© 2018. Published by The Company of Biologists Ltd | Development (2018) 145, dev150375. doi:10.1242/dev.150375

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RESEARCH ARTICLE

Non-cell autonomous control of precerebellar neuron migration by Slit and Robo proteins

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ABSTRACT

During development, precerebellar neurons migrate tangentially from the dorsal hindbrain to the floor plate. Their axons cross it but their cell bodies stop their ventral migration upon reaching the midline. It has previously been shown that Slit chemorepellents and their receptors, Robo1 and Robo2, might control the migration of precerebellar neurons in a repulsive manner. Here, we have used a conditional knockout strategy in mice to test this hypothesis. We show that the targeted inactivation of the expression of *Robo1* and *Robo2* receptors in precerebellar neurons does not perturb their migration and that they still stop at the midline. The selective ablation of the expression of all three Slit proteins in floor-plate cells has no effect on pontine neurons and only induces the migration of a small subset of inferior olivary neurons across the floor plate. Likewise, we show that the expression of Slit proteins in the facial nucleus is dispensable for pontine neuron migration. Together, these results show that Robo1 and Robo2 receptors act non-cell autonomously in migrating precerebellar neurons and that floor-plate signals, other than Slit proteins, must exist to prevent midline crossing.

KEY WORDS: Robo, Slit, Cerebellum, Floor plate, Inferior olive, Migration

INTRODUCTION

First described in human embryos (His, 1891), the rhombic lip, a germinative neuropithelium lining the dorsal edge of the fourth ventricle in the hindbrain, is the source of inferior olivary (IO) neurons and pontine (PN) neurons that both migrate tangentially, parallel to the pial surface, to the ventral midline or floor plate (Altman and Bayer, 1987; Essick, 1907, 1912; Harkmark, 1954). IO and PN neurons are precerebellar neurons that project into the contralateral cerebellum on Purkinje cells and granule cells, respectively. During their migration, they exhibit a unipolar morphology with a long leading process at the front (Bourrat and Sotelo, 1988; Kawauchi et al., 2006; Watanabe and Murakami, 2009; Zelina et al., 2014), which transform into an axon after midline crossing. However, PN neurons do not cross the midline, except for a few that are early born (Kawauchi et al., 2006). Insights onto the mechanisms controlling the migration of precerebellar neurons towards the midline have come from the phenotypic analysis of knockout mice. The current model suggests that precerebellar neuron guidance primarily relies on the same cues, netrin 1 (Ntn1) and Slit proteins (Slits) that control midline crossing of dorsal spinal cord commissural axons (Chédotal, 2011; Sotelo and Chédotal, 2013). In mice lacking Ntn1 or its receptor, deleted in colorectal carcinoma (Dcc), the ventral migration of IO and PN neurons is severely perturbed (Bloch-Gallego et al., 1999; Dominici et al., 2017; Marcos et al., 2009; Yee et al., 1999; Zelina et al., 2014). The abnormal dorsal expression of Ntn1 in the Ezh2 histone methyltransferase knockout induces a premature ventral migration on a subset of PN neurons (Di Meglio et al., 2013). Slit chemorepellents and their cognate receptors, roundabout 1 (Robo1) and Robo2 are also thought to influence precerebellar neuron migration. In both *Slit1;Slit2* and *Robo1;Robo2* double-knockout mice, a significant fraction of IO neurons crosses the floor plate (Di Meglio et al., 2008) and chains of PN neurons prematurely leave the main migratory stream, moving directly to the midline (Geisen et al., 2008). It has also been suggested that Slits released by the facial nucleus force PN neurons to migrate anteriorly before they can turn ventrally (Geisen et al., 2008) (see Fig. 2).

A third Robo receptor, Robo3, is expressed by precerebellar neurons until their leading processes cross the midline (Marillat et al., 2004; Zelina et al., 2014). In *Robo3* knockout (Marillat et al., 2004; Zelina et al., 2014) and in humans carrying mutations in *ROBO3* (Jen et al., 2004), PN neurons are unable to reach the ventral midline. IO neurons reach the floor plate but their axons fail to cross it (Marillat et al., 2004). Robo3 does not bind Slits in mammals and forms a complex with Dcc that promotes PN neuron ventral migration (Zelina et al., 2014). A partial rescue of the IO commissure in *Robo1/2/3* triple knockout suggests that Robo3 might counteract Slit/Robo repulsion, as proposed for spinal cord commissural axons (Di Meglio et al., 2008; Jaworski et al., 2010; Sabatier et al., 2004). Importantly, except for Robo3, the genetic data supporting the actual models come from phenotypic analysis of full knockouts in which Slits, Robo1 and Robo2 are inactivated in all cells. These molecules are broadly expressed throughout the body and the development of many neuronal systems and organs is severely impaired in *Slit1;Slit2* and *Robo1;Robo2* knockouts (Blockus and Chédotal, 2016; Ypsilanti et al., 2010). Therefore, direct genetic evidence validating the current working models could only be provided by a conditional knockout approach. Using this strategy, we show here that Slit/Robo signaling acts on precerebellar neurons in a non-cell-autonomous manner.

RESULTS

To study the role of Slits and Robo receptors in the migration of IO and PN neurons, we used and combined existing knockout lines, including *Slit2* (Rama et al., 2015) and *Robo2* (Gibson et al., 2014) conditional knockouts ($Slit2^{L/L}$ and $Robo2^{L/L}$; see Materials and methods). $Slit2^{L/L}$ mice were crossed to *Slit1* (Plump et al., 2002) and *Slit3* (Yuan et al., 2003) full knockouts and $Robo2^{L/L}$ mice were intercrossed with *Robo1* knockouts (Gibson et al., 2014).

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Received 8 February 2017; Accepted 11 December 2017

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Development (2018) 145, dev150375. doi:10.1242/dev.150375

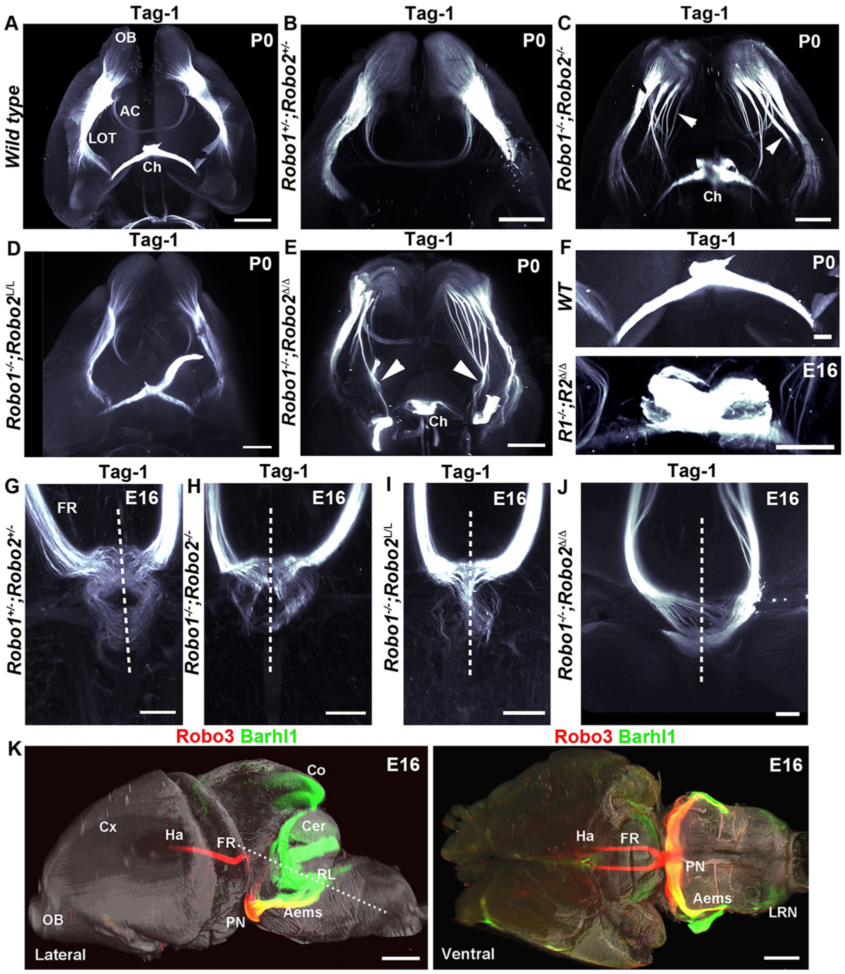

Fig. 1. *Robo1$^{-/-}$;Robo2$^{Δ/Δ}$* mice phenocopy *Robo1$^{-/-}$;Robo2$^{-/-}$* knockouts. (A-E) LSFM images (ventral views) of the forebrain. Tag-1 immunostaining and 3DISCO clearing. The LOT is defasciculated (arrowheads) and closer to the midline in *Robo1$^{-/-}$;Robo2$^{-/-}$* (C) and *Robo1$^{-/-}$;Robo2$^{Δ/Δ}$* (E) mice compared with controls (A,B,D). (F) The chiasm (Ch) is also disorganized in *Robo1$^{-/-}$;Robo2$^{Δ/Δ}$* mutants. (G-J) Tag1^{+} axons from the fasciculus retroflexus (FR) cross the floor plate (dashed line) multiple times in *Robo1$^{+/-}$;Robo2$^{+/-}$* embryos (G) but not in *Robo1$^{-/-}$;Robo2$^{-/-}$* (H), *Robo1$^{-/-}$;Robo2$^{L/L}$* (I) and *Robo1$^{-/-}$;Robo2$^{Δ/Δ}$* (J) mutants. (K) LSFM views (lateral and ventral) of 3DISCO-cleared E16 brains immunostained for Robo3 (red) and Barhl1 (green). AC, anterior commissure; Aems, anterior extramural stream; Cer, cerebellum; Ch, chiasm; Co, colliculus; Cx, cortex; FR, fasciculus retroflexus; Ha, habenula; LRN, lateral reticular nucleus; OB, olfactory bulb; PN, pons; RL, rhombic lip. Scale bars: 1 mm in A; 400 µm in B,D; 300 µm in C,E,H; 200 µm in F,G,I,J; 800 µm in K.

Non-cell-autonomous control of pontine neuron migration by Robo1 and Robo2 receptors

To assess the role of Robo1 and Robo2 receptors in precerebellar neuron migration, we first intercrossed *Robo1$^{-/-}$;Robo2$^{L/L}$* and *Krox20:Cre* mice, which express Cre recombinase in the germline (Voiculescu et al., 2000). The resulting homozygous mutants will be referred to as *Robo1$^{-/-}$;Robo2$^{Δ/Δ}$*. Cre is also expressed in rhombomeres 3 and 5, which do not contain PN neuron progenitors (Di Meglio et al., 2013). In E13 hindbrain, Robo1 and Robo2 antibodies labeled longitudinal axons (Fig. S1B,E). The absence of *Robo2* mRNA and protein in *Robo1$^{-/-}$;Robo2$^{Δ/Δ}$* embryos was confirmed by *in situ* hybridization and immunostaining (Fig. S1A-D). The lateral olfactory tract (LOT), which contains axons projecting from the olfactory bulb to the pyriform cortex is defasciculated in *Robo1/Robo2* double knockouts (Fouquet et al., 2007). In those mutants, severe axon pathfinding defects were also described for the fasciculus retroflexus (FR), which connect the medial habenula to the interpeduncular nucleus (Belle et al., 2014). To visualize the LOT and FR in the various lines, we performed whole-mount immunolabeling with antibodies against transient-associated glycoprotein 1 (Tag-1/contactin 2) (Belle et al., 2014; Wolfer et al., 1994), combined with 3DISCO tissue clearing and light-sheet fluorescence microscopy (LSFM) (Belle et al., 2014). In E16 and P0 control brains from wild type (*n*=3/3), *Robo1$^{+/-}$;Robo2$^{+/-}$* (*n*=3/3), *Robo1$^{+/-}$;Robo2$^{L/+}$* (*n*=3/3) and *Robo1$^{-/-}$;Robo$^{L/L}$* (*n*=5/5) mice, Tag-1^{+} LOT axons form one axon bundle extending on each side of the ventral forebrain (Fig. 1 and not shown). In *Robo1$^{-/-}$;Robo2$^{-/-}$* (Fig. 1C; *n*=4/4) and *Robo1$^{-/-}$;Robo2$^{Δ/Δ}$* mice (Fig. 1E; *n*=8/8), the LOT was defasciculated and some axons extended more medially than in controls. As shown before in *Robo1$^{-/-}$;Robo2$^{-/-}$* knockouts (Plachez et al., 2008), midline crossing was abnormal at the optic chiasm of all *Robo1$^{-/-}$;Robo2$^{Δ/Δ}$* mutants (Fig. 1F). In wild type (*n*=3/3), *Robo1$^{+/-}$;Robo2$^{+/-}$* (*n*=3/3) and *Robo1$^{+/-}$;Robo2$^{L/+}$* (*n*=3/3), FR axons zigzagged at the floor plate upon reaching it (Fig. 1G). In *Robo1$^{-/-}$;Robo$^{L/L}$* (*n*=4/4), midline crossing was perturbed and some axons remained at the floor plate (Fig. 1I). In *Robo1$^{-/-}$;Robo2$^{-/-}$* (*n*=3/3) and *Robo1$^{-/-}$;Robo2$^{Δ/Δ}$* (*n*=3/3) mice, FR axon crossing was more strongly affected and most axons remained on the ipsilateral side (Fig. 1H,J; Belle et al., 2014). These results show that *Robo1$^{-/-}$;Robo2$^{Δ/Δ}$* mice phenocopy *Robo1$^{-/-}$;Robo2$^{-/-}$* double knockouts (Fig. 1F).

To study PN neuron migration, whole-mount double immunostaining for Robo3 and the transcription factor Barhl1 (Zelina et al., 2014) was performed on whole E16 embryos. This was also followed by 3DISCO clearing and LSFM (Fig. 1K; see Materials and methods). In all E16 wild-type (*n*=3/3), *Robo1$^{+/-}$;Robo2$^{+/-}$* (*n*=3/3), *Robo1$^{+/-}$;Robo2$^{L/+}$* (*n*=3/3) and *Robo1$^{-/-}$;Robo$^{L/L}$* (*n*=4/4) embryos (Fig. 2A,C,E; Movie 1 and not shown), PN neurons form a compact stream that migrates rostrally and then ventrally to the floor plate. They strongly express Barhl1 and Robo3. At this stage, Robo3 is also expressed in the FR, which terminates in the interpeduncular nucleus, rostral to the PN. As previously described (Geisen et al., 2008), PN migration was severely

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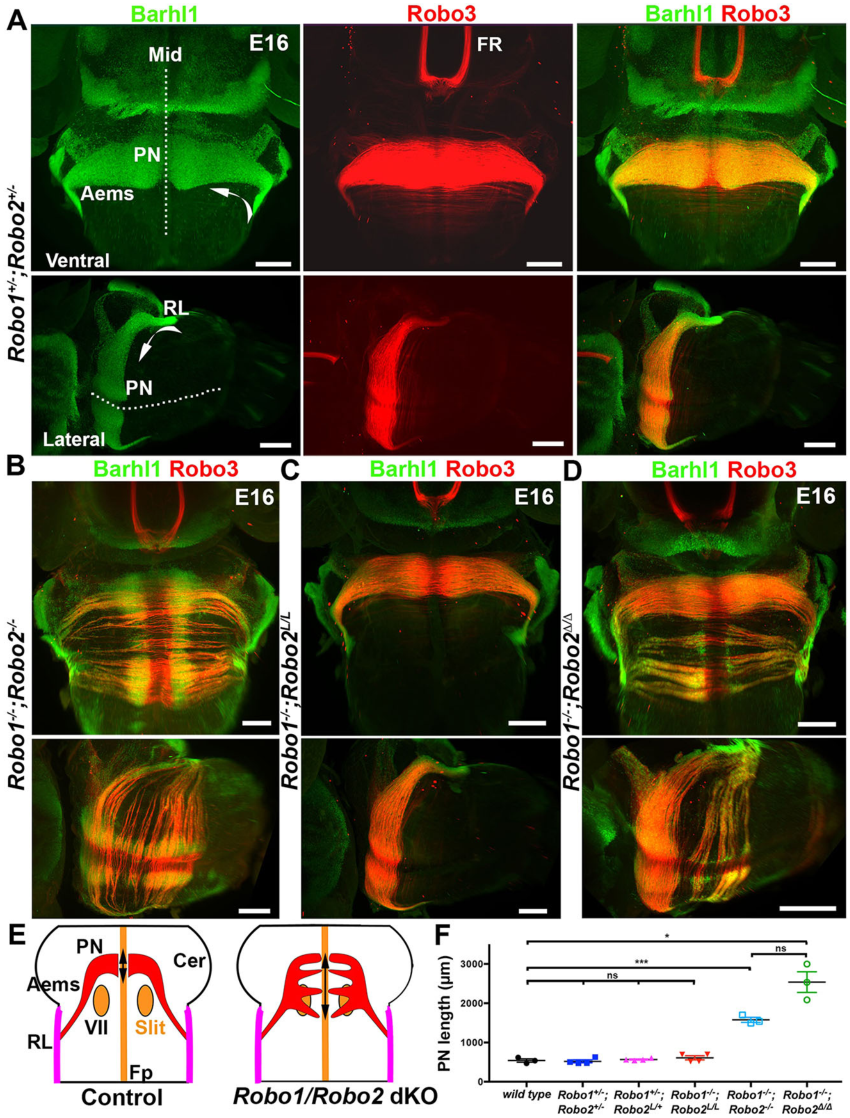

Fig. 2. PN neurons migrate prematurely to the midline in *Robo1*$^{-/-}$*;Robo2*$^{\Delta/\Delta}$ mice. (A-D) LSFM images of 3DISCO-cleared E16 hindbrains immunostained for Barhl1 and Robo3. (A) Ventral views (top panels) and lateral views of a *Robo1*$^{+/-}$*;Robo2*$^{+/-}$ embryo illustrating the normal migration pathway (Aems and curved arrow) followed by PN neurons from the rhombic lip to the midline (dashed line). (B,C) Many PN neurons migrate prematurely to the midline in *Robo1*$^{-/-}$*;Robo2*$^{-/-}$ knockouts (B), unlike in *Robo1*$^{-/-}$*;Robo2*$^{L/L}$ embryos (C). (D) *Robo1*$^{-/-}$*;Robo2*$^{\Delta/\Delta}$ embryos phenocopy *Robo1*$^{-/-}$*; Robo2*$^{-/-}$ mutants. (E) Schematic representations of PN neuron migration (red) in controls and Robo mutants. The black arrows indicate the PN length measured in F. Slits are found in the floor plate and facial nuclei (VII). (F) Quantification of PN length in Robo1/Robo2 mutants. **P*<0.05, ****P*<0.001. ns, not significant (*Robo1*$^{+/-}$*; Robo2*$^{+/-}$, *P*=0.7243; *Robo1*$^{+/-}$*;Robo2*$^{L/+}$, *P*=0.6165; *Robo1*$^{-/-}$*;Robo2*$^{L/L}$, *P*=0.3592; Welch's *t*-test). Error bars indicate s.e.m. Aems, anterior extramural stream; Cer, cerebellum; Fp, floor plate; FR, fasciculus retroflexus; Mid, midbrain; PN, pontine; RL, rhombic lip. Scale bars: 400 μm in A-D (top panels); 500 μm in A-D (bottom panels).

perturbed in *Robo1*$^{-/-}$*;Robo2*$^{-/-}$ (*n*=3/3; Fig. 2B; Movie 2) embryos, and multiple chains of Barhl1^{+}/Robo3^{+} neurons left the normal pathway to prematurely migrate towards the floor plate. However, in *Robo1*$^{-/-}$*;Robo2*$^{-/-}$ mutants, as in controls, Barhl1^{+} PN neurons aggregated on both sides of the floor plate without penetrating it, whereas their axons labeled with Robo3 crossed it. The quantification of the spreading of PN neurons along the floor plate supported the severe defasciculation of migrating PN neurons in *Robo1*$^{-/-}$*;Robo2*$^{-/-}$ mutants (Fig. 2E,F). PN migration defects were strikingly similar in *Robo1*$^{-/-}$*;Robo2*$^{\Delta/\Delta}$ embryos (*n*=3/3; Fig. 2D-F; Movie 3), further validating the *Robo2*$^{L/L}$ conditional knockout line. To determine whether Robo1/Robo2 act cell-autonomously in migrating PN neurons, we next intercrossed *Robo1*$^{-/-}$*;Robo2*$^{L/L}$ mice and *Wnt1:Cre* mice, which are known to drive the expression of Cre recombinase in PN neuron precursors in addition to other hindbrain neurons and neural crest cell derivatives (Di Meglio et al., 2013; Nichols and Bruce, 2006; Rodriguez and Dymecki, 2000; Zelina et al., 2014). In *Wnt1:Cre* E16 embryos (*n*=3/3) and *Wnt1:Cre;Robo1*$^{+/-}$*;Robo2*$^{L/+}$ (*n*=3/3) embryos, all Robo3^{+}/Barhl1^{+} PN neurons migrated to the floor plate as in wild-type embryos, but the shape of the PN nucleus appeared slightly reduced and some FR axons failed to cross the midline (Fig. 3A,B). Surprisingly, the migration of PN neurons was not affected in any of the *Wnt1:Cre;Robo1*$^{-/-}$*;Robo2*$^{L/L}$ (*n*=6/6) embryos, in which all neurons followed the anterior extramural stream. No evidence of a premature migration to the ventral midline was found and PN length was similar to control (Fig. 3C,E,F). By contrast, all PN neurons failed to reach the midline in *Wnt1:Cre;Robo3*$^{L/L}$ embryos (Fig. 3D, E; *n*=3/3), thereby confirming that the *Wnt1:Cre* line efficiently recombines floxed alleles in PN neuron precursors. Although Robo2 mRNA was previously detected in migrating pontine neurons (Geisen et al., 2008), unilateral electroporation in PN neurons of a plasmid encoding GFP (*n*=3 wild-type embryos; see Materials and methods) showed that Robo2 immunoreactivity was detectable only on post-crossing pontine axons (Fig. S1F-I), confirming an earlier report. Together, these data show that Robo1 and Robo2 do not control the migration of PN neurons in a cell-autonomous manner.

Floor plate-derived Slits do not influence PN neuron migration

To study the influence of floor plate-derived Slits on the migration of PN neurons, we next generated *Shh:Cre;Slit1*$^{-/-}$*;Slit2*$^{lox/lox}$*; Slit3*$^{-/-}$ (hereafter referred to as *Shh:Cre;S1*$^{-/-}$*;S2*$^{L/L}$*;S3*$^{-/-}$).

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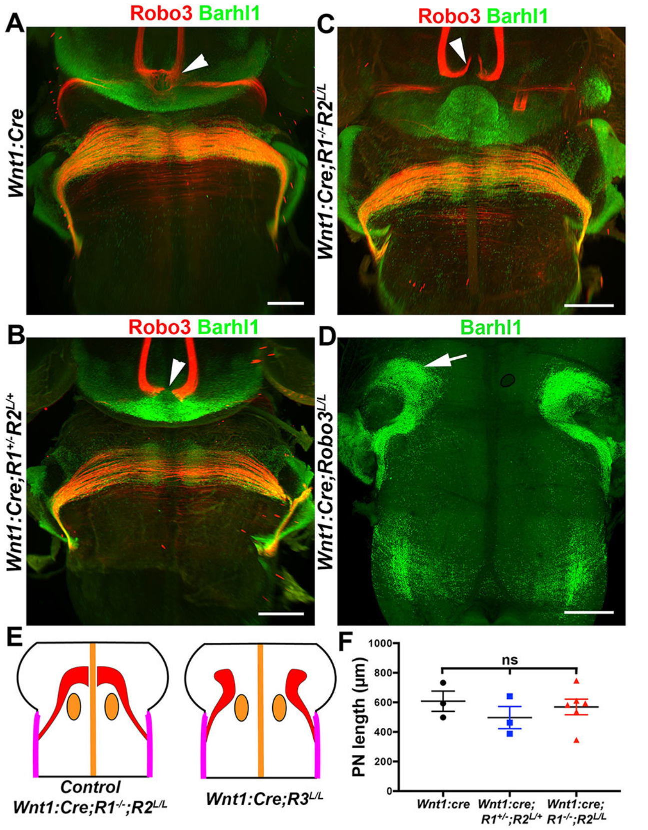

Fig. 3. PN neurons migrate normally in *Wnt1:Cre;Robo1*$^{-/-}$*;Robo2*$^{L/L}$ knockouts. (A-D) LSFM images of 3DISCO-cleared E16 hindbrains immunostained for Barhl1 and Robo3. (A-C) PN neuron migration is normal in *Wnt1:Cre* (A) and *Wnt1:Cre;Robo1*$^{+/-}$*;Robo2*$^{L/+}$ (B). However, FR axons do not properly cross the floor plate (arrowheads). (C) PN migration is not perturbed in *Wnt1:Cre;Robo1*$^{-/-}$*;Robo2*$^{L/L}$ embryos. FR axons do not cross the midline (arrowhead). (D) PN neurons fail to turn ventrally in *Wnt1:Cre; Robo3*$^{L/L}$ embryos (arrow). (E) Schematic representations of PN neuron migration (red) in controls and *Wnt1:Cre;Robo* mutants. (F) Quantification of PN length. ns, not significant (*Wnt1:Cre;Robo1*$^{+/-}$*;Robo2*$^{L/+}$, P=0.3370; *Wnt1: Cre;Robo1*$^{-/-}$*;Robo2*$^{L/L}$, P=0.6744; Welch's t-test). Error bars indicate s.e.m. Scale bars: 300 µm in A; 500 µm in B,C; 600 µm in D.

The viability of *Shh:Cre;S1*$^{-/-}$*;S2*$^{L/L}$ mice was comparable with controls but *Shh:Cre;S1*$^{-/-}$*;S2*$^{L/L}$*;S3*$^{-/-}$ mice died shortly after birth. In *Shh:Cre* mice, Cre recombinase and the reporter green fluorescent protein (GFP) are inserted in the sonic hedgehog locus and are expressed in the floor plate (Harfe et al., 2004; Joksimovic et al., 2009), as seen in E11 spinal cord sections (Fig. 4A). We first wanted to confirm that this line recapitulated the axon guidance defects previously described in *Sli1/Slit2/Slit3* triple knockouts (Long et al., 2004). *In situ* hybridization on E12 spinal cord sections from *Shh:Cre;S1*$^{-/-}$*;S2*$^{L/L}$*;S3*$^{-/-}$ embryos with riboprobes specific for *Slit1*, *Slit2* exon 8 (floxed in *Slit2*^{lox} mice) or *Slit3* showed that they were all deleted from the floor plate, whereas Slit2 persisted in motoneurons (n=4/4; Fig. 4B-E). By contrast, *Ntn1* mRNA was expressed at normal levels in the floor plate of *Shh:Cre;S1*$^{-/-}$*;S2*$^{L/L}$*; S3*$^{-/-}$ embryos. Immunostaining for GFP and Alcam (a floor-plate marker) confirmed that the floor plate appeared normal in *Shh:Cre; S1*$^{-/-}$*;S2*$^{L/L}$*;S3*$^{-/-}$ embryos (Fig. S1J-O). To visualize spinal cord commissural axons, we performed immunolabeling of *S1*$^{-/-}$*;S2*$^{L/L}$*; S3*$^{-/-}$ (n=3), *S1*$^{-/-}$*;S2*$^{L/L}$*;S3*$^{+/-}$ (n=4), *S1*$^{+/-}$*;S2*$^{L/+}$*;S3*$^{+/-}$ (n=3) and *Shh:Cre;S1*$^{-/-}$*;S2*$^{L/L}$*;S3*$^{-/-}$ (n=10) E11-E12 embryos using antibodies against Robo3, Dcc and Robo1 (Jaworski et al., 2010). As previously described in *Slit1/Slit2/Slit3* conventional triple knockout embryos (Long et al., 2004), midline crossing of Robo3^{+} spinal cord commissural axons was similar to controls in *S1*$^{-/-}$*; S2*$^{L/L}$*;S3*$^{-/-}$ embryos (Fig. 4F), further pointing to the redundant activity of Slit1-Slit3 at the floor plate. In *Shh:Cre;S1*$^{-/-}$*;S2*$^{L/L}$*; S3*$^{-/-}$, Robo3^{+} commissural axons reached the midline but crossing was perturbed: the commissure was thicker and axons seemed to project towards the ventricular zone (Fig. 4G and Fig. S1P). LSFM imaging confirmed that the density of commissural axons was increased at the floor plate. Similar observations were made using anti-Dcc antibodies, which also confirmed the thickening of the ventral commissure in *Shh:Cre; S1*$^{-/-}$*;S2*$^{L/L}$*;S3*$^{-/-}$ embryos (n=3/3) compared with *S1*$^{-/-}$*;S2*$^{L/L}$*; S3*$^{-/-}$ embryos (n=3) and *S1*$^{+/-}$*;S2*$^{L/+}$*;S3*$^{+/-}$ (n=3; Fig. 4H,I). The best evidence for abnormal midline crossing came from Robo1 immunolabeling. As previously shown in wild-type embryos (Long et al., 2004), Robo1 was only expressed on post-crossing commissural axons that had started to grow longitudinally and Robo1 staining was absent at the floor plate (Fig. 4H; n=3/3). By contrast, Robo1-positive axons were present in the floor plate of *Shh:Cre;S1*$^{-/-}$*;S2*$^{L/L}$*;S3*$^{-/-}$ embryos (Fig. 4I; n=8/8). Guidance defects were observed at all spinal cord levels. These results support the floor-plate-specific deletion of the three Slits in our mutants and also the importance of floor-plate-derived Slits for spinal cord commissural axon guidance.

To further validate this strategy, we next analyzed the consequence of germline recombination of *Slit2* exon 8 in *S1*$^{-/-}$*; S2*$^{L/L}$*;S3*$^{-/-}$ mice (see Materials and methods). So far, the phenotype of *Slit1/Slit2/Slit3* triple knockouts had just been analyzed in the spinal cord (Long et al., 2004).

We first focused on the LOT and FR projections as they are known to be affected in *Slit1;Slit2* null mice (Belle et al., 2014; Nguyen-Ba-Charvet et al., 2002). In wild type (n=3/3), *S1*$^{-/-}$ (n=4/4) and *S1*$^{-/-}$*; S2*$^{L/L}$ (n=5/5) P0 mice and E16 embryos, the LOT was confined to the lateral part of the forebrain, as shown with anti-Tag-1 labeling (Fig. 5A and not shown). As shown previously (Nguyen-Ba-Charvet et al., 2002), the LOT was defasciculated and bundles of axons invaded a more medial domain of the ventral forebrain in *S1*$^{-/-}$*;S2*$^{-/-}$ double knockouts (Fig. 5B; n=3/3). Similar LOT guidance defects were also seen in *S1*$^{-/-}$*;S2*$^{\Delta/\Delta}$*;S3*$^{+/-}$ embryos (n=4/4; Fig. 5C). At the level of the FR, midline crossing defects were found in *S1*$^{-/-}$*;S2*$^{-/-}$ double knockouts (n=3/3) but not in *S1*$^{-/-}$*;S3*$^{-/-}$ (n=3/3) and *S1*$^{-/-}$*; S2*$^{L/L}$*;S3*$^{-/-}$ embryos (n=4/4; Fig. 5D,E). We found that the FR completely failed to cross the floor plate in *S1*$^{-/-}$*;S2*$^{\Delta/\Delta}$*;S3*$^{-/-}$ embryos (n=4/4; Fig. 5F). These results confirm that axon guidance defects observed after germline deletion of exon 8 in *Slit2*$^{L/L}$ mice faithfully mimic what was previously found using conventional *Slit2* knockouts.

We next studied the migration of PN neurons in *Slit* conditional knockouts. As previously described (Geisen et al., 2008), PN neurons migrated as in wild type in *S1*$^{+/-}$*;S2*$^{L/+}$ (n=3/3), *S1*$^{-/-}$*;S3*$^{+/-}$ (n=4/4), *S1*$^{-/-}$ (n=4/4) and *S1*$^{-/-}$*;S2*$^{L/L}$*;S3*$^{-/-}$ E16 embryos (n=4/4; Fig. 5G and not shown), whereas PN neuron migration was disorganized in *S1*$^{-/-}$*;S2*$^{-/-}$ embryos (n=3/3; Fig. 5H; Movie 4), in which PN neurons directly migrated from the rhombic lip to the floor plate, resulting in a significant caudal extension of Barhl1^{+} PN neurons clustering along the floor plate (Fig. S1Q). A slight rostro-caudal enlargement was also detected in *S1*$^{-/-}$*;S3*$^{-/-}$ embryos but no ectopic migratory chains (Fig. S1Q, n=3/3). The severity of the PN premature migration defects appeared similar in *S1*$^{-/-}$*;S2*$^{-/-}$*;S3*$^{-/-}$ E16 embryos (n=2; Fig. 5I; Movie 5). Likewise, a major disorganization of the PN migratory stream was seen in both

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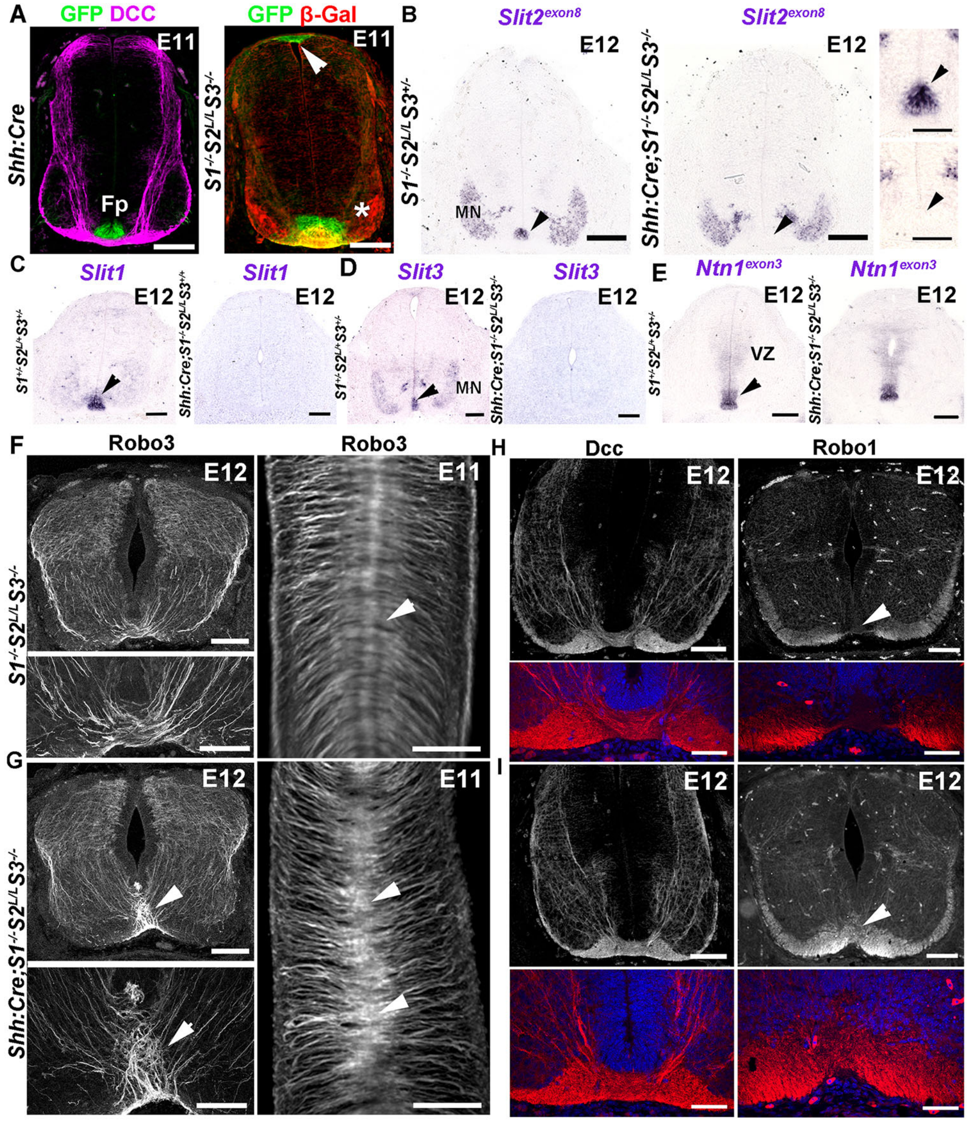

Fig. 4. Floor plate-derived Slits control midline crossing in the spinal cord. (A) Coronal sections of E11 *Shh:Cre* (left panel) and $S1^{-/-};S2^{L/L};S3^{-/-}$ (right panel) spinal cord immunolabeled for Dcc and GFP, or for β-galactosidase (β-gal) and GFP. In *Shh:Cre*, GFP is restricted to the floor plate (Fp) crossed by Dcc⁺ commissural axons. In $S1^{-/-};S2^{L/L};S3^{-/-}$, GFP and β-gal are found in the floor plate. GFP is also in the roof plate (arrowhead) where Slit1 is expressed (GFP was inserted in the *Slit1* locus in the *Slit1* knockout) and β-gal in motoneurons (asterisk). (B) Spinal cord sections of E12 $S1^{-/-};S2^{L/L};S3^{+/-}$ and *Shh:Cre;*$S1^{-/-};S2^{L/L};S3^{-/-}$ embryos hybridized with a *Slit2* exon 8 riboprobe. In $S1^{-/-};S2^{L/L};S3^{-/-}$, *Slit2* is expressed at the floor plate (arrowhead) and in *Shh:Cre;* $S1^{-/-};S2^{L/L};S3^{-/-}$, *Slit2* is detected in motoneurons (MNs) but not in floor plate (arrowhead). Higher magnification views of the floor plate (arrowheads) are shown on the right. (C-E) E12 spinal cord sections. In $S1^{+/-};S2^{L/+};S3^{+/-}$, *Slit1*, *Slit3* and *Ntn1* mRNAs are in the floor plate (arrowhead). *Slit3* is also in motoneurons and *Ntn1* in the ventricular zone (VZ). In *Shh:Cre;*$S1^{-/-};S2^{L/L};S3^{-/-}$, *Slit1* and *Slit3* mRNAs are undetectable, whereas *Ntn1* expression is not affected. (F,G) Robo3 immunolabeling on E12 spinal cord sections (left panels) or on whole-mount E11 spinal cord (right panels; 3DISCO and LSFM). Robo3⁺ axons accumulate at the floor plate (arrowheads in G) in *Shh:Cre;*$S1^{-/-};S2^{L/L};S3^{-/-}$ embryos. Two longitudinal domains (arrowhead in F) with weaker Robo3 expression are absent in the *Shh:Cre;*$S1^{-/-};S2^{L/L};S3^{-/-}$ mutant. (H,I) Confocal images of spinal cord sections from E12 embryos immunolabeled for Dcc (left panels) and Robo1 (right panels). The density of Dcc⁺ axons at the floor plate is increased in *Shh:Cre;*$S1^{-/-};S2^{L/L};S3^{-/-}$ mutant (I) compared with $S1^{-/-};S2^{L/L};S3^{-/-}$ (H). In $S1^{-/-};S2^{L/L};S3^{-/-}$ embryo, Robo1⁺ axons are absent from floor plate (arrowhead in H) unlike in *Shh:Cre;*$S1^{-/-};S2^{L/L};S3^{-/-}$ mutant (arrowhead in I). Scale bars: 100 µm in A-C,H,I; 50 µm in B (right panels); 150 µm in F,G.

$S1^{-/-};S2^{\Delta/\Delta};S3^{+/-}$ (*n*=6/6) and $S1^{-/-};S2^{\Delta/\Delta};S3^{-/-}$ mutants (*n*=5/5; Fig. 5K,L; Movie 6) with multiple chains of Barhl1⁺/Robo3⁺ neurons migrating ventrally, straight to the midline (Fig. S1Q). In addition, in both $S1^{-/-};S2^{\Delta/\Delta};S3^{-/-}$ mutants (*n*=5/5) and $S1^{-/-};S2^{-/-};S3^{-/-}$ E16 embryos (*n*=2/2) PN neurons invaded the floor plate, unlike in $S1^{-/-};S2^{-/-}$ embryos (Fig. 5J, *n*=3/3). As noted before (Geisen et al., 2008), migration defects were observed on both sides, but the right and left PN migratory streams were variably affected in all mutants (ectopic streams do not always appear at the same positions, their number and width also differ), suggesting that loss of Slits partially randomizes PN neuron migration.

To determine whether the floor plate is an important source of Slits for migrating PN, we next studied hindbrain commissures and PN migration in *Shh:Cre;*$S1^{-/-};S2^{L/L};S3^{-/-}$ mutants. GFP immunostaining confirmed that, as in the spinal cord, GFP is expressed by floor plate in the hindbrain of *Shh:Cre* embryos (Fig. 6A). *In situ* hybridization for *Slit2* exon 8 showed that *Slit2*

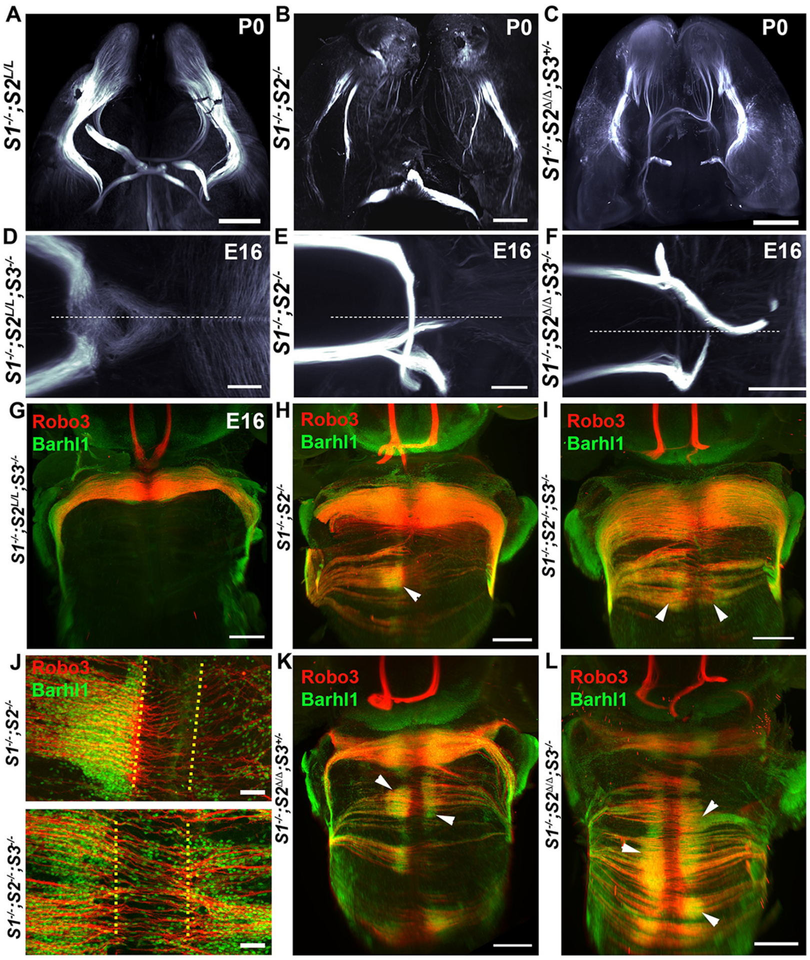

Fig. 5. Abnormal PN migration in *Slit1*/*Slit2*/*Slit3* triple knockouts. (A-F) LSFM images of 3DISCO-cleared E16 and P0 brains (ventral views) immunostained for Tag-1. In *S1*$^{-/-}$;*S2*$^{L/L}$ (A), the LOT is similar to controls, whereas in *S1*$^{-/-}$;*S2*$^{-/-}$ (B) and *S1*$^{-/-}$;*S2*$^{Δ/Δ}$;*S3*$^{+/-}$ (C) embryos, LOT axons are defasciculated and project medially. (D-F) LSFM images illustrating FR axon midline (broken line) crossing defects in *S1*$^{-/-}$;*S2*$^{-/-}$ and *S1*$^{-/-}$;*S2*$^{Δ/Δ}$;*S3*$^{-/-}$ mutant embryos. (G-L) LSFM images (G-I,K,L) and confocal images (J) of 3DISCO-cleared E16 embryo hindbrains immunolabeled for Barhl1 and Robo3. PN migration is normal in *S1*$^{-/-}$;*S2*$^{L/L}$;*S3*$^{-/-}$ (G). By contrast, in *S1*$^{-/-}$;*S2*$^{-/-}$ (H), *S1*$^{-/-}$;*S2*$^{-/-}$;*S3*$^{-/-}$ (I), *S1*$^{-/-}$;*S2*$^{Δ/Δ}$;*S3*$^{+/-}$ (K) and *S1*$^{-/-}$;*S2*$^{Δ/Δ}$;*S3*$^{-/-}$ (L) mutants, multiple chains of PN neurons migrate directly from the rhombic lip to the floor plate, forming ectopic clusters (arrowheads). (J) Most ectopic PN neurons stop at the floor plate (between dotted lines) in *S1*$^{-/-}$;*S2*$^{-/-}$ knockout (upper panel), whereas they enter it in great numbers in the *S1*$^{-/-}$;*S2*$^{-/-}$;*S3*$^{-/-}$ knockout (lower panel). Scale bars: 500 µm in A-C,G-I,K,L; 200 µm in D-F; 50 µm in J.

mRNA was deleted from the floor plate in *Shh:Cre;S1*$^{-/-}$;*S2*$^{L/L}$;*S3*$^{-/-}$ (*n*=3) and *Shh:Cre;S1*$^{-/-}$;*S2*$^{L/L}$ (*n*=3) embryos, but that *Slit2* expression in the facial nuclei was as that in *S1*$^{-/-}$;*S2*$^{L/L}$;*S3*$^{+/-}$ embryos (*n*=3). To study hindbrain commissures, we first, performed immunostaining for Dcc, which showed that the thickness of hindbrain commissures was significantly increased at the floor plate in *Shh:Cre;S1*$^{-/-}$;*S2*$^{L/L}$;*S3*$^{-/-}$ (*n*=3; Fig. 6C and Fig. S1R). As in the spinal cord, commissural axon midline crossing defects were best seen after Robo1 immunostaining. LSFM on 3DISCO-cleared E12 hindbrains showed that Robo1 labels longitudinal axons along the floor plate in *S1*$^{-/-}$;*S2*$^{L/L}$;*S3*$^{-/-}$ embryos (*n*=3/3), whereas aberrant crossing of Robo1^{+} axons was observed in *Shh:Cre;S1*$^{-/-}$;*S2*$^{L/L}$;*S3*$^{-/-}$ mutants (*n*=3/3; Fig. 6D). Confocal imaging of hindbrain sections also showed this abnormal accumulation of Robo1^{+} axons at the floor plate of *Shh:Cre;S1*$^{-/-}$;*S2*$^{L/L}$;*S3*$^{-/-}$ embryos (*n*=3/3; Fig. 6D). Strikingly, we could not detect any PN migration defects in *Shh:Cre;S1*$^{-/-}$;*S2*$^{L/L}$;*S3*$^{+/-}$ (*n*=4/4) or *Shh:Cre;S1*$^{-/-}$;*S2*$^{L/L}$;*S3*$^{-/-}$ (*n*=4/4) mutants, and the shape and size of the PN migratory stream were comparable with *Shh:Cre;S1*$^{-/-}$;*S2*$^{L/L}$;*S3*$^{-/-}$ controls: *Shh:cre,S1*$^{+/-}$;*S2*$^{L/+}$ (*n*=3), *S1*$^{+/-}$;*S2*$^{L/+}$ (*n*=3/3), *S1*$^{-/-}$;*S3*$^{-/-}$ (*n*=3/3), *S1*$^{-/-}$;*S3*$^{+/-}$ (*n*=4/4), *S1*$^{-/-}$ (*n*=3/3) and *S1*$^{-/-}$;*S2*$^{L/L}$;*S3*$^{-/-}$ (*n*=3/3) (Fig. 6E and Fig. S1S; Movie 7). The abnormal midline crossing of FR axons in these embryos (Fig. 6F) confirmed that *Slit2* was efficiently inactivated in *Shh:Cre;S1*$^{-/-}$;*S2*$^{L/L}$;*S3*$^{-/-}$ mice. Together, the lack of PN migration defects reveals that floor-plate-derived Slits do not play an important role in PN neuron migration.

Neurons in the facial nucleus express Slit2 and Slit3 (Geisen et al., 2008), and it was proposed that this is a main source of chemorepellents for PN neurons, during their longitudinal migration along the hindbrain. To test this hypothesis, we generated *Phox2b:Cre;S1*$^{-/-}$;*S2*$^{/L/L}$ and *Phox2b:Cre;S1*$^{-/-}$;*S2*$^{L/L}$;*S3*$^{-/-}$ mice. Phox2b is a transcription factor that controls the specification of the facial motor nucleus (Pattyn et al., 2000). The expression of Cre recombinase in embryonic facial neurons was first validated by crossing *Phox2b:Cre* and *Tau*^{GFP} mice in which a membrane-tethered GFP is expressed in axons in the presence of Cre (Hippenmeyer et al., 2005) (Fig. 7A). *In situ* hybridization with a *Slit2* exon 8 probe on E12 *Phox2b:Cre;S1*$^{-/-}$;*S2*$^{L/L}$ (*n*=3/3) and *Phox2b:Cre;S1*$^{-/-}$;*S2*$^{L/L}$;*S3*$^{-/-}$ mutants (*n*=3/3) showed that, unlike in *S1*$^{-/-}$;*S2*$^{L/L}$ embryos (*n*=3/3), *Slit2* expression was prevented in the facial nucleus but maintained in the floor plate (Fig. 7B). The lack of Slit2 did not perturb the development of the facial nucleus, which had a similar position and morphology to that in *Phox2b:Cre;S1*$^{-/-}$;*S2*$^{L/L}$;*S3*$^{-/-}$ mutants (*n*=3/3) and *S1*$^{-/-}$;*S2*$^{L/L}$ embryos (*n*=3/3). Next, we analyzed the migration of PN neurons using Barhl1/Robo3 double immunolabeling and LSFM. We could not detect any PN migration defects in *S1*$^{-/-}$;*S2*$^{L/L}$;*S3*$^{-/-}$ (*n*=3/3), *Phox2b:Cre;S1*$^{-/-}$;*S2*$^{L/L}$ (*n*=3/3) and *Phox2b:Cre;S1*$^{-/-}$;*S2*$^{L/L}$;*S3*$^{-/-}$ (*n*=3/3) embryos (Fig. 7D-F and Fig. S2A; Movie 8). These results show that PN neurons migrate normally in the absence of Slit2 and Slit3 in the facial nucleus.

To determine whether the simultaneous inactivation of Slit1-Slit3 at the level of the floor plate and facial nucleus perturbs PN neuron

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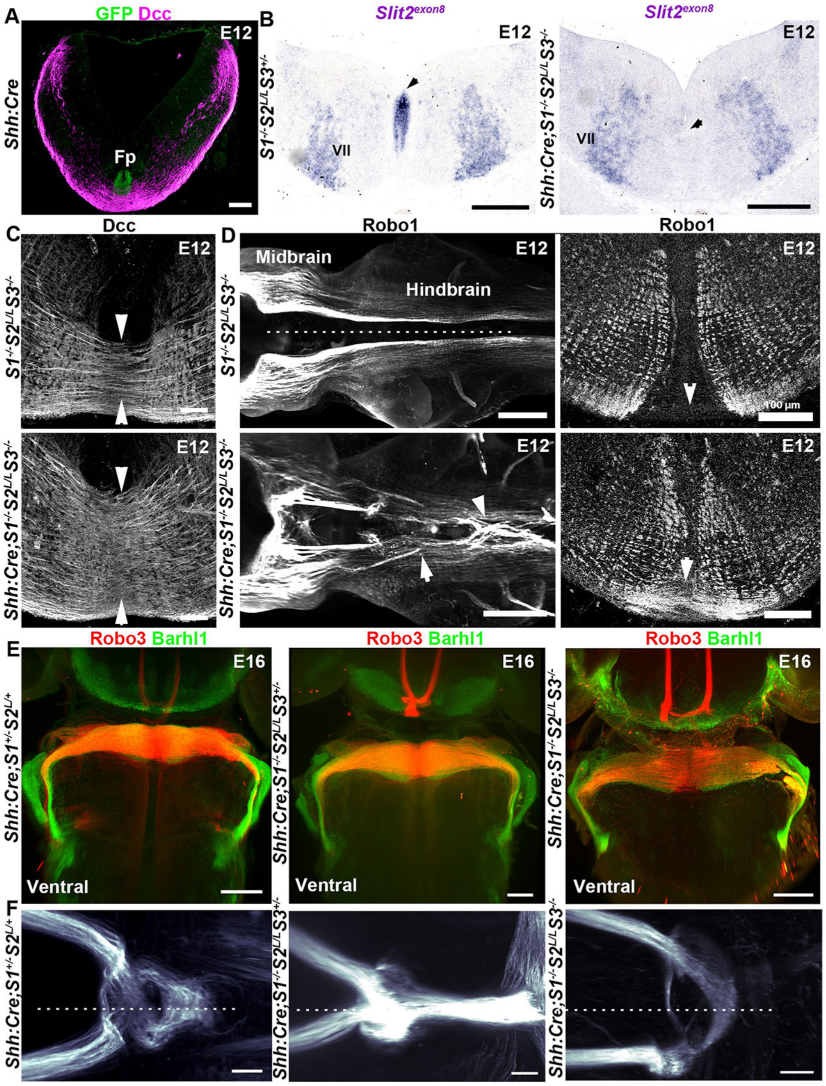

Fig. 6. Normal PN migration in *Shh:Cre;S1*$^{-/-}$*;S2*$^{L/L}$*;S3*$^{-/-}$ mutants. (A) Coronal sections of an E12 *Shh:Cre* embryo immunolabeled for Dcc and GFP. GFP^{+} floor plate (Fp) is crossed by Dcc^{+} commissural axons. (B) *In situ* hybridization with a *Slit2* exon 8 probe on E12 coronal sections at the level of the facial nucleus (VII) of *S1*$^{-/-}$*;S2*$^{L/L}$*;S3*$^{+/-}$ (left) and *Shh:Cre;S1*$^{-/-}$*;S2*$^{L/L}$*;S3*$^{-/-}$ (right) embryos. Slit2 is absent from Fp in the *Shh:Cre;S1*$^{-/-}$*;S2*$^{L/L}$*;S3*$^{-/-}$ embryo (arrowheads), but still present in facial nuclei. (C) Confocal images of E12 hindbrain commissures labeled using anti-Dcc antibody. The commissure (between arrowheads) is enlarged in *Shh:Cre;S1*$^{-/-}$*;S2*$^{L/L}$*;S3*$^{-/-}$ (bottom panel) compared with *S1*$^{-/-}$*;S2*$^{L/L}$*;S3*$^{-/-}$. (D) LSFM (left panels) and confocal (right panels) images of *S1*$^{-/-}$*;S2*$^{L/L}$*;S3*$^{-/-}$ (upper panels) and *Shh:Cre;S1*$^{-/-}$*;S2*$^{L/L}$*;S3*$^{-/-}$ (lower panels) embryos immunolabeled for Robo1. In *Shh:Cre;S1*$^{-/-}$*;S2*$^{L/L}$*;S3*$^{-/-}$ mutants, longitudinal Robo1^{+} axons extending from the midbrain to the hindbrain abnormally enter the midline (arrowheads). (E,F) LSFM images (ventral views) of E16 hindbrain after Barhl1/Robo3 immunostaining. PN migration is similar in *Shh:Cre;S1*$^{+/-}$*;S2*$^{L/+}$*;S3*$^{+/-}$ controls and in *Shh:Cre;S1*$^{-/-}$*;S2*$^{L/L}$*;S3*$^{+/-}$ or *Shh:Cre;S1*$^{-/-}$*;S2*$^{L/L}$*;S3*$^{-/-}$ mutants (E). By contrast, severe FR axon midline crossing defects exist in *Shh:Cre;S1*$^{-/-}$*;S2*$^{L/L}$*;S3*$^{+/-}$ and *Shh:Cre;S1*$^{-/-}$*;S2*$^{L/L}$*;S3*$^{-/-}$ mutants (F). Scale bars: 100 µm in A,C,D (right panels); 250 µm in B; 400 µm in D (left panels); 500 µm in E; 200 µm in F.

migration, we generated E16 *Shh:Cre;Phox2b:Cre;S1*$^{-/-}$*;S2*$^{L/L}$*;S3*$^{-/-}$. Interestingly, a few streams of ectopic and prematurely migrating PN neurons were found in *Shh:Cre;Phox2b:Cre;S1*$^{-/-}$*;S2*$^{L/L}$*;S3*$^{+/-}$ embryos (*n*=3/3; Fig. 7G) and in *Shh:Cre;Phox2b:Cre;S1*$^{-/-}$*;S2*$^{L/L}$*;S3*$^{-/-}$ embryos (*n*=2/3; Fig. 7H; Movie 9). Confocal imaging of the ectopic PN clusters showed axons crossing the midline but also extending within the floor plate (Fig. 7I,J). Although minor PN migratory defects exist in mice simultaneously depleted of Slits in the floor plate and facial nucleus, the phenotype is much milder than after ubiquitous inactivation of all Slits (Fig. 7K).

Revisiting the role of Slit/Robo signaling in IO development

The IO nucleus, does not develop properly in *Robo1/Robo2* and *Slit1/Slit2* double knockouts (Di Meglio et al., 2008; Geisen et al., 2008). To study the 3D organization of the IO nucleus in Slit and Robo conditional knockouts, we performed whole-mount immunostaining for Foxp2 (Fujita and Sugihara, 2012) followed by 3DISCO clearing and LSFM (Figs 8 and 9). The IO is adjacent to the floor plate and comprises several subnuclei organized in a lamellated pattern (Azizi and Woodward, 1987) (Fig. 8A,B). At P0, the 3D structure of the IO was comparable with wild type in *Robo1*$^{+/-}$*;Robo2*$^{+/-}$ mice (*n*=3/3; Fig. 8C), whereas in *Robo1*$^{-/-}$*;Robo2*$^{-/-}$ (*n*=3/3; Fig. 8D) and *Robo1*$^{-/-}$*;Robo2*$^{\Delta/\Delta}$ mice (*n*=3/3; Fig. 8E), the IO was elongated and more compact. The circumferential migration of IO neurons starts around E11 and finishes by E14-E15 (Di Meglio et al., 2008). Optical sectioning with Imaris (see Materials and methods) was used to visualize migrating IO neurons at E13 (Fig. 8F; Movies 10-13). In wild type, Foxp2^{+} IO neurons stopped at the midline, whereas in *Robo1*$^{-/-}$*;Robo2*$^{-/-}$ double knockouts and *Robo1*$^{-/-}$*;Robo2*$^{\Delta/\Delta}$ mice, a significant fraction of IO neurons migrated into the floor plate (*n*=3/3; Fig. S2B). Unilateral DiI injection into the cerebellum resulted in bilateral retrograde labeling of IO neurons in both *Robo1*$^{-/-}$*;Robo2*$^{-/-}$ and *Robo1*$^{-/-}$*;Robo2*$^{\Delta/\Delta}$ mutants, whereas in wild type, labeled neurons are only contralateral (*n*=3/3 for each genotype; Figs 8G and 9I). To assess the cell-autonomous function of Robo1/2 receptors in IO neuron migration, we crossed the *Robo1*$^{-/-}$*;Robo2*$^{L/L}$ mice to the *Ptf1a:Cre* line. Ptf1a is a transcription factor expressed by IO progenitors and the *Ptf1a:Cre* line drives Cre expression in IO neurons (Badura et al., 2013; Renier et al., 2010). *Ptf1a:Cre;Robo1*$^{-/-}$*;Robo2*$^{L/L}$ mice were fully viable and did not exhibit any obvious motor deficits, unlike other IO mutants (Badura et al., 2013; Renier et al., 2010). At E12, the downregulation of Robo2 expression in a large fraction of hindbrain neurons was confirmed by immunostaining and *in situ* hybridization

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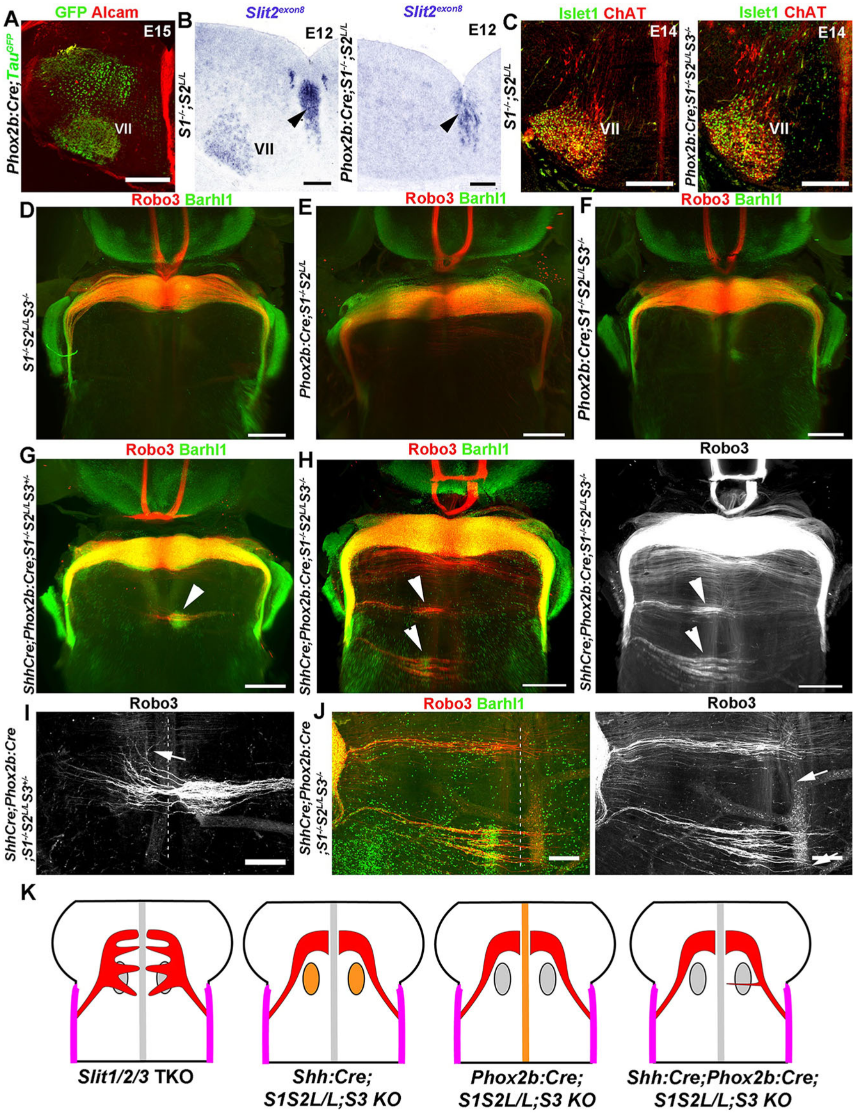

Fig. 7. PN migration in absence of Slit expression in the facial nucleus. (A) Coronal section at the level of the facial nucleus (VII) in a *Phox2b:Cre;*Tau^{GFP} E15 embryo immunolabeled for GFP and Alcam, a motoneuron and floor-plate marker (Weiner et al., 2004). GFP is highly expressed in facial nuclei (VII). (B) E12 coronal sections of $S1^{-/-};S2^{L/L}$ (left) and *Phox2b:Cre;*$S1^{-/-};S2^{L/L};S3^{-/-}$ (right) embryos hybridized with a *Slit2* exon 8 probe. *Slit2* is in the floor plate (arrowheads), but absent from the VII nucleus in *Phox2b:Cre;*$S1^{-/-};S2^{L/L};S3^{-/-}$ mutants. (C) Coronal sections of E12 $S1^{-/-};S2^{L/L}$ (left) and *Phox2b:Cre;*$S1^{-/-};S2^{L/L};S3^{-/-}$ (right) embryos immunolabeled for the two motoneuron markers ChAT and islet 1. The VII nucleus labeling is similar. (D-H) LSFM images of E16 hindbrains labeled with Barhl1 and Robo3. The PN migration pathway is as in controls in $S1^{-/-};S2^{L/L};S3^{-/-}$ (D), *Phox2b:Cre;*$S1^{-/-};S2^{L/L}$ (E) and *Phox2b:Cre;*$S1^{-/-};S2^{L/L};S3^{-/-}$ (F) mutants. A few small streams of PN neurons (arrowheads) migrate prematurely to the midline in *Shh:Cre;Phox2b:Cre;*$S1^{-/-};S2^{L/L};S3^{+/-}$ (G) and *Shh:Cre;Phox2b:Cre;*$S1^{-/-};S2^{L/L};S3^{+/-}$ (H) mutants. (I,J) Confocal images of ectopic PN clusters. Some $Robo3^{+}$ PN axons extend along the floor plate (arrows). (K) Schematic representations of PN neuron migration (red) in *Slit* mutants. Scale bars: 250 µm in A; 100 µm in B; 150 µm in C,I,J; 500 µm in D-H.

(Fig. S2C,D). At P0, the shape of the IO in *Pft1a:Cre;*$Robo1^{-/-};Robo2^{L/L}$ mice was similar to controls (*n*=4/4; Fig. 8H) and IO neurons projected to the contralateral cerebellum (*n*=5/5; Fig. 8H). In P15 and adult *Pft1a:Cre;*$Robo1^{-/-};Robo2^{L/L}$ mice, the lamellation of the IO nucleus was also normal (*n*=3/3; Fig. 8I and not shown). These data suggest that the migration of IO neurons does not require Robo1/Robo2 receptors.

We next analyzed the role of floor-plate-derived Slits. At P0, LSFM confirmed that the IO shape was abnormal in $S1^{-/-};S2^{-/-}$ double knockouts (*n*=3/3; Fig. 9A; Di Meglio et al., 2008) with a lateromedial compaction similar to *Robo1*/*Robo2* knockouts. This was also the case in $S1^{-/-};S2^{\Delta/\Delta};S3^{+/-}$ mice (*n*=3/3) but not in $S1^{-/-};S2^{L/L};S3^{+/-}$ (*n*=5/5), $S1^{-/-};S2^{L/L}$ (*n*=3/3), $S1^{-/-};S3^{-/-}$ (*n*=3/3) or $S1^{-/-}$ (*n*=3/3) mice (Fig. 9B,C). In *Shh:Cre;*$S1^{-/-};S2^{L/L};S3^{-/-}$ (*n*=3/3) and *Shh:Cre;*$S1^{-/-};S2^{L/L}$ mice (*n*=3/3), the overall IO shape was similar to controls, but gaps devoid of Foxp2 IO neurons could be seen (Fig. 9D). DiI-labeled IO neurons were contralateral in $S1^{-/-};S2^{L/L}$ (*n*=4/4) and $S1^{-/-};S2^{L/L};S3^{-/-}$ (*n*=3/3) newborn mice (Fig. 9E,I). By contrast, DiI-labeled neurons were also observed ipsilaterally in *Shh:Cre;*$S1^{-/-};S2^{L/L};S3^{+/-}$ embryos (*n*=4/4) and to a larger extent in *Shh:Cre;*$S1^{-/-};S2^{L/L};S3^{-/-}$ mutants (*n*=3/3), suggesting that some neurons might have crossed the floor plate. Accordingly, Foxp2+IO neurons were observed in the floor plate of $S1^{-/-};S2^{-/-}$ (*n*=3/3), $S1^{-/-};S2^{\Delta/\Delta};S3^{+/-}$ (*n*=3/3) and $S1^{-/-};S2^{\Delta/\Delta};S3^{-/-}$ (*n*=4/4) E13 embryos, as well as in E14 *Shh:Cre;*$S1^{-/-};S2^{L/L};S3^{+/-}$ (*n*=3/3) and *Shh:Cre;*$S1^{-/-};S2^{L/L};S3^{-/-}$ (*n*=3/3) embryos but not in $S1^{-/-};S2^{L/L}$ embryos (*n*=3/3) and $S1^{-/-};S2^{L/L};S3^{-/-}$ (*n*=4/4) (Fig. 9F,G). The abnormal morphology of the IO complex in *Shh:Cre;*$S1^{-/-};S2^{L/L}$ was still observed in P25 and adult animals (*n*=4/4; Fig. 9H and not shown), indicating that lamellation defects were not corrected after birth.

DISCUSSION

Robo receptors were discovered because of their role in commissural axon guidance in the *Drosophila* nerve cord (Kidd et al., 1998; Seeger et al., 1993). *Drosophila* Robo receptors (Robos) bind Slit and trigger a repulsive signal (Kidd et al., 1999). This function of Slits and Robos in the regulation of midline crossing seems to be conserved across evolution from worm to humans (Brose et al., 1999; Fricke et al., 2001; Jen et al., 2004; Zallen et al., 1999). In rodents, genetic evidence supporting a repulsive activity of Slit/Robo signaling for commissural axons has been obtained in various neuronal systems through the phenotypic analysis of Slit and Robo knockout mice (Bagri et al., 2002; Fouquet

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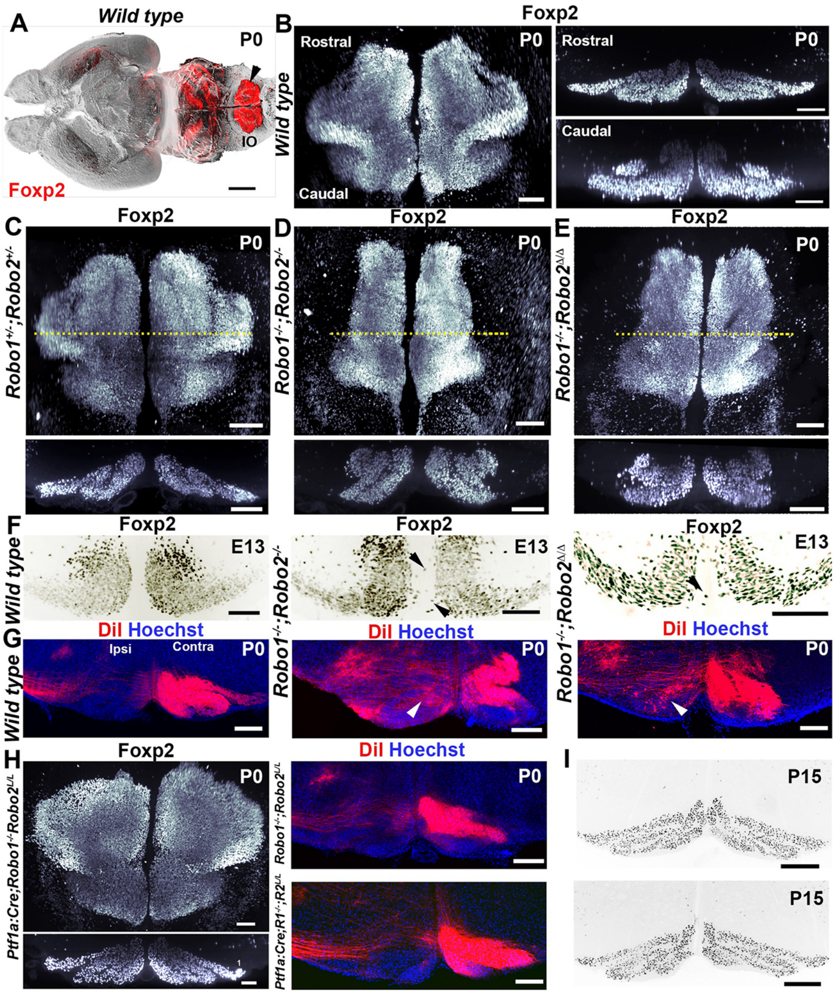

Fig. 8. Robo1/2 receptors do not control cell-autonomous midline crossing of IO neurons. (A) LSFM image (ventral view) of a wild-type P0 brain immunolabeled for Foxp2. The inferior olive (IO, arrowhead) strongly expresses Foxp2. (B) High magnification of the IO (left) and optical sections (right) illustrating IO lamellation. (C-E) LSFM images the IO in *Robo1*$^{+/-}$*;Robo2*$^{+/-}$ (C), *Robo1*$^{-/-}$*;Robo2*$^{-/-}$ (D) and *Robo1*$^{-/-}$*;Robo2*$^{\Delta/\Delta}$ (E) mice. IO morphology is abnormal and more compact in mice deficient for Robo1 and Robo2. (F) Optical coronal sections through the IO of wild-type, *Robo1*$^{-/-}$*;Robo2*$^{-/-}$ or *Robo1*$^{-/-}$*;Robo2*$^{\Delta/\Delta}$ E13 embryos labeled with Foxp2. In *Robo1*$^{-/-}$*;Robo2*$^{-/-}$ and *Robo1*$^{-/-}$*;Robo2*$^{\Delta/\Delta}$ mutants, some Foxp2 IO neurons enter the floor plate (arrowheads). (G) Coronal IO sections of wild type, *Robo1*$^{-/-}$*;Robo2*$^{-/-}$ or *Robo1*$^{-/-}$*;Robo2*$^{\Delta/\Delta}$ mutants unilaterally injected with DiI into the cerebellum. In *Robo1*$^{-/-}$*;Robo2*$^{-/-}$ and *Robo1*$^{-/-}$*;Robo2*$^{\Delta/\Delta}$ mutants, retrogradely traced IO neurons are found on both the contralateral (contra) and ipsilateral sides (ipsi, arrowhead). (H) IO development in *Ptf1a:Cre;Robo1*$^{-/-}$*;Robo2*$^{L/L}$ mutants. 3D LSFM view (left) of the IO labeled for FoxP2. Note the absence of compaction of the nucleus. The right panels are IO coronal sections of a DiI-injected P0 mouse. DiI-labeled neurons are contralateral to the site of injection. (I) P25 IO coronal sections labeled for Foxp2. The morphology and lamellation of the IO nucleus are similar in *Robo1*$^{-/-}$*;Robo2*$^{L/L}$ and in *Ptf1a:Cre;Robo1*$^{-/-}$*;Robo2*$^{L/L}$ mice. Scale bars: 100 µm.

et al., 2007; Jaworski et al., 2010; Long et al., 2004; López-Bendito et al., 2007; Shu et al., 2003). Spinal cord commissural axons accumulate at the floor plate in *Robo1/Robo2* double knockouts and *Slit1/Slit2/Slit3* triple knockouts (Jaworski et al., 2010; Long et al., 2004), and this is also the case for fasciculus retroflexus axons (Belle et al., 2014). In the cortex, callosal axons fail to cross the midline in Slit and Robo knockouts (Conway et al., 2011; López-Bendito et al., 2007; Shu et al., 2003; Unni et al., 2012).

Apart from one exception (Belle et al., 2014), all these *in vivo* data were obtained in classic Slit and Robo knockouts in which the genes were inactivated in all cells. Slit and Robo knockouts display developmental defects outside the CNS, such as in the kidney (Grieshammer et al., 2004), heart (Mommersteeg et al., 2013, 2015) and vasculature (Rama et al., 2015). Therefore, some of the CNS defects could be related to abnormal function or development of non-neuronal cells that could secondarily alter axon outgrowth and cell migration. Previous work on retinoblastoma (RB) showed that the CNS development was severely perturbed in RB-knockout embryos, including massive apoptosis and precerebellar neuron migration defects (Jacks et al., 1992; Lee et al., 1992). Interestingly, later experiments using chimera and conditional knockouts showed that these anomalies were caused by abnormal placenta development leading to hypoxia (MacPherson et al., 2003; Maandag et al., 1994). This underlines the importance of performing cell-specific inactivation of broadly expressed genes.

Although PN and IO neuron migration is severely perturbed in classic *Robo1/Robo2* knockouts, the selective silencing of Robo1 and Robo2 expression in PN or IO neurons does not lead to significant migration anomalies. PN neurons do not form ectopic chains and IO neurons stop at the midline. This suggests that the defects previously seen in the classic Slit and Robo knockouts are non-cell autonomous. Hoxa2 binds Robo2 genomic sequences and PN migration defects are very similar in *Hoxa2* and *Robo1/Robo2* knockouts (Geisen et al., 2008). However, only a few ectopic PN neurons migrate prematurely in *Wnt1:Cre;Hoxa2*$^{lox/lox}$ mutants (Geisen et al., 2008). These results, together with previous studies in *Drosophila* (Evans et al., 2015; Ordan and Volk, 2015) and mouse (Kaneko et al., 2010) support the existence of non-cell-autonomous function of Slits and Robos in developing neurons. Slits and Robos are expressed in glial cells and neuronal progenitors (Borrell et al., 2012) (and data not shown). In the rostral migratory stream, Slits help sculpting migratory tunnels for neuroblasts (Kaneko et al., 2010) and this could also be the case in the embryonic hindbrain. Interestingly, Ntn1 released at the pial surface by neuronal progenitors rather than floor plate was recently shown to control precerebellar neuron migration (Dominici et al., 2017). These results question the role of floor plate in precerebellar neuron development.

In *Drosophila*, Robo receptors are not expressed at the surface of commissural axons until they have crossed the midline (Kidd et al., 1998). In mouse embryos, our results and previous ones also show

Development (2018) 145, dev150375. doi:10.1242/dev.150375

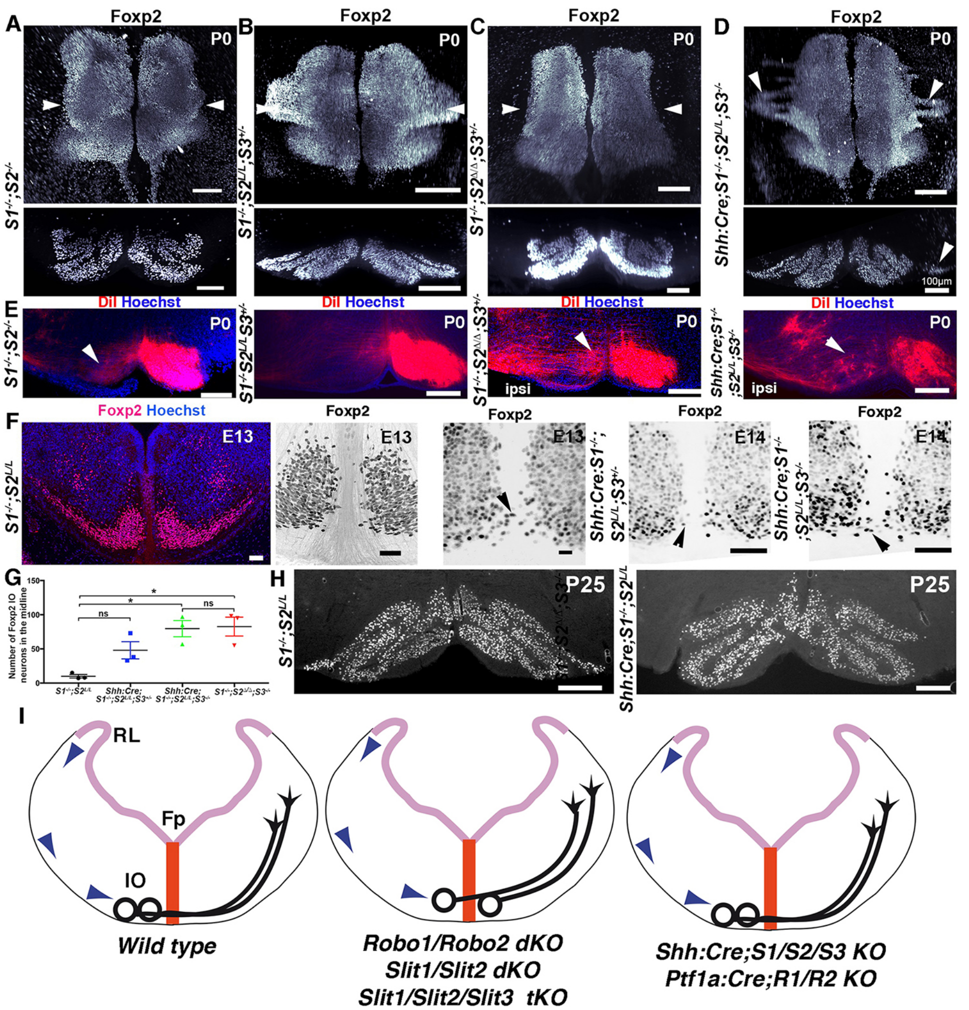

Fig. 9. Slits control the development of the IO nucleus. (A-D) LSFM images of the P0 IO (top panels) and optical sections (lower panels) immunolabeled for Foxp2 and cleared with 3DISCO. In *S1*$^{-/-}$*;S2*$^{-/-}$ (A) and, *S1*$^{-/-}$*;S2*$^{\Delta/\Delta}$*;S3*$^{+/-}$ (C) mutants, the IO is abnormal and more compact than wild type (see Fig. 8). IO morphology is normal in *S1*$^{-/-}$*;S2*$^{L/L}$*;S3*$^{+/-}$ (B) mice. (D) In the *Shh:Cre;S1*$^{-/-}$*;S2*$^{L/L}$*;S3*$^{-/-}$ mutant, the morphology of the IO is also perturbed, and neuron-free gaps are seen laterally (arrowheads). (E) Coronal IO section from P0 Slit mutants unilaterally injected with DiI in the cerebellum. In *S1*$^{-/-}$*;S2*$^{L/L}$*;S3*$^{+/-}$mice, all retrogradely labeled neurons are on the contralateral side, as in wild type. DiI-labeled IO neurons are found in the ipsilateral IO of *S1*$^{-/-}$*;S2*$^{-/-}$, *S1*$^{-/-}$*;S2*$^{\Delta/\Delta}$*;S3*$^{+/-}$ and *Shh:Cre; S1*$^{-/-}$*;S2*$^{L/L}$*;S3*$^{-/-}$ mutants (arrowheads). (F) Coronal IO sections of E13 and E14 embryos labeled with Foxp2. In *S1*$^{-/-}$*;S2*$^{L/L}$ embryo, IO neurons stop at the midline, whereas in all the other mutants, Foxp2$^+$ IO neurons enter the floor plate (arrowheads). (G) Quantification of the number of Foxp2$^+$ IO neurons invading the midline. *P<0.05. ns, not significant (*Shh:Cre;S1*$^{-/-}$*;S2*$^{L/L}$*;S3*$^{+/-}$, P=0.0891; Welch's *t*-test). Error bars indicate s.e.m. (H) Coronal IO sections of P25 mice immunolabeled for Foxp2. IO morphology is perturbed in *Shh:Cre;S1*$^{-/-}$*;S2*$^{L/L}$ mutant compared with *S1*$^{-/-}$*;S2*$^{L/L}$ mice. The IO is more compact and lamellation is abnormal. (I) Schematic representation of IO defects in Robo and Slit mutants, supporting a non-cell-autonomous action. Scale bars: 200 µm in A, B,E; 100 µm in C,D; 50 µm in F,H.

that Robo1/Robo2 proteins are only detectable in post-crossing axons (Jaworski et al., 2010; Tamada et al., 2008). Therefore, the absence of PN migration defect could be expected if PN neurons do not express Robo1/Robo2 protein during their migration to the midline. Likewise, there is no evidence supporting an expression of Robo1/Robo2 proteins by IO neurons (Di Meglio et al., 2008), and we show here that IO neurons develop normally in *Ptf1a:Cre; Robo1*$^{-/-}$*;Robo2*$^{lox/lox}$ mice. Therefore, the previously described PN and IO migration defects are most likely non-cell-autonomous. Although the selective deletion of Slits in the floor plate, does not seem to have any major effect on precerebellar neurons, it induces significant midline crossing defects at the level of the spinal cord, as reported in *Slit1/Slit2/Slit3* triple knockouts (Long et al., 2004). These results suggest that the sensitivity of commissural neurons to floor-plate-derived guidance cues differs between the hindbrain and the spinal cord.

Interestingly, PN and IO defects in *Slit* conditional knockouts do not phenocopy those found in Robo knockouts. This suggests that Slits might act on precerebellar neurons through other receptors. Slits are cleaved by an unknown protease, into a long N-terminal fragment (Slit-N) and a short C-terminal fragment (Slit-C). Recent studies have show that Slit2-C binds to plexin A1 (Delloye-Bourgeois et al., 2014) and dystroglycan (Wright et al., 2012). In the spinal cord, the floor plate secretes semaphorins and cell-adhesion molecules (CAMs), which are also involved in the control of midline crossing. For example, the gain in responsiveness of commissural axons to semaphorin 3B repulsion after midline crossing (Nawabi et al., 2010) is stimulated by a soluble form of the neural cell-adhesion molecule (NrCAM). Precerebellar neurons express some components of the receptor complex for semaphorins (Backer et al., 2002; Chen et al., 1997; Gesemann et al., 2001; Vilz et al., 2005) but their function in precerebellar neuron migration is largely unknown and should be studied. For example, NrCAM is expressed by migrating IO neurons (not by PN neurons) but the IO is normal in *Nrcam* knockouts (Backer et al., 2002). Although *in vitro* assays have also confirmed that the floor plate acts as a stop signal for migrating precerebellar neurons (de Diego et al., 2002), our results suggest that repellents other than Slits are involved. It will now be important to extend this strategy to other systems, and re-assess Robo1/Roco2 function in commissural axon guidance in the optic nerve, spinal cord and cortex.

MATERIALS AND METHODS

Mouse strains and genotyping

Slit1/Slit2 (Plump et al., 2002), *Slit3* (Yuan et al., 2003), *Robo1* (Long et al., 2004), *Robo2* (Grieshammer et al., 2004), *Robo3lox* (Renier et al., 2010), *Robo2lox* (Gibson et al., 2014) and *Slit2lox* (Rama et al., 2015) knockouts, and *Shh:Cre* (Harfe et al., 2004), *Wnt1:Cre* (Danielian et al., 1998), *Krox20: Cre* (Voiculescu et al., 2000), *Ptf1a:Cre* (Kawaguchi et al., 2002), *Phox2b: Cre* (Pattyn et al., 2000) and *Tau*^{GFP} (Hippenmeyer et al., 2005) lines were previously described and genotyped by PCR. Wild-type mice were from the C57BL6 background (Janvier, France). Compound mutants were obtained by intercrosses. The day of the vaginal plug was counted as E0.5 and the day of the birth as postnatal day 0 (P0). From E16 to P0, the nervous system was dissected and fixed at 4°C overnight by immersion in 4% paraformaldehyde (PFA; Merck) in 0.1 M phosphate buffer (pH 7.4). P15-P25 mice were anesthetized with ketamine (100 mg/ml) and xylazine (10 mg/ml), and perfused using 4% PFA. All animal procedures were carried out in accordance to institutional guidelines (UPMC Comité Charles Darwin). Mice of either sex were used and no animals were excluded. All data quantification was carried out by an observer blinded to the experimental conditions. We did not perform randomization into groups.

Immunohistochemistry on brain sections

Embryos and adult brains were cryoprotected in 10% sucrose (in 0.1 M phosphate buffer) for cryostat sectioning. Cryostat sections (20 µm) were blocked in PBS containing 0.2% gelatin and 0.25% Triton X-100, and incubated overnight at room temperature with primary antibodies against goat anti-Robo3 (1:300, R&D System, AF3076), rabbit anti-Barhl1 (1:500, Sigma, HPA004809), goat anti-ChAT (1:100, Millipore, AB144P), goat anti-Dcc (1:500, Santa Cruz, sc-6535), rabbit anti-Foxp2 (1:1000, Abcam, ab16046), chicken anti-GFP (1:800, Abcam, ab13970), goat anti-Robo1 (1:500, R&D System, AF1749), rabbit anti-Robo2 (against peptide QNQSQRPRPTKKHKGGRMDP; 1:800, Biotem), goat anti-Tag-1 (1:1000, R&D Systems, AF4439) and rabbit anti-islet1 (1:500, Abcam, ab20670). The following secondary antibodies were used: bovine anti-goat and donkey anti-rabbit, coupled to CY3 or CY5 (1:600, Jackson Laboratories; 805-165-180, 805-605-180, 711-165-152 and 711-175-152), donkey anti-chicken coupled to Alexa Fluor 488 (1:600, Invitrogen, A-11039) and donkey anti-rabbit coupled to Alexa Fluor 647 (1:600, Jackson Laboratories, 711-605-152). Sections counterstained with Hoechst 33258 (10 mg/ml, Sigma, B2883) were imaged with a fluorescent microscope (DM6000, Leica) coupled to a CoolSnapHQ camera (Roper Scientific), a Nanozoomer (Hamamatsu) or an upright confocal microscope (Olympus FV1000).

In utero electroporation

In utero electroporation of pCX-EGFP (1 µg/µl) plasmid (provided by Dr M. Okabe, Osaka University, Japan) PN neurons was performed as described previously (Zelina et al., 2014).

DiI tracing

The 4% PFA-fixed P0 animals were injected into the cerebellum with small crystals of 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine (DiI; Invitrogen) using glass micropipettes. Injected brains were kept at 37°C for 3 weeks. Brains were cut at 100 µm with a vibratome (Leica), counterstained with Hoechst and imaged by an upright confocal microscope (Olympus FV1000).

In situ hybridization

Antisense riboprobes were labeled with DIG (digoxigenin-11-UTP, Roche) as described previously (Marillat et al., 2002) by *in vitro* transcription of mouse cDNA encoding *Slit1* (Nguyen-Ba-Charvet et al., 2004), *Slit2* exon 8 (Rama et al., 2015), *Slit3* (Rama et al., 2015), *Ntn1* (Serafini et al., 1996) and *Robo2* exon 5 (Gibson et al., 2014). *In situ* hybridization was performed as described previously (Marillat et al., 2002) and tissue sections were imaged using a Nanozoomer slide scanner (Hamamatsu).

Whole-mount immunostaining and tissue clearing

The procedure was similar for single and multiple labeling and has been described previously (Belle et al., 2014). Clearing was performed according to the 3DISCO procedure (Ertürk et al., 2011), with slight modifications (Belle et al., 2014).

3D imaging and image processing

3D imaging was primarily performed with an ultramicroscope (LaVision BioTec) using ImspectorPro software (LaVision BioTec) or with an upright confocal microscope (Olympus FV1000). 3D volume images were generated using Imaris ×64 software (version 7.6.1, Bitplane). Stack images were first converted to imaris file (.ims) using Imaris FileConverter. File size was next reduced to 8 bits. 3D reconstruction of the samples was performed using ‘volume rendering’ (Imaris). The sample could be optically sliced in any angle using the ‘orthoslicer’ or ‘obliqueslicer’ tools. Air bubbles and crystals that might form at the surface of the samples could be eliminated using the ‘surface’ tool by creating a mask around each volume. 3D pictures and movies were generated using the ‘snapshot’ and ‘animation’ tools. Finally, images were cropped and, if required, their brightness was adjusted evenly using Photoshop CS4 (Adobe).

Statistical analysis

To quantify the number of $Foxp2^+$ IO neurons invading the midline, eight series of 20 µm cryosections were obtained from E13 and E14 hindbrains. On each section, a 40 µm region at the midline was chosen and the number of $Foxp2^+$ IO neurons was counted using ImageJ Software (n=3 embryos for each genotype). For statistical analyses, an unpaired t-test was used. To quantify PN migration defects, the total distance separating the caudal-most and rostral-most $Barhl1^+$/Robo3 neurons along the midline was measured using the measurement tool (Imaris, Bitplane Software; n=3-5 cases for each genotype). For statistical analysis, a Welch's t-test was used. Compiled data are expressed as mean±s.e.m. Statistical analyses were performed with Prism 7 (GraphPad Software).

Acknowledgements

We thank Le Ma, Marc Tessier-Lavigne, Patrick Charnay and Jean-François Brunet for providing mouse lines.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: A.C.; Methodology: C.D., Q.R., P.Z., S.F., A.C.; Validation: S.F., A.C.; Formal analysis: C.D., Q.R., P.Z., A.C.; Investigation: C.D., Q.R., P.Z., S.F., A.C.; Resources: A.C.; Data curation: C.D., Q.R., P.Z., S.F., A.C.; Writing - original draft: A.C.; Writing - review & editing: C.D., P.Z., A.C.; Visualization: A.C.; Supervision: A.C.; Project administration: A.C.; Funding acquisition: A.C.

Funding

C.D. was a recipient of a fellowship from the Fondation pour la Recherche Médicale (FRM). This work was supported by grants from the Fondation pour la Recherche Médicale (DEQ20120323700), the Agence Nationale de la Recherche (ANR-14-CE13-0004-01). It was performed in the frame of the Labex Lifesenses (ANR-10-LABX-65) supported by French state funds managed by the Agence Nationale de la Recherche within the Investissements d'Avenir programme (ANR-11-IDEX-0004-02).

Supplementary information

Supplementary information available online at
http://dev.biologists.org/lookup/doi/10.1242/dev.150375.supplemental

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