

Figure S1

Supplementary Figure 1

Commissural axons exit the CNS by different nerve roots in absence of netrin-1

A-F, Coronal sections of E11 spinal cords immunolabelled for Robo3 and counterstained with DAPI. In wild type (A, B; n=3) Robo3 axons are only found in the CNS. In *Ntn1* ^{β geo/ β geo} (C, D; n=5) and *Ntn1*^{-/-} (E, F; n=6) mutants, Robo3+ commissural axons invade the dorsal root ganglia (drg, arrowhead and dotted lines). G-J, LSM images of ventral views of *Ntn1*^{-/-} E11 embryo illustrating the invasion of the trigeminal (V) and auditory (VIII) nerves and ganglia. Trigeminal neurons are immunolabelled for Islet1 (H, J, n=3). K-R, are coronal sections of the hindbrain of E16 embryos. In wild type, Pax6+ (K, L; n=5) and Barhl1+ (O, P; n=5) pontine neurons (arrowheads) migrate under the CNS/PNS boundary (dotted line). In *Ntn1* ^{β geo/ β geo} (M, Q; n=4) and *Ntn1*^{-/-} (N, R; n=5) mutants, both Pax6 and Barhl1+ cells exit the brainstem and migrate in the PNS (arrows). They also express Robo3, confirming their commissural neuron identity (M, N). S, T, Quantification of the volume of Robo3+ axons invading the trigeminal nerve at E11 (S) and E13 (T) in *Ntn1* ^{β geo/ β geo}, *Ntn1*^{-/-} and *Nes:Cre; Ntn1*^{fl/fl} embryos (Table S1). U, Quantification of the number of Barhl1+ cells in the auditory and trigeminal nerve roots in *Ntn1* ^{β geo/ β geo}, *Ntn1*^{-/-} and *Nes:Cre; Ntn1*^{fl/fl} E16 cryosections (Table S2). Abbreviations: Mes, mesencephalon; Hind, hindbrain. Dotted lines delineate the CNS limit. Scale bars, 50 μ m (B, D, F), 100 μ m (A, C, E, K-R), 200 μ m (H, I, J).

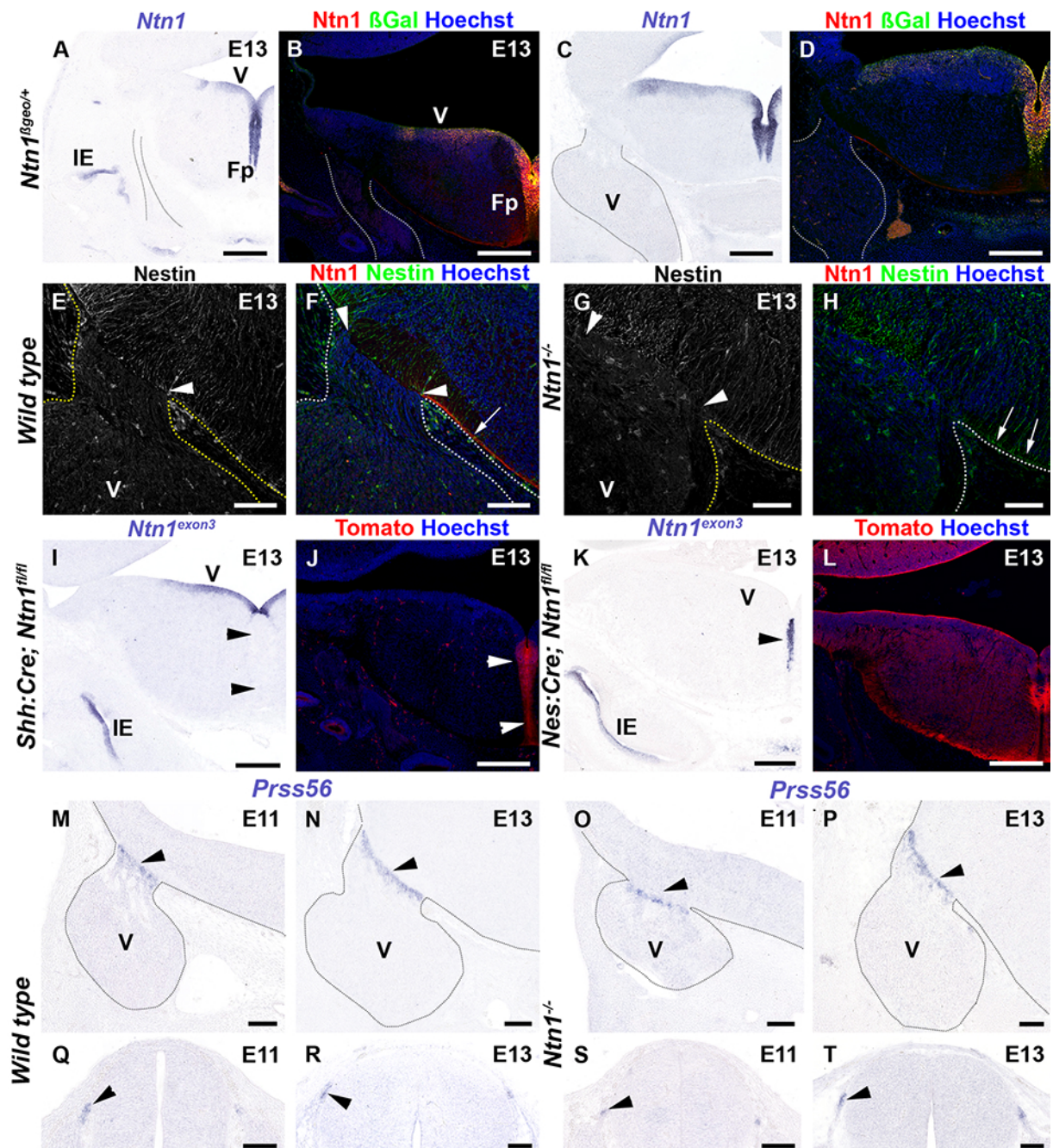


Figure S2

Supplementary Figure 2

Netrin-1 is not expressed at the level of hindbrain sensory roots.

Coronal sections at the level of the hindbrain (A-P) and spinal cord (Q-T).

A-D, In E13 *Ntn1* ^{β geo/+} control embryo, *netrin-1* mRNA, β -galactosidase and netrin-1 immunoreactivity are found at the floor plate (Fp), ventricular zone (V) and the inner ear (IE). No netrin-1 is detected in nerves and ganglia (dotted lines, n=6). E-H, In E13 wild type embryos, the radial processes of Nestin+ ventricular zone progenitors extend to the pial surface at the level of the trigeminal nerve root (between arrowheads). Netrin-1 protein is found along the basal lamina (arrow in F, n=6) except at the trigeminal nerve root (arrowheads in F). In *Ntn1*^{-/-} (G and H; n=6) mutants, Nestin+ fibers still reach the pial surface (arrowheads in G) even if netrin-1 protein is absent (arrows in H). I-J, In *Shh:Cre; Ntn1*^{fl/fl} mutants, *netrin-1* mRNA is deleted from the floor plate (arrowheads in I; n=5) where tomato expression is induced by Cre recombinase (arrowheads in J; n=3). K-L, In *Nes:Cre;Ntn1*^{fl/fl} mutants, *netrin-1* mRNA expression is ablated from the ventricular zone (v) and maintained in the floor plate (arrowhead in J; n=5) and the inner ear (IE). Tomato expression is induced throughout the CNS except the floor plate (L; n=3). M-T, In wild type E11 and E13 embryos, boundary cap cells express *Prss56* mRNA (arrowheads) at nerve roots in the hindbrain (M, N; n=3) and spinal cord (Q, R; n=3). This is also the case in *Ntn1*^{-/-} mutants embryos (arrowheads in O, P, S, T; n=3). Abbreviation: IE, inner ear; V, trigeminal ganglion. Scale bars, 100 μ m (E, H, M-T), 250 μ m (A-D, I-L).

Supplementary table 1**Volume of Robo3+ axons in the trigeminal ganglion at E11 and E13 in *Ntn1* mutants**

Genotype (E11)	Number of cases	Mean \pm SD ($\times 10^6 \mu\text{m}^3$)
<i>CTL</i>	n = 5	0 \pm 0
<i>Ntn1</i> ^{$\beta\text{Geo}/\beta\text{Geo}$}	n = 4	0.3 \pm 0.15
<i>Ntn1</i> ^{-/-}	n = 6	2.72 \pm 0.90
Genotype comparison	Mann-Whitney test	Kruskal-Wallis test
<i>CTL</i> vs <i>Ntn1</i> ^{$\beta\text{Geo}/\beta\text{Geo}$}	p-value = 0.008 (**)	p-value < 0.0001 (***)
<i>CTL</i> vs <i>Ntn1</i> ^{-/-}	p-value = 0.004 (**)	
<i>Ntn1</i> ^{$\beta\text{Geo}/\beta\text{Geo}$} vs <i>Ntn1</i> ^{-/-}	p-value = 0.009 (**)	

Genotype (E13)	Number of cases	Mean \pm SD ($\times 10^6 \mu\text{m}^3$)
<i>CTL</i>	n = 5	0 \pm 0
<i>Ntn1</i> ^{$\beta\text{Geo}/\beta\text{Geo}$}	n = 4	4.25 \pm 1.33
<i>Ntn1</i> ^{-/-}	n = 7	8.26 \pm 2.61
<i>Nes:Cre; Ntn1</i> ^{fl/fl}	n = 5	6.55 \pm 1.08
Genotype comparison	Mann-Whitney test	Kruskal-Wallis test
<i>CTL</i> vs <i>Ntn1</i> ^{$\beta\text{Geo}/\beta\text{Geo}$}	p-value = 0.008 (**)	p-value = 0.0013 (**)
<i>CTL</i> vs <i>Ntn1</i> ^{-/-}	p-value = 0.003 (**)	
<i>CTL</i> vs <i>Nes:Cre; Ntn1</i> ^{fl/fl}	p-value = 0.008 (**)	
<i>Ntn1</i> ^{$\beta\text{Geo}/\beta\text{Geo}$} vs <i>Ntn1</i> ^{-/-}	p-value = 0.024 (*)	
<i>Ntn1</i> ^{$\beta\text{Geo}/\beta\text{Geo}$} vs <i>Nes:Cre; Ntn1</i> ^{fl/fl}	p-value = 0.032 (*)	
<i>Ntn1</i> ^{-/-} vs <i>Nes:Cre; Ntn1</i> ^{fl/fl}	p-value = 0.149 (ns)	

Supplementary table 2**Number of Barhl1+ cells in the auditory and trigeminal nerve roots at E16 in*****Ntn1* mutants**

Genotype	Number of cases	Mean \pm SD (n° cells)
<i>CTL</i>	n = 5	0 \pm 0
<i>Ntn1</i> ^{βGeo/βGeo}	n = 4	106.1 \pm 6.77
<i>Ntn1</i> ^{-/-}	n = 4	218.8 \pm 50.82
<i>Nes:Cre; Ntn1</i> ^{fl/fl}	n = 5	127.4 \pm 14.94
Genotype comparison	Mann-Whitney test	Kruskal-Wallis test
<i>CTL</i> vs <i>Ntn1</i> ^{βGeo/βGeo}	p-value = 0.008 (**)	p-value < 0.0001 (***)
<i>CTL</i> vs <i>Ntn1</i> ^{-/-}	p-value = 0.008 (**)	
<i>CTL</i> vs <i>Nes:Cre; Ntn1</i> ^{fl/fl}	p-value = 0.008 (**)	
<i>Ntn1</i> ^{βGeo/βGeo} vs <i>Ntn1</i> ^{-/-}	p-value = 0.029 (*)	
<i>Ntn1</i> ^{βGeo/βGeo} vs <i>Nes:Cre; Ntn1</i> ^{fl/fl}	p-value = 0.064 (ns)	
<i>Ntn1</i> ^{-/-} vs <i>Nes:Cre; Ntn1</i> ^{fl/fl}	p-value = 0.016 (*)	

Supplementary table 3**Volume of Robo3+ axons in the trigeminal ganglion at E11 and E13 in *Dcc* KO**

Genotype (E11)	Number of cases	Mean \pm SD (n° cells)
<i>CTL</i>	n = 4	0 \pm 0
<i>Dcc</i> ^{-/-}	n = 4	1.078 \pm 1.24
Genotype comparison	Mann-Whitney test	Kruskal-Wallis test
<i>CTL</i> vs <i>Dcc</i> ^{-/-}	p-value = 0.029 (*)	Not necessary

Genotype (E13)	Number of cases	Mean \pm SD (n° cells)
<i>CTL</i>	n = 4	0 \pm 0
<i>Dcc</i> ^{-/-}	n = 4	5.258 \pm 2.054
Genotype comparison	Mann-Whitney test	Kruskal-Wallis test
<i>CTL</i> vs <i>Dcc</i> ^{-/-}	p-value = 0.029 (*)	Not necessary

Supplementary table 4

Number of Barhl1+ cells in the auditory and trigeminal nerve roots at E16 in Dcc mutants

Genotype	Number of cases	Mean \pm SD (n° cells)
<i>CTL</i>	n = 5	0 \pm 0
<i>Dcc</i> ^{-/-}	n = 5	146 \pm 36.83
<i>Wnt1:Cre; Dcc</i> ^{fl/fl}	n = 4	28.3 \pm 9.25
Genotype comparison	Mann-Whitney test	Kruskal-Wallis test
<i>CTL</i> vs <i>Dcc</i> ^{-/-}	p-value = 0.008 (**)	p-value < 0.0001 (***)
<i>CTL</i> vs <i>Wnt1:Cre; Dcc</i> ^{fl/fl}	p-value = 0.008 (**)	
<i>Dcc</i> ^{-/-} vs <i>Wnt1:Cre; Dcc</i> ^{fl/fl}	p-value = 0.016 (*)	

Movies



Movie 1

3D movie of 3DISCO-cleared spinal cord from an E11 wild type embryo showing the restriction of Robo3-immunoreactive commissural axons to the CNS.



Movie 2

3D movie of 3DISCO-cleared spinal cord from an E11 *Ntn1*^{-/-} embryo showing the invasion of dorsal root ganglia (Drg) by a subset of Robo3-immunoreactive commissural axons.



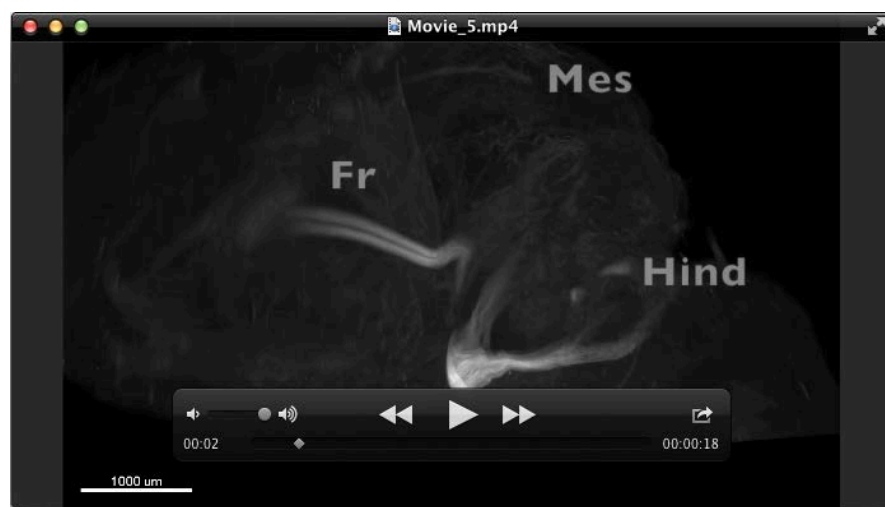
Movie 3

3D movie of 3DISCO-cleared E13 wild type embryo. All Robo3+ axons are confined to the CNS. Abbreviations: Ce, cerebellum; Hind, hindbrain; Mes, mesencephalon; Fr; Fasciculus retroflexus.



Movie 4

3D movie of 3DISCO-cleared hindbrain from an E13 *Ntn1*^{-/-} embryo showing the massive invasion of trigeminal and auditory nerves by Robo3-immunoreactive commissural axons. Abbreviations: Ce, cerebellum; Hind, hindbrain; Mes, mesencephalon; Fr; Fasciculus retroflexus; V, trigeminal nerve; VIII, auditory nerve.



Movie 5

3D movie of the migrating stream of Robo3-immuoreactive pontine neurons in a wild type E16 embryo. Abbreviations: Hind, hindbrain; Mes, mesencephalon; Fr; Fasciculus retroflexus;



Movie 6

3D movie of the migrating stream of Robo3-immuoreactive pontine neurons in a *Ntn1*^{-/-} E16 embryo. Robo3 pontine neurons fail to reach the midline and a large fraction migrate outside the CNS in to the trigeminal and auditory nerves.

Abbreviations: Hind, hindbrain; Mes, mesencephalon; Fr; Fasciculus retroflexus; V, trigeminal nerve; VIII, auditory nerve.



Movie 7

3D Movies of an E16 *Ntn1*^{-/-} embryo electroporated unilaterally with GFP at E13. Whole-mount immunolabeling for Barhl1 (Green) and Robo3 (Magenta) and 3DISCO clearing. GFP+/Barhl1+ pontine neurons invade the PNS. Abbreviations: Hind, hindbrain; Mes, mesencephalon; Fr, Fasciculus retroflexus; V, trigeminal nerve; VIII, auditory nerve.