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Dorsally derived netrin 1 provides an inhibitory cue and elaborates the 'waiting period' for primary sensory axons in the developing spinal cord

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Dorsal root ganglion (DRG) neurons extend axons to specific targets in the gray matter of the spinal cord. During development, DRG axons grow into the dorsolateral margin of the spinal cord and projection into the dorsal mantle layer occurs after a 'waiting period' of a few days. Netrin 1 is a long-range diffusible factor expressed in the ventral midline of the developing neural tube, and has chemoattractive and chemorepulsive effects on growing axons. Netrin 1 is also expressed in the dorsal spinal cord. However, the roles of dorsally derived netrin 1 remain totally unknown. Here, we show that dorsal netrin 1 controls the correct guidance of primary sensory axons. During the waiting period, netrin 1 is transiently expressed or upregulated in the dorsal spinal cord, and the absence of netrin 1 results in the aberrant projection of sensory axons, including both cutaneous and proprioceptive afferents, into the dorsal mantle layer. Netrin 1 derived from the dorsal spinal cord, but not the floor plate, is involved in the correct projection of DRG axons. Furthermore, netrin 1 suppresses axon outgrowth from DRG in vitro. *Unc5c*^{rcm} mutant shows abnormal invasion of DRG axons as observed in netrin 1 mutants. These results are the first direct evidence that netrin 1 in the dorsal spinal cord acts as an inhibitory cue for primary sensory axons and is a crucial signal for the formation of sensory afferent neural networks.

KEY WORDS: Network formation, Dcc, Unc5c, RCM, Dorsal funiculus, DRG, Mouse

INTRODUCTION

Primary sensory axons from the dorsal root ganglion (DRG) convey different types of sensory submodalities. The projections of sensory axons to specific targets are crucial for the accurate perception of and reflex to external sensory information (Sprague, 1958). The central projections of DRG axons in the spinal cord are tightly regulated spatially and temporally, and different functional types of sensory neurons send afferents to different target areas in the spinal cord (Snider, 1994). During development, DRG axons enter the spinal cord at the dorsal root entry zone (DREZ) and then grow to the marginal zone of the spinal cord longitudinally to form the dorsal funiculus without penetrating the dorsal mantle layer. After a few days, proprioceptive afferents, which are involved in the muscle stretch reflex, penetrate the mantle layer and project ventrally through the dorsal layers. Subsequently, cutaneous sensory afferents start to send collaterals into the dorsal mantle layer and terminate in the dorsal-most laminae of the cord (Ozaki and Snider, 1997). Therefore, the projection pattern of DRG axons shows a delay between the formation of the dorsal funiculus and the extension of collaterals into the dorsal mantle layer, called the 'waiting period'. Based on this growth pattern of DRG axons, it has been presumed that repellant and/or inhibitory cues transiently prevent sensory afferents from penetrating the dorsal spinal cord during the waiting period (Ozaki and Snider, 1997). In other words, inhibitory cues are apparently required for the correct patterning of sensory afferents. For example, semaphorin (Sema) proteins are one of the candidates expressed in the spinal cord, and repel DRG axons in vitro (Messersmith et al., 1995; Shepherd et al., 1997; Masuda et al., 2003). However, the trajectory of dorsal root afferents is apparently normal in *Sema3a* and its receptor neuropilin 1 mutant embryos (Kitsukawa et al., 1997; Taniguchi et al., 1997). Thus, the functions of Sema proteins cannot fully explain the waiting period in vivo.

Netrin 1 was originally identified in the ventral midline of the neural tube as a bifunctional guidance molecule during early embryogenesis (Serafini et al., 1994; Kennedy et al., 1994); it is a long-range diffusible factor that exerts chemoattractive or chemorepulsive effects for distinct developing neural cells depending on the specific combination of Dcc and Unc5 receptors, thus regulating axon outgrowth and cell migration (Serafini et al., 1994; Colamarino and Tessier-Lavigne, 1995a; Keino-Masu et al., 1996; Ackerman et al., 1997; Leonardo et al., 1997; Hong et al., 1999; Yee et al., 1999). Netrin secreted from cells in the floor plate directs many axons to the midline (Colamarino and Tessier-Lavigne, 1995b; Tessier-Lavigne and Goodman, 1996). Netrin 1 is also weakly expressed in the developing dorsal spinal cord (Serafini et al., 1996). However, the function of dorsally derived netrin 1 is unknown.

In this study, we examined the mechanisms regulating the neural network formation of primary sensory axons in the spinal cord. Our results demonstrate that netrin 1 is transiently expressed near the DREZ during the waiting period, and that loss of netrin 1 results in aberrant invasion of cutaneous and proprioceptive afferents into the dorsal mantle layer without first growing along the marginal zone. Furthermore, netrin 1 suppresses axon outgrowth from DRG explants in vitro. Mutation of a netrin receptor *Unc5c* results in the aberrant projection of DRG axons. These findings clearly demonstrate that netrin 1 expressed in the dorsal spinal cord is

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necessary to prevent premature extension of primary sensory axons into the mantle layer and thus serves as a crucial signal for the proper formation of sensory neural networks.

MATERIALS AND METHODS

Animals

Timed-pregnant ICR mice were obtained from Japan SLC. Mice heterozygous for netrin 1 and for deleted in colorectal carcinoma (*Dcc*) were kindly provided by Dr M. Tessier-Lavigne (Genentech) (Serafini et al., 1996; Fazeli et al., 1997). Netrin 1 mutant mice were originally generated on a CD1 background but were mated onto C57BL6 or ICR background. Mice homozygous for netrin 1, *Dcc* and *Unc5c*^{rcm} mutations were obtained from heterozygote matings and were identified as described previously (Serafini et al., 1996; Ackerman et al., 1997; Fazeli et al., 1997). *Gli2* heterozygous mice were kindly provided by Dr C. C. Hui (University of Toronto) (Mo et al., 1997). These mice in a mixed background of 129/Sv and CD1 were mated onto an ICR background. The genotyping of *Gli2*-deficient mice was performed as described previously (Mo et al., 1997). All procedures were approved by the Animal Research Committee of the National Institute for Physiological Sciences.

The plug date was considered to be embryonic day 0.5 (E0.5). Embryos at E11.5-18.5 were harvested from anesthetized pregnant mice. For histological analysis, the embryos were fixed with 4% paraformaldehyde (PFA) in PBS overnight at 4°C and then incubated in PBS containing 20% sucrose at 4°C, embedded in OCT compound (Sakura Finetechnical). Frozen sections (18 μm) were cut on a cryostat (CM3050; Leica) and mounted onto MAS-coated glass slides (Matsunami). All analyses in this study were performed on the spinal cord at cervical and thoracic levels.

In situ hybridization

The following cDNAs were generated by RT-PCR and used as probes: mouse netrin 1 (GenBank Accession Number, NM_008744), *Dcc*, *Unc5a*, *Unc5b*, *Unc5c* (Sugimoto et al., 2001), *lacZ*, *Ebf1* (gifts from Dr H. Takebayashi), *Pax3* (a gift from Dr O. Chisaka), *Math1* (a gift from Dr R. Kageyama), *Lmx1b* (a gift from Dr B. Lee) and *Brn3a* (a gift from Dr E. E. Turner). Digoxigenin-labeled RNA probes were synthesized using DIG RNA-labeling kit (Roche Diagnostics). In situ hybridization was performed as described previously (Ding et al., 2005a). DIG-labeled RNA hybrids were reacted with alkaline phosphatase-conjugated anti-DIG antibody (Roche). Reaction product was visualized by incubating the sections with nitrobluetetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate (Roche).

Immunohistochemistry and X-gal staining

Cryostat sections were immunohistochemically stained as previously described (Ono et al., 2004). Sections were incubated with primary antibodies overnight at 4°C, and were then processed with the ABC method (Vector Lab.), following the manufacturer's protocol. Detection with horseradish peroxidase was performed by incubation in 0.05% diaminobenzidine and 0.015% hydrogen peroxide in PBS. For immunofluoresence, sections were labeled with species-specific secondary antibodies conjugated to Alexa Fluor 488 or 594 (Molecular Probes) and with Hoechst 33342 (Sigma) to visualize nuclei. The primary antibodies used were: mouse anti-neurofilament M (1C8; culture supernatant; 1:5), rabbit anti-TrkA (a gift from Dr L. F. Reichardt, UCSF; 1:2000) and rabbit anti-TrkC (Santa Cruz Biotechnology; 1:200) antibodies. Immunostaining of whole embryos and DRG explants was performed using the same method. For double labeling, in situ hybridization with *lacZ* probe was carried out, followed by immunostaining for neurofilament. X-gal staining was performed as previously described (Ding et al., 2005a).

BrdU labeling

Pregnant mice were given an intraperitoneal injection of BrdU (50 μg/g body weight; Sigma) at E10.5 or E11.5. Embryos were isolated at E12.5. BrdU was detected by immunostaining using mouse anti-BrdU antibody (BD Pharmingen; 1:500). For quantification, we used Photoshop (Adobe Systems) to divide the dorsal spinal cord into two, dorsal and ventral, halves, and BrdU-labeled neurons in the mantle layer were enumerated in two halves in six sections from three wild-type and netrin 1 mutant mice.

DiI tracing of DRG axons

To label DRG axons, E12.5 and E13.5 embryos were fixed in 4% PFA, and small crystals of 1,1′-dioctadecyl-3,3,3′,3″-tetramethylindocarbocyanine perchlorate (DiI; Molecular Probes) were put on the DRG. After incubation for 1-2 weeks at 37°C, 60 μm transverse sections were cut with a tissue slicer (DSK Microslicer). Free-floating sections were counterstained by Hoechst 33342 and mounted onto glass slides.

Collagen gel culture

An in vitro assay using collagen gel culture was performed as previously described with a slight modification (Kennedy et al., 1994; Masuda et al., 2003). Recombinant Sindbis virus for the expression of netrin 1 or EGFP was used, and BHK cell aggregates were prepared as previously described (Furuta et al., 2001; Sugimoto et al., 2001). DRG was dissected from the E13.5 ICR mouse embryos. DRG explants and BHK-cell aggregates were embedded in rat-tail collagen gels separated by a distance of 200-1000 μm. The explants in gels were incubated for 24-48 hours in DMEM containing 10% FBS, 50 ng/ml 7S nerve growth factor (NGF; Chemicon) and 50 ng/ml neurotrophin 3 (NT3; Sigma) at 37°C under 5% CO_2. Anti-netrin 1 rabbit polyclonal antibody (Oncogene Research Products) was added at a concentration of 2 μg/ml to neutralize the effect of netrin 1 secreted from BHK cells. After incubation, these explants were fixed with 4% PFA overnight at 4°C and placed in PBS for immunostaining. At least three independent cultures were carried out for statistical analysis (see below).

Quantification

For quantification of the disorganization of the dorsal funiculus, areas occupied by the dorsal funiculus formed in the marginal zone and ectopic axon bundles invading the mantle layer were measured by tracing the edges of bundles, which were identified by staining with neurofilament antibody on transverse sections of the spinal cord using ImageJ software (NIH). The boundary between the marginal zone and the mantle layer of the dorsal spinal cord was identified by NeuroTrace fluorescent Nissl stain (Molecular Probes). Eighteen sections from three embryos were quantified.

To quantify the axon outgrowth from DRG explants, the total axon surface area out of the explants stained by anti-neurofilament antibody was measured using Photoshop (Adobe) and Matlab software (Media Cybernetics), and analyzed using Student's *t*-test. In addition, axons from DRG explants co-cultured with BHK cells were grouped into four quadrants: proximal (p), distal (d) and two lateral quadrants. The data were expressed as the ratio between the area of axons present in the proximal and distal quadrants (p/d ratio) (Masuda et al., 2003).

RESULTS

Expression of netrin 1 and its receptors in the developing spinal cord

To find clues to the function of dorsally expressed netrin 1, we examined the temporal expression pattern of netrin 1, and its receptors Dcc and members of the Unc5 family by in situ hybridization. We focused on their expression during the early stage of sensory afferents projecting into the dorsal spinal cord (Ozaki and Snider, 1997). At E11.5, when many DRG axons have reached the DREZ, netrin 1 mRNA was not detected dorsally, although its signal was detected intensely in the floor plate and the ventral ventricular zone (Fig. 1A). At E12.5, when sensory axons grow longitudinally along the dorsolateral margin of the cord, and few collaterals enter the dorsal gray matter, netrin 1 expression was observed in the dorsolateral region adjacent to the DREZ (Fig. 1B,D). Signal intensity seemed to be weaker in the dorsal spinal cord than in the floor plate or even in the ventricular zone. In the E12.5 DRG, netrin receptors *Unc5a* and *Unc5c* were expressed, whereas *Dcc* and *Unc5b* transcripts were not detectable (Fig. 1F-I). At this stage, all receptors were expressed in the spinal cord as reported by others (data not shown) (Keino-Masu et al., 1996; Ackerman et al., 1997; Leonardo et al., 1997). In the E13.5 spinal cord, when many collaterals have entered the mantle layer, netrin 1 mRNA intensity

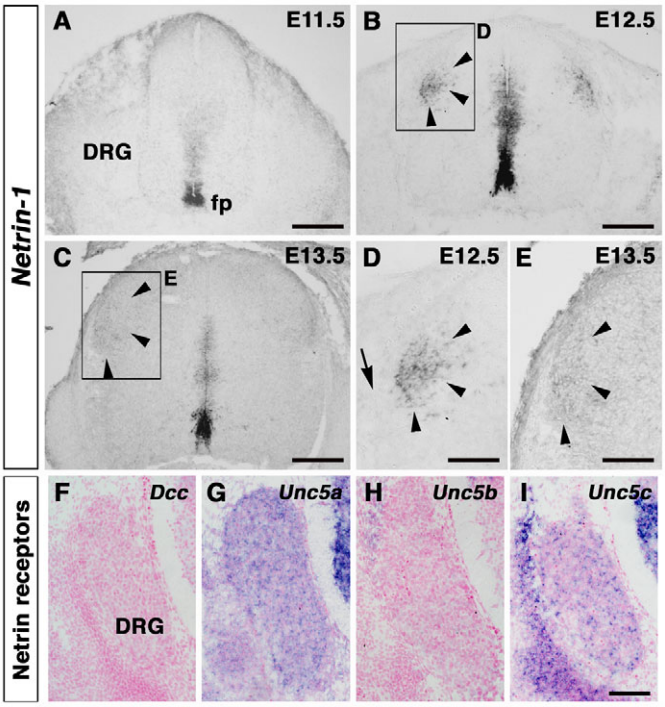

Fig. 1. Expression of netrin 1 and its receptors in the developing spinal cord and DRG. (**A-E**) Expression of netrin 1 mRNA visualized by in situ hybridization on transverse sections of the mouse spinal cord. At E11.5 (A), netrin 1 is expressed in the floor plate and the ventral-most part of the ventricular zone of the neural tube. At E12.5 (B,D), netrin 1 mRNA is localized in the dorsolateral region of the cord (arrowheads in B,D) as well as the floor plate and the ventral ventricular zone. The boxed area is magnified in D. At E13.5 (C,E), netrin 1 expression in the dorsal spinal cord is decreased (arrowheads in C,E). The boxed area is magnified in E. (D) Netrin 1-expressing cells are localized near the DREZ (arrow). fp, floor plate. (**F-I**) In situ hybridization for *Dcc* (F), *Unc5a* (G), *Unc5b* (H) and *Unc5c* (I) in the E12.5 mouse DRG. *Unc5a* and *Unc5c* are expressed in the DRG, whereas *Dcc* and *Unc5b* cannot be detected. Sections were counterstained with Nuclear Fast Red. Scale bar: 200 μm in A-C; 100 μm in D-I.

in the dorsal spinal cord was decreased when compared with that at E12.5 (Fig. 1C,E). Therefore, netrin 1 is transiently expressed or upregulated in the dorsal spinal cord during the period of sensory axon outgrowth but prior to the entry of these axons into the gray matter, suggesting that netrin 1 may have a role in primary sensory axon patterning in the spinal cord.

Disorganization of the dorsal funiculus in netrin 1 mutant mice

To verify the involvement of netrin 1 in primary sensory afferent patterning, we next examined whether netrin 1 mutant mice have defects in the projection of sensory axons into the dorsal spinal cord. As netrin 1 mutant mice die at birth, mutant embryos between E11.5 and E18.5 were used. Whole-mount immunostaining of E11.5 embryos with anti-neurofilament antibody demonstrated that DRG axons projected towards the spinal cord from the DRG in a similar fashion in both wild-type and netrin 1 mutant mice (Fig. 2A,B). In addition, the dorsal funiculus extended longitudinally in both animals (Fig. 2A,B).

Next, the axonal pattern was visualized on transverse sections of the spinal cord. In the E11.5 wild-type mice, the dorsal funiculus was formed in the dorsolateral margin of the spinal cord (data not shown). Subsequently, the dorsal funiculus developed well as a crescent-shape with a sharp inner border facing the dorsal mantle layer, and very few collaterals invaded the mantle layer at E12.5 (Fig. 2C). By E13.5, collaterals had begun entering the mantle layer from the dorsomedial region of the dorsal funiculus, projecting ventrally (Fig. 2G). This projection pattern was compatible with a previous observation by Ozaki and Snider (Ozaki and Snider, 1997). In netrin 1 mutant mice at E11.5, the formation of the dorsal funiculus was almost identical to that observed in wild-type animals except for slight dorsal extension of the funiculus (data not shown). At E12.5, the dorsal funiculus was dramatically defasciculated with an unclear inner border, and neurofilament-positive axons dispersed within the superficial part of the dorsal mantle layer (Fig. 2D). As the netrin 1 mutant allele harbors a *lacZ* insertion, *lacZ* expression recapitulates the pattern of endogenous netrin 1 (Serafini et al., 1996; Charron et al., 2003). We performed immunostaining for neurofilament after in situ hybridization with a *lacZ* probe in netrin 1 heterozygous and homozygous mice (Fig. 2E,F). In netrin 1-deficient mice, many axon bundles directly invaded the *lacZ*-expressing zone of the dorsal spinal cord (Fig. 2F), whereas very few axons entered the netrin 1-*lacZ*-expressing areas in heterozygous animals (Fig. 2E). The quantitative analysis demonstrated that the dorsal funiculus in the correct superficial position was markedly decreased in netrin 1-deficient mice, less than in heterozygous or wild-type animals at E12.5 (Table 1). By contrast, aberrant projections in the dorsal gray matter were more common than normal projections in the mutants. The dorsal funiculus in netrin 1 heterozygous mice was normally formed with a sharp inner border, and was only slightly thinner than that of wild-type mice (Table 1). At E13.5, the dorsal funiculus in the marginal zone of netrin 1 mutant mice is thinner than that of wild-type mice (Fig. 2G,H). Moreover, thick axon bundles were observed within the dorsal mantle layer in netrin 1 mutants (Fig. 2H). The ectopic axon bundles extended towards the ventricular zone and turned dorsally. In spite of the disorganization of the dorsal funiculus, some neurofilament-positive fibers extended ventrally from the ectopic dorsal funiculus as observed in the normal spinal cord (Fig. 2G,H). Disorganization of the dorsal funiculus was observed throughout the spinal cord from E12.5 onwards, and the extent of disorganization became more severe as the embryos matured (Fig. 2, see Fig. S1 in the supplementary material).

Aberrant projections of both cutaneous and proprioceptive afferents in the absence of netrin 1

To further confirm that fibers affected by netrin 1 deficiency are DRG axons, we performed DiI labeling of DRG axons in wild-type and netrin 1 mutant mice. In the E12.5 wild-type animals, DRG axons bifurcated at the DREZ and extended longitudinally for more than three segments (Fig. 3A) (Ozaki and Snider, 1997). In netrin 1 mutant mice, longitudinal tracts were established as observed in wild-type mice (Fig. 3B).

We next examined axonal trajectories on transverse sections. In the E12.5 netrin 1 mutants, DRG axons stacked near the DREZ, and some axons entered the lateral part of the dorsal gray matter (data not shown). The defects of axonal patterning were more severe at E13.5 than at E12.5 (Fig. 2). At E13.5, the dorsal funiculus had been formed in the dorsolateral margin of the wild-type spinal cord (Fig. 3C). By contrast, thick axon bundles directly invaded the dorsal mantle layer without elongating along the dorsolateral margin of the cord in netrin 1-deficient mice, as observed in neurofilament staining (Fig. 3D).

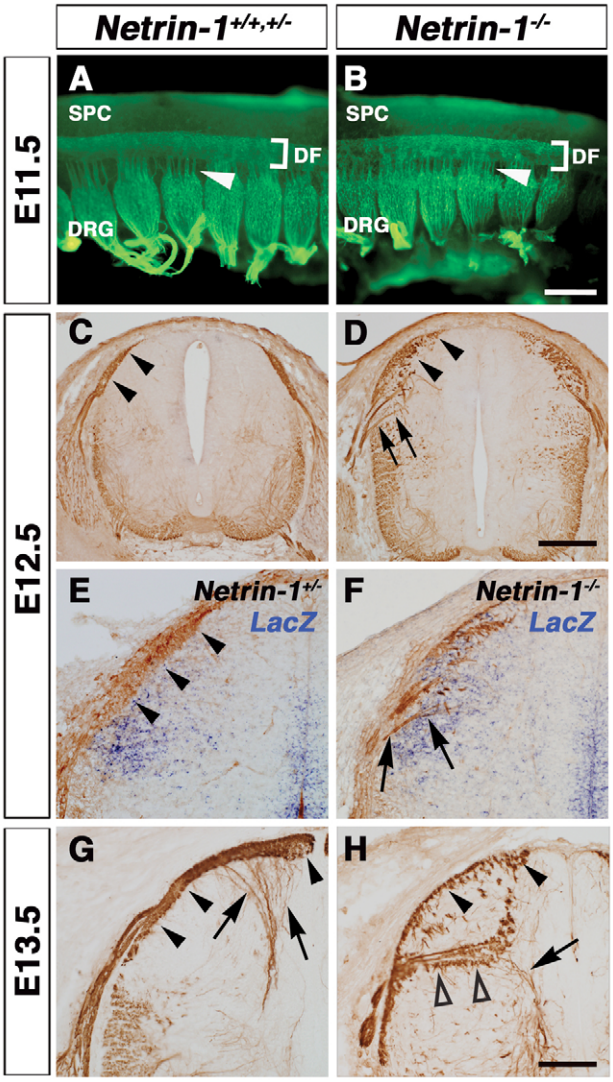

Fig. 2. The dorsal funiculus is disorganized in the developing spinal cord of netrin 1 mutant mice. Whole-mount preparations (A,B) and transverse sections (C-H) stained with anti-neurofilament antibody from wild-type (A,C,G), netrin 1 heterozygous (E) and homozygous (B,D,F,H) mice. (**A**,**B**) E11.5. In both animals, DRG axons correctly project from the DRG to the spinal cord to form dorsal rootlets (arrowheads) and extend longitudinally in the dorsal spinal cord. DF, dorsal funiculus; SPC, spinal cord. (**C-F**) E12.5. DRG axons grow to the dorsolateral margin of the spinal cord forming the dorsal funiculus, and very few axons invade the dorsal mantle layer of wild-type and netrin 1 heterozygous mice (arrowheads in C,E). By contrast, the dorsal funiculus is severely disorganized with the loss of a clear inner border in netrin 1 mutant mice (arrowheads in D). Many axon bundles directly enter the dorsal mantle layer (arrows in D,F). Sections in E and F are processed for *lacZ* in situ hybridization followed by neurofilament immunohistochemistry. In netrin 1 mutants, direct invasion of axons into the dorsolateral part of the spinal cord in which netrin 1 expression is lost (demarcated by *lacZ* expression) is observed (arrows in F). (**G**,**H**) E13.5. In wild-type mice (G), the dorsal funiculus extends to the most dorsomedial region of the spinal cord (arrowheads in G), and some collaterals enter the dorsal spinal cord from the dorsomedial region of the dorsal funiculus (arrows in G). In netrin 1 mutant mice (H), the dorsal funiculus in the marginal zone is thinner than that in wild-type mice (arrowheads in G,H), and the inner border is less sharp. Moreover, thick axon bundles are observed within the dorsal mantle layer of netrin 1 mutants (open arrowheads in H). As observed in wild-type animals, some neurofilament-positive fibers extend ventrally from this ectopic funiculus (G,H arrows). Scale bar: 250 μm in A,B; 200 μm in C,D; 100 μm in E-H.

To elucidate which types of sensory axons aberrantly project into the dorsal spinal cord of netrin 1 mutants, subclasses of DRG axons were labeled by TrkA as a marker of thermoceptive and nociceptive cutaneous afferents and by TrkC for proprioceptive afferents (Huang and Reichardt, 2001). In wild-type mice, both TrkA- and TrkC-positive afferents grew into the marginal zone of the spinal cord, and no axons entered the dorsal gray matter at E12.5 (Fig. 3E,F). In netrin 1 mutant mice, TrkA-positive axons stayed near the DREZ, and several axons aberrantly entered the dorsal gray matter (Fig. 3G). TrkC-positive axon bundles also penetrated the dorsal mantle layer (Fig. 3H). These results suggest that netrin 1 is required for the appropriate guidance of primary sensory axons, including both cutaneous and proprioceptive afferents.

Suppression of axon outgrowth from DRG by netrin 1 in vitro

The above results strongly suggest that netrin 1 is necessary for the accurate projection of DRG axons. To determine the function of netrin 1 in the outgrowth of DRG axons, DRG explants were co-cultured in collagen gels with aggregates of BHK cells expressing EGFP or netrin 1 via Sindbis virus transfection. After 24-48 hours, total axonal outgrowth from DRG cultured adjacent to netrin 1-expressing cells was dramatically inhibited when compared with control cell aggregates that expressed EGFP (Fig. 4A,B,D). Although less axon outgrowth was observed in the presence of netrin 1-expressing cells, there did not seem to be a specific effect on either axon attraction or repulsion (p/d values: EGFP, 0.9; netrin 1, 1.0; not statistically significant; see Materials and methods). Axon outgrowth was rescued by the application of anti-netrin 1 antibody to the DRG co-cultures with netrin 1-expressing cells (Fig. 4B,C,D). These results support the hypothesis that netrin 1 provides an inhibitory cue for DRG axons, preventing these axons from penetrating the dorsal gray matter directly.

Evidence that dorsally derived netrin 1 is crucial for the correct projection of sensory afferents

As netrin 1 expressed in the floor plate apparently influences the dorsal spinal cord in the early stages (Tessier-Lavigne et al., 1988), we next examined whether netrin 1 secreted from the dorsal spinal cord regulates the DRG axon pathfinding. To do this, we took advantage of *Gli2* mutant mice, which lack netrin 1 expression in the floor plate because of a marked decrease in floor-plate cells (Matise

Table 1. Quantification of the area occupied by the dorsal funiculus in the E12.5 spinal cord

Genotype	Defect*	Dorsal funiculus area±s.d. (μm²)	
		Correct projections	Aberrant projections
Wild type	0/4	13272±1017	0
Netrin 1$^{+/-}$	1/9	9163±2549	33±99
Netrin 1$^{-/-}$	5/5	4676±1763	8692±3087

*Defects indicate the number of embryos that show direct invasion of the axons into the dorsal horn.

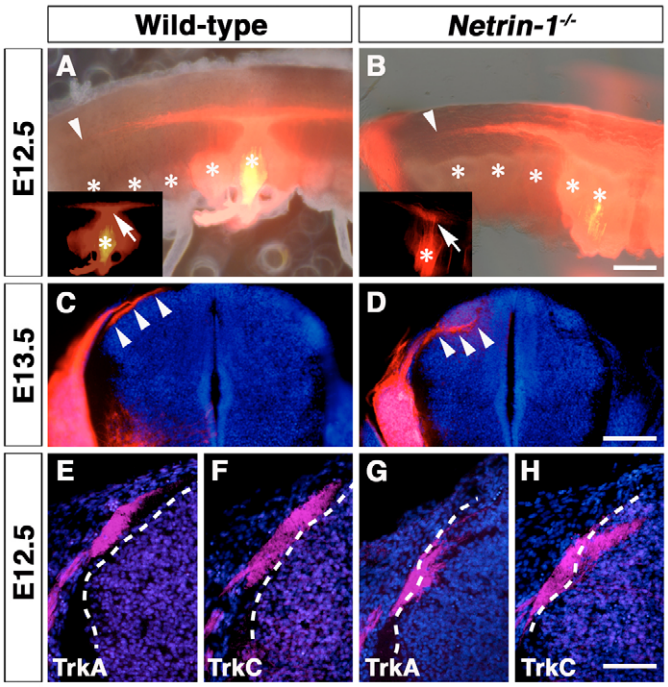

Fig. 3. Both cutaneous and proprioceptive afferents directly invade the dorsal mantle layer in the absence of netrin 1. (A,C,E,F) Wild-type mice. (B,D,G,H) Netrin 1 mutant mice. DRG axons in the lateral views of whole-mount E12.5 spinal cord preparations (A,B) and on transverse sections of the E13.5 spinal cord (C,D) labeled by DiI, and immunohistochemically labeled with anti-TrkA (E,G) or TrkC (F,H) antibodies at E12.5. (**A**,**B**) In both animals, DRG axons bifurcate at DREZ (arrow in insets) and extend longitudinally more than three segments (arrowheads). An epifluorescent picture is merged onto a differential interference contrast (DIC) picture. Insets indicate the site of DiI injection in the DRG. Asterisks indicate DRG. (**C**,**D**) In E13.5 wild-type animals, the dorsal funiculus has been formed in the marginal zone, which is composed of DiI-labeled fibers (arrowheads in C). However, in netrin 1 mutant mice, most DRG axons directly invade the dorsal mantle layer forming the ectopic dorsal funiculus (arrowheads in D). (**E-H**) In wild-type mice, TrkA- (E) and TrkC-positive (F) fibers project to the marginal zone, and their projection into the dorsal mantle layer is not observed. In netrin 1 mutants, TrkA-positive axon bundles stay near the DREZ (G), and many TrkA- and TrkC-positive (H) fibers aberrantly project into the dorsal mantle layer. White broken lines indicate the boundary between the marginal zone and the dorsal mantle layer. Sections were counterstained with Hoechst 33342 (blue). Scale bar: 250 μm in A,B; 200 μm in C,D; 100 μm in E-H.

et al., 1999; Charron et al., 2003). Netrin 1 expression was obviously weakened in the ventral region of the E12.5 *Gli2*-deficient spinal cord (Fig. 5A,B), while netrin 1 mRNA appeared to be normally expressed in the dorsal spinal cord of mutants when compared with wild-type and heterozygous mice (Fig. 5A,B). Although the overall shape of the spinal cord was extremely abnormal in the *Gli2* knockout mice, the dorsal funiculus was correctly formed with a sharp inner border, and lacked defasciculation or axonal invasion defects as observed in netrin 1 mutant embryos (Fig. 5C,D). These results indicate that dorsally derived netrin 1 influences the axonal patterning of primary sensory afferents.

Expression of netrin 1 in early-born neurons of the dorsal spinal cord

Dorsal interneurons are born in two waves of neurogenesis: early-born neurons (E10-12.5) predominantly settle in the deep layer of the dorsal horn, whereas late-born neurons (E11-13) populate the superficial layer (Caspary and Anderson, 2003; Helms and Johnson, 2003; Ding et al., 2005b). Moreover, early-born neurons are important for the correct projection of DRG axons (Ding et al., 2005b). To examine netrin 1 expression in early-born neurons, we performed a BrdU labeling experiment. We carried out immunostaining for BrdU after *lacZ* in situ hybridization in netrin 1 heterozygous and homozygous mice. Pregnant mice were given an injection of BrdU at E10.5 and allowed to develop for another 48 hours. Many BrdU-positive cells in the dorsolateral spinal cord were co-labeled with *lacZ* (see Fig. S2A,B in the supplementary material). Moreover, we observed that *lacZ*-expressing cells were correctly localized in the E12.5 netrin 1 heterozygous and homozygous dorsolateral spinal cord (Fig. 2E,F), whereas many X-gal-positive cells were aberrantly distributed in the dorsolateral spinal cord of the mutants at later stages such as E14.5 (Fig. S2C,D). Therefore, early-born neurons express dorsally derived netrin 1 and are correctly settled in the dorsolateral spinal cord around E12.5 in netrin 1 mutants.

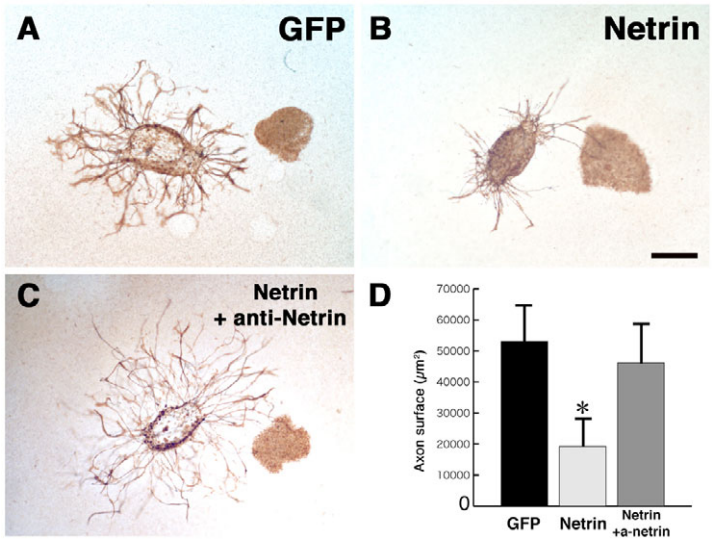

Fig. 4. Netrin 1 suppresses axon outgrowth from DRG explants in vitro. E13.5 mouse DRG explants were co-cultured for 24-48 hours with aggregates of BHK cells expressing EGFP (**A**) or netrin 1 (**B**,**C**) in collagen gel. Explants were stained with anti-neurofilament antibody. Axon outgrowth from DRG explants co-cultured with netrin 1-expressing cells is inhibited (B) compared with that of control DRG explants cultured with EGFP-expressing cells (A). (C) The inhibitory effects are neutralized by the application of 2 μg/ml anti-netrin 1 rabbit antibodies. Scale bar: 250 μm. (**D**) Quantification of axon growth from DRG. The areas covered by neurofilament-positive axons were measured. **P*<0.05.

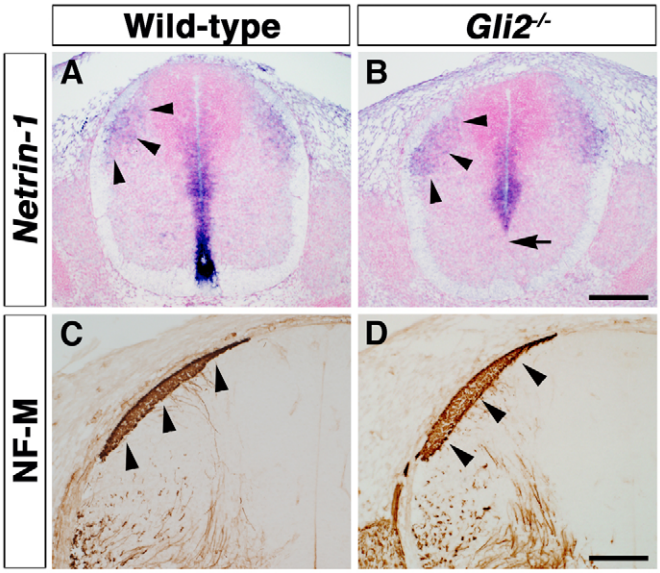

Fig. 5. Dorsally derived netrin 1 is important for the projection of sensory afferents. In situ hybridization for netrin 1 (A,B) and neurofilament immunohistochemistry (C,D) in transverse sections of the E12.5 spinal cord from wild-type (A,C) and *Gli2* mutant (B,D) mice. (**A**) Netrin 1 is expressed in the floor plate and the ventral ventricular zone of the neural tube and at low levels in the dorsolateral spinal cord (arrowheads) in wild-type mice. (**B**) Netrin 1 expression in the ventral-most spinal cord is lost in *Gli2* mutant mice (arrow), while the dorsolateral spinal cord expresses netrin 1 similarly to wild-type mice (arrowheads). (**C**,**D**) In both animals, the dorsal funiculus is correctly established in the marginal zone of the spinal cord with a sharp inner border (arrowheads). Scale bar: 200 μm in A,B; 100 μm in C,D.

Normal neural patterning in the dorsal spinal cord of netrin 1 mutant mice

In netrin 1 mutant mice, a small island of ectopic neurons was found in the dorsolateral spinal cord (Fig. 6A,B). To determine if the aberrant projection of sensory afferents observed in netrin 1 mutant mice is a result of abnormalities in the embryonic patterning of dorsal neurons, we analyzed the arrangement of dorsal neural cells by the expression of several cell type-specific markers in the E11.5 and E12.5 spinal cord (see Materials and methods) (Goulding et al., 1991; Ninkina et al., 1993; Akazawa et al., 1995; Wang et al., 1997; Chen et al., 1998). We were unable to detect any obvious alternation in the expression pattern of these markers between wild-type and netrin 1 mutant mice at the stages observed. For example, *Math1* and *Pax3* expressions at E12.5 were observed in the dorsal ventricular zone of the cord in both animals (*Pax3*, Fig. 6C,D; *Math1*, data not shown). *Brn3a*, *Lmx1b* and *Ebf1* were also expressed in the lateral margin of the dorsal ventricular zone and in the dorsal mantle layer of both animals (*Lmx1b*, Fig. 6E,F; others, data not shown).

As mice lacking *Dcc* show abnormal ventral migration of early-born neurons (Ding et al., 2005b), we next performed a BrdU labeling experiment to examine the ventral migration of dorsal neurons. We analyzed the distribution of E10.5 or E11.5 BrdU-labeled neurons in the E12.5 dorsal spinal cord. As a result, we could not detect any significant change in the ratio of BrdU-positive cells in the dorsal half and ventral half of the dorsal spinal cord between wild-type and netrin 1 mutant mice (Fig. 6G-K), indicating that the ventral migration of dorsal neurons is nearly normal in netrin 1-deficient mice. Although the majority of early-born neurons migrated ventrally (Fig. 6G,H), a few BrdU-positive cells were

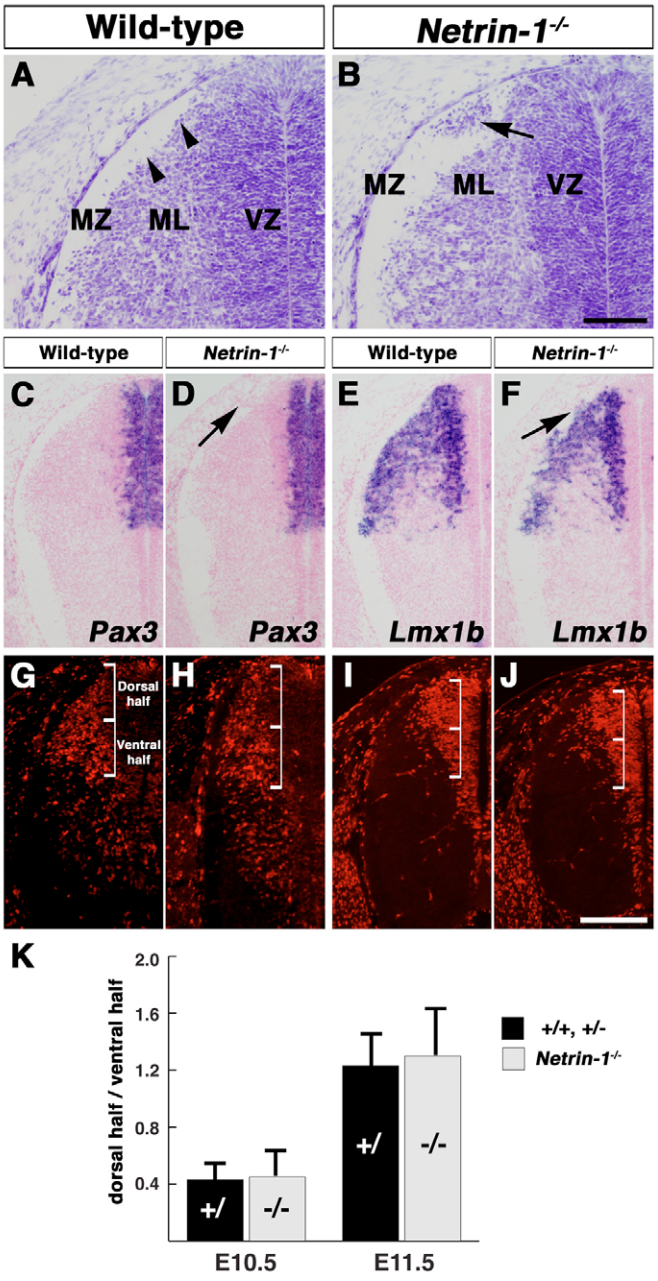

Fig. 6. Dorsal neural cell patterning is largely normal in netrin 1 mutant mice. Cellular patterning in the dorsal spinal cord demonstrated by Nissl staining (A,B) and in situ hybridization for *Pax3* (C,D) and *Lmx1b* (E,F) on transverse sections from the E12.5 spinal cord. (G-K) BrdU labeling experiment. (A,C,E,G,I) Wild-type mice. (B,D,F,H,J) Netrin 1 mutant mice. (**A**) In wild-type mice, Nissl staining reveals a three-layered structure, and few cells are observed in the marginal zone (dorsal funiculus) which shows a sharp inner border (arrowheads). (**B**) In netrin 1 mutant mice, an ectopic small island is observed in the marginal zone (arrows in B,D,F), although a three-layered structure is formed. VZ, ventricular zone; ML, mantle layer; MZ, marginal zone. (**C-F**) *Pax3* labels dorsal ventricular cells (C,D), and *Lmx1b* expression is found in cells in the dorsal mantle layer (E,F), both of which show few differences between wild-type and netrin 1 mutant mice. (**G-J**) The distribution of E10.5 (G,H) or E11.5 (I,J) BrdU-labeled cells in the E12.5 spinal cord. Many BrdU-labeled cells are observed in the dorsal mantle layer. (**K**) Quantification analysis of BrdU-labeled cells between the dorsal half and the ventral half of the dorsal spinal cord. Scale bar: 100 μm in A,B; 200 μm in C-J.

found in the superficial part of the wild/heterozygous dorsolateral mantle layer and in the ectopic cell island of the mutant spinal cord at E12.5, following E10.5 BrdU injection (data not shown). Therefore, local patterning of early-born neurons seems similar between the two groups in this stage. These results suggest that defects of axonal pathfinding in netrin 1 mutants are not secondary to abnormal neural patterning in the dorsal spinal cord, especially in the early stage.

Aberrant projection of DRG axons in *Unc5c*^{rcm} mutant mice

To elucidate the netrin receptors on which the projection of sensory afferents is dependent, we next analyzed the trajectories of sensory axons in *Dcc* mutant and *Unc5c*^{rcm} mutant mice (Ackerman et al., 1997; Fazeli et al., 1997). In the E12.5 spinal cord of *Dcc*-deficient mice, the dorsal funiculus was established in the correct position, and both TrkA- and TrkC-positive afferents grew into the marginal zone as wild-type mice (Fig. 7A,C,D; Figs 2, 3). The dorsal funiculus of *Dcc* mutant mice seemed to be slightly defasiculated when compared with that of wild-type mice, and some neurofilamant-positive fibers were observed in the mantle layer (Fig. 7A; Fig. 2, data not shown). However, the direct invasion of thick axon bundles into the dorsal spinal cord, which was observed in netrin 1 mutant mice, was not found in *Dcc* mutants at this stage, and thus the inner border of the dorsal funiculus was relatively sharp (Fig. 7A,C,D; Figs 2, 3). As previously reported, in E13.5 *Dcc* mutants, many fibers entered the dorsal mantle layer from the lateral dorsal funiculus, whereas fibers also projected from the dorsomedial region as seen in wild-type mice (see Fig. S3 in the supplementary material) (Ding et al., 2005b).

In E12.5 *Unc5c*^{rcm} mutant mice, neurofilament-positive axon bundles containing TrkA- and TrkC-positive fibers aberrantly invaded the mantle layer identified by NeuroTrace staining, resulting in an unclear inner border, whereas the dorsomedial region of the dorsal funiculus had a sharp inner border to the mantle layer (Fig. 7B,E,F; Fig. 2). As a result, the dorsal funiculus of *Unc5c*^{rcm} mutants had a triangle-shaped protrusion to the dorsal mantle layer at E12.5 (Fig. 7B). Quantitatively, 14.6±6.1% of axon bundles abnormally existed in the mantle layer of the *Unc5c*^{rcm} spinal cord. These results, together with the expression of *Unc5* in the DRG, suggest that Unc5 receptor is required for the correct projection of DRG axons.

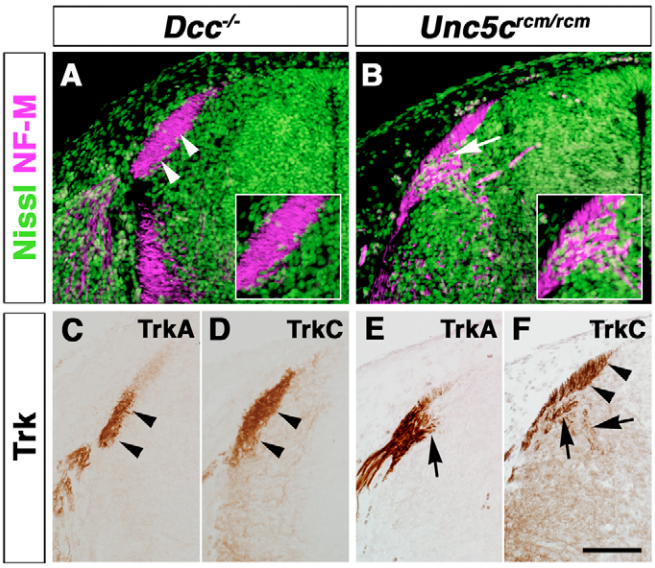

Fig. 7. Mutation of *Unc5c* results in the disorganization of the dorsal funiculus. Cytoarchitecture and axonal patterning of the E12.5 *Dcc* mutant and *Unc5c*^{rcm} mutant dorsal spinal cord revealed by double staining with Nissl method and neurofilament immunohistochemistry (A,B), and by immunohistochemistry for TrkA (C,E) and TrkC (D,F). (A,C,D) *Dcc* mutant mice. (B,E,F) *Unc5c*^{rcm} mutant mice. (**A**) The dorsal funiculus is normally formed in *Dcc* mutant mice with a sharp inner border (arrowheads). (**B**) The *Unc5c*^{rcm} mutant dorsal spinal cord contains an ectopic cell island in the marginal zone (arrow), and neurofilament-positive axons form a triangle dorsal funiculus with an irregular inner border at the ventral part of the dorsal funiculus. Insets indicate higher magnification of the dorsal funiculus. (**C**,**D**) Both TrkA- and TrkC-positive afferents grow into the marginal zone without invading the dorsal gray matter, both of which show a sharp inner border (arrowheads). (**E**,**F**) TrkA- and TrkC-positive axons in *Unc5c*^{rcm} mutant mice form an aberrant dorsal funiculus, slightly spreading into the dorsal gray matter (arrows). The medial region of the dorsal funiculus has a sharp border to the dorsal mantle layer (arrowheads in F). Scale bar: 100 μm.

DISCUSSION

It has previously been reported that netrin 1 is expressed not only in the ventral midline but also in the dorsolateral spinal cord, raising the possibility that netrin 1 plays a role in axon guidance in the dorsal local region. However, this possibility has not been examined previously. In this study, we substantiated the role of dorsally derived netrin 1; our results provide the first direct evidence of the involvement of netrin 1 in regulating growing DRG axons in the dorsal spinal cord (Fig. 8).

Netrin 1 is an inhibitory cue for growing DRG axons, elaborating the waiting period

The most important finding in this study is that dorsal netrin 1 provides an inhibitory cue for DRG axons. Netrin 1 is expressed in the dorsal spinal cord during the middle time window of the waiting period for DRG axons. Loss of dorsal netrin 1 leads to premature invasion of cutaneous and proprioceptive afferents into the mantle layer. Thus, netrin 1-deficient mice show loss of this waiting period at E12.5 onwards (right box in Fig. 8). We also suggest that aberrant projections of DRG axons are not due to abnormal cell migration or patterning in the dorsal spinal cord, which is reported in the spinal

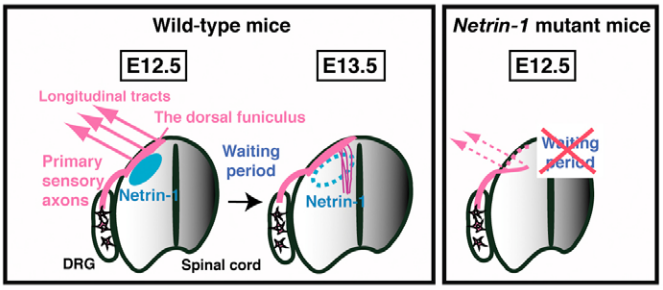

Fig. 8. Schematic diagram summarizing the function of dorsally derived netrin 1 on primary sensory afferents. (**Left**) In the E12.5 wild-type spinal cord, primary sensory axons have formed the dorsal funiculus in the dorsolateral margin of the spinal cord and extend longitudinally without extending collaterals into the dorsal spinal cord. At this stage, netrin 1 is expressed in the dorsal spinal cord and prevents the axons from entering the mantle layer. At E13.5, netrin 1 expression is decreased, allowing some collaterals (probably proprioceptive afferents) to enter the dorsal spinal cord. (**Right**) In the absence of netrin 1, the 'waiting period' is abolished at this developmental stage (E12.5), and many sensory axons directly enter the dorsal mantle layer.

cord development of *Dcc* knockout mice (see Fig. 6) (Ding et al., 2005b). Furthermore, our in vitro studies elucidated the inhibitory effect of netrin 1 on axon outgrowth from DRG explants (see Fig. 4). Taken together, our results provide strong evidence that dorsally derived netrin 1 transiently inhibits the direct invasion of DRG axons into the dorsal mantle layer of the spinal cord and thus elaborates the waiting period.

Netrin 1 may act as a short-range guidance cue for primary sensory axons

In mouse DRG, *Unc5* family receptors, but not *Dcc*, are predominantly expressed during development (see Fig. 1) (Keino-Masu et al., 1996; Ackerman et al., 1997; Leonardo et al., 1997). *Unc5c*^{rcm} mice show aberrant projections of DRG axons, whereas the dorsal funiculus of *Dcc* mutant mice is normally established at E12.5 (see Fig. 7). As netrin 1 mutant mice show more severe defects than *Unc5c*^{rcm} mutants, it is probable that the netrin 1 signal is mediated not only by Unc5c receptor but also by other receptors such as Unc5a, which is also expressed in the DRG, and that moderate defects in the *Unc5c*^{rcm} mutant dorsal spinal cord may be caused by functional redundancy with such receptors. Thus, dorsal netrin 1 probably functions in DRG axons through Unc5 family receptors around E12.5. In our studies, netrin 1 expressed in the dorsal spinal cord is not as intense as that in the floor plate and thus could be localized only close to the DREZ. We observed slightly impaired projections of DRG axons in netrin 1$^{+/-}$ mice (see Table 1), suggesting the dose dependency of the defects in the dorsal spinal cord. Such a dose-dependent response of DRG axons to netrin 1 was also examined in vitro; BHK cells expressing netrin 1 were diluted with uninfected BHK cells, which were co-cultured with DRG explants in the same experiment (Liu et al., 2005). BHK cell aggregates diluted at 1:10 (netrin 1-expressing cells: uninfected cells) had an inhibitory effect, whereas those at 1:100 had no inhibitory effect (data not shown). Therefore, netrin 1 may have an inhibitory effect at relatively higher concentrations on DRG axons. Interestingly, it has been reported that the ectopic expression of UNC5 elicits a short-range response in the absence of the *Drosophila* Dcc homolog Frazzled, in which UNC5 causes the axons to stop elongation, rather than directing them to repel away from the source of netrin (Keleman and Dickson, 2001). Dorsal netrin 1 may act as a short-range inhibitory cue for sensory afferents through Unc5 family receptors without cooperation of the Dcc. The dorsal netrin 1 signal to DRG axons functions to form the dorsal funiculus in the correct position: the marginal zone of the dorsolateral spinal cord.

Multiple guidance molecules may elaborate the waiting period for sensory afferents in the developing spinal cord

Dorsal netrin 1 is expressed or upregulated in the restricted time window of the waiting period at E12.5, while the arrest of axon collateral extension into the dorsal spinal cord is observed from E10.5 to E14.5, depending on the axon subclasses (Ozaki and Snider, 1997). Therefore, it is apparent that molecules other than netrin 1 also inhibit the invasion of axons into the dorsal spinal cord during early and late phases of the waiting period. For example, Sema3a repels NGF-responsive axons in vitro (Messersmith et al., 1995). In addition, Wang et al. (Wang et al., 1999) suggested that slit controls the initial collateral branching of DRG axons in vitro, whereas netrin 1 has no effect on this branching. Furthermore, transcription factors and cell surface molecules, such as Drg11, Runx3, Er81 and F11, are involved in the correct projection of DRG axons (Arber et al., 2000; Chen et al., 2001; Inoue et al., 2002; Perrin et al., 2001). These observations suggest that multiple molecules orchestrate to elaborate the total waiting period for sensory afferents. Further studies are required to fully elucidate the mechanisms underlying the whole waiting period.

Dorsally derived netrin 1 is involved in dorsal spinal cord formation in both direct and indirect manners

During development, the waiting period has been demonstrated in many regions (Schreyer and Jones, 1982; Ghosh and Shatz, 1992; Renzi et al., 2000; Wang and Scott, 2000). The waiting period is thought to be important for the formation of proper neural networks. In netrin 1 mutant mice, which lack the middle phase of the waiting period, disorganization of the cytoarchitecture in the dorsal spinal cord becomes more severe at E13.5 and later, although defects in the early patterning of dorsal cells are subtle (see Figs 2, 6; Fig. S1 in the supplementary material; data not shown). Furthermore, in netrin 1, *Dcc* and *Unc5c*^{rcm} mutants, corticospinal tract abnormalities are observed, and the dorsal funiculus is more disorganized in the perinatal stage (Finger et al., 2002). It is probable that the misrouting of pioneer DRG axons in the netrin 1-deficient dorsal spinal cord directs the following DRG fibers and other axons to a more abnormal course, which may prevent dorsal cells from localizing in the appropriate positions in later stages. Recently, Ding et al. (Ding et al., 2005b) demonstrated that the abnormal migration of dorsal cells in *Dcc*-deficient mice induces aberrant patterning of DRG axons through Sema3a signaling. netrin 1 may control the axonal pathfinding of DRG axons by affecting the migration of spinal cord neurons through Dcc and the outgrowth of DRG axons through Unc5 during different developmental stages. Therefore, the functional architecture of neural tissues is formed at least in part by neuron-axon interactions. In this study, we elucidated that netrin 1 directly regulates the timing and patterning of early DRG axons in the dorsal spinal cord. In addition, netrin 1 may also influence dorsal cell patterning indirectly at a later stage by regulating axonal pathfinding.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/133/7/1379/DC1

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