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Supplemental Information

Phox2a Defines a Developmental Origin of the Anterolateral System in Mice and Humans

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SUPPLEMENTAL INFORMATION:

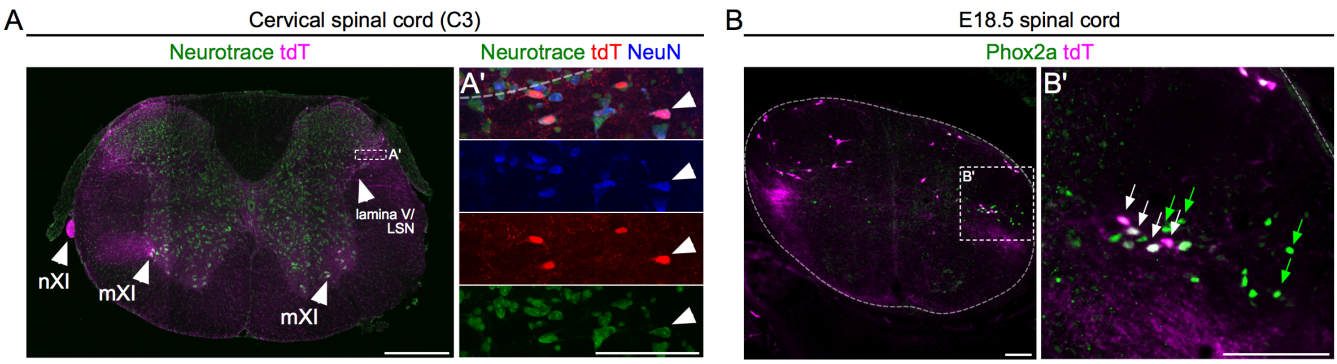
A
Cervical spinal cord (C3)
Neurotrace tdT
Neurotrace tdT NeuN
A'
lamina V/
LSN
nXI
mXI
mXI
B
E18.5 spinal cord
Phox2a tdT
B'
B'

Figure S1: Spinal Phox2a^{Cre} neurons reside in lamina I, V and LSN. Related to Figure 1.

All images are of *Phox2a*^{Cre}; *R26*$^{LSL\text{-}tdT/+}$ mice. (A) TdT+ neurons in the cervical spinal cord of adult mice. (A') Magnified box from (A) showing lamina V/LSN Neurotrace, tdT and NeuN co-labelling. (B) Expression of tdT in Phox2a+ neurons in an E18.5 mouse embryo.
Numbers: (A) n=3 adult mice, (B) n=3 E18.5 embryos.
Scale bars: (A) 500 μm, (A', B) 100 μm.
Abbreviations: nXI (accessory motor nerve).

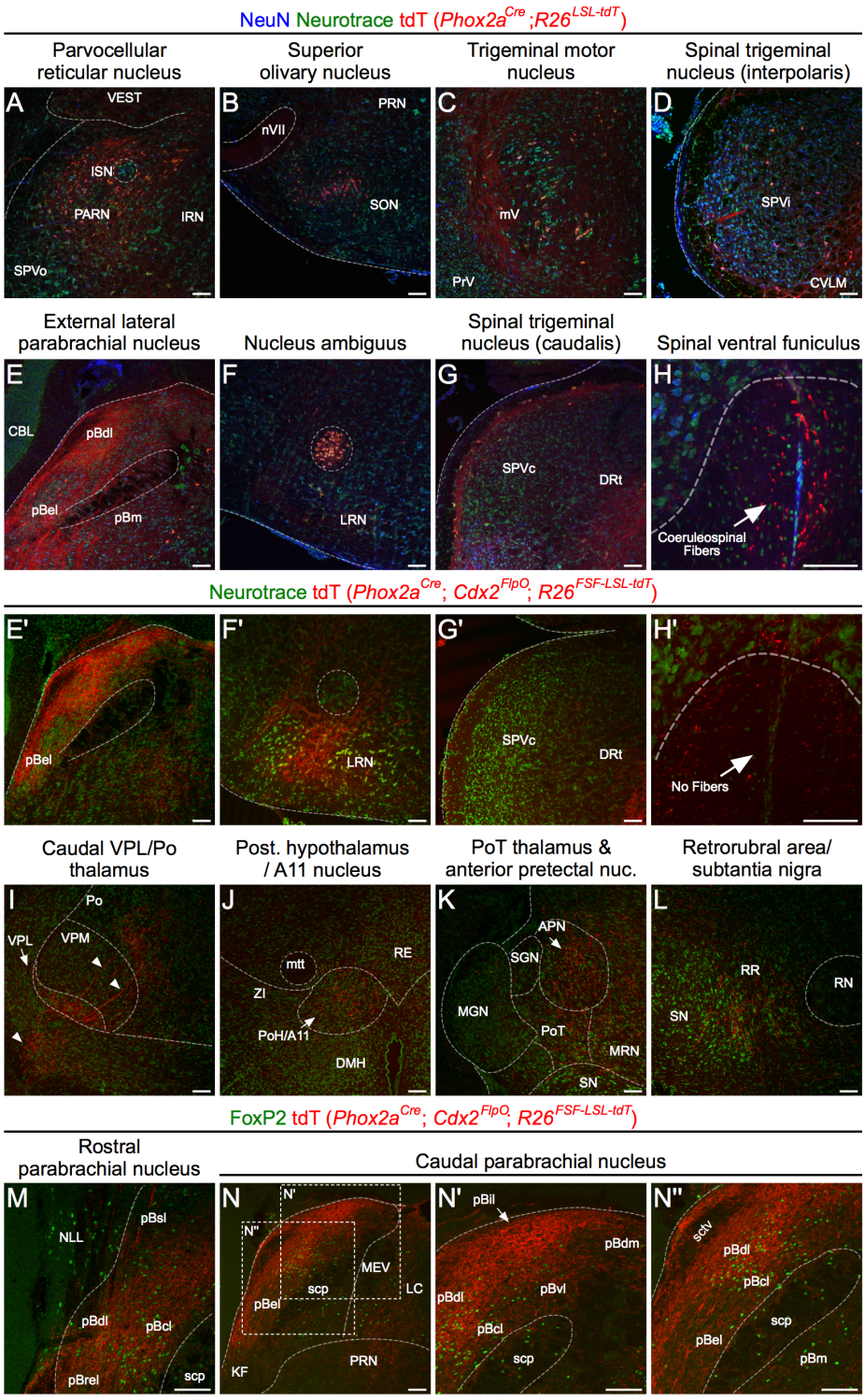
NeuN Neurotrace tdT (Phox2a Cre ;R26 LSL-tdT)
Parvocellular reticular nucleus
Superior olivary nucleus
Trigeminal motor nucleus
Spinal trigeminal nucleus (interpolaris)
A
VEST
ISN
PARN
IRN
SPVo
B
PRN
nVII
SON
C
mV
PrV
D
SPVi
CVLM
External lateral parabrachial nucleus
Nucleus ambiguus
Spinal trigeminal nucleus (caudalis)
Spinal ventral funiculus
E
CBL
pBdl
pBel
pBm
F
LRN
G
SPVc
DRt
H
Coeruleospinal Fibers
Neurotrace tdT (Phox2a Cre ; Cdx2 FlpO ; R26 FSF-LSL-tdT)
E'
pBel
F'
LRN
G'
SPVc
DRt
H'
No Fibers
Caudal VPL/Po thalamus
Post. hypothalamus / A11 nucleus
PoT thalamus & anterior pretectal nuc.
Retrorubral area/ subtantia nigra
I
Po
VPL
VPM
J
mtt
RE
ZI
PoH/A11
DMH
K
APN
SGN
MGN
PoT
MRN
SN
L
RR
RN
SN
FoxP2 tdT (Phox2a Cre ; Cdx2 FlpO ; R26 FSF-LSL-tdT)
Rostral parabrachial nucleus
Caudal parabrachial nucleus
M
NLL
pBsl
pBdl
pBcl
pBrel
scp
N
N'
N''
MEV
LC
scp
pBel
KF
PRN
N'
pBil
pBdm
pBdl
pBvl
pBcl
scp
N''
scv
pBdl
pBcl
scp
pBel
pBm

Figure S2: Spinal Phox2a^{Cre} neurons innervate AS targets. Related to Figure 2.

(A–G) NeuN, Neurotrace and tdT staining in brain regions expressing *Phox2a^{Cre}*; *R26$^{LSL\text{-}tdT/+}$* not depicted in Fig. 2. (E'–G') Lack of cellular tdT expression in *Phox2a^{Cre}; Cdx2^{FLPo}; R26$^{FSF\text{-}LSL\text{-}tdT/+}$* mice compared to the same brain regions of *Phox2a^{Cre}*; *R26$^{LSL\text{-}tdT/+}$* mice (E–G). (H, H') Large axons in the spinal ventral funiculus of *Phox2a^{Cre}*; *R26$^{LSL\text{-}tdT/+}$* mice (H), absent in *Phox2a^{Cre}; Cdx2^{FLPo}; R26$^{FSF\text{-}LSL\text{-}tdT/+}$* mice (H'), are likely coeruleospinal. (I–L) Additional brain regions receiving Phox2a^{Cre}+ spinofugal axons not depicted in Fig. 2. (M–N'') Spinal Phox2a^{Cre}+ axons in parabrachial subnuclei identified by FoxP2 expression (present in pBdl, pBcl but absent from pBel), in *Phox2a^{Cre}; Cdx2^{FLPo}; R26$^{FSF\text{-}LSL\text{-}tdT/+}$* mice. N' and N'': higher magnification of boxes in N.

Numbers: (A–H) n=3 *Phox2a^{Cre}*; *R26$^{LSL\text{-}tdT/+}$* adult mice, (E'–H', I–N, N', N'') n=3 *Phox2a^{Cre}; Cdx2^{FLPo}; R26$^{FSF\text{-}LSL\text{-}tdT/+}$* adult mice.

Scale bars: 100 µm.

Abbreviations: APN (anterior pretectal nucleus), CBL (cerebellum), DMH (dorsomedial hypothalamus), DRt (dorsal reticular nucleus), IRN (intermediate reticular nucleus), ISN (inferior salivatory nucleus), KF (Kölliker-Fuse nucleus), LC (locus coeruleus), MGN (medial geniculate nucleus), MRN (midbrain reticular nucleus), mtt (mamillothalamic tract), mV (trigeminal motor nucleus), nVII (facial motor nerve), pBrel (rostral external-lateral parabrachial nucleus), PoH (posterior hypothalamus), PoT (posterior triangular thalamus), RE (Reuniens nucleus), RN (red nucleus), RR (retrorubral nucleus), sctv (ventral spinocerebellar tract), SGN (suprageniculate nucleus), SN (substantia nigra), SON (superior olivary nucleus), SPVc (spinal trigeminal nucleus, caudalis), SPVi (spinal trigeminal nucleus, interpolaris), SPVo (spinal trigeminal nucleus, oralis), VEST (vestibular nuclei), VPM (ventral posteromedial thalamus).

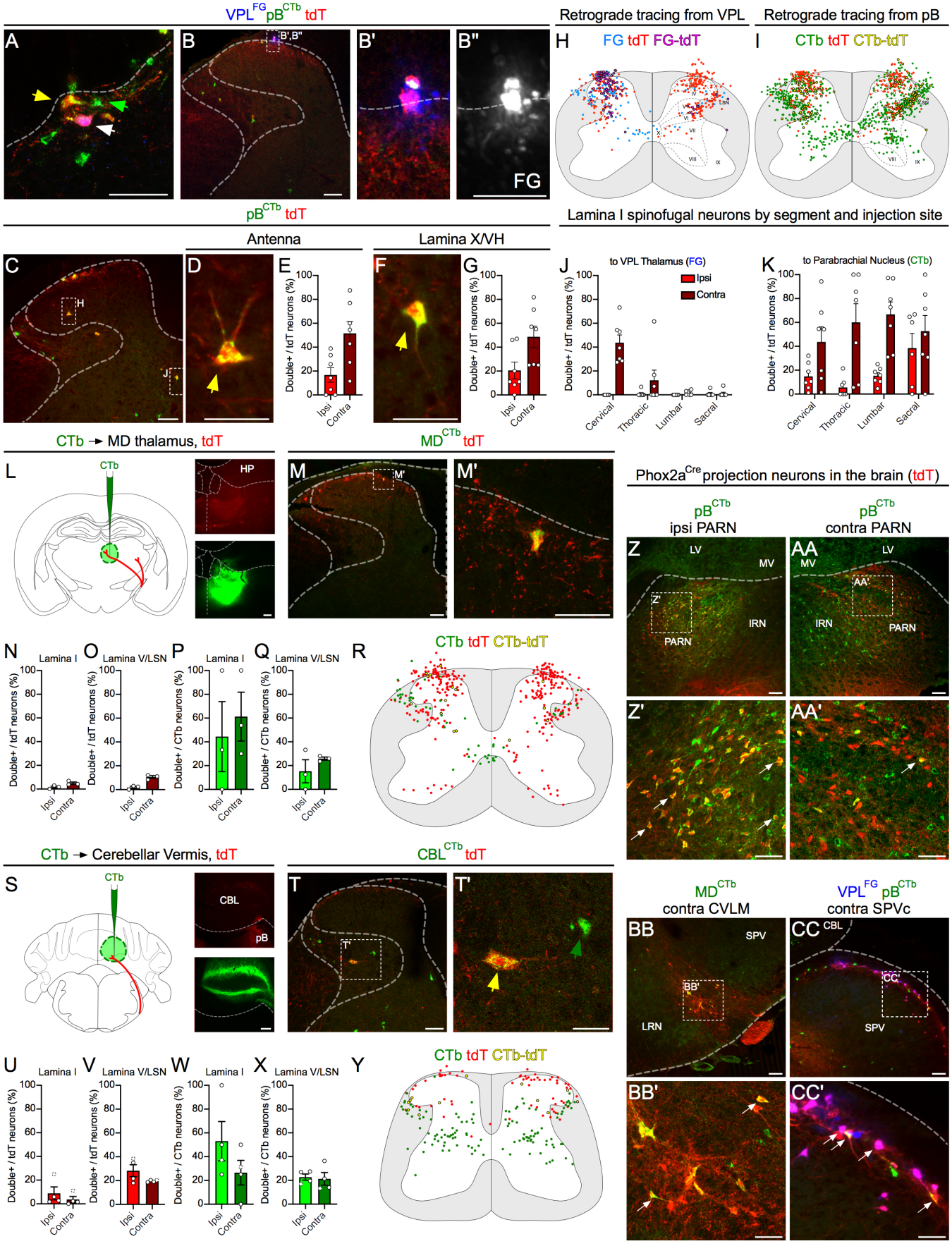
VPL FG pB CTb tdT
A
B
B'
B''
B',B"
FG
Retrograde tracing from VPL
Retrograde tracing from pB
H
FG tdT FG-tdT
I
CTb tdT CTb-tdT
pB CTb tdT
Lamina I spinofugal neurons by segment and injection site
Antenna
Lamina X/VH
C
D
E
F
G
H
J
J
to VPL Thalamus (FG)
Ipsi
Contra
K
to Parabrachial Nucleus (CTb)
Double+ / tdT neurons (%)
Cervical
Thoracic
Lumbar
Sacral
CTb → MD thalamus, tdT
MD CTb tdT
L
CTb
HP
M
M'
Phox2a Cre projection neurons in the brain (tdT)
pB CTb
ipsi PARN
pB CTb
contra PARN
Z
Z'
AA
AA'
LV
MV
IRN
PARN
N
Lamina I
O
Lamina V/LSN
P
Lamina I
Q
Lamina V/LSN
Double+ / tdT neurons (%)
Double+ / CTb neurons (%)
Ipsi
Contra
R
CTb tdT CTb-tdT
CTb → Cerebellar Vermis, tdT
CBL CTb tdT
S
CBL
pB
T
T'
MD CTb
contra CVLM
VPL FG pB CTb
contra SPVc
BB
BB'
CC
CC'
SPV
LRN
CBL
U
Lamina I
V
Lamina V/LSN
W
Lamina I
X
Lamina V/LSN
Y
CTb tdT CTb-tdT

Figure S3: Spinal Phox2a^{Cre} neurons are predominantly AS neurons. Related to Figure 3.

(A) Inset of Fig. 3G, demonstrating tdT and retrograde tracer co-localization in lamina I of *Phox2a*^{Cre}; *R26*$^{LSL\text{-}tdT/+}$ mice with VPL-injected FG and pB-injected CTb. White arrow: tdT+ cell labelled with both FG and CTb; green arrow: CTb-only cell; yellow arrow: tdT+ cell labelled with CTb. (B–B'') Examples of FG-labelled neurons. As FG+ cells were difficult to detect with the confocal microscope, an epifluorescence microscope was used to photograph them prior to cover-slipping (B'') after which, CTb and tdT were imaged using a confocal microscope. FG and CTb/tdT images were then overlaid in register and merged; (B', B'') are higher magnifications of boxed lamina I area in B. White arrow: tdT+ cell doubly labelled with FG and CTb; magenta arrow: tdT+ cell labelled with FG; yellow arrow: tdT+ cell labelled with CTb. (C–G) Rare Phox2a^{Cre} neuron types such as antenna neurons (D) and lamina X/VH neurons (F). Percent of tdT+ neurons labeled with either or both tracers in Antenna (E) or lamina X/VH (G) neurons. (H, I) Diagram of the location of tdT+ neurons (red) and VPL-injected FG-labelled neurons (H, FG+ neurons in blue, and tdT+/FG+ co-labelled neurons in magenta) or pB-injected CTb-labelled neurons (I, CTb+ neurons in green and tdT+/CTb+ neurons in yellow) in five, non-sequential 25 µm sections of the cervical spinal cord of one representative *Phox2a*^{Cre}; *R26*$^{LSL\text{-}tdT/+}$ mouse. (J, K) Percent of lamina I tdT+ neurons labelled with VPL-injected FG (J) or pB-injected CTb (K) in cervical, thoracic, lumbar and sacral spinal segments. (C–G) (L–R) Analysis of *Phox2a*^{Cre}; *R26*$^{LSL\text{-}tdT/+}$ mice injected in the MD thalamus with CTb. (L) Representative image of a CTb injection site. HP: Hippocampus (M, M') Representative cervical spinal cord section with a lamina I tdT+ neuron labelled with CTb (M'). (N–O) Percent of tdT neurons labelled with CTb in lamina I (N) or lamina V/LSN (O) of all spinal segments. (P–Q) Percent of CTb-labelled neurons expressing tdT in lamina I (P) or lamina V/LSN (Q) of all spinal segments. (R) Diagram of the location of tdT+ only (red), CTb+ only (green) or tdT+/CTb+ co-labelled (yellow) neurons, in 5 non-sequential 25 µm sections of the cervical spinal cord compiled from all 3 animals analysed. (S–Y) Analysis of *Phox2a*^{Cre}; *R26*$^{LSL\text{-}tdT/+}$ mice injected in the cerebellar (CBL) vermis with CTb (green). (S) Representative image of a CTb injection site. (T, T') Representative lumbar spinal cord section where a lamina V/LSN tdT+ neuron is labelled with CTb and magnified in (T'; yellow arrow). Green arrow shows a CTb-only neuron. (U–V) Percent of tdT neurons labelled with CTb in lamina I (U) or lamina V/LSN (V) at different spinal levels. (W–X) Percent of CTb-labelled neurons expressing tdT in lamina I (W) or lamina V/LSN (X) of all spinal segments. (Y) Diagram of the location of tdT+ only (red), CTb+ only (green) or tdT+/CTb+ co-labelled (yellow) neurons, in 10 non-sequential 25 µm sections of the lumbar spinal cord compiled from one representative animal. A CTb injection in one of the four mice spread into the dorsal pB – data points from this animal are represented by circles with dashed borders; increased incidence of lamina I tdT+ neurons labeling with

CTb is notable. (Z–CC) Examples of tdT+ neurons in various brain regions (arrows), labelled by retrograde tracer injections in the pB, VPL and MD thalamus. (Z, Z') tdT+ parvocellular reticular nucleus (PARN) neurons project to the ipsilateral parabrachial nucleus (AA, AA') but rarely to the contralateral parabrachial nucleus, (BB, BB') tdT+ caudal ventrolateral medulla (CVLM) neurons project to the MD Thalamus, and (CC, CC') lamina I/paratrigeminal neurons of the spinal trigeminal nucleus (SPVc) project to the VPL thalamus and, much more sparsely, to the parabrachial nucleus.
Numbers: (S3A–K, Z–CC) n=7 *Phox2a*^{Cre}; *R26*$^{LSL\text{-}tdT/+}$ adult mice (4 male, 3 female), (S3L–R) n=3 *Phox2a*^{Cre}; *R26*$^{LSL\text{-}tdT/+}$ adult mice (1 male, 2 female), (S3S-Y) n=4 *Phox2a*^{Cre}; *R26*$^{LSL\text{-}tdT/+}$ adult mice (2 male, 2 female).
Data are represented as mean ± SEM.
Scale bars: 100 μm, except (L, S) 250 μm, and (A, B', B'', D, F, M', T', Z'–CC') 50 μm.
Abbreviations: HP (Hippocampus).

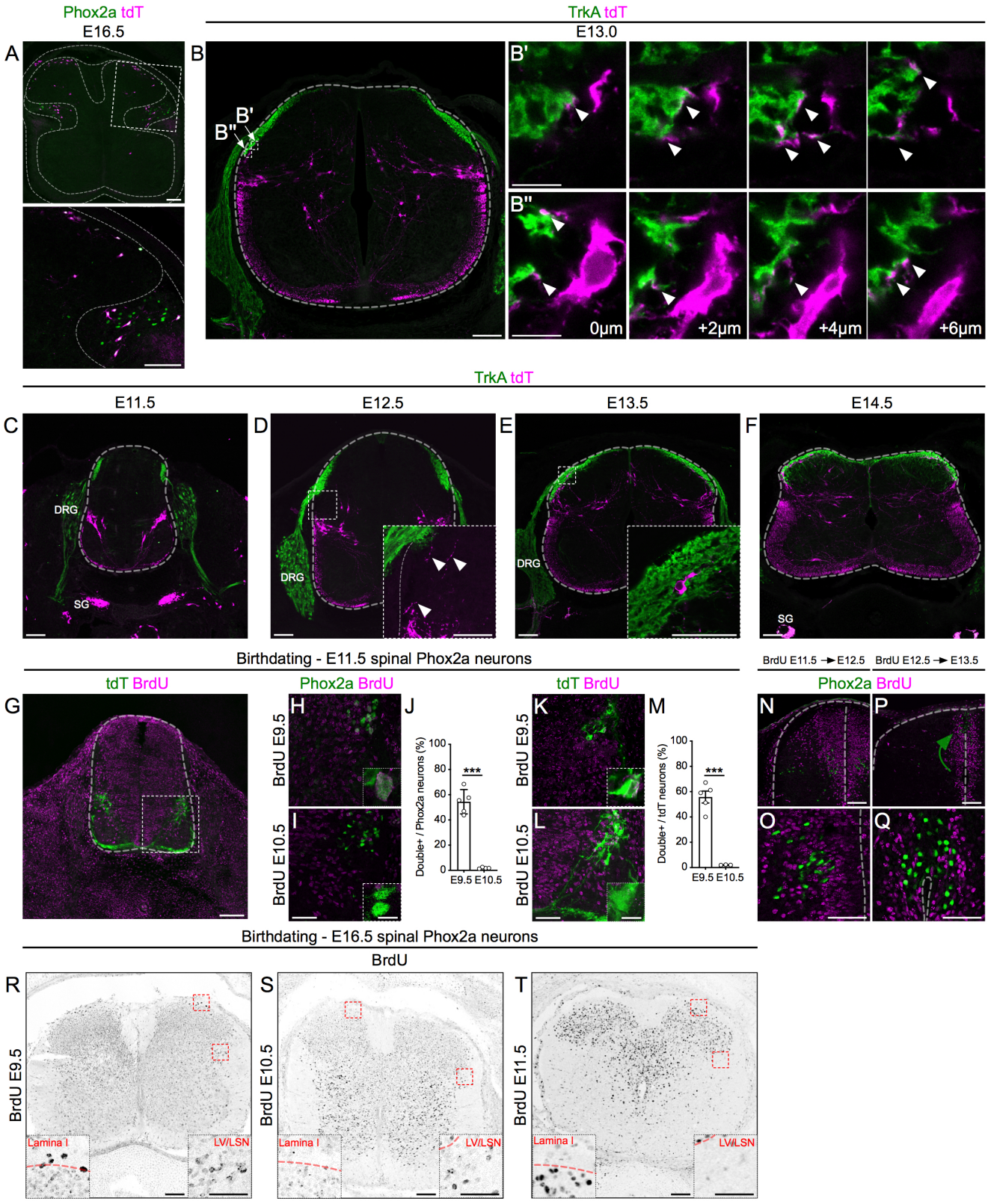
Phox2a tdT
E16.5
A
B
B'
B''
TrkA tdT
E13.0
B'
B''
0µm
+2µm
+4µm
+6µm
TrkA tdT
C
E11.5
DRG
SG
D
E12.5
DRG
E
E13.5
DRG
F
E14.5
SG
Birthdating - E11.5 spinal Phox2a neurons
BrdU E11.5 ➞ E12.5
BrdU E12.5 ➞ E13.5
tdT BrdU
G
Phox2a BrdU
H
BrdU E9.5
I
BrdU E10.5
J
Double+ / Phox2a neurons (%)
***
E9.5 E10.5
tdT BrdU
K
BrdU E9.5
L
BrdU E10.5
M
Double+ / tdT neurons (%)
***
E9.5 E10.5
Phox2a BrdU
N
P
O
Q
Birthdating - E16.5 spinal Phox2a neurons
BrdU
R
BrdU E9.5
Lamina I
LV/LSN
S
BrdU E10.5
Lamina I
LV/LSN
T
BrdU E11.5
Lamina I
LV/LSN

Figure S4: Heterogeneity of spinal Phox2a neuron migration, sensory afferent interaction and birth time. Related to Figure 4.

(A) Position of Phox2a+, tdT+ and Phox2a+ tdT+ (white) neurons in E16.5 embryonic spinal cords of *Phox2a*^{Cre}; *R26*$^{LSL\text{-}tdT/+}$ mice. (B–F) Development of contacts between lamina I Phox2a neurons and TrkA+ primary afferents in E13.0 (B), E11.5 (C), E12.5 (D), E13.5 (E) and E14.5 (F) *Phox2a*^{Cre}; *R26*$^{LSL\text{-}tdT/+}$ mouse spinal cords. (B', B'') Consecutive confocal microscopy z-stack images of lamina I neuron (magenta) contacts with TrkA primary afferents in E13.0 *Phox2a*^{Cre}; *R26*$^{LSL\text{-}tdT/+}$ mouse embryos, magnified boxed areas from (B). (D) inset: Phox2a neuron fibres near the dorsal root entry zone, prior to Phox2a cell soma entry into the dorsal horn. (E) inset: a lamina I Phox2a cell surrounded by TrkA afferents. (G–M) Birthdating of spinal Phox2a neurons in E11.5 *Phox2a*^{Cre}; *R26*$^{LSL\text{-}tdT/+}$ mouse embryos, labelled by BrdU exposure at E9.5 (H, K) or E10.5 (I, L). (G) The spinal cord of an E11.5 embryo exposed to BrdU at E10.5, showing BrdU and tdT labelling. (J, M) Percent of all tdT cells labelled with BrdU (J) and the percent of all Phox2a+ cells labelled with BrdU (M), in E11.5 *Phox2a*^{Cre}; *R26*$^{LSL\text{-}tdT/+}$ embryos exposed to BrdU at E9.5 or E10.5 (n=4-5). (N, O) E12.5 spinal cord of *Phox2a*^{Cre}; *R26*$^{LSL\text{-}tdT/+}$ embryos showing Phox2a^{DeepLate} neurons (Phox2a+) intermingled with newly born (BrdU+) neurons exposed to BrdU at E11.5. (P, Q) E13.5 spinal cord of *Phox2a*^{Cre}; *R26*$^{LSL\text{-}tdT/+}$ mouse embryos with Phox2a^{DeepLate} neurons neurons clustered near the roof of the central canal, separate from other newborn (BrdU+) neurons, labelled with BrdU at E12.5. (R–T) E16.5 spinal cord of *Phox2a*^{Cre}; *R26*$^{LSL\text{-}tdT/+}$ mouse embryos pulsed with BrdU at E9.5 (R), E10.5 (S) or E11.5 (T), showing BrdU+ cells in lamina I and lamina V/LSN (insets). Lamina I cells are labelled by a BrdU pulse at E9.5 but E10.5 and E11.5 BrdU pulses result in sparser labelling. Lamina V/LSN cells show labelling by E9.5 and E10.5 BrdU pulses but minimal labelling by a E11.5 BrdU pulse.

Numbers: *Phox2a*^{Cre}; *R26*$^{LSL\text{-}tdT/+}$ embryos: (A) n=3 E16.5, (B) n=3 E13.0, (C) n=3 E11.5, (D) n=3 E12.5, (E) n=3 E13.5, (F) n=3 E14.5, (G–M) n=3-5 E11.5 per condition, (N, O) n=3 E12.5, (P, Q) n=3 E13.5, (R) n=3 E16.5, (S) n=3 E16.5, (T) n=3 E16.5.

Statistics: (J, M) Unpaired t-test, ***: $p<0.0001$. Data are represented as mean ± SEM.

Scale bars: (A–G, N, P, R–T) 100 μm. (H, I, K, L, O, Q) and insets of (D, E, R, S, T) 50 μm, (B', B'') and insets of (H, I, K, L) 10 μm.

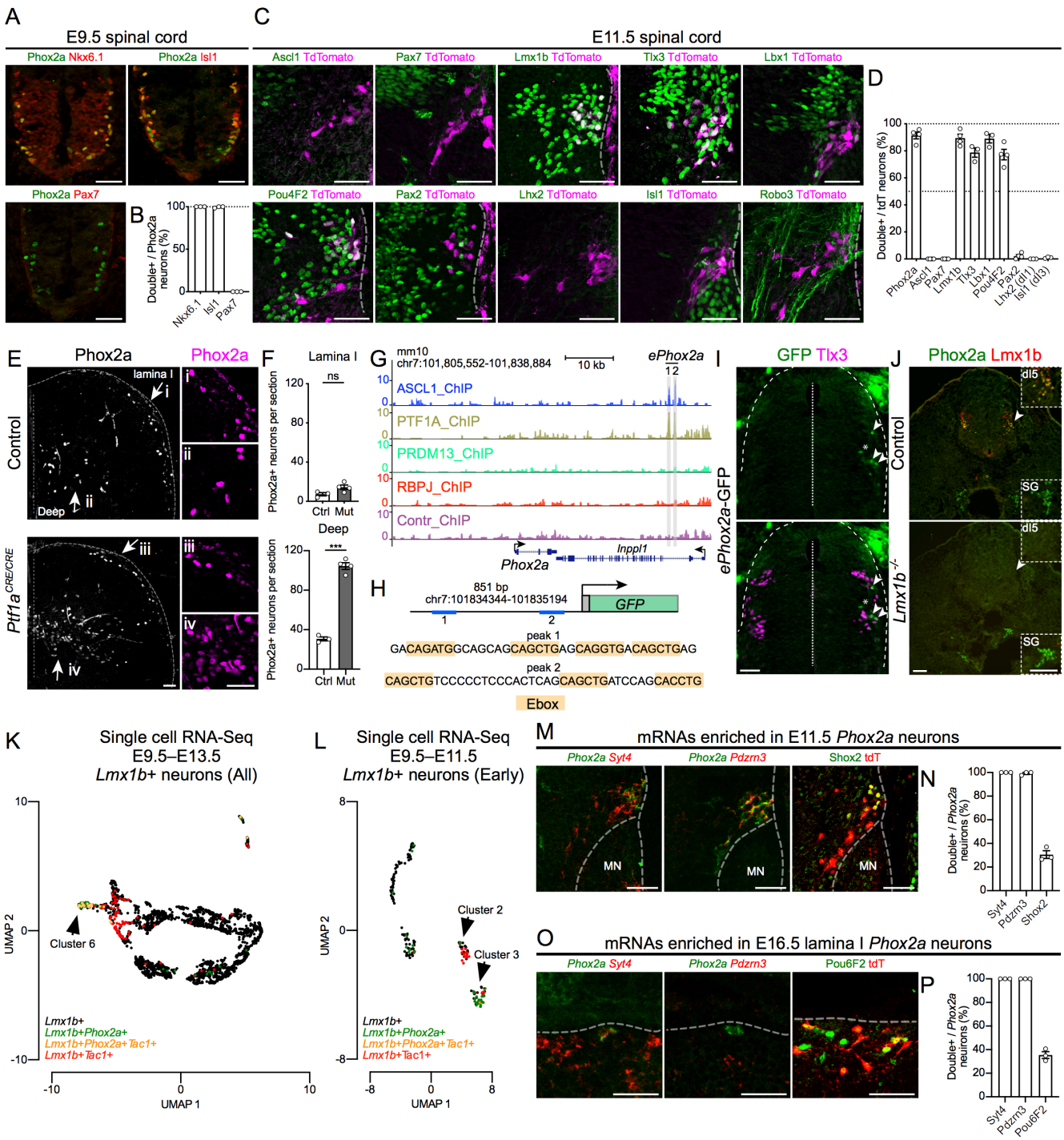
A
E9.5 spinal cord
Phox2a Nkx6.1
Phox2a Isl1
Phox2a Pax7
B
Double+ / Phox2a neurons (%)
Nkx6.1
Isl1
Pax7
C
E11.5 spinal cord
Ascl1 TdTomato
Pax7 TdTomato
Lmx1b TdTomato
Tlx3 TdTomato
Lbx1 TdTomato
Pou4F2 TdTomato
Pax2 TdTomato
Lhx2 TdTomato
Isl1 TdTomato
Robo3 TdTomato
D
Double+ / tdT neurons (%)
Phox2a
Ascl1
Pax7
Lmx1b
Tlx3
Lbx1
Pou4F2
Pax2
Lhx2 (dI1)
Isl1 (dI3)
E
Phox2a
Phox2a
Control
lamina I
i
ii
Deep
Ptf1a CRE/CRE
iii
iv
F
Lamina I
ns
Phox2a+ neurons per section
Ctrl
Mut
Deep
***
G
mm10
chr7:101,805,552-101,838,884
10 kb
ePhox2a
ASCL1_ChIP
PTF1A_ChIP
PRDM13_ChIP
RBPJ_ChIP
Contr_ChIP
Inppl1
Phox2a
H
851 bp
chr7:101834344-101835194
GFP
peak 1
GACAGATGGCAGCAGCAGCTGAGCAGGTGACAGCTGAG
peak 2
CAGCTGTCCCCCTCCCACTCAGCAGCTGATCCAGCACCTG
Ebox
I
GFP Tlx3
ePhox2a-GFP
Control
Lmx1b -/-
J
Phox2a Lmx1b
dI5
SG
K
Single cell RNA-Seq
E9.5–E13.5
Lmx1b+ neurons (All)
UMAP 2
UMAP 1
Cluster 6
Lmx1b+
Lmx1b+Phox2a+
Lmx1b+Phox2a+Tac1+
Lmx1b+Tac1+
L
Single cell RNA-Seq
E9.5–E11.5
Lmx1b+ neurons (Early)
Cluster 2
Cluster 3
M
mRNAs enriched in E11.5 Phox2a neurons
Phox2a Syt4
Phox2a Pdzrn3
Shox2 tdT
MN
N
Double+ / Phox2a neurons (%)
Syt4
Pdzrn3
Shox2
O
mRNAs enriched in E16.5 lamina I Phox2a neurons
Phox2a Syt4
Phox2a Pdzrn3
Pou6F2 tdT
P
Double+ / Phox2a neurons (%)
Syt4
Pdzrn3
Pou6F2

Figure S5: The molecular identity and specification of spinal Phox2a neurons. Related to Figure 5.

(A, B) Molecular characterisation of spinal Phox2a neurons in the E9.5 *Phox2a*^{Cre}; *R26*$^{LSL\text{-}tdT/+}$ spinal cord. Phox2a neurons co-express ventral motor neuron markers Nkx6.1, Isl1, but not the progenitor marker Pax7, and are most likely accessory motor neurons. The percent of Phox2a+ cells co-expressing Nkx6.1, Isl1 and Pax7 is quantified. (C, D) Co-expression of tdT with embryonic spinal neuron markers in the in the E11.5 *Phox2a*^{Cre}; *R26*$^{LSL\text{-}tdT/+}$ spinal cord using immunohistochemistry, with (D) quantification of each marker done as a percent of tdT+ neurons. (E) Detection of Phox2a cells in spinal cords of control and *Ptf1a*$^{Cre/Cre}$ E14.5 mice. Panels with Roman numerals are magnifications of regions marked by arrows. (F) Number of Phox2a+ cells per average section in lamina I and deep laminae of E14.5 control and *Ptf1a*$^{Cre/Cre}$ (mut) mice. (G, H) Genome sequences ChIP-Seq hits detected using antibodies against Ascl1, Ptf1a, Prdm13, and Rbpj at the *Phox2a* locus (G). The two sites bound by both Ascl1 and Ptf1a are highlighted in gray. (H) Diagram of the *ePhox2a* GFP reporter construct with the cis-regulatory sequence containing Ascl1 and Ptf1a sites. The sequence at the center of each ChIP-seq peak is shown with the E-box basic helix-loop-helix transcription factors binding motif is highlighted in orange. The active Ptf1a containing transcription complex requires a TC-Rbpj binding sequence that is not present in this enhancer. (I) Transverse sections of embryonic day 4 (E4) chicken neural tubes electroporated with the *ePhox2a-GFP* reporter at embryonic day 2. *ePhox2a* directs dI5-specific GFP expression in Tlx3+ cells. (J) E11.5 *Lmx1b*$^{-/-}$ spinal cords lack Phox2a expression (dI5 inset), but sympathetic ganglia maintain it (SG inset). (K, L) UMAP plots of E9.5-E13.5 *Lmx1b*+ neurons (K) and E9.5-E11.5 *Lmx1b*+ neurons (L) showing *Lmx1b*+/*Phox2a*+, *Lmx1b*+/*Tac1*+, and *Lmx1b*+/*Phox2a*+/*Tac1*+ cell clusters. (M, N) Validation of selected dI5-enriched mRNAs at E11.5 by RNA-Scope in situ detection of *Synaptotagmin 4*, *Pdzrn3* mRNAs and Shox2 protein immunofluorescence (Z). (N) Percent of E11.5 Phox2a+ or *Phox2a* mRNA+ neurons co-expressing the selected mRNAs or proteins. (O, P) Validation of selected dI5-enriched mRNAs at E16.5 in lamina I, by RNA-Scope in situ detection of *Synaptotagmin 4* and *Pdzrn3* mRNAs and Pou6F2 protein immunofluorescence. (P) Percent of E16.5 Phox2a+ or *Phox2a* mRNA+ neurons co-expressing the selected mRNAs or proteins.
Numbers: *Phox2a*^{Cre}; *R26*$^{LSL\text{-}tdT/+}$ embryos (A, B) n=3 E9.5, (C, D) n=3-4 E11.5, (M, N) n=3 E11.5, (O, P) n=3 E16.5. (E, F) n=3 control, n=4 *Ptf1a*$^{Cre/Cre}$ E14.5 embryos, (I) n=6 E4 chicken embryos, (J) n=3 control, n=3 Lmx1b$^{-/-}$ E11.5 embryos. Statistics: (F) Groups compared with Student's t-test, ***: $p<0.001$. (K, L) Data derived from Delile et al., (2019); data processing and statistics described in STAR Methods. Data are represented as mean ± SEM.Scale bars: All 50 μm, except (J) 100 μm.

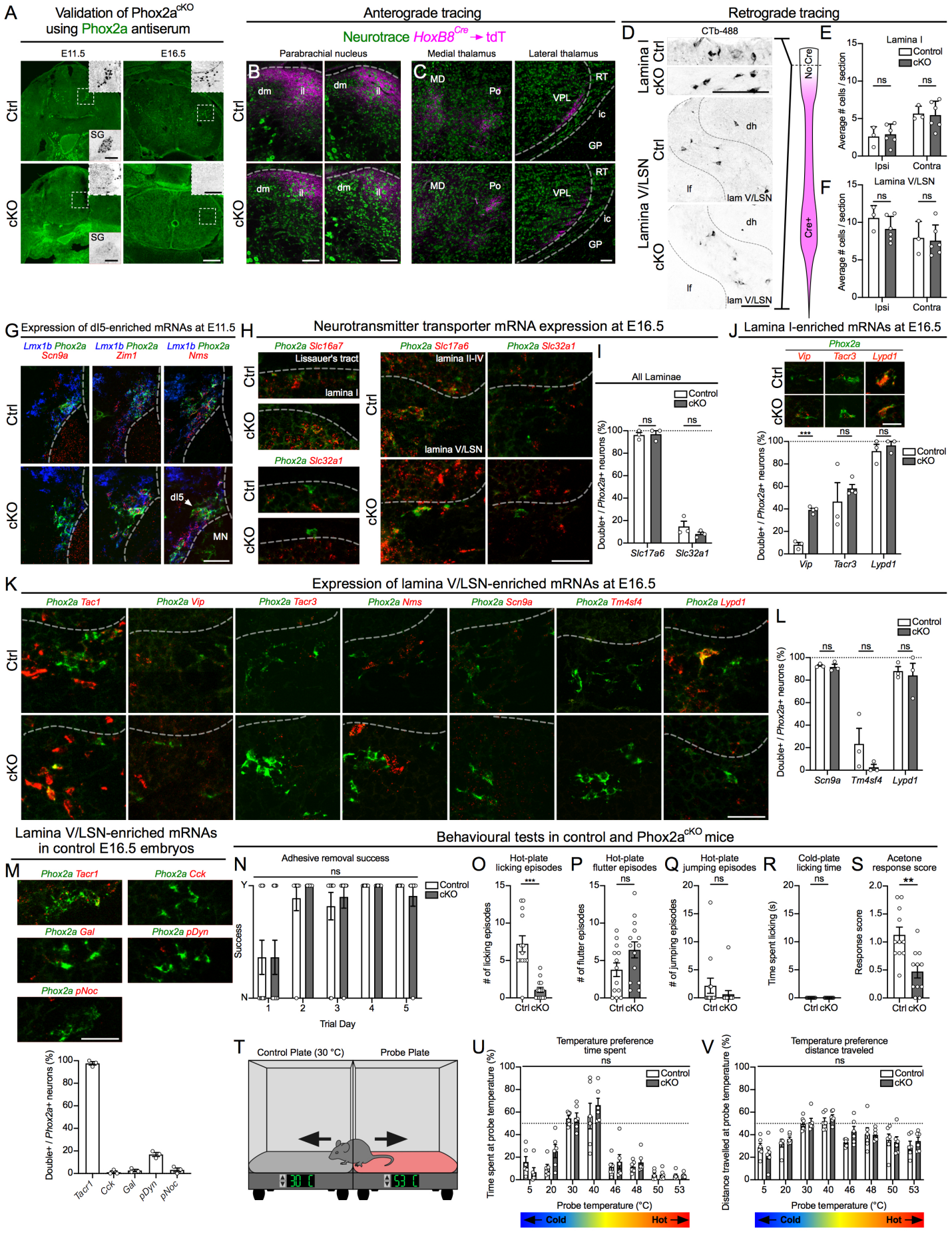
A
Validation of Phox2a^cKO using Phox2a antiserum
E11.5
E16.5
Ctrl
cKO
SG
Anterograde tracing
Neurotrace HoxB8^Cre → tdT
Parabrachial nucleus
Medial thalamus
Lateral thalamus
B
C
dm
il
MD
Po
VPL
RT
ic
GP
Retrograde tracing
D
CTb-488
Lamina I
Lamina V/LSN
dh
lf
lam V/LSN
No Cre
Cre+
E
Lamina I
Control
cKO
Average # cells / section
ns
Ipsi
Contra
F
Lamina V/LSN
G
Expression of dI5-enriched mRNAs at E11.5
Lmx1b Phox2a Scn9a
Lmx1b Phox2a Zim1
Lmx1b Phox2a Nms
dI5
MN
H
Neurotransmitter transporter mRNA expression at E16.5
Phox2a Slc16a7
Lissauer's tract
lamina I
Phox2a Slc17a6
lamina II-IV
lamina V/LSN
Phox2a Slc32a1
I
All Laminae
Double+ / Phox2a+ neurons (%)
Slc17a6
Slc32a1
J
Lamina I-enriched mRNAs at E16.5
Phox2a
Vip
Tacr3
Lypd1
***
K
Expression of lamina V/LSN-enriched mRNAs at E16.5
Phox2a Tac1
Phox2a Vip
Phox2a Tacr3
Phox2a Nms
Phox2a Scn9a
Phox2a Tm4sf4
Phox2a Lypd1
L
Scn9a
Tm4sf4
Lypd1
M
Lamina V/LSN-enriched mRNAs in control E16.5 embryos
Phox2a Tacr1
Phox2a Cck
Phox2a Gal
Phox2a Pdyn
Phox2a pNoc
Tacr1
Cck
Gal
pDyn
pNoc
Behavioural tests in control and Phox2a^cKO mice
N
Adhesive removal success
Success
Trial Day
O
Hot-plate licking episodes
# of licking episodes
P
Hot-plate flutter episodes
# of flutter episodes
Q
Hot-plate jumping episodes
# of jumping episodes
R
Cold-plate licking time
Time spent licking (s)
S
Acetone response score
Response score
T
Control Plate (30 °C)
Probe Plate
U
Temperature preference time spent
Time spent at probe temperature (%)
Probe temperature (°C)
Cold
Hot
V
Temperature preference distance traveled
Distance travelled at probe temperature (%)

Figure S6: Phox2a is required for AS neuron development and function. Related to Figure 6.

(A) Phox2a expression in E11.5 (left) and E16.5 (right), control (top row, Ctrl) and Phox2a^{cKO} (bottom row; cKO) spinal cords. Insets at the top right are magnified boxed areas, depicting the loss of Phox2a expression (black) in cKO. Bottom right inset shows loss of Phox2a expression in sympathetic ganglia. (B, C) Brains of *HoxB8^{Cre}*; *Phox2a$^{+/+}$*; *R26$^{LSL\text{-}tdT/+}$* (Ctrl, top row) and *HoxB8^{Cre}*; *Phox2a$^{f/f}$*; *R26$^{LSL\text{-}tdT/+}$* (Phox2a^{cKO} or cKO, bottom row) mice, in which spinofugal axons are labelled via *HoxB8^{Cre}*-driven axonal tdT (magenta). (B) Brain sections from two different animals per genotype show decreased Pb innervation in the parabrachial nucleus of cKO mice (as in Fig. 6). (C) Normal innervation of the medial (left) and lateral (right) thalamus was noted in both control and cKO mice. (D–F) CTb-labeled neurons in lamina I (upper panels, quantified in E) and lamina V/LSN (lower panels, quantified in F), in the cervical spinal cord of control and Phox2a^{cKO} mice, rostral to HoxB8^{Cre} expression domain, diagrammed in magenta. (G) Expression of dI5-enriched mRNAs in E11.5 control (top row) and Phox2a^{cKO} (bottom row) spinal cords not shown in Fig. 6 but quantified in Fig. 6G. (H) Expression of *Slc17a6* and *Slc32a1* mRNAs encoding, respectively, the neurotransmitter transporters vGlut2 and vGAT, in lamina I and lamina V/LSN of E16.5 control and Phox2a^{cKO} embryos. (I) Percent of control and Phox2a^{cKO} *Phox2a*+ neurons expressing *Slc17a6* and *Slc32a1* mRNAs (n=3 control, n=3 Phox2a^{cKO}). (J) Expression and quantification of candidate AS-enriched mRNAs in lamina I *Phox2a*+_neurons of E16.5 control and Phox2a^{cKO} embryos (n=3 control, n=3 Phox2a^{cKO}). (K) Expression of mRNAs encoding neuropeptides and neuropeptide receptors in lamina V/LSN *Phox2a*+ neurons of E16.5 control (top row) and Phox2a^{cKO} (bottom row) spinal cords not shown in Fig. 6 but quantified in Fig. 6M. (L) Expression and quantification of mRNAs encoding neuropeptides expressed in lamina V/LSN *Phox2a*+ neurons, not depicted in Fig. 6L. (M) Expression and quantification of mRNAs encoding neuropeptides and neuropeptide receptors in *Phox2a*+ neurons of control E16.5 embryos – high co-expression of *Phox2a* and *Tacr1* (a known AS marker) is noted. (N) Adhesive removal success ratio between control and Phox2a^{cKO} mice over 5 days. (O–S) Responses to non-injected noxious stimuli: episodes of (O) licking, (P) hind paw flutter, and (Q) jumping during the hot-plate test (n=13 control, n=14 Phox2a^{cKO}) – though jumping frequency was not significantly different between groups, 4/13 control mice exhibited jumping versus 1/14 cKO mice. (R) Time spent licking during the cold-plate test (n=9 control, n=10 Phox2a^{cKO}). (S) The acetone response score after hind paw application of acetone (n=6 control, n=6 Phox2a^{cKO}). (T–V) The two-choice temperature preference assay (T), demonstrating time spent at the probe temperature versus 30 °C (U), and the distance traveled in the probe temperature compartment versus 30 °C (V) (n=6 control, n=6 Phox2a^{cKO}).

Numbers: (A) n=3 control, n=3 Phox2a^{cKO} E11.5 and E16.5 mice. (B, C) n=4 control, n=4 Phox2a^{cKO} adult mice; (D–F) n=3 control, n=6 Phox2a^{cKO} adult mice; (G) n=3 control, n=5 Phox2a^{cKO} E11.5 mice; (H, I) n=3 control, n=3 Phox2a^{cKO} E16.5 mice; (J) n=3 control, n=4 Phox2a^{cKO} E16.5 mice; (K) n=3 control, n=3 Phox2a^{cKO} E16.5 mice; (L) n=3 control E16.5 mice; (N–V) Numbers described above.
Statistics: (E, F) Two-way ANOVA with Tukey's multiple comparisons test, (I, J, K) multiple t-tests using Holm-Sidak method, (M) mixed-effects analysis with Sidak's multiple comparisons test, (O, P, Q, R, S) Mann–Whitney test, and (U, V) two-way ANOVA with Sidak's multiple comparisons test. ns: non-significant, **: $p<0.01$, ***: $p<0.001$. Data are represented as mean ± SEM.
Scale bars: (A) 200 μm, insets in (A) and (B–D) 100 μm, (G, H, K, M) 50 μm and (J) 25 μm.
Images in Fig. 5Q have been re-used in Fig. S6G.
Abbreviations: dh (dorsal horn), lf (lateral funiculus).

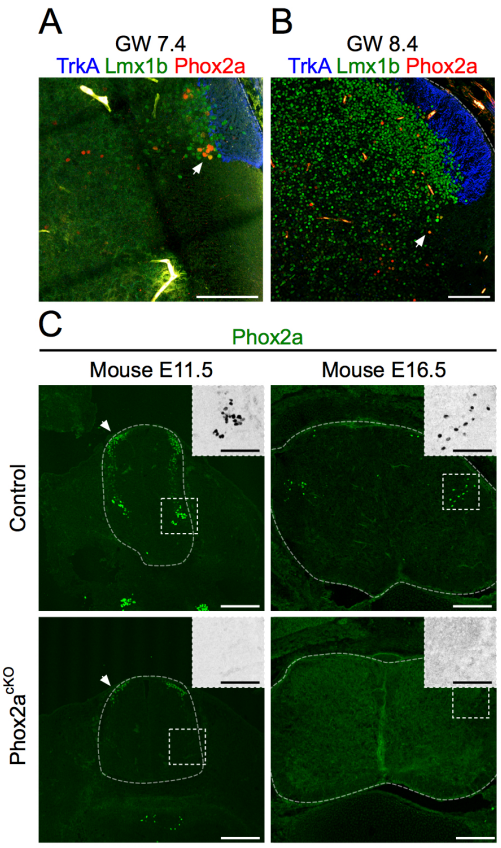
A
GW 7.4
TrkA Lmx1b Phox2a
B
GW 8.4
TrkA Lmx1b Phox2a
C
Phox2a
Mouse E11.5
Mouse E16.5
Control
Phox2a^cKO

Figure S7: Phox2a neuron molecular identity is conserved in the developing human spinal cord. Related to Figure 7.

(A, B) Insets from G.W 7.4 (A) and G.W 8.4 (B) human spinal cords in Fig. 7, demonstrating Phox2a and Lmx1b co-expression, and apposition of TrkA fibres with Phox2a neurons. (C) Validation of the Abcam Phox2a antibody in mouse Phox2a^{cKO} tissue at both E11.5 and E16.5. Arrows show immunostaining in dorsal dI neurons (likely dI1), which was not observed with the Phox2a antibody used in previous experiments (from JF Brunet) or by RNA in situ detection of *Phox2a* mRNA, and it is not eliminated in the Phox2a^{cKO}. These neurons may contribute to some of the Phox2a+/Lmx1b-negative neurons observed in Fig. 7.
Numbers: (A) Single G.W 7.4 human spinal cord, (B) single G.W 8.4 human spinal cord, (C) n=3 E11.5 control, n=3 E11.5 Phox2a^{cKO}, n=3 E16.5 control, n=3 E16.5 Phox2a^{cKO} mouse embryos.
Scale bars: (A, B) and insets in (C) 100 μm, (C) 200 μm.

Tables:

Table S1: mRNAs enriched in embryonic AS neurons. Related to Figure 5.
Top 25 enriched mRNAs per three clusters identified in Fig. 5L and 5N, when compared to all other spinal neurons in the dataset at the same embryonic ages. For each cluster, the transcript name, log_{10}fold change (log_{10}FC) and -log_{10}p values are represented. P-values rounded to 0 (<2.22x10^-308) were recorded as having a -log_{10}P value of ">307.65". Transcripts with high -log_{10}P values that were not expressed in non-Lmx1b+ neurons were considered to be useful markers for Phox2a neurons.

E9.5-E13.5 *Lmx1b* Cluster 6			E9.5-E11.5 *Lmx1b* Cluster 2			E9.5-E11.5 *Lmx1b* Cluster 3		
Name	**log_{10}FC**	**-log_{10}p**	**Name**	**log_{10}FC**	**-log_{10}p**	**Name**	**log_{10}FC**	**-log_{10}p**
Phox2a	0.97	>307.65	*Lmx1b*	0.75	218.80	*Lmx1b*	0.74	270.53
Nms	0.44	>307.65	*Tac1*	1.16	94.60	*Nms*	0.65	177.44
Syt13	0.66	271.21	*C13002*	0.53	48.54	*Phox2a*	1.11	158.15
Zim1	0.20	176.74	*Pou4f2*	0.45	37.76	*C13002*	0.67	99.06
633040	0.28	146.78	*Gabra2*	0.50	29.19	*Car10*	0.64	88.30
Arhgdig	0.66	138.31	*Tlx3*	0.60	24.04	*Tm4sf4*	0.14	86.40
Snca	1.01	123.62	*Syt13*	0.42	18.00	*Syt13*	0.66	63.69
Syt4	2.18	120.03	*Chl1*	0.86	17.43	*Mar1*	0.49	50.42
Lmx1b	0.56	119.42	*Tcerg1l*	0.51	16.68	*Rit2*	0.31	48.88
Scn9a	0.50	112.81	*Mab21l2*	1.07	16.43	*Pde8b*	0.14	46.44
Sema3	0.44	107.43	*Pou4f1*	1.26	15.95	*Fam19a*	0.47	46.09
Kctd4	0.33	103.94	*Tmeff2*	0.57	14.85	*Tcerg1l*	0.74	41.81
Sncg	1.77	103.89	*Kif26b*	0.36	13.68	*Mdga1*	0.41	40.92
Tac1	0.48	101.61	*Cadm2*	0.40	13.52	*Pdzrn3*	0.91	39.54
Rit2	0.45	101.60	*Slc17a6*	0.54	13.39	*Nrxn1*	1.26	38.57
Car10	0.50	99.29	*Tshz2*	1.03	12.96	*Tmeff2*	0.67	37.60
Pcp4	1.18	94.51	*Mab21l1*	0.42	12.76	*Negr1*	0.58	36.64
Tcerg1l	0.62	92.38	*Ntrk3*	0.50	12.00	*Scn3b*	0.65	31.99
Pde8b	0.12	91.23	*Ppp3ca*	0.78	11.09	*Syt4*	1.91	30.60
Tspan1	0.65	88.46	*Nrxn1*	0.77	10.56	*Stk39*	0.86	27.30
Scn3b	0.63	83.81	*Nms*	0.16	10.19	*Shox2*	0.85	26.70
Adap1	0.27	81.32	*Mtus2*	0.70	10.11	*Stk32b*	0.55	25.69
Fam19a	0.48	80.39	*Scn3b*	0.46	9.95	*Chl1*	0.88	25.24
Nptxr	0.28	76.58	*Stk39*	0.69	9.84	*Sema3a*	0.45	25.20
Gap43	1.91	76.21	*Hs3st2*	0.10	9.56	*Necab2*	0.50	24.31