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. Unc5Ccom 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 repellent 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 (Kitakawa 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 (Serailini 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 (Serailini 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 (Serailini 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 C57BL/6 or ICR background but were mated onto C57BL/6 or ICR background. Mice homozygous for netrin 1, Dcc and Unc5cNull mutations were obtained from heterozygote matings and were identified as described previously (Serafini et al., 1996; Ackerman et al., 1997; Fazeli et al., 1997). Gilz 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 C57B1 were mated onto an ICR background. The genotyping of Gilz-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 Brm3a (a gift from Dr E. E. Turner). Digoxigenin-labeled RNA probes were synthesized using DIG RNA-labelling 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 nitroblue tetrazolium 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 then were processed with the ABC method (Vector Lab.), following the manufacturer’s protocol. Detection with horseradish peroxidase was performed by incubation in 0.05% dianisobenzidine and 0.015% hydrogen peroxide in PBS. For immunofluorescence, 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.

**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 EGF 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% CO2. 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 foniculus 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 Imaged 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 area surface area out of the explants stained by anti-neurofilament antibody was measured using Photoshop (Adobe) and Matlab software (Mathworks), 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

**Dil 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'-tetramethylindocarbocyanine perchlorate (Dil; 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.
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 dorso medial 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 spinal cord (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 Dil 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).
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.

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

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Defect*</th>
<th>Dorsal funiculus area±s.d. (μm²)</th>
<th>Correct projections</th>
<th>Aberrant projections</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>0/4</td>
<td>1327±1017</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Netrin 1+/–</td>
<td>1/9</td>
<td>9163±2549</td>
<td>33±899</td>
<td></td>
</tr>
<tr>
<td>Netrin 1–/–</td>
<td>5/5</td>
<td>4676±1763</td>
<td>8692±3087</td>
<td></td>
</tr>
</tbody>
</table>

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

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 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 (pd 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.

Table 2. Summary of the area occupied by the dorsal funiculus in the E12.5 spinal cord

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Defect*</th>
<th>Dorsal funiculus area±s.d. (μm²)</th>
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*Defects indicate the number of embryos that show direct invasion of the axons into the dorsal horn.
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 (Caspar 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 dorsal 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. 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 Dil, 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 Dil 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 Dil-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.

**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.
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
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<sup>crem</sup> 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<sup>crem</sup> 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 neurofilament-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 Unc5<sup>crem</sup> 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 Unc5<sup>crem</sup> 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 Unc5<sup>crem</sup> 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.

**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...
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). Unc5crcm mice show aberrant projections of DRG axones, 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 Unc5crcm 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 Unc5crcm mutant dorsal spinal cord may be caused by functional redundancy with such receptors. Thus, dorsal netrin 1 probably functions in DRG axones 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 axones 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 axones to netrin 1 was also examined in vitro; BHK cells expressing netrin 1 were dilated 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 high concentrations on DRG axones. 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 axones 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 axones into the dorsal spinal cord during early and late phases of the waiting period. For example, Sema3a repels NGF-responsive axones 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 axones 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 axones (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 Unc5crcm 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 axones in the netrin 1-deficient dorsal spinal cord directs the following DRG fibers and other axones 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 axones through Sema3a signaling. netrin 1 may control the axonal pathfinding of DRG axones by affecting the migration of spinal cord neurons through Dcc and the outgrowth of DRG axones 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 axones 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

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References


