the development of mdDA neurons. In the adult mouse brain, Phox2b is expressed in the hindbrain in branchiomotor and visceromotor neurons [6,11,20]. In addition, it is expressed in the noradrenergic system [locus ceruleus and lateral tegmental area] and in oculomotor and trochlear neurons [10,21–25]. Here we investigated the postnatal Phox2b midbrain expression, to determine whether Phox2b is present in mature mdDA neurons.

Therefore, we performed Phox2b, Lmx1b and Th ISH analysis on P14 coronal sections of wild-type mice (Fig. 3). Lmx1b expression has been analyzed before [26,27], and we confirmed Lmx1b expression in the (dorsal) raphe nucleus (DR), the parabrachial nucleus (PB) and the principal sensory trigeminal nucleus (PSV) (Fig. 3a).

In adjacent sections, no Phox2b expression was observed in these domains. However, close to the expression domains of Lmx1b in the PB and PSV, Phox2b was expressed in neurons of the motor nucleus of the trigeminal nerve (V) (Fig. 3b). More rostrally, in the anterior hindbrain and posterior midbrain, Phox2b expression was confirmed in the oculomotor nuclei (OM) (Fig. 3E,H,K). Importantly, no Lmx1b expression or Th expression was observed in the OM (Fig. 3D,G,F,I). Clear expression of Th and Lmx1b was found in the VTA and SNc, and the latter gene was also expressed in the supramammillary nucleus (SuM) (Fig. 3l,o,r and J,M,P). However, no mdDA specific Phox2b expression was detected in these areas (Fig. 3K,N,Q), indicating that Phox2b is not expressed in mature mdDA neurons.

Phox2b is restricted to a caudal subset of mdDA neurons at E14.5

To further substantiate Phox2b expression in developing mdDA neurons, we performed Phox2b ISH analysis, together with TH protein analysis, on E14.5 sagittal wild-type tissue (Fig. 4). We confirmed the specific expression of Phox2b in a selective TH positive domain of the dorsal-caudal midbrain (Fig. 4a–c), as was observed previously in coronal sections. Moreover, medially, many cells in this domain co-expressed Phox2b mRNA and TH protein (Fig. 4a–b', arrowheads).

To investigate this co-expression into more detail, we analyzed TH and bGAL protein expression in E12.5 and E14.5 Phox2b–LacZ mouse midbrains [14]. Importantly, in these LacZ knock-in mice, we observed TH expressing neurons that clearly co-expressed bGAL (Fig. 4d–f', arrowheads). In line with this, also at E14.5, co-expression of TH and bGAL was demonstrated, in a small group of cells in the dorsal-caudal midbrain (Fig. 4g–i'). Altogether, the combined data from ISH and IHC expression

Figure 3. Phox2b transcript is absent in mdDA neurons at P14. ISH analysis of Lmx1b, Th and Phox2b in postnatal day 14 (P14) coronal mid-and hindbrain tissue. (a–p) Lmx1b expression in the hindbrain (pons) in the dorsal raphe nucleus (DR), the parabrachial nucleus (PB) and the principal sensory trigeminal nucleus (PSV). In the anterior hindbrain, Lmx1b is expressed in the superior central raphe nucleus (CS), and more rostrally, in the posterior midbrain, in mdDA neurons of the ventral tegmental area (VTA). Most rostrally, in the anterior midbrain, expression is observed in mdDA neurons of the substantia nigra pars compacta (SNc) and in the supramammillary nucleus (SuM). (b–q) in the pons, Phox2b is expressed in the motor nucleus of the trigeminal nerve (V), which is in the same domain as the PB and PSV but represents a different set of neurons. In the anterior hindbrain and posterior midbrain, Phox2b is expressed in neurons of the trochlear and oculomotor nuclei (OM). Phox2b is not expressed in neurons of the VTA or SNc. (c–q) Th is expressed in the periaqueductal gray (F), the retrorubral field (I) and the VTA and SNc. Some Th positive cells are located near the OM nuclei, but not in the same domain (U, L, C, caudal, R, rostral).

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Phox2b Influences DopaMergic Development

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Taken together, *Phox2b* is expressed in a subset of neurons that expresses mild levels of TH, in the dorsal-caudal midbrain and in a small group of TH positive cells located near the isthmic organizer. This isthmic group is spared whereas the more anterior located TH positive cells lose TH expression as a consequence of *Phox2b* ablation, suggesting that *Phox2b* is involved in the correct specification of this small subset of mdDA neurons.

**Discussion**

*Phox2a*, and its parologue *Phox2b*, play important roles in the development of branchiomotor and visceromotor neurons in the ventral hindbrain and are both expressed in noradrenergic centers [6,10,11,20,25,28–30]. In the main noradrenergic center, the locus ceruleus, both genes can fully compensate for each other [25]. In addition, both factors are also specifically expressed in...
midbrain oculomotor neurons and in trochlear neurons, where Phox2a expression precedes that of Phox2b [4,6,8,12].

Since Phox2a displayed an expression pattern that was largely overlapping with the dorsal-caudal mdDA neuronal field, in an earlier study (unpublished data), we suspected a role for Phox2a,
and Phox2b, in the development of these neurons, in addition to the proposed function in oculomotor neuron (OMN) development. Indeed, our current analysis of Phox2b provides insight in a role of this gene in the development of a subset of mdDA neurons.

**Phox2b is temporarily expressed in a subset of developing mdDA neurons**

Our detailed analysis of the expression pattern of Phox2b during several developmental stages, confirms that Phox2b is expressed in a specific pattern overlapping with the dorsal-caudal mdDA neuronal field. However, in this midbrain area, motorneurons are formed as well. Moreover, a known marker for motorneurons, Isl1, is expressed in this area in a similar expression pattern as Phox2a and Phox2b [8,31].

In this study, we aimed to investigate in detail whether Phox2b is truly confined to OMNs, or in addition plays a role in mdDA neuronal development. Intriguingly, we clearly showed that many Phox2b expressing cells in the midbrain area, co-express TH protein, which was confirmed in Phox2b-LacZ positive neurons, and by RT-PCR on FAC-sorted Pitx3-positive neurons. In contrast to this, analysis of postnatal mouse brains revealed that Phox2b is not expressed in mature mdDA neurons. We confirmed expression in known sites of Phox2b, in the ventral hindbrain, and in the OM. However, no expression rostral to this nucleus was observed. Since Phox2b is clearly expressed in the developing mdDA neuronal field, the lack of expression in the postnatal mdDA system indicates a role for Phox2b in a small but specific subset of the mdDA domain during development of these neurons.

**References**

1. Smidt MP, Ashbrook CH, Cox JJ, Chen H, Johnson RL, et al. (2000) A second independent pathway for development of mesencephalic dopaminergic neurons requires Lmx1b. Nat Neurosci 3: 337–341. doi:10.1038/73902.
2. Guo C, Qui H-Y, Huang Y, Chen H, Yang R-Q, et al. (2007) Lmx1b is essential for Fgf8 and Wnt1 expression in the isthmic organizer during tectum and cerebellum development in mice. Development 134: 317–323. doi:10.1242/dev.02745.
3. Adams KA, Maida JM, Golden JA, Riddle RD (2000) The transcription factor Lmx1b maintains Wnt1 expression within the isthmic organizer. Development 127: 1057–1067.
4. Deng Q, Anderson E, Hellmund E, Alekszenko Z, Coppola E, et al. (2011) Specific and integrated roles of Lmx1a, Lmx1b and Phox2a in ventral midbrain development. Development 138: 3399–3408. doi:10.1242/dev.065482.
5. Guo S, Brush J, Teraoka H, Goddard A, Wilson SW, et al. (1999) Development of noradrenergic neurons in the zebraline hindbrain requires BMP, FGF8, and the homeodomain protein soulless/Phox2a. Neuron 24: 555–566.
6. Pattyn A, Morin X, Cremer H, Goridis C, Brunet JF (1999) Expression and interactions of the two closely related homeobox genes Phox2a and Phox2b during neurogenesis. Development 124: 4065–4070.
7. Goridis C, Brunet JF (1999) Transcriptional control of neurotransmitter phenotype. Curr Opin Neurobiol 9: 47–53.
8. Hasam KB, Agarwala S, Ragdale CW (2010) PHOX2A regulation of oculomotor complex neurogenesis. Development 137: 1203–1213. doi:10.1242/dev.041251.
9. Pa A, Hirsch MR, Le Cron S, Reiprich S, Harley VR, et al. (2008) Identification of Phox2b-regulated genes by expression profiling of cranial motorneuron precursors. Neural Dev 3: 14. doi:10.1186/1749-8104-3-14.
10. Pattyn A, Goridis C, Brunet JF (2009) Significance of the central noradrenergic phenotype by the homeobox gene Phox2b. Mol Cell Neurosci 45: 235–245. doi:10.1006/mcne.1999.0826.
11. Pattyn A, Hirsch M, Goridis C, Brunet JF (2000) Control of hindbrain motor neuron differentiation by the homeobox gene Phox2b. Development 127: 1549–1558.
12. Coppola E, Pattyn A, Guthrie SC, Goridis C, Studer M (2005) Reciprocal gene replacements reveal unique functions for Phox2 genes during neurulation. EMBO J 24: 4392–4403. doi:10.1038/sj.emboj.7600897.
13. Forca A, Larchetti H, Benfante R, Goridis C, Clementi F, et al. (2001) Sp proteins and Phox2b regulate the expression of the human Phox2a gene. J Neurosci 21: 7037–7045.
14. Pattyn A, Morin X, Cremer H, Goridis C, Brunet JF (1999) The homeobox gene Phox2b is essential for the development of autonomic neural crest derivatives. Nature 399: 366–370. doi:10.1038/20570.
15. Jacobs FMJ, Veenvliet JV, Almirza WH, Hoekstra EJ, von Oerthel L, et al. (2011) Retinoic acid-dependent and -independent gene-regulatory pathways of Pitx3 in meso-diencephalic dopaminergic neurons. Development 138: 5213–5222. doi:10.1242/dev.071704.
16. Smidt MP, Smits SM, Bouwmeester H, Hamers FP, van der Linden AJA, et al. (2004) Early developmental failure of substantia nigra dopamine neurons in mice lacking the homeodomain gene Pitx3. Development 131: 1145–1155. doi:10.1242/dev.01022.
17. Smits SM, Ponsioen T, Connelly OM, Burbach JPH, Smidt MP (2003) Involvement of Nurr1 in specifying the neurotransmitter identity of ventral midbrain dopaminergic neurons. Eur J Neurosci 18: 1731–1738.
18. Grima B, Lamorsou A, Blaust F, Bigert NF, Mallo J (1985) Complete coding sequence of rat tyrosine hydroxylase mRNA. Proc Natl Acad Sci USA 82: 617–621.
19. Jacobs FMJ, van der Linden AJA, Wang Y, von Oerthel L, Sul HS, et al. (2009) Identification of Dlk1, Ptpru and Klhl1 as novel Nurr1 target genes in mesodiencephalic dopaminergic neurons. Development 136: 2363–2373. doi:10.1242/dev.035756.
20. Pattyn A, Vallstedt A, Dias JM, Samad OA, Kraulund R, et al. (2003) Coordinated temporal and spatial control of motor neuron and serotonergic neuron generation from a common pool of CNS progenitors. Genes Dev 17: 729–737. doi:10.1101/gad.253803.
21. Zellmer E, Zhang Z, Greco D, Rhodes J, Cassel S, et al. (1995) A homeodomain protein selectively expressed in noradrenergic tissue regulates transcription of neurotransmitter biosynthetic genes. J Neurosci 15: 8109–8120.
22. Swanson DJ, Zellmer E, Lewis EJ (1997) The homeodomain protein Arix interacts synergistically with cyclic AMP to regulate expression of neurotransmitter biosynthetic genes. J Biol Chem 272: 27302–27309.
23. Swanson DJ, Adachi M, Lewis EJ (2000) The homeodomain protein Arix promotes protein kinase A-dependent activation of the dopamine beta-hydroxylase promoter through multiple elements and interaction with the

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In line with the expression of Phox2b in a dorsal-caudal subset of developing mdDA neurons, a loss of TH was observed in the homozygous Phox2b mutant, in this specific domain. Furthermore, a small decrease in the rostral expression domain (diencephalon) of TH was identified, suggesting that impaired expression in the dorsal caudal part of the developing mdDA neuronal field, might additionally influence a rostral subset of neurons. This may be a consequence of failure of migration of neurons from the medial-caudal region, or represent a more general, non-cell autonomous defect.

To conclude, our data not only identified Phox2b temporal expression in a select group of developing mdDA neurons, but also revealed a role for this gene in the development of these neurons. Thus, besides the known role of both Phox2a-genes in OM development, our data suggest that Phox2b is involved in the correct specification of a small caudal subset of mdDA neurons.

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**Author Contributions**

Conceived and designed the experiments: EJH MPS. Performed the experiments: EJH LvO AJAL. Analyzed the data: EJH MPS. Contributed reagents/materials/analysis tools: MPS. Wrote the paper: EJH MPS.
coactivator cAMP-response element-binding protein. J Biol Chem 275: 2911–2923.

24. Yang C, Kim HS, Seo H, Kim CH, Brunet JF, et al. (1998) Paired-like homeodomain proteins, Phox2a and Phox2b, are responsible for noradrenergic cell-specific transcription of the dopamine beta-hydroxylase gene. J Neurochem 71: 1813–1826.

25. Coppola E, d’Autreux F, Rijli FM, Brunet J-F (2010) Ongoing roles of Phox2 homeodomain transcription factors during neuronal differentiation. Development 137: 4211–4220. doi:10.1242/dev.056747.

26. Ashbreuk CHJ, Vogelaar CF, Hellemons A, Smith MP, Burbach JPH (2002) CNS expression pattern of Lmx1b and coexpression with ptx genes suggest functional cooperativity in the development of forebrain motor control systems. Mol Cell Neurosci 21: 410–420.

27. Dai J-X, Hu Z-L, Shi M, Guo C, Ding Y-Q (2008) Postnatal ontogeny of the transcription factor Lmx1b in the mouse central nervous system. J Comp Neurol 509: 341–355. doi:10.1002/cne.21759.

28. Brunet J-F, Pattyn A (2002) Phox2 genes – from patterning to connectivity. Curr Opin Genet Dev 12: 435–440.

29. Dubreuil V, Hirsch Mr, Pattyn A, Brunet JF, Goridis C (2000) The Phox2b transcription factor coordinates neuronal cell cycle exit and identity. Development 127: 5191–5201.

30. Dubreuil V, Hirsch Mr, 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.

31. Jessell TM (2000) Neuronal specification in the spinal cord: inductive signals and transcriptional codes. Nat Rev Genet 1: 20–29. doi:10.1038/35049541.