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RESEARCH ARTICLE

Netrin 1 and Dcc signalling are required for confinement of central axons within the central nervous system

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ABSTRACT

The establishment of anatomically stereotyped axonal projections is fundamental to neuronal function. While most neurons project their axons within the central nervous system (CNS), only axons of centrally born motoneurons and peripherally born sensory neurons link the CNS and peripheral nervous system (PNS) together by navigating through specialized CNS/PNS transition zones. Such selective restriction is of importance because inappropriate CNS axonal exit could lead to loss of correct connectivity and also to gain of erroneous functions. However, to date, surprisingly little is known about the molecular-genetic mechanisms that regulate how central axons are confined within the CNS during development. Here, we show that netrin 1/Dcc/Unc5 chemotropism contributes to axonal confinement within the CNS. In both *Ntn1* and *Dcc* mutant mouse embryos, some spinal interneuronal axons exit the CNS by traversing the CNS/PNS transition zones normally reserved for motor and sensory axons. We provide evidence that netrin 1 signalling preserves CNS/PNS axonal integrity in three ways: (1) netrin 1/Dcc ventral attraction diverts axons away from potential exit points; (2) a Dcc/Unc5c-dependent netrin 1 chemoinhibitory barrier in the dorsolateral spinal cord prevents interneurons from being close to the dorsal CNS/PNS transition zone; and (3) a netrin 1/Dcc-dependent, Unc5c-independent mechanism that actively prevents exit from the CNS. Together, these findings provide insights into the molecular mechanisms that maintain CNS/PNS integrity and, to the best of our knowledge, present the first evidence that chemotropic signalling regulates interneuronal CNS axonal confinement in vertebrates.

KEY WORDS: Netrin 1, Dcc, Spinal cord, CNS exit, Unc5, Axonal confinement, Mouse

INTRODUCTION

Nervous system function is dependent on appropriate neuronal connectivity and is contingent on the formation of a stereotyped anatomical organization of axons. A core feature of axonal organization is that neurons generally project axons to targets within the central nervous system (CNS). Therefore, axons are confined to the CNS and generally do not cross the border between the CNS and periphery. There are two exceptions to this rule: (1) motoneurons, which are centrally born and project out of the CNS, largely through the motor exit point (MEP), into the periphery to connect with their target muscle (Bravo-Ambrosio and Kaprielian, 2011); and (2) neurons from the peripheral nervous system (PNS), which convey sensory information from the body to the CNS by projecting axons into the CNS (Fig. 1) (de Nooij et al., 2013).

Thus, tightly controlled mechanisms must restrict axons of all CNS interneurons to project within the CNS despite the presence of sensory axon entry and motor axon exit points. Two mechanisms have been postulated to regulate such axonal confinement: physical and chemotrophic barriers. The physical barrier consists of a basal lamina that surrounds the neural tube and, within the spinal cord, a layer of glial end-feet (Bravo-Ambrosio and Kaprielian, 2011; Fraher et al., 2007). The PNS/CNS transition zones within the spinal cord exit (MEP) and entry (dorsal root entry zone, DREZ) points are characterized by a break in the basal lamina and peripheral boundary cap cells that prevent cell bodies from leaving the CNS, as well as by gaps between the glial end-feet (Fraher et al., 2007; Mauti et al., 2007; Snider and Palavali, 1990; Vermeren et al., 2003). Additionally, one study in *Drosophila* has shown that the chemotrophic molecule dephrin provides a molecular barrier at the CNS/PNS interface that prevents CNS interneuron axons from exiting the CNS (Bossing and Brand, 2002). However, very little is known about how chemotropic signals influence interneuron axons to project only within the CNS, particularly in vertebrates (Bossing and Brand, 2002; Dorsten et al., 2010; Keleman and Dickson, 2001; Layden et al., 2006).

Using the mouse embryonic spinal cord as a model system, the aim of this study was to examine how central axons are confined within the CNS in the vertebrate nervous system, particularly focusing on the potential role of netrin 1 (Ntn1) chemotropism. The developing mouse spinal cord is composed of motoneurons that transmit motor information and of a range of interneurons that relay sensory information from the body to the CNS (Goulding et al., 2002). Here, we focus on spinal interneurons, all of which project to targets within the CNS. Netrin 1 is a bi-functional secreted chemotropic ligand: it acts as a chemoattractant via its receptors deleted in colorectal cancer (Dcc) or neogenin, or as a chemorepellent via Dcc/Unc5 or Unc5/Unc5 receptor dimers (Cirulli and Yebra, 2007; Hedgecock et al., 1990; Ishii et al., 1992; Kolodkin and Tessier-Lavigne, 2011; Mitchell et al., 1996; Serafini et al., 1996). Netrin 1 has an evolutionarily conserved role in spinal commissural axon attraction to the midline but is also expressed in a region adjacent to the DREZ, raising the possibility that it could also contribute to the confinement of CNS interneuron axons via its chemoattractive and/or chemorepulsive properties (Bovolenta, 2005; Cirulli and Yebra, 2007; Serafini et al., 1994; Vallstedt and Kullander, 2013; Watanabe et al., 2006).

We show that manipulation of netrin 1 signalling leads to spinal interneuron axonal exit from the CNS. We provide evidence that this is regulated by a combination of at least three mechanisms: (1) strong ventral attraction by netrin 1/Dcc diverts axons away from PNS/CNS transition zones; (2) a netrin 1 chemotropic barrier in the dorsolateral spinal cord that prevents interneurons from being close

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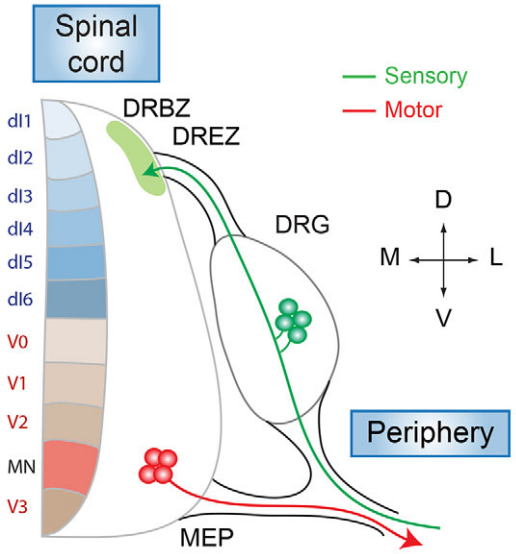

Fig. 1. Schematic representation of the central nervous system/peripheral nervous system (CNS/PNS) transition zones in the embryonic spinal cord. Motoneurons (red) exit the CNS predominantly at the motor exit point (MEP). Peripheral neurons (green) with cell bodies in the peripherally located dorsal root ganglia (DRG) project axons in to the dorsal root bifurcation zone (DRBZ) and the grey matter of the CNS via the dorsal root entry zone (DREZ). The dorsoventral origin of spinal interneurons dI1-6, V0-3 and motoneurons (MNs) are depicted. D, dorsal; V, ventral; M, medial; L, lateral.

to the DREZ; and (3) a netrin 1/Dcc-dependent, Unc5c-independent mechanism for preventing axonal exit from the CNS. Together, these findings provide insights into part of a larger mechanism for maintaining CNS/PNS integrity in vertebrates.

RESULTS

Axons expressing markers typical of CNS interneurons are present in the PNS of *Ntn1*$^{LacZ/LacZ}$ and *Dcc*$^{-/-}$ embryos

From embryonic day (E) 10.5-12.5, mouse embryo interneurons project to their targets, bypassing the DREZ and MEP, a process that is coincident with expression of netrin 1 and its receptors Dcc and Unc5 (Dillon et al., 2007; Fazeli et al., 1997; Helms and Johnson, 2003; Palmesino et al., 2012). We first focussed on netrin 1 and its receptor Dcc, as Dcc has been implicated in both attractive and repellent functions of netrin 1. We examined *Ntn1*$^{LacZ/LacZ}$ and *Dcc*$^{-/-}$ mouse embryos for possible ectopic axonal exit through the DREZ and MEP (Fazeli et al., 1997; Hong et al., 1999; Keino-Masu et al., 1996).

To mark centrally born axons, we used immunofluorescence labelling of Robo3, which delineates all commissural neurons within the spinal cord (Sabatier et al., 2004). In addition, we used immunofluorescence of GFP in *Barhl2*^{GFP} transgenic embryos, which labels commissurally projecting dI1c and ipsilaterally projecting dI1i neurons (Wilson et al., 2008). In wild-type embryos, ectopic axonal exit of spinal interneurons was never observed. However, in the DRG of *Ntn1*$^{LacZ/LacZ}$;*Barhl2*^{GFP} and *Dcc*$^{-/-}$;*Barhl2*^{GFP} embryos, we observed a small but consistent number (100% penetrance) of Robo3- and GFP-expressing axons (Fig. 2). These ectopic axons were Robo3^{-}/GFP^{+} (dI1i ipsilateral), Robo3^{+}/GFP^{+} (dI1c commissural) and Robo3^{+}/GFP^{-} (non-dI1c commissural) neurons (Fig. 2D,I,N). In *Ntn1*$^{LacZ/LacZ}$;*Barhl2*^{GFP} and *Dcc*$^{-/-}$;*Barhl2*^{GFP}, but not wild-type, embryos, we also observed Robo3^{+} and GFP^{+} axons in the ventral root and the MEP (Fig. 2E,J,O). This ventral root observation was documented in every knockout embryo examined but not in every section, making it a reliable but rare observation.

Ntn1$^{LacZ/LacZ}$ embryos showed no statistically significant increase in the number of spinal interneuronal axons in the DRG at older ages or at a different axial level (Fig. 3C). The exception was a statistically significant increase in GFP^{+}/Robo3^{-} dI1i axons between E11.5 and E12.5 (Fig. 3C). This was in sharp contrast to *Dcc*$^{-/-}$ embryos, which showed an overall stronger phenotype compared with *Ntn1*$^{LacZ/LacZ}$ for all subtypes examined. In E12.5 *Dcc*$^{-/-}$ embryos, the number of ectopic axons in the DRG was ~4.5-fold higher than in *Ntn1*$^{LacZ/LacZ}$ embryos of the same age (Fig. 3B,C). The average number of ectopic axons in the DRG of *Dcc*$^{-/-}$ embryos increased fourfold between E11.5 and E12.5 (Fig. 3B). In the DRG of E12.5 *Dcc*$^{-/-}$ embryos, there was also a fivefold statistically significant increase in peripherally located ectopic CNS axons in a lumbar (low) to cervical (high) gradient (Fig. 3H). A similar increase was observed for all axonal types examined.

A statistically significant increase in the number of Robo3^{+}/GFP^{-} axons was observed in the ventral root of in *Ntn1*$^{LacZ/LacZ}$ embryos between E11.5 and E12.5 (Fig. 3F). However, the overall frequency of ectopic axons observed in ventral roots of *Dcc*$^{-/-}$ and *Ntn1*$^{LacZ/LacZ}$ embryos were generally similar at all axial levels and ages examined (Fig. 3J-L). These data provide evidence that Robo3^{+} and GFP^{+} spinal interneuron axons that were never detected outside the CNS in control embryos were observed in the periphery of *Ntn1*$^{LacZ/LacZ}$;*Barhl2*^{GFP} and *Dcc*$^{-/-}$;*Barhl2*^{GFP} embryos.

The peripherally located ectopic axons in *Dcc*$^{-/-}$ and *Ntn1*$^{LacZ/LacZ}$ embryos are of CNS origin

The above findings have at least two possible explanations: in *Ntn1*$^{LacZ/LacZ}$ and *Dcc*$^{-/-}$ embryos, (1) CNS axons were abnormally exiting the CNS or (2) peripheral neurons underwent a fate change to express CNS markers such as Robo3 and Barhl2. To distinguish between these possibilities, we took three approaches: retrograde tracing of axons from the periphery, anterograde labelling of central axons and detection of spinal interneuron marker expression in cell bodies of DRGs. The peripheral nerves of E12.5 control, *Ntn1*$^{LacZ/LacZ}$ and *Dcc*$^{-/-}$ embryos were labelled with biotin-conjugated dextran. These embryos were analysed for retrograde transport of dextran into the CNS (Fig. 4A-E). Motoneurons, which have cell bodies in the ventral spinal cord, normally exit through the MEP and project axons into the peripheral nerve and were therefore labelled using this method in all genotypes (Bravo-Ambrosio and Kaprielian, 2011). Consequently, we analysed the dorsal spinal cord for neurons that projected through the DREZ. Consistent with previous findings, we also observed the premature entry of peripheral axons into the spinal cord of *Ntn1*$^{LacZ/LacZ}$ embryos (Watanabe et al., 2006), but not of *Dcc*$^{-/-}$ or control embryos (Fig. 4C-E). Additionally, we found ectopic dextran-labelled cell bodies within the dorsolateral spinal cord of every *Dcc*$^{-/-}$ (n=6/6) and *Ntn1*$^{LacZ/LacZ}$ (n=5/5), but not control (n=0/20), embryos (Fig. 4C-E). These data suggest that centrally located neurons do project axons into the periphery of *Ntn1*$^{LacZ/LacZ}$ and *Dcc*$^{-/-}$ embryos.

To confirm that the peripherally located Robo3^{+} axons in the mutant embryos were derived from the CNS, we transgenically labelled spinal cord but not peripherally derived neurons by electroporating *CMV-mCherry* and *Barhl2*^{GFP} (which expresses GFP in dI1 neurons) plasmids into the spinal cord of E10.5 *Dcc*$^{-/-}$, *Ntn1*$^{LacZ/LacZ}$ or control mouse embryos. Electroporated embryos were subsequently cultured for 48 hours by whole embryo culture (WEC)

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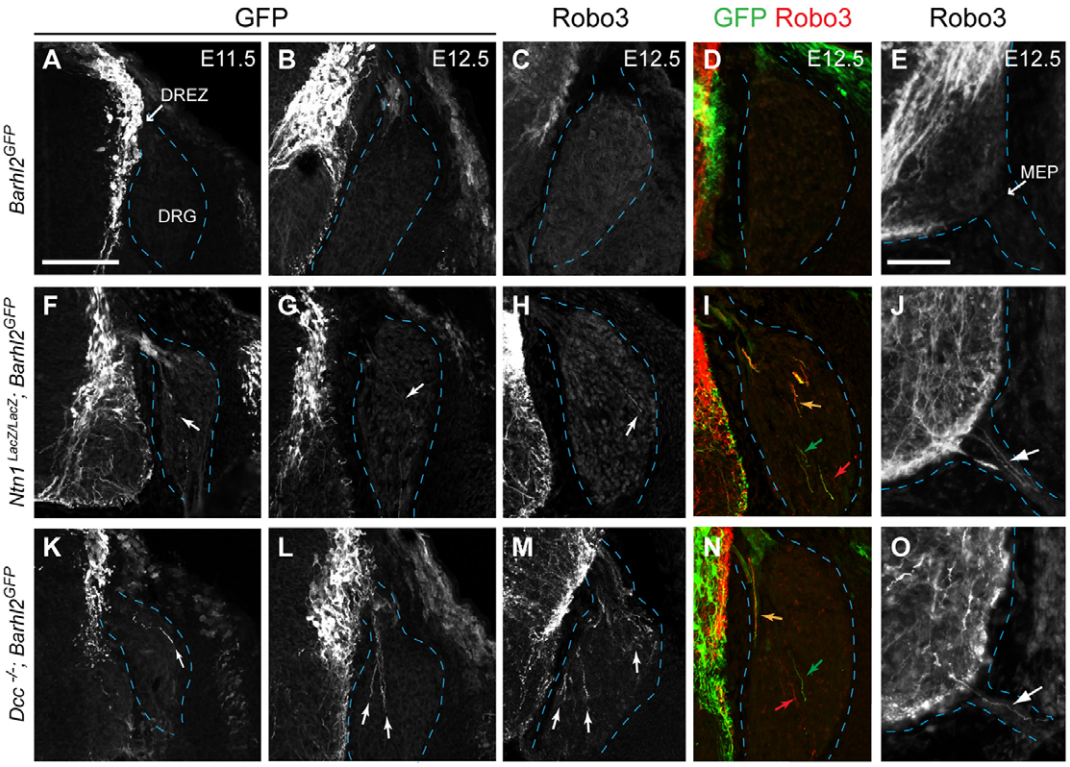

Fig. 2. CNS interneuronal axons were observed in the periphery of neurons of *Ntn1*$^{LacZ/LacZ}$ and *Dcc*$^{-/-}$ embryos. (A-O) Photomicrographs of GFP and Robo3 immunolabelling in spinal cord transverse sections of *Barhl2*^{GFP} (A-E), *Ntn1*$^{LacZ/LacZ}$;*Barhl2*^{GFP} (F-J) and *Dcc*$^{-/-}$;*Barhl2*^{GFP} (K-O) mouse embryos at thoracic levels at E11.5 and E12.5 show ectopic axons (arrows) that expressed markers typical of CNS neurons in the dorsal root ganglia (DRG) and motor exit point (MEP) (both indicated by dashed lines) of *Ntn1*$^{LacZ/LacZ}$ and *Dcc*$^{-/-}$ but not control embryos. Scale bar: in A, 100 µm for A-D,F-I,K-N; in E, 50 µm for E,J,O.

and analysed for the presence of mCherry and/or GFP fluorescence in the periphery (Fig. 4F-M). We detected mCherry^{+} and GFP^{+} axons in the DRG of every *Dcc*$^{-/-}$ (n=5/5) and *Ntn1*$^{LacZ/LacZ}$ (n=3/3) embryo but not in control embryos (n=0/20) (Fig. 4H-M).

Finally, we performed *in situ* hybridization of *Robo3* and *Barhl2* mRNA in control, *Ntn1*$^{LacZ/LacZ}$ and *Dcc*$^{-/-}$ embryos to examine whether DRG neurons had undergone a fate change. We did not observe expression of *Robo3* or *Barhl2* mRNA in the DRG of any genotype examined, suggesting that, in the mutants, DRG neurons had not been respecified as central neurons (Fig. 4N-S). Taken together, these data strongly support the notion that, in *Ntn1*$^{LacZ/LacZ}$ and *Dcc*$^{-/-}$ embryos, some central neurons project axons out of the CNS.

CNS interneuron axons project into and through the DRBZ in *Ntn1*$^{LacZ/LacZ}$ and *Dcc*$^{-/-}$ embryos

Around E11.0, the peripheral sensory neurons send axons towards the spinal cord through the DREZ and start to form the dorsal root bifurcation zone (DRBZ), where they project in an anterior-posterior direction within the tract (Fig. 1) (Altman and Bayer, 1984). Therefore, the DRBZ is in direct apposition to the DREZ (Fig. 1). Thus, if CNS-derived axons were indeed projecting out of the CNS into the periphery via the DREZ in *Ntn1*$^{LacZ/LacZ}$ and *Dcc*$^{-/-}$ embryos, these axons would be present in or projecting through the DRBZ. We therefore examined the DRBZ of *Ntn1*$^{LacZ/LacZ}$ and *Dcc*$^{-/-}$ embryos. To delineate the DRBZ we used an antibody against a neurofilament-associated protein antibody (2H3) and CNS-derived axons were distinguished by their expression of Robo3 in commissural neurons and GFP in dI1 neurons (Sabatier et al., 2004; Wilson et al., 2008). We observed Robo3^{+} and GFP^{+} axons in the DRBZ of both *Netrin1*$^{LacZ/LacZ}$ and *Dcc*$^{-/-}$ embryos but only occasionally in wild-type embryos (Fig. 5A-O). Unlike the axonal exit phenotype, the number of CNS axons in DRBZ appeared to increase between E11.5 and E12.5 in both *Ntn1*$^{LacZ/LacZ}$ and *Dcc*$^{-/-}$ embryos (supplementary material Fig. S1; Fig. 5). In *Dcc*$^{-/-}$ embryos, we observed some Robo3^{+} axons projecting in an anterior-posterior direction within the DRBZ (Fig. 5P-T). These data are further evidence that CNS neurons are in a spatial position that allows them to send their axons abnormally into the periphery of *Ntn1*$^{LacZ/LacZ}$ and *Dcc*$^{-/-}$ embryos.

Ntn1 mRNA is expressed in the dorsolateral region of the spinal cord from E11.5

To address whether netrin 1 signalling restricts CNS axons by either forming a chemotropic repellent boundary surrounding the dorsally located DREZ and ventrally located MEP or by redirecting axons away from this potential exit point, we first examined the detailed expression of *Ntn1* mRNA in mouse embryos during the initial interneuron axon outgrowth period, between E10.5 and E12.5 (Fig. 6). To identify the DRBZ, we co-immunolabelled the sections with a Tag-1 antibody, which labels centrally born commissural neurons and peripheral sensory neurons that project within the DRBZ (Dodd et al., 1988; Vaughn et al., 1992). As reported previously, at E10.5 within the spinal cord, *Ntn1* mRNA is largely restricted to the floor plate and ventricular zone (Fig. 6A) (Wang et al., 1999). This pattern persisted at E11.5 and E12.5 (Fig. 6B-E). In addition, at anterior levels of E11.5 embryos and at all levels of E12.5 embryos, *Ntn1* mRNA was detected in the mantle zone adjacent to the DRBZ (Fig. 6). This *Ntn1* mRNA expression domain followed an anterior-posterior high to low expression gradient,

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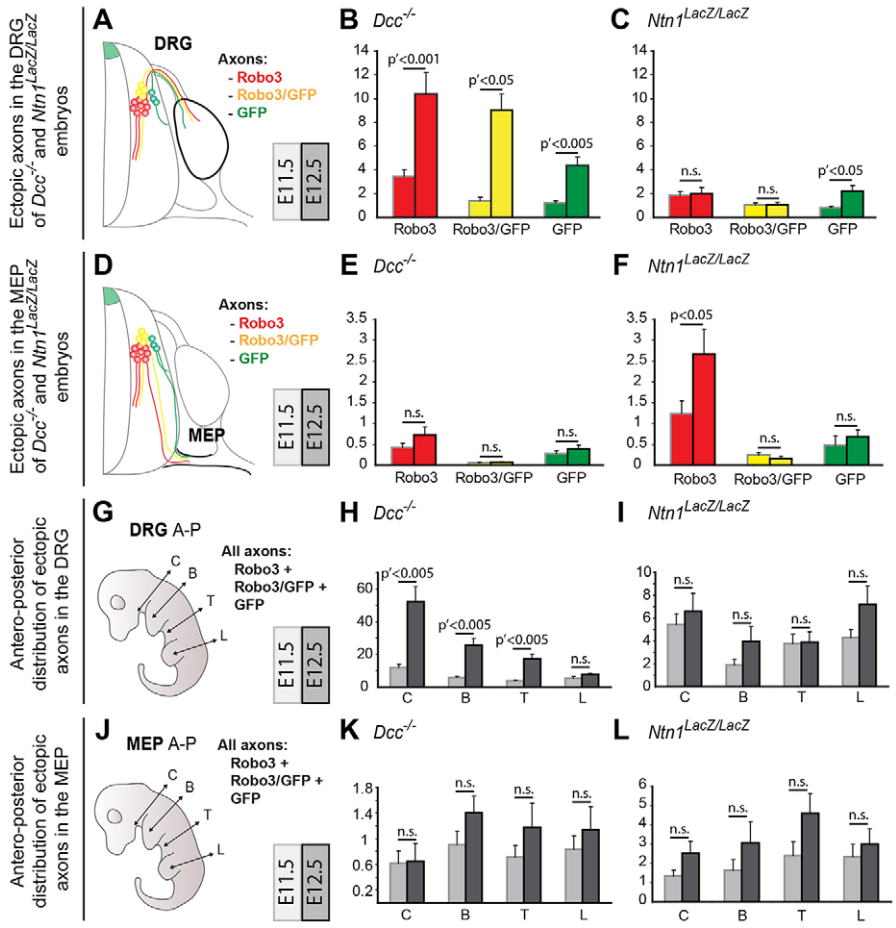

Fig. 3. Quantification of ectopically located CNS axons within the periphery of *Ntn1*$^{LacZ/LacZ}$ and *Dcc*$^{-/-}$ embryos. (A-F) Quantification of the overall average numbers of Robo3^{+}/GFP^{-} (non-dI1 commissural, red), Robo3^{+}/GFP^{+} (dI1 commissural, yellow) and Robo3^{-}/GFP^{+} (dI1 ipsilateral, green) ectopic axons per section in the dorsal root ganglia (DRG) (A-C) and motor exit point (MEP) (D-F) at E11.5 (grey outline) and E12.5 (black outline) in *Dcc*$^{-/-}$ (B,E) and *Ntn1*$^{LacZ/LacZ}$ (C,F) embryos. The phenotypes for the DRG and MEP are represented as schematics in A and D. The phenotype in the DRG of *Dcc* mutant embryos increases between E11.5 and E12.5, and is more pronounced than the phenotype in *Ntn1* mutant embryos. (G-L) Average numbers of Robo3^{+} and/or GFP^{+} ectopic neurites located in the DRG (G-I) or MEP (J-L) at cervical (C), brachial (B), thoracic (T) and lumbar (L) axial levels at E11.5 (grey outline) and E12.5 (black outline) in *Dcc*$^{-/-}$ (H,K) and *Ntn1*$^{LacZ/LacZ}$ (I,L) embryos. The level of sections analysed are represented schematically in G and J. This shows an increase in CNS axons in the periphery in cervical versus more posterior axial levels of *Dcc* mutant embryos (H). Ectopic projections were not observed in the DRG or MEP of wild-type embryos (I,L). At least five embryos for each group were counted. *P* values and *P'* values (with Welch correction) are indicated. n.s., not significant. Error bars indicate s.e.m.

reflecting the spinal cord anterior-posterior developmental progression. *Ntn1* mRNA expression was not observed surrounding the MEP (Fig. 6A-E). These data suggest that netrin 1 might have a role in the axonal confinement at the DREZ but not at the MEP, by forming a chemotropic inhibitory barrier that prevents axons from entering the DBRZ inappropriately.

Robo3^{+} axons ectopically enter the DRBZ in *Unc5c*$^{-/-}$ embryos but do not exit the CNS

The dorsolateral *Ntn1* expression domain levels increased in a time frame similar to the appearance of ectopic axons in the DRBZ in *Dcc* and *Ntn1* mutant embryos (Figs 2, 5, 6; supplementary material Fig. S1). This led us to consider whether the dorsolateral expression of netrin 1 was preventing ectopic entry of spinal interneuronal axons into the DBRZ. Unc5 receptors convey the chemorepulsion from netrin 1 (Cirulli and Yebra, 2007), and *Unc5a* and *Unc5c* mRNAs are expressed in the dorsal mouse spinal cord at the time the axon exit phenotypes were observed (Dillon et al., 2007; Masuda et al., 2008). Thus, to test whether spinal interneuron axons were being repelled from the dorsolateral domain of netrin 1 expression, we examined *Unc5a*$^{-/-}$, *Unc5c*$^{-/-}$ and control E12.5 embryonic dorsal commissural axons. In *Unc5a*$^{-/-}$ and control embryos, we observed occasional Robo3^{+} axons in the DRBZ; however, strikingly, in *Unc5c*$^{-/-}$ embryos, many Robo3^{+} axons and cell bodies entered the DRBZ and penetrated further into the DBRZ compared with *Unc5a*$^{-/-}$ and wild-type embryos (Fig. 7C,G,I-L). These observations were robust, with 100% of *Unc5c*$^{-/-}$ embryos displaying this phenotype (*n*=8). Unlike in *Dcc*$^{-/-}$ and *Ntn-1*$^{-/-}$ embryos, Robo3^{+} axons were never observed exiting the CNS in *Unc5c*$^{-/-}$, *Unc5c*$^{+/-}$, *Unc5a*$^{-/-}$, *Unc5a*$^{+/-}$ or wild-type embryos [Figs 2, 7; for *Unc5c*$^{-/-}$, *Unc5c*$^{+/-}$, *Unc5a*$^{-/-}$ and *Unc5a*$^{+/-}$(*n*=8, *n*=9, *n*=10, *n*=8, respectively)]. The severity of the *Unc5c*$^{-/-}$ phenotype mirrored the relative dorsolateral expression level of *Ntn1*, with the most anterior regions being most strongly affected, and mimicked the extent and progression of the DRBZ phenotype in *Ntn-1*$^{-/-}$ embryos (Fig. 5N; Fig. 7I-L and supplementary material Fig. S1). Taken together, these data are consistent with the idea that the dorsolateral expression of netrin 1 forms a chemoinhibitory boundary in the mantle zone surrounding the DRBZ/DREZ and that chemorepulsion mediated by Unc5c/Dcc receptors normally prevents commissural axons and cell bodies from projecting into the DRBZ and therefore being in a spatial position to exit the CNS.

Ectopic CNS axonal exit occurs before the onset of dorsolateral netrin 1 expression

As dorsolateral netrin 1 expression was not observed until late E11.5, by which time we observed ectopic axonal exit from the CNS in *Ntn1*$^{LacZ/LacZ}$ and *Dcc*$^{-/-}$ embryos, we next aimed to examine whether dorsolateral repulsion by netrin 1 was solely responsible for the netrin 1 loss of axon confinement phenotype or whether other mechanisms were coming into play. To test this idea, we analysed the precise time course of netrin 1 expression with respect to the onset of the misprojection phenotype. The DREZ was identified by morphological appearance. To localize netrin 1 expression, we used β-galactosidase immunolabelling in *Ntn1*$^{LacZ/LacZ}$ embryos. The mutant *Ntn1* allele produces a fusion protein of exons 1 and 2 of *Ntn1* and β-galactosidase, which is expressed under the control of the endogenous *Ntn1* gene (Serafini et al., 1996).

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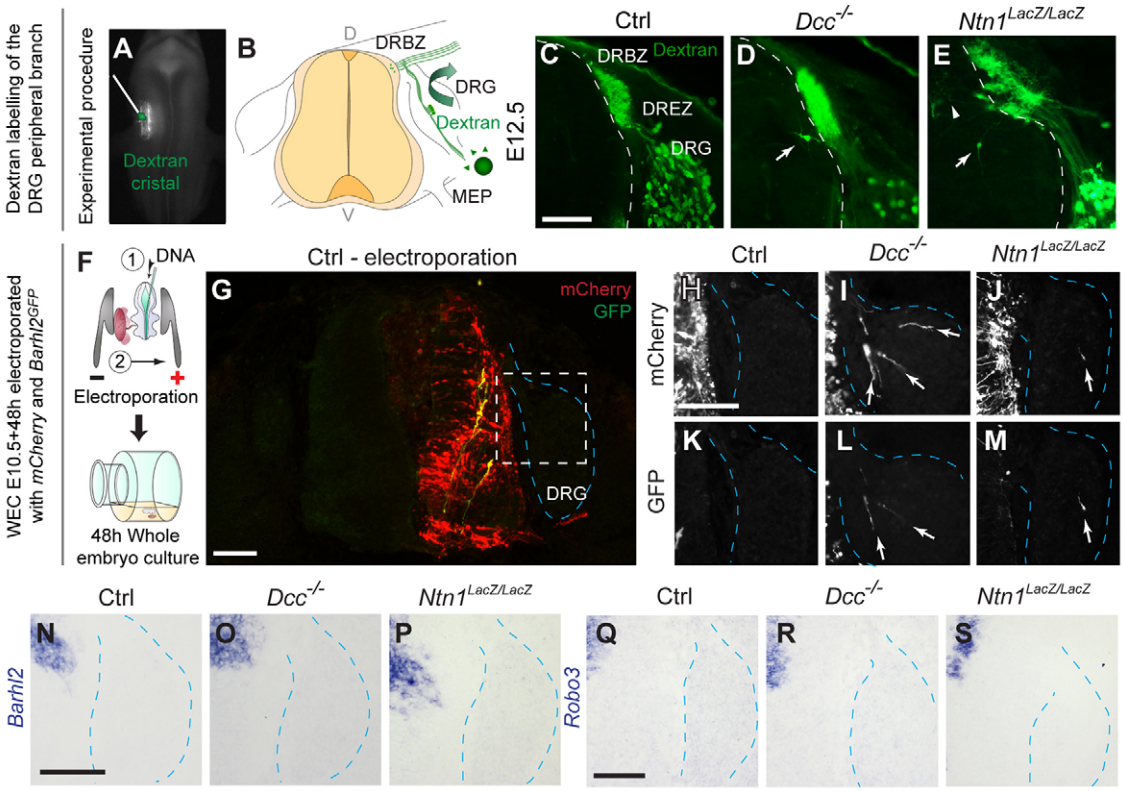

Fig. 4. Ectopic peripherally located axons in *Ntn1*$^{LacZ/LacZ}$ and *Dcc*$^{-/-}$ embryos originate in the CNS. (A,B) Whole-mount dorsal view of an embryo showing the position of implant of the biotin-conjugated dextran (A) and schematic of the experimental procedure (B). (C-E) Photomicrographs of transverse sections of E12.5 control, *Dcc*$^{-/-}$ and *Ntn1*$^{LacZ/LacZ}$ mouse embryos following peripheral labelling with biotin-conjugated dextran (green). The dashed line represents the border of the spinal grey matter. Arrowhead in E indicates ectopic peripheral sensory neuron collateral as described previously (Watanabe et al., 2006). Arrows in D and E indicate dorsally located CNS neurons that have been labelled from peripherally injected dextran, indicating that axons from the CNS project out into the peripheral nerve in the mutant embryos. (F) Schematic representation of the whole-embryo culture (WEC) experimental procedure. (G-M) Photomicrographs of transverse sections of control (G,H,K), *Dcc*$^{-/-}$ (I,L) and *Ntn1*$^{LacZ/LacZ}$ (J,M) mouse embryos following electroporation with *mCherry* and *Barhl2*^{GFP} plasmids and culture show GFP- and mCherry-expressing axons (arrows) derived from the CNS projecting into the periphery (the DRG is indicated by dashed lines). (N-S) Photomicrographs of *in situ* hybridization of *Barhl2* and *Robo3* in the DRG (dashed outline) of control (N,Q), *Dcc*$^{-/-}$ (O,R) and *Ntn1*$^{LacZ/LacZ}$ (P,S) mouse embryos reveals that CNS markers *Robo3* and *Barhl2* mRNA expression was undetectable in the DRG of embryos of all genotypes examined, suggesting that the peripheral neurons have not been respecified in the mutant embryos. Scale bars: in C, 100 µm for C-E; in G, 100 µm; in H, 50 µm for H-M; in N, 100 µm for N-P; in Q 100 µm for Q-S.

Similar to the mRNA expression of *Ntn1* in wild-type embryos, in *Ntn1*$^{LacZ/LacZ}$ and *Ntn1*$^{+/LacZ}$ embryos, the onset of β-galactosidase labelling in the dorsolateral spinal cord was detected at E11.5 (Fig. 6H and Fig. 8C). By contrast, the onset of the first peripherally located CNS interneuron axons in *Ntn1*$^{LacZ/LacZ}$ and *Dcc*$^{-/-}$ embryos was at E10.5 (Fig. 8). Taken together, these data provide evidence that while the onset and increase of netrin 1 expression surrounding the DRBZ/DREZ contributes to axonal confinement, other mechanisms, such as strong ventral attraction diverting axons away from potential exit points, are likely to contribute to CNS/PNS axonal fidelity (Fig. 9).

DISCUSSION

We have examined how chemotropism regulates CNS axonal confinement, particularly focusing on the role of netrin 1/Dcc signalling using knockout mouse embryos. Our data showing some similarities and some differences in the phenotypes of *Ntn1*, *Dcc* and *Unc5* mutant embryos reveals key aspects of the mechanism of axonal confinement. First, the appearance of ectopic axons in the DRBZ in *Dcc*, *Ntn1* and *Unc5c* mutant embryos increases in a similar way to the increase in the dorsolateral expression of *Ntn1*. However, CNS axons exit ectopically in only *Dcc* and *Ntn1* (but not *Unc5c*) mutant embryos. Furthermore, the development of the exit phenotype in *Dcc* mutant embryos is more pronounced than in *Ntn1*$^{-/-}$ embryos. We interpret these differences as meaning that netrin 1/Dcc/Unc5 signalling preserves CNS/PNS integrity in different steps. The first stage involves regulating the proximity of the axon to the CNS/PNS transition zones and the second part regulates the ability of an axon to take advantage of the 'CNS/PNS transition zone molecular and physical anatomical architecture' and exit the CNS. To our knowledge, these findings provide the first evidence that chemotropic signalling is responsible for the confinement of interneuron axons within the vertebrate CNS.

The first stage – regulating the axons proximity to the CNS/PNS transition zone

Our data suggest that netrin 1 signalling drives axons away from the CNS/PNS transition zones by two mechanisms. At early stages strong netrin 1/Dcc ventral attraction diverts axons away from potential exit points. Given that a number of chemotropic signals have been detected in the periphery of mid-gestation mouse embryos, it seems plausible that, in the absence of strong attractive signals diverting them away from potential exit points, axons could be instead potentially be attracted to other chemotactic

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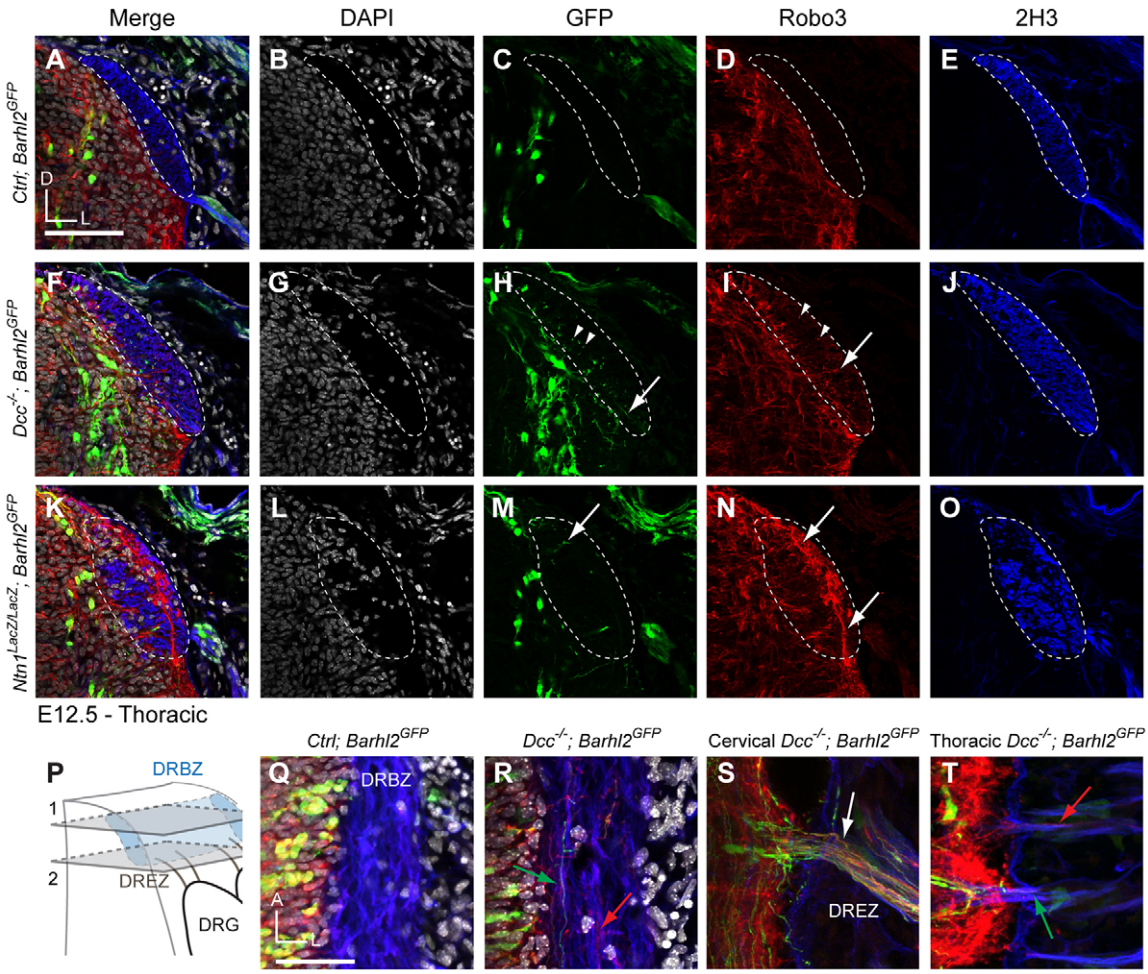

Fig. 5. Robo3^{+} axons project into the DRBZ in *Ntn1*$^{LacZ/LacZ}$ and *Dcc*$^{-/-}$ embryos. (A-O) Photomicrographs of transverse sections of the dorsal spinal cord of control *Barhl2*^{GFP} (A-E), *Dcc*$^{-/-}$;*Barhl2*^{GFP} (F-J) and *Ntn1*$^{LacZ/LacZ}$;*Barhl2*^{GFP} (K-O) embryos at E12.5 showing DAPI (white), Robo3 (red), GFP (green) and 2H3 (blue). This reveals that CNS axons ectopically enter the DRBZ (indicated by dashed lines) abutting the DREZ in the mutant embryos (arrows). In *Dcc* mutant embryos, a number of axons appear to project within the DRBZ (arrowheads). (P-T) Schematic of the region of the dorsal spinal cord imaged (P) and photomicrographs of horizontal sections of the dorsal spinal cord of control and *Dcc*$^{-/-}$;*Barhl2*^{GFP} embryos at E12.5, showing GFP^{+} and Robo3^{+} axons projecting within the DRBZ in *Dcc*$^{-/-}$;*Barhl2*^{GFP} embryos (arrows in R). An increased number of GFP^{+} and Robo3^{+} axons project in to the periphery in cervical versus thoracic levels in *Dcc*$^{-/-}$;*Barhl2*^{GFP} embryos (arrows in S and T). D, dorsal; V, ventral; M, medial; L, lateral. Scale bars: in A, 50 µm for A-O; in Q, 25 µm for Q-T.

signals known to be present in the periphery (Wang et al., 1999). At later stages, the dorsolateral expression of *Ntn1* increased approximately proportionally to the increased appearance of ectopic axons in the DRBZ in *Dcc*, *Ntn1* and *Unc5c* mutant embryos (Figs 2, 5, 7; supplementary material Fig. S1). This supports the notion that the dorsolateral expression of netrin 1 forms an inhibitory barrier at the DRBZ/DREZ that prevents axons and cell bodies from entering the DRBZ, and therefore being in a spatial position to exit the CNS. This dorsolateral expression of netrin 1 has been previously reported to be an Unc5c-dependent chemoinhibitory signal for peripheral sensory axon entry into the spinal cord (Watanabe et al., 2006). This provides a consolidated mechanism of how PNS/CNS projection fidelity is coordinated using the same signal.

However, the concept that netrin 1 is acting as a repellent for pre-crossing commissural axons produces a dichotomy because netrin 1 is a well-established attractant of spinal commissural neurons (Charron et al., 2003; Fazeli et al., 1997; Okada et al., 2006; Serafini et al., 1996). It is generally recognized that growth cones can switch attractive/repellent responsiveness to chemotropic ligands by a dynamic change in receptor expression or presentation. Both the netrin 1 attractant and repellent receptors, including *Dcc* and *Unc5s* mRNAs, are expressed in overlapping regions of the spinal cord at mid-gestation ages, raising the possibility that either the attractive or repellent influence of netrin 1 could come into play (Dillon et al., 2007; Fazeli et al., 1997). Our finding that commissural axons ectopically enter the DRBZ in netrin 1 repellent receptor *Unc5c* mutant embryos supports the idea that netrin 1 initially act as a repellent of subsets of later-born commissural neurons. As the axons grow more ventrally past this region, they could then become attracted to netrin 1, perhaps by a dynamic change in proportion or presentation of Unc5 and Dcc receptors at the growth cone (Fig. 9). This repellent function of netrin 1 may also be achieved through synergy with a molecule not examined in this study.

The second stage – regulating exit of the CNS via the CNS/PNS transition zones

The observation of ectopic axonal exit in *Ntn1*$^{LacZ/LacZ}$ and *Dcc*$^{-/-}$ but not in *Unc5c*$^{-/-}$ embryos and the early onset of the phenotype suggested there is a distinct netrin 1/Dcc-dependent, Unc5c-

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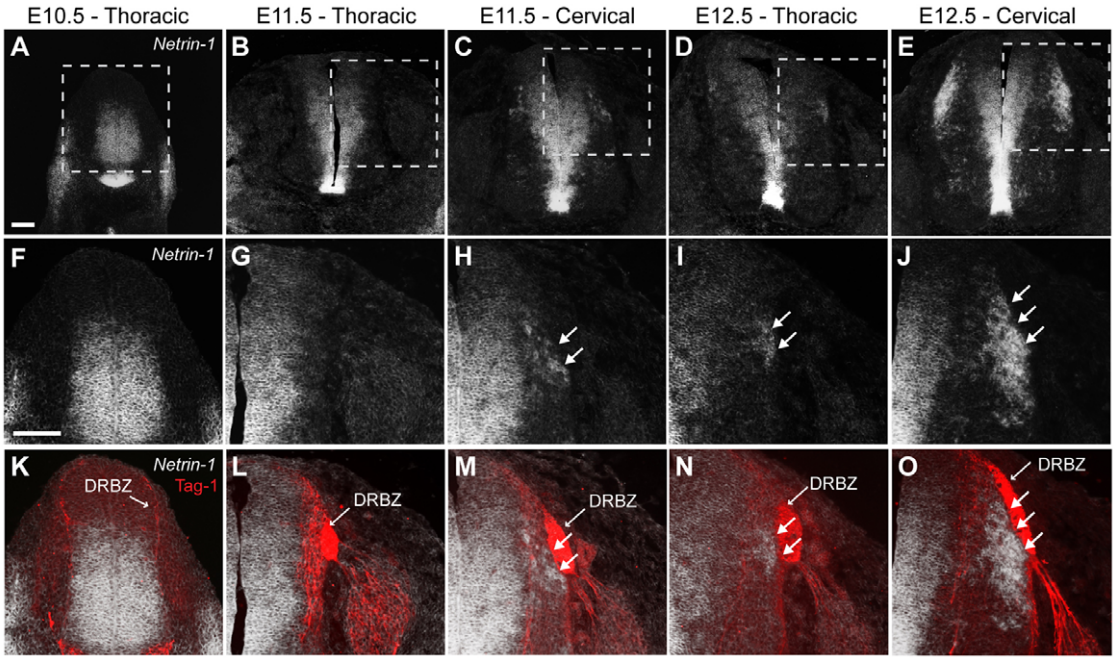

Fig. 6. *Ntn1* expression. (A-O) *In situ* hybridization with *Ntn1* (white) combined with immunolabelling of Tag-1 (red) on wild-type mouse embryo sections. F-O are enlarged version of the boxed areas in A-E. Arrows in H-J,M-O indicate *Ntn1* expression at the border of the DRBZ. Scale bars: in A, 100 µm for A-E; in F, 50 µm for F-O.

independent mechanism that prevents axonal exit. Importantly, the phenotype in *Dcc*$^{-/-}$ embryos was more pronounced and increased with age compared with *Ntn1*$^{LacZ/LacZ}$ embryos (Fig. 3). Several possibilities could account for this difference, including the following: (1) the *Ntn1*^{LacZ} allele is a hypomorph, whereas the *Dcc*$^{-}$ allele is null (Serafini et al., 1996); (2) the cellular organization of *Dcc*$^{-/-}$ compared with *Ntn1*$^{LacZ/LacZ}$ embryos could differ, which may lead to differential responses of local axons; however, the fact that we observed ectopic cell bodies in the DRBZ of *Unc5c*$^{-/-}$ embryos but did not observe exit of interneurons in that mutant argues against this idea; and (3) (our favoured possibility) of the molecules we have examined here, Dcc signalling could have the central role in preserving the fidelity of CNS axons to remain within the CNS. Dcc could be acting via a ligand other than netrin 1 or in conjunction with molecules that modulate its response to netrin 1. One possibility is draxin, which has been shown to produce a repellent activity in neurons via Dcc (Ahmed et al., 2011). Although there is currently no evidence to suggest that draxin is involved in CNS axon restriction, future studies will be required to examine this idea. In retinal axons, laminin 1 has been shown to modulate the

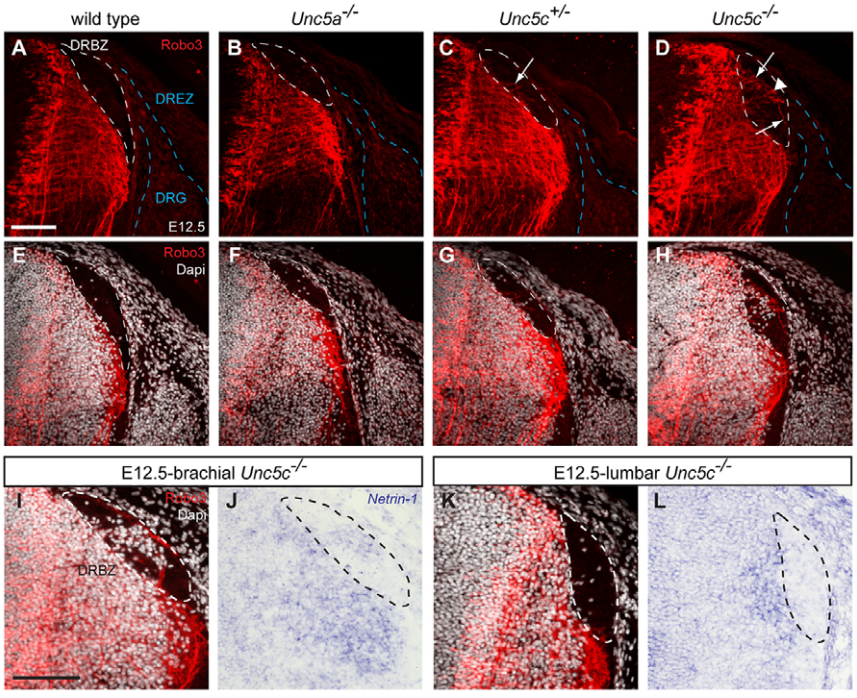

Fig. 7. Robo3^{+} axons misproject into the DRBZ but not into the periphery in *Unc5c*$^{-/-}$ embryos. (A-H) Photomicrographs of transverse sections of the dorsal spinal cord of wild-type (A,E), *Unc5a*$^{-/-}$ (B,F), *Unc5c*$^{+/-}$ (C,G) and *Unc5c*$^{-/-}$ (D,H) E12.5 embryos showing Robo3^{+} (red) axons (arrows) entering the dorsal root bifurcation zone (DRBZ, outlined in white) in *Unc5c*$^{-/-}$ and *Unc5c*$^{+/-}$ but only occasionally in *Unc5a*$^{-/-}$ or wild-type embryos. This reveals that CNS axons ectopically enter the DRBZ abutting the DREZ in the in *Unc5c* mutant embryos, a phenotype that is more pronounced in homozygote than in heterozygote embryos. Robo3^{+} axons were not observed in the dorsal root ganglia (DRG, outlined in dashed blue lines) in any genotype examined. Arrowhead in D indicates ectopic neuronal somata. (I-L) DAPI (white), Robo3^{+} (red) axons and *Ntn1* mRNA (purple) in an *Unc5c*$^{-/-}$ embryo. The strength of the phenotype in *Unc5c*$^{-/-}$ embryos corresponds to the relative level of *Ntn1* expression at a higher brachial (I,J) versus lower lumbar (K,L) levels. Scale bars: in A, 100 µm for A-H; in I, 100 µm for I-L.

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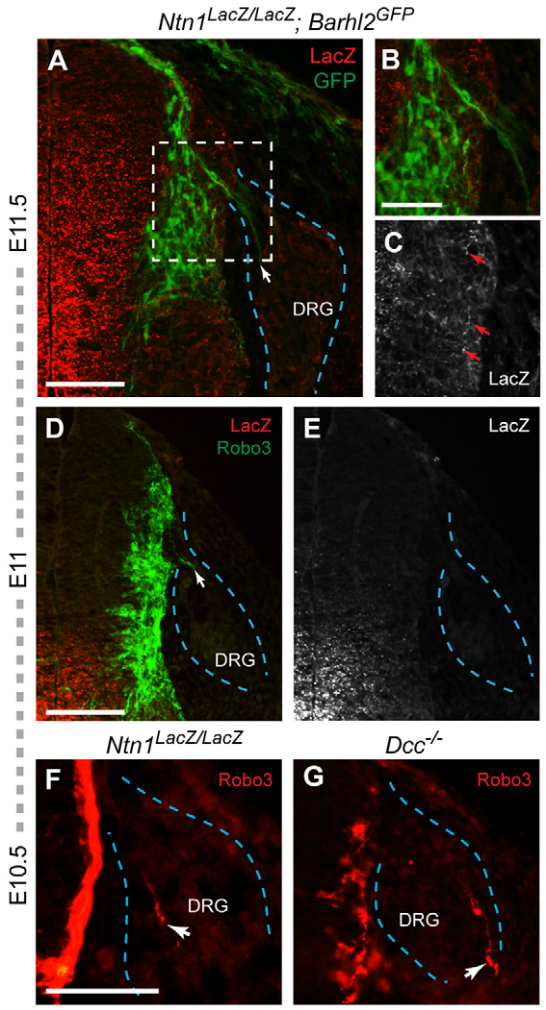

Fig. 8. The onset of β-galactosidase/netrin 1 expression is first detected after CNS axons exit the CNS in *Ntn1*$^{LacZ/LacZ}$;*Barhl2*^{GFP} embryos. (A-E) Immunohistochemical labelling of β-galactosidase (red or white), GFP (green in A and B) and Robo3 (green in D) in E11.5 (A-C) and E11.0 (D-E) *Ntn1*$^{LacZ/LacZ}$;*Barhl2*^{GFP} embryos shows that netrin 1 expression is first apparent next to the DRBZ at E11.5 (red arrows in C). (F,G) Immunohistochemical labelling of Robo3 (red) in E10.5 *Ntn1*$^{LacZ/LacZ}$ (F) and *Dcc*$^{-/-}$ (G) embryos shows that the first CNS axons (white arrows in A,D,F,G) were detected in the mutants at E10.5 before the onset of dorsolateral netrin 1 expression, indicating that the dorsolateral expression of netrin 1 is not solely responsible for the CNS-confining properties of netrin 1/Dcc. The boxed area in A is depicted in B and C. Scale bars: in A, 100 µm; in D, 100 µm for D,E; in B, 50 µm for B,C; in F, 50 µm for F,G.

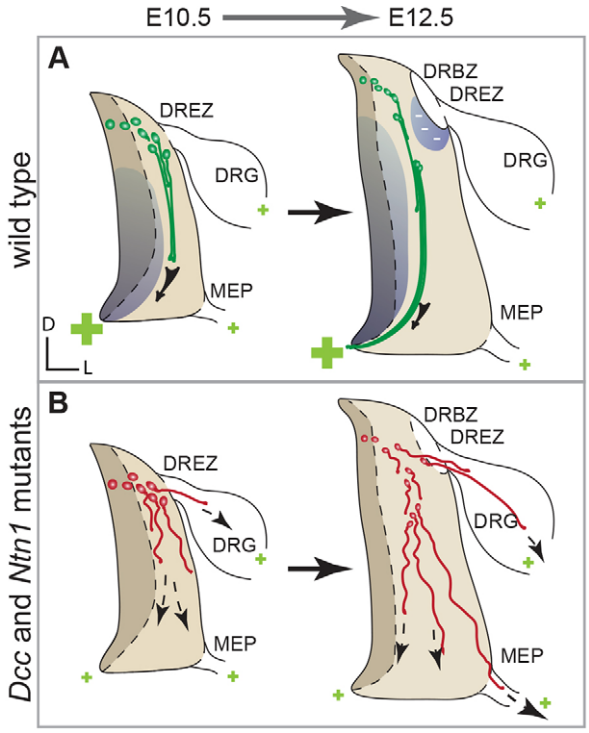

Fig. 9. Summary of the model. (A) In wild-type embryos at E10.5, strong chemoattractive signalling by ventral midline netrin 1 (grey) directs interneuron axons (green) ventrally. Between E11.5 and E12.5, a lateral chemoinhibitory boundary of netrin 1 ligand forms adjacent to the DRBZ/DREZ (grey with white lines). This repels axons away from this region, which prevents axons from being in the vicinity of the DREZ. (B) In *Ntn1*$^{LacZ/LacZ}$ and *Dcc*$^{-/-}$ embryos at E10.5, lack of strong ventral attraction by netrin 1/Dcc signalling could enable CNS-derived interneurons to grow towards weaker attractive signals in the periphery, leading to CNS exit through the DREZ or MEP. In E11.5 to E12.5 *Dcc*$^{-/-}$ embryos, in addition to the loss of direction caused by loss of ventral attraction, axons are no longer sensitive to the netrin 1 chemoinhibitory boundary adjacent to the DRBZ/DREZ. This is Unc5c dependent. Once axons are in the DBRZ, either because of loss of ventral attraction or loss or dorsolateral repulsion by netrin 1, there is a separate netrin 1/Dcc-dependent, Unc5c-independent mechanism for CNS exit.

response of Dcc to netrin 1, converting it from an attractant to repellent activity (Höpker et al., 1999). Thus, the presence of laminin in the basal lamina encasing the CNS/PNS transition zones could therefore modulate the response of Dcc to netrin 1 at the DREZ. Alternatively, as motoneurons are the only CNS-derived neurons that normally exit the CNS, in the *Ntn1* and *Dcc* mutant embryos the misprojecting axons could commandeer the physical architecture and the chemotactic signals normally present that ensure motoneuron exit (Bravo-Ambrosio and Kaprielian, 2011; Bravo-Ambrosio et al., 2012; Lieberam et al., 2005). For example, spinal accessory motoneuron (SACMN) growth cones becomes long and slender, a property that could facilitate their passage through the small gaps in the end-feet distribution (Bravo-Ambrosio and Kaprielian, 2011; Snider and Palavali, 1990). Whether spinal interneurons that exit the CNS in the *Dcc*$^{-/-}$ and *Ntn1*$^{LacZ/LacZ}$ embryos also respond to cues that normally instruct motor exit remains to be determined.

Is netrin 1/Dcc signalling an evolutionary conserved mechanism for axonal confinement between invertebrates and vertebrates?

Two studies in *Drosophila* have described embryos with genetic modifications in the netrin 1 signalling pathway that have axonal confinement defects (Dorsten et al., 2010; Keleman and Dickson, 2001). As in vertebrates, CNS commissural axons within the ventral nerve cord (VNC) of *Drosophila* embryos are attracted to the midline by Netrin signalling via the attractive Netrin receptor Frazzled (Fra; Dcc in vertebrates) (Kolodziej et al., 1996). Embryonic overexpression of the repellent netrin 1 receptor Unc5 in *Drosophila* commissural neurons resulted in reversal of the response of axons to netrin 1 from attractive to repellent at the midline. This manipulation resulted in axons projecting in an opposite direction: away from the centrally located axonal tracts and instead into the periphery (Keleman and Dickson, 2001). Although this study was seminal in understanding of netrin 1

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receptor signalling, it did not address the issue of axonal confinement. Based on our new findings in mouse embryos, we have reinterpreted this aspect of the paper and suggest it supports our findings that loss of attractive midline signalling from netrin 1 results in loss of axonal confinement. It also provides proof that exit from the CNS is either permissive (i.e. through lack of attraction elsewhere) or that netrin 1 receptors are actively involved in regulating this process. A separate study revealed that, in *Drosophila*, *Fra* (the orthologue of vertebrate *Dcc*) is a sensitized allele for ectopic exit of CNS-derived axons (Dorsten et al., 2010). Although the mechanisms underlying this remain obscure, these studies, together with the study presented here raise interesting evolutionary parallels between vertebrates and invertebrates. The idea that mechanistic similarities exist between flies and mice is quite striking given the structural differences at the CNS/PNS interface in *Drosophila* and mouse embryos (Tripodi and Arber, 2012). This suggests that we have discovered a crucial piece of a larger mechanism for maintaining CNS/PNS integrity in vertebrates that may well be evolutionarily conserved.

MATERIALS AND METHODS

Mice

Animal experiments were performed according to institutional guidelines, national laws and approved by the regional ethical committees for animal experimentation. The *Ntn1* (referred to as $Ntn1^{LacZ/LacZ}$ and $Ntn1^{Gt(pGT1.8TM)629Wcs}$) (Serafini et al., 1996), *Dcc* (Dcc^{tm1Wbg}) (Fazeli et al., 1997), *Unc5c* ($Unc5c^{tvmTg(Ucp)1.23Kz}$) (Jackson Laboratories) (Ackerman et al., 1997) and *Unc5a* ($Unc5^{atm1Lhck}$) (Williams et al., 2006) mouse lines and a transgenic mouse line expressing GFP in dI1 neurons under the control of a *Barhl2* enhancer $Barhl2^{GFP}$ [*Tg(Barhl2-GFP)1Dodd*] (Wilson et al., 2008) were maintained on 129S6/Swiss Webster or C57Bl/6 mixed backgrounds. Staged embryos were obtained as previously described (Kropp and Wilson, 2012). $Ntn1^{LacZ/LacZ}$ embryos were genotyped by gPCR for neomycin using neo forward (5′ggagaggctattcggctatga3′) and neo reverse (5′ctttctcggcag-gagcaaggt3′) oligonucleotide primers, and *in situ* hybridization of spinal cord sections using a probe against exons 3-7 of mouse *Ntn1*. $Dcc^{+/-}$ and $Dcc^{-/-}$ embryos were genotyped by genomic PCR (gPCR) using wild-type forward (5′ggccattgaggttccttt3′), wild-type reverse (5′aagacgaccacacgcgag3′) and mutant reverse (5′tcctcgtgctttacggtatc3′) primers. *Unc5a* and *Unc5c* mutant mice and embryos were genotyped as previously described (Manitt et al., 2010; Williams et al., 2006). $Barhl2^{GFP}$ embryos were identified by epifluorescence.

Embryo processing

Staged embryos (confirmed by morphology) were pinned onto a silicone plate to straighten the spinal cord prior to fixation (75 minutes) in 4% (w/v) PFA in PBS and cryoprotected overnight in 30% (w/v) sucrose in PBS at 4°C. Embryos were mounted in 7.5% (w/v) gelatin in PBS, frozen and sectioned at 20 or 50 µm on a HM 505 E cryostat (Microm, Thermo Scientific, Walldorf, Germany).

Ex utero electroporation and mouse whole-embryo culture

Whole-embryo culture (WEC) and electroporation were performed as described previously with the following modifications (Osumi and Inoue, 2001; Takahashi et al., 2008): a BTC precision incubator rotating culture system was used (BTC Engineering, Cambridge, UK). Embryos were cultured using rat serum obtained from Harlan USA. Embryos were harvested at E10.5, DNA was injected into the neural tube at the thoracic level, then they were electroporated and cultured for 48 hours before fixation and processing as described above. *mCherry* (CMV-mCherry) and $Barhl2^{GFP}$ plasmid (which expresses GFP under the control of a *Barhl2* enhancer to label dI1 neurons) were used (Gray et al., 2006; Wilson et al., 2008).

Retrograde dextran labelling of the peripheral nerve

Biotin-conjugated dextran (MW3000, Molecular Probes) was used for retrograde labelling of the peripheral branch of the DRG in E12.5 mouse embryos. Embryos were dissected in Tyrode's solution at 37°C and dextran crystals applied in a surgically created pouch on the peripheral side of the DRG, followed by incubation in oxygenated rat serum for 4 hours and then processed as described above.

Immunohistochemistry

Immunohistochemistry was performed as described previously (Yamada et al., 1993). The following primary antibodies were used: chicken α-GFP (1/1000, GFP-1020; Aves Labs), β-galactosidase (1/1000, BGL-1040; Aves Labs), rabbit α-Robo3 (1/1000) (Sabatier et al., 2004), α-Tag-1 (1/10,000) (Dodd et al., 1988) and mouse α-2H3 (1/10) (Dodd et al., 1988). Cy5, Cy3 and FITC secondary antibodies and conjugated streptavidin-FITC were obtained from Jackson Immunochemicals and Aves Labs [anti-chicken fluorescein (1/400, F1005), anti-mouse Cy5 (1/400, 115-175-146), anti-rabbit Cy3 (1/1000, 111-165-144), DTAF streptavidin (1/500, 016-010-084), anti-chicken Cy3 (1/1000, 703-165-155)].

In situ hybridization

In situ hybridization was performed as previously described using a probe against mouse *Ntn1* exons 3-7, *mRobo 3.1* or *mBarhl2* (Schaeren-Wiemers and Gerfin-Moser, 1993). Double immunohistochemistry/*in situ* hybridization was performed as previously described (Kropp and Wilson, 2012).

Imaging

Samples were imaged using a Nikon Eclipse E800 fluorescence microscope and Zeiss LSM 710 confocal microscopes. Images were processed using Adobe Photoshop CS4 and assembled in Adobe Illustrator CS4. In Fig. 6, *Ntn1 in situ* hybridization data were imaged under bright-field illumination, converted to greyscale and colour-inverted for better visualization.

Axonal counts and statistical analysis

Axonal misprojections in E11.5 and E12.5 $Dcc^{-/-}$ and $Ntn1^{LacZ/LacZ}$ embryos were counted. No misprojecting axons were observed in wild-type embryos. At least five embryos for each group were counted. In Fig. 3A-F, the average number of ectopic neurites in the periphery per section were counted. Six to 14 sections distributed throughout cervical to lumbar levels were counted per embryo. The mean of sections within the same embryo was calculated. The values shown in Fig. 3A-F are means of these means from at least five embryos for each data point. For Fig. 3H,I,K,L, the data were sampled in a similar way except the sections were pooled into bins according the axial level. This resulted in each axial level from each individual embryo representing the mean value from 2 to 6 sections per embryo. Statistical significance was analysed using InStat 3 software (GraphPad). Pairs of data (the same axial level, axon type and genotype but different ages) were compared with using Student's *t*-test, two-tailed *P* value (*P*) with a Welch correction when the standard deviation of the two groups were significantly different (P′) (Fig. 3), whereas groups (the same genotype and age but different axial levels) were compared with one-way ANOVA with post-hoc Student-Newman-Keuls test (Fig. 3H,I,K,L).

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Competing interests

The authors declare no competing financial interests.

Author contributions

S.I.W. conceived and supervised the study. S.I.W. and C.L. designed and analysed the experiments, and wrote the manuscript. C.L., R.V.S., A.K. and S.I.W. edited the

manuscript. R.V.S. generated the *Unc5a* and *Unc5c* mutant embryos. S.I.W. carried out the *in situ* hybridization. C.L. performed all the remaining experimental work and assembled the figures.

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Supplementary material
Supplementary material available online at
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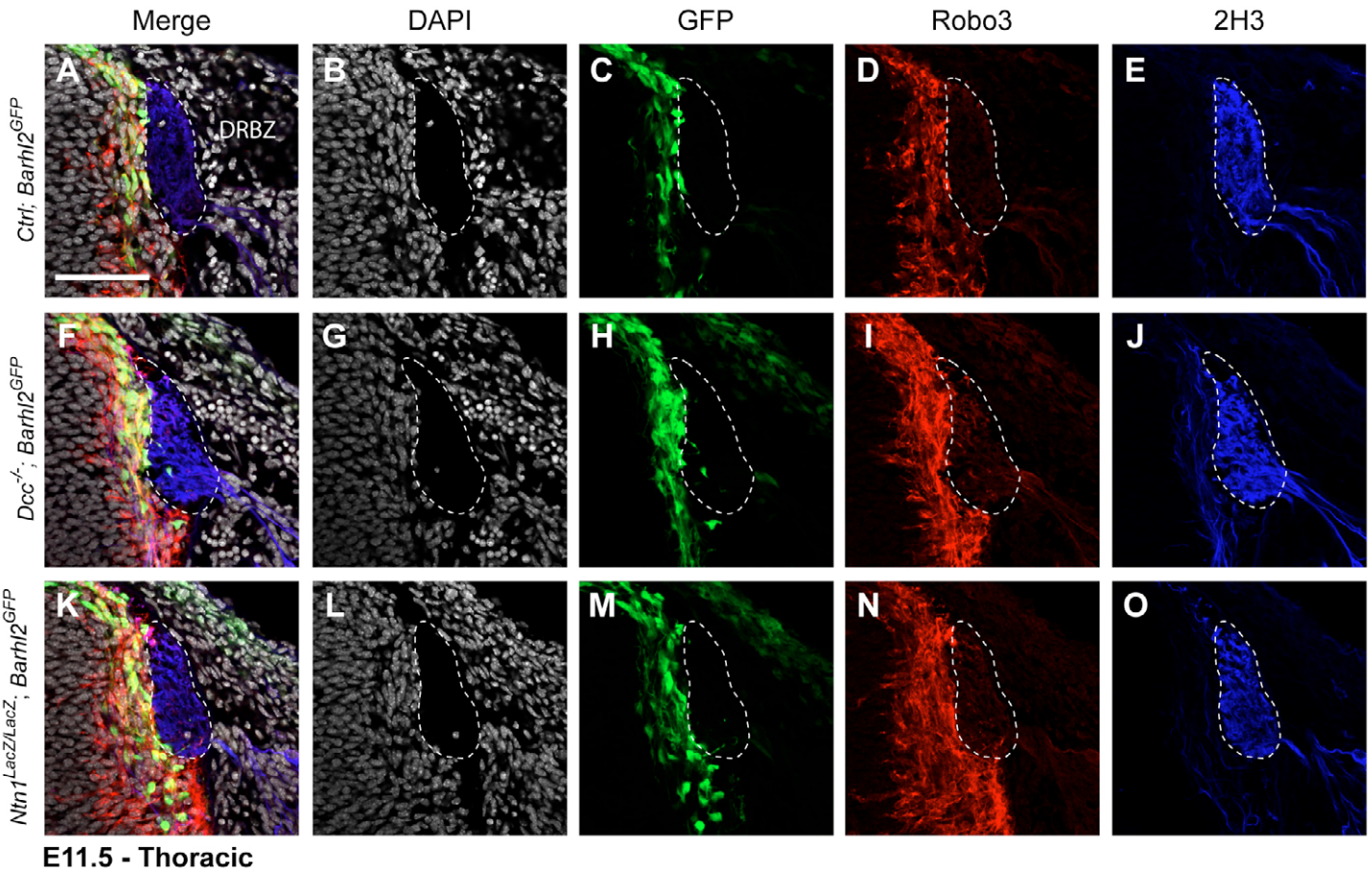

Fig. S1. CNS derived axons ectopically project into the DRBZ in *Dcc*$^{-/-}$ and *Ntn1*$^{LacZ/LacZ}$ embryos at E11.5. (A – O) Photomicrographs of transverse sections of the dorsal spinal cord of control; *Barhl2*^{GFP} (A - E), *Dcc*$^{-/-}$; *Barhl2*^{GFP} (F - J) and *Ntn1*$^{LacZ/LacZ}$; *Barhl2*^{GFP} embryos (K - O) at E11.5 showing DAPI (white), Robo3 (red), GFP (green) and 2H3 (blue). This shows relatively few CNS axons that ectopically enter the DRBZ abutting the DREZ at E11.5 in any genotype. Scale bar in A is 50 μm.