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Deleted in Colorectal Cancer (*DCC*) Encodes a Netrin Receptor

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Summary

The guidance of developing axons in the nervous system is mediated partly by diffusible chemoattractants secreted by axonal target cells. Netrins are chemoattractants for commissural axons in the vertebrate spinal cord, but the mechanisms through which they produce their effects are unknown. We show that Deleted in Colorectal Cancer (DCC), a transmembrane protein of the immunoglobulin superfamily, is expressed on spinal commissural axons and possesses netrin-1-binding activity. Moreover, an antibody to DCC selectively blocks the netrin-1-dependent outgrowth of commissural axons in vitro. These results indicate that DCC is a receptor or a component of a receptor that mediates the effects of netrin-1 on commissural axons, and they complement genetic evidence for interactions between DCC and netrin homologs in C. elegans and Drosophila.

Introduction

The establishment of neuronal connections involves the accurate guidance of developing axons to their targets through the combined actions of attractive and repulsive guidance cues in the extracellular environment. Accumulating evidence has indicated the importance of long-range mechanisms for axon guidance, involving diffusible chemoattractants secreted by target cells that attract axons to their targets, and diffusible chemorepellents secreted by nontarget cells which generate exclusion zones that axons avoid (Keynes and Cook, 1995). Two recently identified families of guidance molecules, the netrins and semaphorins, comprise members that can function as diffusible attractants or repellents for developing axons, but the receptors and signal transduction mechanisms through which they produce their effects are poorly understood (Goodman, 1996).

The netrins comprise a phylogenetically conserved family of long-range guidance cues related to the extracellular matrix molecule laminin, with members implicated in attraction and repulsion of axons in Caenorhabditis elegans (UNC-6; Ishii et al., 1992), in vertebrates (netrin-1 and netrin-2; Kennedy et al., 1994; Serafini et al., 1994; Colamarino and Tessier-Lavigne, 1995), and in Drosophila melanogaster (netrin-A and netrin-B; Harris et al., 1996; Mitchell et al., 1996). In chicks, the netrin-1 and netrin-2 proteins have been implicated in guiding commissural axons in the spinal cord along a circumferential pathway from the dorsal spinal cord to floor plate cells at the ventral midline. The two proteins were originally purified from embryonic chick brain on the basis of their ability to mimic an outgrowth-promoting effect of floor plate cells on commissural axons in collagen matrices in vitro (Serafini et al., 1994). In vivo, *netrin-1* is expressed in floor plate cells and *netrin-2* is expressed at lower levels in the ventral two-thirds of the chick spinal cord, suggesting that there is a decreasing ventral-to-dorsal gradient of netrin protein that functions to attract commissural axons to the ventral midline of the spinal cord (Kennedy et al., 1994). A netrin gradient may also contribute to repelling some axons, like trochlear motor axons, away from the ventral midline, since netrin-1 can repel these axons in vitro (Colamarino and Tessier-Lavigne, 1995). In C. elegans, UNC-6 is likewise thought to attract ventrally directed axons and to repel dorsally directed axons, since guidance of these axons is impaired in *unc-6* mutants (Hedgecock et al., 1990), and since UNC-6 appears to be concentrated in the ventral portion of the nematode (Wadsworth et al., 1996).

Insights into the mechanisms of action of netrins have come from C. elegans, where two genes, *unc-5* and *unc-40*, have been implicated in *unc-6*-dependent guidance of circumferential migrations of axons and mesodermal cells. Several lines of evidence have indicated that the *unc-5* gene product, a transmembrane protein, is part of a receptor mechanism that mediates dorsal migrations away from sources of UNC-6 (Hedgecock et al., 1990; Leung-Hagesteijn et al., 1992; Hamelin et al., 1993, Wadsworth et al., 1996). The mechanisms involved in mediating presumed attractive effects of UNC-6 on ventrally directed axons are less well understood, but several studies have suggested an involvement of the *unc-40* gene product. Mutations in the *unc-40* gene affect ventral migrations in the same way as *unc-6* mutations, and *unc-6:unc-40* double mutants do not display any enhanced defects compared to the single mutants (Hedgecock et al., 1990). Moreover, the *unc-40* gene product is a predicted transmembrane protein (Chan et al., 1996 [this issue of *Cell*]), raising the possibility that UNC-40 is an UNC-6 receptor involved in directing ventral migrations. However, mutations in *unc-40* also affect dorsal migrations as well as several other patterning events in the nematode (Hedgecock et al., 1990), and *unc-40* appears to be expressed in some neurons whose

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axonal migrations are not affected in *unc-6* mutants (Chan et al., 1996). Thus the precise function of UNC-40 in mediating responses to UNC-6 and, in particular, whether UNC-40 is an UNC-6 receptor are not fully elucidated at present, though available evidence is consistent with a role for UNC-40 as an UNC-6 receptor involved in directing ventral migrations (Chan et al., 1996).

UNC-40 is a C. elegans homolog of two previously identified vertebrate proteins, Deleted in Colorectal Cancer (DCC) and neogenin, which form a subgroup of the immunoglobulin (Ig) superfamily characterized by the presence of four Ig domains and six fibronectin type III repeats in their extracellular domains (Fearon et al., 1990; Hedrick et al., 1994; Vielmetter et al., 1994). The *DCC* gene was originally identified as a candidate tumor suppressor gene located on human chromosome 18q that is lost at high frequency in colorectal cancers (Fearon et al., 1990). *DCC* is frequently deleted in several different kinds of cancers and has been proposed to be involved in mediating the transition from proliferation to terminal differentiation (Cho and Fearon, 1995). *DCC* transcripts are present at low levels in almost all normal adult tissues, with highest levels in neural tissues (Reale et al., 1994; Cooper et al., 1995). *DCC* is also expressed in the nervous system in developing mouse, chick, and Xenopus embryos, though the precise cell types expressing *DCC* have not been identified (Chuong et al., 1994; Pierceall et al., 1994; Cooper et al., 1995). Neogenin expression in embryonic brain and retinal cells in chicks correlates with the onset of neuronal differentiation and neurite extension, suggesting that neogenin is involved in terminal differentiation or axon guidance (Vielmetter et al., 1994). However, the actual functions of DCC and neogenin in the nervous system have not been identified.

The high sequence homology and apparent conservation of function between vertebrate and fly netrins and UNC-6 raises the question of whether the receptor and signal transduction mechanisms involved in mediating netrin responses are also conserved across species. Here we provide evidence for a conservation in signal transduction pathways by implicating DCC in mediating the effects of netrin-1 on spinal commissural axons. Additional evidence for conservation is provided by Kolodziej et al. (1996 [this issue of *Cell*]) through analysis of a DCC homolog, Frazzled, in Drosophila.

Results

DCC and *neogenin* Are Expressed in the Developing Rat Spinal Cord

The finding that UNC-40 is a homolog of DCC and neogenin (Chan et al., 1996) prompted us to search for related molecules in the rat spinal cord, where netrin proteins act in commissural axon guidance. Degenerate primers to sequences conserved among DCC and neogenin were used to amplify sequences from embryonic day 12 (E12) rat spinal cord mRNA and brain mRNA by the reverse transcriptase polymerase chain reaction (RT–PCR). The products were found to encode fragments of the presumed rat homologs of DCC and neogenin; no novel sequences were isolated. cDNAs for rat DCC and neogenin were isolated from an E18 rat brain cDNA library. The amino acid sequence of rat DCC is 98%, 96%, and 81% identical with those of mouse, human, and Xenopus DCC, respectively, whereas that of rat neogenin is 82% identical to that of chick neogenin. Overall, the homology between rat DCC and rat neogenin is 51%.

To obtain clues to the function of DCC and neogenin in the spinal cord, we examined the spatial distribution of *DCC* and *neogenin* transcripts in embryonic rat spinal cord at forelimb levels by in situ hybridization histochemistry. At embryonic day 11 (E11), when the first neuronal populations are differentiating, *DCC* mRNA is strongly expressed in the developing motor columns. Substantial hybridization was also observed in the dorsal spinal cord over the cell bodies of commissural neurons (the only differentiated neurons present in this region at this stage; Altman and Bayer, 1984); no significant signal was observed over undifferentiated neuroepithelial cells (Figure 1A). At E13, intense expression of *DCC* was observed in a pattern that corresponds to the cell bodies of commissural neurons (and possibly also association neurons: see below). Weaker hybridization was also detected in a subpopulation of cells in the developing motor columns (Figure 1D). *Neogenin* mRNA was observed in ventricular neuroepithelial cells at E11, with highest expression midway along the dorsal-ventral axis (Figure 1C). The expression of *neogenin* becomes widespread in the E13 spinal cord and highest in the ventral third of the ventricular zone, but is almost absent in commissural neurons, so that the expression patterns of *DCC* and *neogenin* at E13 are strikingly complementary (Figures 1D and 1F). The finding of *DCC* but not *neogenin* expression by commissural neurons led us to focus on the characterization of DCC, since commissural neurons are the only spinal cord neurons whose axons are known to be affected by netrin proteins (Serafini et al., 1994; unpublished data).

The *DCC* Gene Product Is Expressed on Commissural Axons

We next examined the expression of DCC in the developing rat spinal cord (Figures 1B and 1E) using an antibody to DCC. Immunoreactivity was detected predominantly on the axons of neuronal cells, with faint or undetectable expression on the cell bodies of these neurons. At E11, DCC protein was detected in the ventral roots of the spinal cord, which contain the axons of motor neurons. Expression was also detected on commissural axons at this stage (Figure 1B). Axonal staining of commissural neurons becomes very intense at E13 and was observed on these axons both as they project toward the floor plate, and also in the ventral funiculi where these axons extend longitudinally after crossing the midline (Figure 1E). Weaker staining was observed on motor axons. We cannot exclude that DCC might also be expressed on association neurons, which are found in a similar distribution to commissural neurons but do not project to the floor plate (Altman and Bayer, 1984). Thus, the sites of DCC protein expression corre-

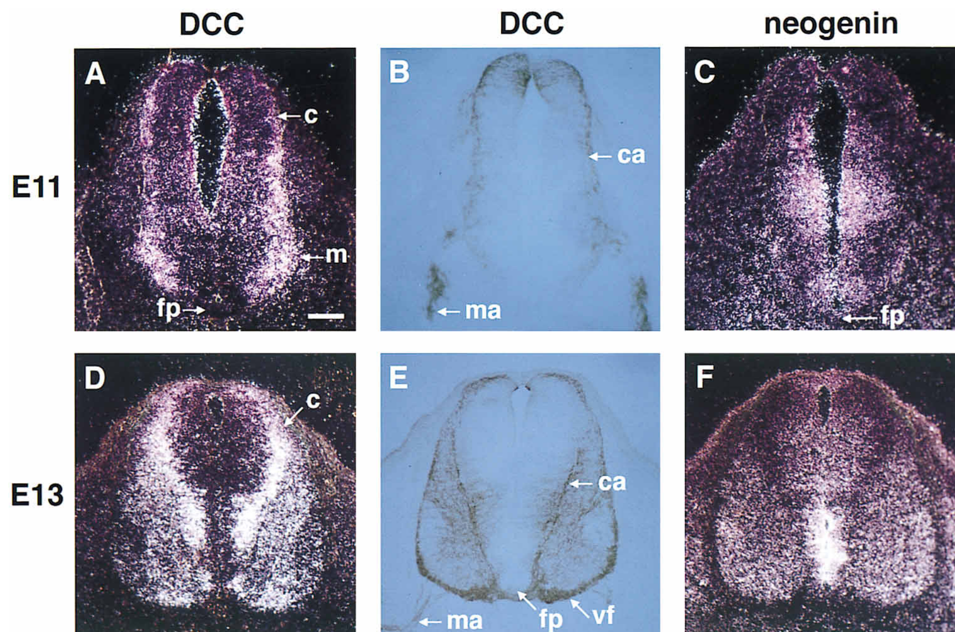


Figure 1. Expression of DCC and neogenin in the Developing Rat Spinal Cord

Expression of *DCC* and *neogenin* mRNA in transverse sections of the rat spinal cord visualized by in situ hybridization (A, C, D, and F), and expression of DCC protein visualized by immunohistochemistry (B and E), at E11 (A–C) and E13 (D–F). Semiadjacent sections from the forelimb level were used at each age.

(A and B) At E11, *DCC* mRNA (A) is detected over the cell bodies of commissural (c) and motor (m) neurons, and its protein product (B) is detected on the axons of these neurons (ca, commissural axons; fp, floor plate; ma, motor axons).

(C) At this stage, neogenin mRNA is concentrated in three regions of the ventricular zone (but is excluded from the floor plate).

(D and E) At E13, intense expression of *DCC* mRNA (D) is detected over the cell bodies of commissural neurons (c). Lower levels of expression are detected more widely in the intermediate zone, including in some portions of the motor columns. DCC protein (E) is detected on commissural axons, both in the transverse plane (ca) and in the ventral funiculus (vf), and is still detected to some extent on motor axons (ma).

(F) At this stage, *neogenin* mRNA is detected at a high level in the ventral aspect of the ventricular zone and at a lower level more diffusely in a pattern that is roughly complementary to that of *DCC* mRNA.

Scale bar, 50 μm in (A)–(C), 100 μm in (D)–(F).

spond precisely to the cells that express *DCC* transcripts, and their axonal processes.

To determine whether DCC protein is expressed over the entire extent of commissural axons, we took advantage of a culture system using explants of E11 or E13 dorsal spinal cord in which profuse outgrowth of bundles of commissural axons can be evoked by purified netrin-1 (Serafini et al., 1994; see also Figure 5). In such cultures, DCC immunoreactivity was detected not just on commissural axons that grew out in response to netrin-1, but also on their growth cones (Figure 2 and data not shown), consistent with a potential role for DCC in mediating responses of these axons to netrin-1.

Binding of netrin-1 to DCC-Expressing Cells

To characterize potential interactions between DCC and netrin-1, we examined whether netrin-1 binds cells expressing DCC. Transfected human embryonic kidney 293 cells expressing recombinant rat DCC (Figure 3A) showed significant binding of netrin-1 above background, as assessed using an antibody specific to netrin-1 (Figure 3E). In these experiments, background

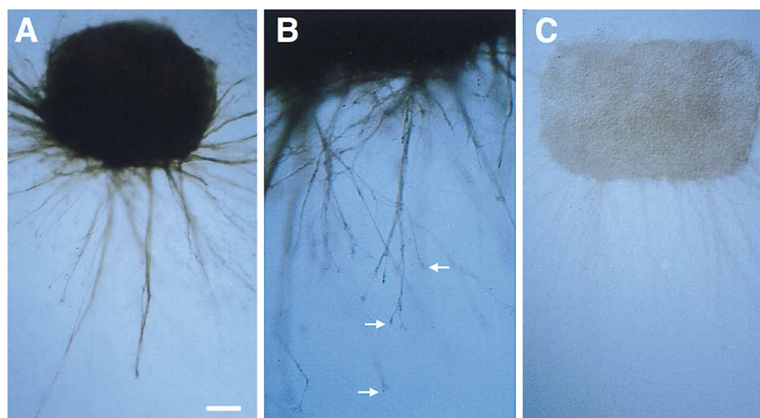


Figure 2. DCC Is Expressed by Commissural Axons and Their Growth Cones

E11 dorsal spinal cord explants were cultured for 40 hr in collagen matrices with netrin-1 to evoke commissural axon outgrowth (Serafini et al., 1994) and immunostained with an antibody to DCC and an HRP-conjugated secondary antibody (A and B) or with secondary antibody alone (C). DCC immunoreactivity is detected on commissural axons (A) and their growth cones (B, arrows). Scale bar, 100 μm in (A) and (C), 25 μm in (B).

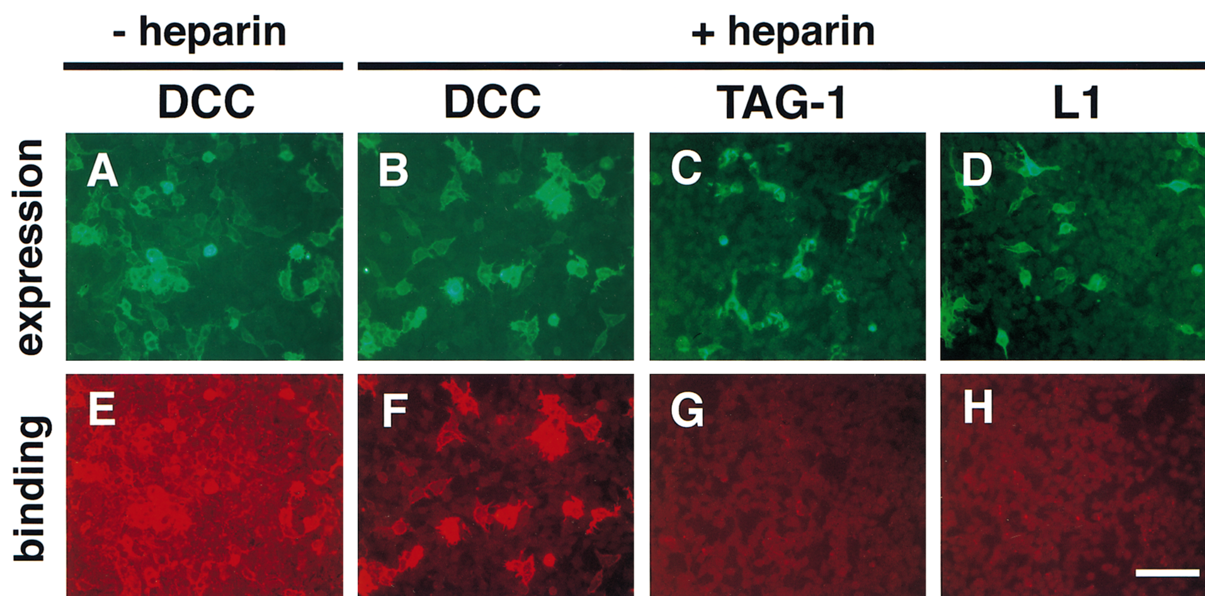


Figure 3. Netrin-1 Binds 293 Cells Expressing DCC but Not TAG-1 or L1

Fluorescence photomicrographs of 293 cells transfected with DCC (A, B, E, and F), TAG-1 (C and G), or L1 (D and H) expression constructs. (A–D) Protein expression was detected using antibodies specific to each protein and FITC-conjugated secondary antibodies. (E–H) Binding of netrin-1 protein on the cells was detected using an antibody specific to netrin-1 and a Cy3-conjugated secondary antibody, after incubating cells with 2 μg/ml netrin-1 in the absence (E) or presence (F–H) of 2 μg/ml heparin (which reduces background binding). (A) and (E) show the same field of cells, as do panels (B) and (F), and (C) and (G), respectively. Scale bar, 100 μm.

netrin-1 binding was detected on nonexpressing 293 cells (Figure 3E), consistent with the fact that netrins are heparin-binding proteins that can associate nonspecifically with cell membranes (Serafini et al., 1994; Kennedy et al., 1994). When the binding reaction was performed in the presence of heparin, which releases netrin proteins from cell surfaces in vitro (K. Brose, C. Mirzayan, and M. T.-L., unpublished data), specific binding of netrin-1 to DCC-expressing cells was maintained, but nonspecific binding was completely eliminated. Under these conditions, the degree of netrin-1 binding to cell surfaces appeared to correlate closely with the degree of DCC protein expression (Figures 3B and 3F). Netrin-1 also bound to 293 cells expressing neogenin to the same apparent extent as to cells expressing DCC (data not shown).

To address the specificity of netrin-1 binding to DCC- or neogenin-expressing cells, we examined whether netrin-1 could bind two other members of the Ig superfamily, TAG-1 and L1 (Moos et al., 1988; Furley et al., 1990), which are also expressed on growing commissural axons (Dodd et al., 1988). Transiently transfected 293 cells expressing TAG-1 or L1 (Figures 3C and 3D) did not bind netrin-1 (Figures 3G and 3H). Thus, binding of netrin-1 to DCC- and neogenin-expressing cells is specific and does not reflect a generalized interaction of netrin-1 with members of the Ig superfamily.

The affinity of DCC for netrin-1 was estimated in equilibrium binding experiments. Figure 4A shows total binding of ^{125}I-labeled netrin-1 to DCC-expressing and to control 293 cells. The specific binding curve (obtained as the difference between these two curves) could be fitted to the Hill equation (Figure 4B). Two separate experiments yielded values for the dissociation constant and Hill coefficient of $K_d = 42 \pm 1.6$ nM and $n_H = 1.5 \pm 0.1$ (mean ± SEM), respectively. The interpretation of this result is, however, complicated by the fact that, at physiological salt concentrations, purified netrin-1 appears to aggregate and to precipitate out of solution at concentrations above ~10 μg/ml (125 nM) (unpublished data), making the binding data at high concentrations unreliable. To circumvent this problem, we used a more soluble netrin-1 derivative. Netrins possess three structural domains termed domains VI, V, and C (Serafini et al., 1994). Domain C represents the major heparin-binding domain of the netrins but appears to be partly dispensable for function since a chimeric protein comprising domains VI and V of netrin-1 fused to the constant (Fc) region of a human immunoglobulin molecule can evoke outgrowth of commissural axons from E13 rat dorsal spinal cord explants, with a specific activity similar to that of native netrin-1 (C. Mirzayan and M. T.-L., unpublished data). This netrin(VI•V)-Fc fusion also binds DCC-expressing cells but is less prone to aggregation than native netrin-1 at high concentrations (data not shown). Specific binding of netrin(VI•V)-Fc to DCC-expressing cells showed saturation (Figure 4D), and the binding curve was fitted to the Hill equation, with $K_d = 5.2 \pm 0.2$ nM and $n_H = 1.2 \pm 0.01$ (mean ± SEM, n = 2). Together, our results suggest an order of magnitude for the K_d of $\sim 10^{-8}$ M (~1 μg/ml). This value is consistent with the effective dose for netrin-1 effects on commissural axons (Serafini et al., 1994) and is of a similar order of magnitude to the dissociation constant for the

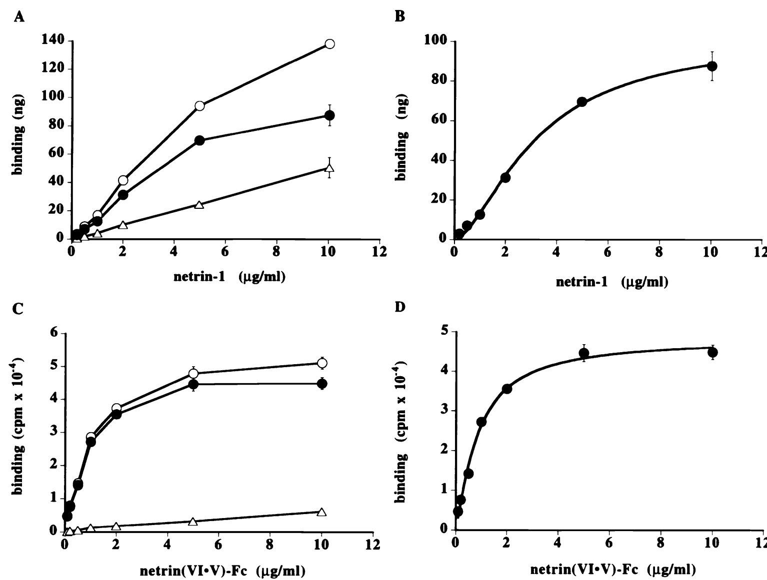


Figure 4. Equilibrium Binding of netrin-1 and a netrin(VI·V)-Fc Fusion Protein to DCC-Expressing Cells

Equilibrium binding to rat DCC of radiolabeled netrin-1 (A and B) and netrin(VI•V)-Fc (see text) (C and D). 293 cells transfected with a rat DCC expression construct or vector alone were incubated with the indicated concentrations of ligand in PBS supplemented with 2 μg/ml heparin for 3 hr. Total binding of labeled netrin-1 was determined by measuring radioactivity associated with the cells after centrifugation and washing (A). Binding of the unlabeled netrin (VI•V)Fc was determined by measuring the radioactivity associated with the cells after subsequent incubation with radiolabeled anti-human IgG antibody (C). Open circles: total binding to DCC-expressing cells; open triangles: total binding to nonexpressing cells; closed circles: specific binding (defined as the difference between these two values at each concentration). Bars indicate SEM for triplicates. Results shown are from one representative of two experiments performed for each ligand. Specific binding curves were fitted using the Hill equation, yielding K_d values of 40 nM for netrin-1 (B) and 5.4 nM for netrin (VI•V)-Fc (D). (For netrin-1 and netrin(VI•V)-Fc, 1 μM corresponds to about 80 μg/ml and 160 μg/ml, respectively).

interaction of the α1β2 integrin with laminin-1 (Pfaff et al., 1994).

DCC Function Is Required for Axon Outgrowth Evoked by netrin-1

To test directly whether DCC is involved in mediating the effects of netrin-1, we examined the effect of perturbing DCC function on commissural axon outgrowth evoked by netrin-1 from explants of E11 or E13 rat dorsal spinal cord (Figures 5A and 5E). Addition of 0.1–10 μg/ml of a monoclonal antibody against the extracellular domain of DCC (see Experimental Procedures) resulted in a dose-dependent reduction in the extent of commissural axon outgrowth evoked by netrin-1 in both assays, whereas 10 μg/ml of normal mouse immunoglobulin had no apparent effect (Figures 5C, 5D, 5F, and 5K and data not shown). The antibody mainly affected the number and length of axon bundles, but not the extent of fasciculation of the axons into bundles. At the highest dose, the outgrowth was reduced to <10% of control (Figure 5K).

We next examined whether the anti-DCC antibody could interfere with the outgrowth of commissural axons from E13 dorsal explants that is evoked by floor plate cells (Figures 6A and 6B) and that appears to be due to netrin-1 secreted by these cells (Kennedy et al., 1994; T. Serafini et al., submitted). At 10 μg/ml, the antibody almost completely eliminated this outgrowth (Figure 6D), whereas normal mouse immunoglobulin (10 μg/ml) had no effect (Figure 6C). Thus, the anti-DCC antibody can block the outgrowth of commissural axons in vitro that is evoked by netrin-1, whether presented as a pure protein or secreted from floor plate cells.

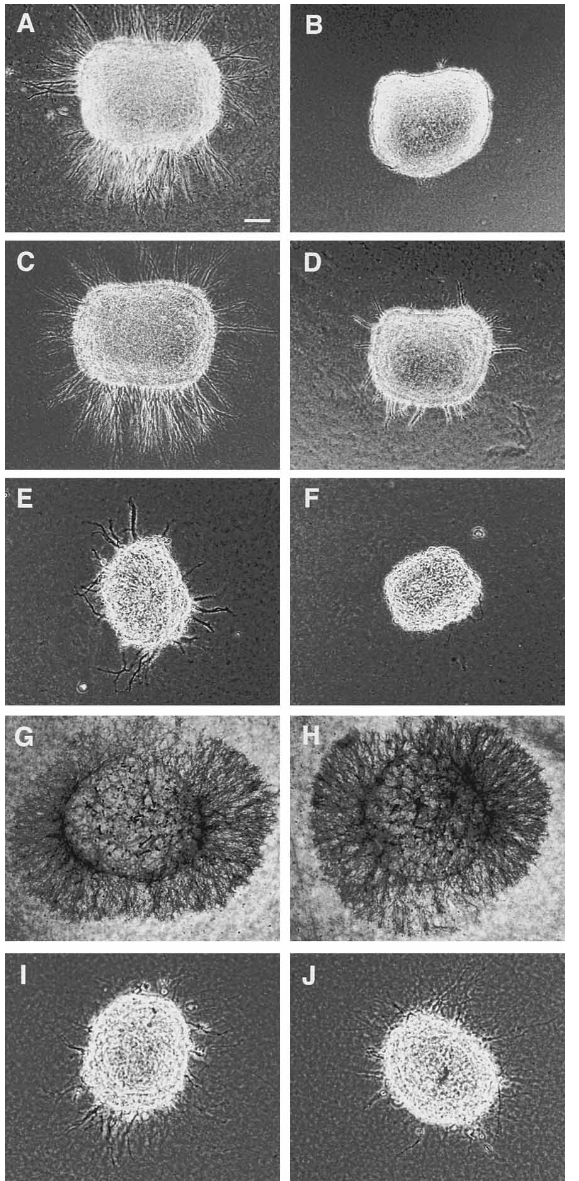
Two different types of controls were performed to test the specificity of the blocking effect of the antibody. First, we examined the effect of the antibody on the radial growth of sensory axons from E15 dorsal root ganglia (DRG) into collagen gels that is evoked by nerve growth factor (NGF; Figures 5G and 5H). These axons do not express DCC either in vivo or in vitro (data not shown). This outgrowth was not affected by the presence of anti-DCC antibody (up to 20 μg/ml; Figure 5H), showing that the antibody does not simply have a generalized inhibitory effect on axons. Second, we sought to test whether the antibody was simply interfering with commissural axon growth in a general way, independent of the involvement of netrin-1. For this, we took advantage of the observation that, whereas little axon outgrowth is visible from E13 dorsal spinal cord explants in the absence of netrin-1 after 16 hr of incubation (Figure 6A), profuse outgrowth is observed from these explants by 40 hr in culture even in the absence of netrin-1 (Figure 5I). These axons express DCC (data not shown) but are less fasciculated than those that project out in the presence of netrin-1. When E13 explants were cultured in the absence of netrin-1 but with the anti-DCC antibody (10 μg/ml), no obvious change in the pattern or extent of outgrowth was observed compared to control explants cultured without antibody, or cultured in the presence of normal mouse immunoglobulin (10 μg/ml; Figures 5I and 5J). To quantitate this apparent lack of effect, we measured the sum of the lengths of all axons projecting from the explants. For explants cultured with the anti-DCC antibody, with normal mouse immunoglobulin, and with no addition, the summed lengths per explant were, respectively, 10.99 ± 0.54 mm, 10.61 ± 0.53 mm, and 10.94 ± 0.22 mm (mean ± SEM, $n = 6$, 6, and 7, respectively). No significant difference was detected between values compared pairwise ($p > 0.5$ for each pair, Student's t test). Thus, the anti-DCC antibody blocks the netrin-dependent outgrowth of commissural axons without apparent effect on the netrin-independent outgrowth of these axons.

In addition to their ability to evoke outgrowth of commissural axons into collagen gels, both netrin-1 and the

floor plate can reorient the growth of these axons within E11 dorsal spinal cord explants. In E11 explants cultured alone for 40 hr, commissural axons grow along their normal dorsoventral trajectory, but when explants are cultured with either a floor plate explant or an aggregate of cells secreting netrin-1 placed adjacent to the dorsoventral axis of the explant, commissural axons within ~100–300 μm of the target are deflected from this trajectory and turn to the target within the explant (Placzek et al., 1990; Kennedy et al., 1994). The anti-DCC antibody (10 μg/ml) did not block commissural axon growth along a dorsoventral trajectory within explants or turning of these axons toward either target (data not shown). This indicates either that DCC function is not required for the turning response, or that the antibody did not penetrate the tissue explant effectively.

Other Sites of DCC Expression

Together, our results indicate that DCC is a component of the receptor mechanism through which netrin-1 produces its effects on commissural axons. However, netrin-1 expression is not restricted to the spinal cord (Kennedy et al., 1994; Skarnes et al., 1995; T. Serafini et al., submitted), and other axonal populations have been shown to respond to netrin-1 at other axial levels in the nervous system (Shirasaki et al., 1995). To obtain further insight into the neuronal populations that might use DCC to respond to netrin-1, we investigated the expression pattern of DCC in embryos at E11–13. *DCC* transcript expression was observed in many types of neurons in various regions (see also Cooper et al., 1995), and DCC protein expression was consistent with the sites of transcript expression (Figure 7 and data not shown). DCC was in particular observed on the axons



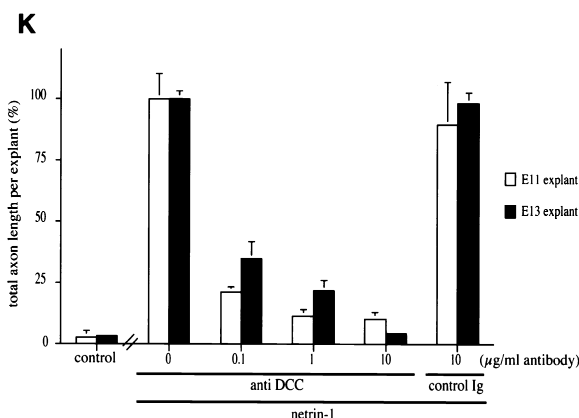


Figure 5. DCC Function Is Required for Axon Outgrowth Evoked by netrin-1

(A–D) E11 dorsal spinal cord explants cultured for 40 hr with 1.2 μg/ml netrin-1 (A), without netrin-1 (B), with netrin-1 and 10 μg/ml normal mouse immunoglobulin (C), or with netrin-1 and 1.0 μg/ml of the monoclonal antibody directed against the extracellular portion of DCC (D). Outgrowth evoked by netrin-1 (A) is blocked by addition of anti-DCC antibody (D), but not by addition of control immunoglobulin (C). Little outgrowth is observed from explants cultured alone (B).

(E and F) E13 dorsal explants cultured for 16 hr with 300 ng/ml netrin-1. Robust outgrowth elicited by netrin-1 (E) is blocked by the addition of 10 μg/ml anti-DCC antibody (F). Little outgrowth is observed in controls (see Figure 6A).

(G and H) E15 dorsal root ganglia (DRG) cultured for 16 hr with 25 ng/ml NGF and stained with anti-p75 antibody. Anti-DCC antibody (20 μg/ml) has no apparent effect on axon outgrowth (H), as compared to control (G).

(I and J) Netrin-1-independent outgrowth from E13 dorsal explants cultured for 40 hr in the absence of netrin-1 (data not shown) is not affected by the addition of 10 μg/ml anti-DCC antibody (J), or of 10 μg/ml normal mouse immunoglobulin (I).

(K) Quantification of the blocking effects of anti-DCC antibody on netrin-1-dependent outgrowth of commissural axons. E11 and E13 explants were cultured for 40 hr and 16 hr, respectively, either in the absence of netrin-1, or in the presence of netrin-1 (1.2 μg/ml and 300 ng/ml, respectively) and the indicated concentrations of anti-DCC antibody or normal mouse immunoglobulin. The total length of axons growing into the collagen matrix was measured (see Experimental Procedures) for each explant and normalized to the values obtained from E11 and E13 explants cultured in the absence of anti-DCC antibody. Values shown are means ± SEM (n = 4). Scale bar, 100 μm in (A)–(D) and (G)–(H), 50 μm in (E)–(F) and (I)–(J).

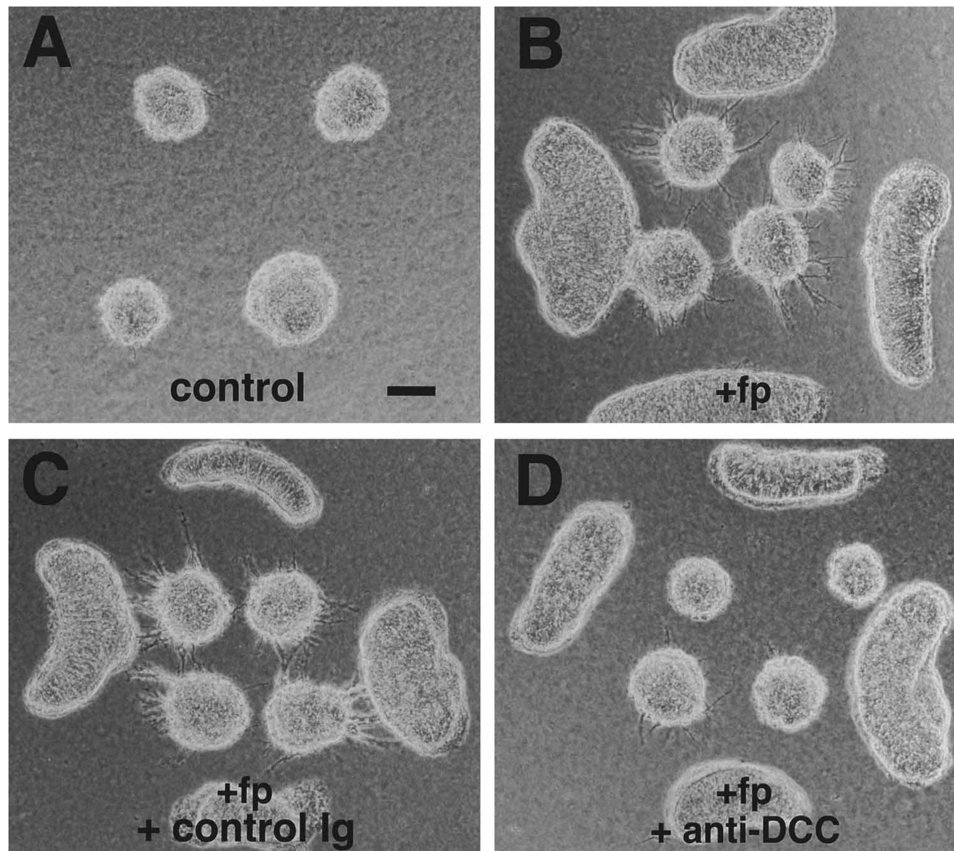


Figure 6. DCC Function Is Required for Axon Outgrowth Evoked by Floor Plate Cells

Each panel shows four E13 dorsal spinal cord explants cultured for 14 hr in collagen matrices either alone (A) or surrounded by four explants of E13 rat floor plate (B–D). Explants were cultured either without antibody (A and B), with normal mouse immunoglobulin (10 μg/ml) (C) or with anti-DCC antibody (10 μg/ml) (D). Axon outgrowth evoked by floor plate cells (B) is reduced to control levels (A) by anti-DCC antibody (D) but not control immunoglobulin (C).
Scale bar, 100 μm.

of spinal commissural neurons, as well as neurons in the hindbrain (cerebellar plate neurons), and neurons in the midbrain and forebrain. The DCC-positive fibers in the midbrain and diencephalon appear to include the axons in the first tracts that develop, such as the mesencephalic tract of the trigeminal nerve, the circumferential descending axons (or tecto-bulbar tract), the posterior commissure, and the medial longitudinal fasciculus (Easter et al., 1993). The DCC-positive cells in the superficial layer of the cerebral cortex appear to be Cajal-Retzius cells. Weaker staining was observed on spinal motor axons and some cranial nerves, in the paraxial mesoderm, and in the dorsal aspect of the eyeball. In addition, DCC is expressed on axons in the region where the anterior commissure and optic chiasm are forming, and on axons in the tract of postoptic commissure (TPOC) (data not shown).

Discussion

DCC Is a Netrin-1 Receptor on Commissural Axons

We have shown that DCC is expressed on the axons and growth cones of spinal commissural neurons as they extend to and across the floor plate, and then during their subsequent growth in the ventral funiculus. Our studies also indicate that DCC is a netrin-1-binding protein that is required to mediate the outgrowth-promoting effect of netrin-1 on commissural axons in vitro. Since we have not tested the ability of DCC to bind netrin-1 in a cell-free environment, we cannot exclude that netrin-1 binding to DCC requires a cofactor contributed by the expressing cells. We also cannot exclude the formal possibility that in our transfection experiments netrin-1 was binding to a distinct surface component induced by DCC expression rather than to DCC itself, though this seems unlikely given that antibodies to DCC block the outgrowth-promoting effects of netrin-1 on commissural axons. In vivo, netrin-1 has been directly implicated in guidance of commissural axons from the dorsal spinal cord to the ventral midline of the spinal cord (T. Serafini et al., submitted), and our results therefore suggest that DCC functions as a receptor that is involved in mediating this guidance in vivo.

The finding of biochemical interactions between DCC and netrin-1 in vertebrates complements recent studies in C. elegans and Drosophila. In C. elegans, the DCC homolog UNC-40 is required for a subset of axon guid-

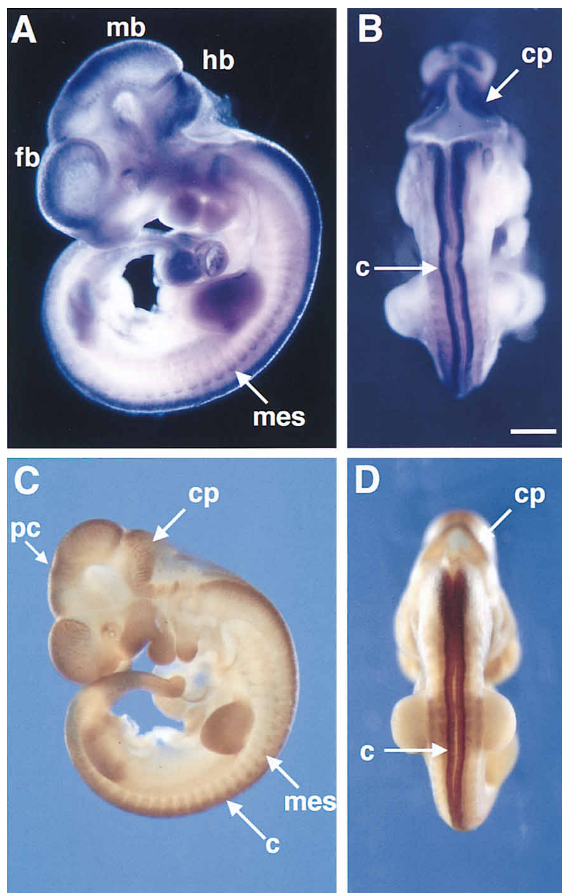


Figure 7. Sites of DCC Expression at Midgestation

Lateral views (A and C) and dorsal (back) views (B and D) of E12 rat embryos are shown.

(A and B) *DCC* mRNA expression visualized by whole-mount in situ hybridization. Intense hybridization is seen to spinal commissural neurons (c) and to cells in the hindbrain (hb), midbrain (mb), forebrain (fb), and paraxial mesoderm (mes).

(C and D) DCC protein expression visualized by whole-mount immunohistochemistry. DCC protein expression corresponds to sites of *DCC* mRNA expression site but is observed predominantly on axons, including spinal commissural axons and axons in hindbrain, midbrain and forebrain. Expression is also visible in the paraxial mesoderm, some cranial nerves, and the dorsal aspect of the eyeball. Additional abbreviations: cp, cerebellar plate; pc, posterior commissure. Scale bars, 1.1 mm in (A) and (B), 1.3 mm in (C) and (D).

ance events that are directed by UNC-6, a netrin-1 homolog. In particular, ventrally directed migrations, which are presumed attractive responses to UNC-6 (Wadsworth et al., 1996), are strongly affected by loss of UNC-40 function (Hedgecock et al., 1990). Moreover, *unc-40* appears to act cell-autonomously (Chan et al., 1996). Together, these studies have led to the suggestion that UNC-40 is a receptor involved in mediating attractive effects of UNC-6 on ventrally directed axons. Likewise, in Drosophila, the loss-of-function phenotype of the gene encoding the DCC homolog Frazzled (Kolodziej et al., 1996) strongly resembles that of a deletion of the two Drosophila *Netrin* genes (Mitchell et al., 1996; Harris et al., 1996), suggesting that Frazzled is a netrin receptor in flies. It remains to be shown directly in each species that these DCC and netrin homologs can interact biochemically, but this possibility is supported by the finding of interactions between DCC and netrin-1 reported here.

Although DCC function appears to be necessary for outgrowth of commissural axons in response to netrin-1, our studies have not determined whether DCC functions alone to mediate these responses or whether instead it is one component of a multimeric netrin-1 receptor complex on the surface of commissural axons. DCC is expressed on spinal motor axons and on axons that form the posterior commissure, which do not appear to show either attractive or repulsive responses to netrin-1 in vitro (K. Brose and M. T.-L., unpublished data; R. Shirasaki et al., submitted), suggesting that expression of DCC alone is not sufficient to confer netrin-responsiveness to axons. It is possible that additional cell-surface or intracellular signaling components are required for netrin-responsiveness and are present in commissural axons, or alternatively nonresponsive axons might express cell-surface or intracellular components that block netrin-responsiveness of these axons despite their expression of DCC. In this regard, it is interesting that the anti-DCC antibody that blocks netrin-1 effects does not interfere with the binding of netrin-1 to 293 cells expressing DCC (data not shown); the antibody could prevent an essential conformational change in DCC, but it could also prevent an essential interaction of DCC with another surface protein. Our studies have also not determined whether expression of DCC is necessary to mediate all actions of the netrins. In particular, our test of whether DCC is required to mediate turning of commissural axons toward a source of netrin-1 was inconclusive. In addition, netrin-1 has been shown to function as a chemorepellent for trochlear motor axons in vitro (Colamarino and Tessier-Lavigne, 1995). DCC is expressed by trochlear motor axons but at very low levels (data not shown), suggesting that DCC may not be required to mediate the repellent action of netrin-1 on these axons.

Other Functions of DCC Subfamily Members in the Nervous System?

DCC is expressed by a number of different classes of axons at all axial levels during early stages of neural development. At E13, DCC is highly expressed in the cerebellar plate, which contains axons whose outgrowth is elicited by netrin-1 in vitro (Shirasaki et al., 1995). DCC is also abundant on axons in the TPOC, whose navigation pathway is marked by high expression of netrin-1 (Skarnes et al., 1995). The DCC-positive axons in the midbrain and diencephalon also appear to include axons of the circumferential descending (or tecto-bulbar) tract, which extend ventrally and many of which cross the midline at the floor plate. Thus, DCC might be involved in the attraction of these axons by netrin-1, extending to other axial levels the role documented here in the spinal cord.

As discussed above, however, DCC is also expressed by motor axons and posterior commissure axons that do not appear to be netrin responsive. This raises the question whether DCC is involved in mediating the responses of these axons to nonnetrin guidance cues.

DCC could in principle interact with different ligands through different domains, as shown for the receptor-type protein tyrosine phosphatase β (RPTPβ), which interacts with F3/F11/contactin and with a glial cell surface molecule through distinct domains (Peles et al., 1995).

The DCC-relative neogenin also binds netrin-1. Neogenin is expressed on developing chick retinal ganglion cell axons (Vielmetter et al., 1994). Since *netrin-1* is expressed in the optic stalk in the chick (Kennedy et al., 1994), it is possible that neogenin functions as a netrin receptor involved in axon guidance in the chick visual system. In the E11 rat spinal cord, *neogenin* is mainly expressed in a restricted region in the ventricular (proliferative) zone, and later becomes expressed widely but not by commissural neurons. One possibility is that neogenin functions as a passive netrin-1-binding protein that binds and stabilizes a gradient of netrin-1 in the spinal cord. It is also conceivable that neogenin transduces a signal from netrin-1 that influences the proliferation, differentiation, or migration of neuronal precursor cells in the spinal cord. Such an involvement would also be consistent with the suggestion that DCC is normally involved in mediating the transition from proliferation to terminal differentiation in various tissues (Cho and Fearon, 1995).

Ig Superfamily Members as Axon Guidance Receptors

Studies on Ig superfamily members expressed in the nervous system have focused for the most part on their homophilic or heterophilic interactions with other Ig superfamily members (reviewed in Brümmendorf and Rathjen, 1994). There is, however, accumulating evidence that Ig superfamily members can also in some cases bind extracellular matrix (ECM) proteins: (i) Ng-CAM, a chick homolog of L1, can bind laminin-1 (Grumet et al., 1993); (ii) Gicerin, a transmembrane Ig superfamily member with five Ig repeats, binds the laminin-related molecule neurite outgrowth factor (NOF) (Taira et al., 1994); (iii) chick F11/contactin and its mouse homolog F3, GPI-linked proteins with a similar structure to TAG-1, bind to members of the tenascin family and are implicated in mediating repulsive actions of these ligands (Nörenberg et al., 1992; Zisch et al., 1992; Pesheva et al., 1993). Although netrins can function as diffusible chemoattractants (Kennedy et al., 1994), in structure they are related to portions of the laminin molecules (Ishii et al., 1992; Serafini et al., 1994). Thus, the finding of interactions between DCC and netrin-1 parallels other observations on interactions between Ig superfamily proteins and ECM molecules and indicates that such interactions can be involved in mediating outgrowth-promoting effects of the ligands.

The signal transduction pathways that are triggered by homophilic interactions between Ig superfamily members are beginning to be elucidated (reviewed in Brümmendorf and Rathjen, 1994), but nothing is known yet about signaling triggered by binding of Ig superfamily members to ECM molecules. The identification of DCC as a netrin-1 receptor provides a clear biological context for the elucidation of downstream components of DCC signaling that are important for mediating axon guidance.

Experimental Procedures

Search for Relatives of DCC and neogenin

Eight degenerate primers that cover all possible codons encoding the conserved amino acid sequences between human DCC and chick neogenin were made. The sequences of the forward primers corresponded to the amino acid sequences KNG(D/E)VV, DEG(F/Y)YQC, KV(A/V)TQP, and DLWIHH. Those for the reverse primers corresponded to TGYKIR, MTVNGTG, NIVVRG, and EGLMK(Q/D). PCR was performed using cDNA reverse-transcribed from total RNA from E12 rat spinal cord or brain (E0 is the day of vaginal plug). PCR products with sizes close to those calculated for DCC and neogenin were subcloned, and their sequences were determined. An E18 rat brain cDNA library (a gift of Dr. S. Nakanishi) was screened with the PCR fragments for DCC and neogenin as probes. Searches of the DNA databases were performed using the BLAST server service, and sequence analysis and alignments were performed using GeneWorks software (Intelligenetics). GenBank database accession numbers for rat DCC and rat neogenin are U68725 and U68726, respectively.

In Situ Hybridization

Cryostat sections (10 μm) were processed for in situ hybridization as described (Frohman et al., 1990). [^{35}S]UTP-labeled antisense riboprobes were synthesized using the PCR products as templates. Two nonoverlapping probes were used with identical results for both DCC and neogenin. Whole-mount in situ hybridization was as described (Kennedy et al., 1994; Shimamura et al., 1994), using BM purple (Boehringer-Mannheim) as an alkaline phosphatase substrate.

Immunohistochemistry

Immunostaining of 10 μm cryostat sections was carried out with anti-DCC antibody (an IgG1 mouse monoclonal antibody, clone AF5, Oncogene Science, Inc., used at 1 μg/ml), a biotinylated anti-mouse IgG antibody (1:200 dilution, Vector), and a Vectastain Elite ABC kit (Vector). Diaminobenzidine (Sigma) was used as a chromogen. Whole-mount immunohistochemistry was done using the same reagents, as described previously (Shimamura et al., 1994). According to the vendor's information, the anti-DCC antibody is raised against the extracellular domain of the human DCC protein, but the region of the extracellular portion that reacts with the antibody is unknown. It also binds rat DCC (but not rat neogenin) expressed in 293 cells (Figure 3 and data not shown).

Purification of Recombinant netrin-1

cDNAs encoding chick netrin-1 tagged with a c-myc epitope at its C-terminus (Serafini et al., 1994), or domains VI and V of chick netrin-1 fused to the constant (Fc) region of the human IgG1, were subcloned into the expression vector pCEP4 (Invitrogen) and used to transfect 293-EBNA cells (Invitrogen). Cell lines permanently expressing either netrin-1 or netrin(VI•V)-Fc were established after drug selection (R. Shirasaki et al., submitted; C. Mirzayan and M. T.-L., unpublished data). Proteins were purified from conditioned media by heparin affinity chromatography to 85%–90% homogeneity, as assessed by silver staining.

Binding Experiments

Transfections of cDNAs for rat DCC, L1, and TAG-1 (the latter two gifts of Dr. A. Furley) into 293-EBNA cells were performed using LipofectAMINE (GIBCO–BRL). Forty-eight hours after transfection, the cells were incubated with 2 μg/ml chick netrin-1 protein in PBS supplemented with 10% horse serum and 0.1% sodium azide in the presence or absence of 2 μg/ml heparin at room temperature for 90 min. After washing three times with PBS, the cells were fixed with methanol. Double staining of transfected gene products and bound netrin-1 were carried out using the antibodies to DCC, or to TAG-1 (4D7 mouse monoclonal antibody of IgM class, a gift of Dr. M. Yamamoto, at 1:100 dilution), and an anti-netrin-1 antibody

(affinity-purified rabbit polyclonal antibody, T.E. Kennedy and M. T.-L., unpublished data, used at 1:1000 dilution). Staining of L1 protein and bound netrin-1 for the L1-transfected cells was done in separate wells because the anti-L1 antibody (gift of Dr. C. Lagenaur) is also a rabbit polyclonal antibody. Expression of the transfected proteins was detected using FITC-labeled secondary antibodies (anti-mouse IgG and anti-rabbit IgG from Boehringer Mannheim; anti-mouse IgM, CAPPEL), while bound netrin-1 was visualized using a Cy3-labeled secondary antibody (Jackson Immunological laboratories).

Equilibrium-Binding Experiments

Low passage 293-EBNA cell lines permanently expressing rat DCC were used for binding studies, and untransfected or mock-transfected cells were used as negative controls. Netrin-1 was radiolabeled with ^{125}I using Iodo-beads (Pierce) according to the manufacturer's instructions, to a specific activity of 17 mCi/μg protein. Cells (3×10^5) were incubated in triplicate with different concentrations of labeled netrin-1 in PBS containing 2 μg/ml heparin and 1 mg/ml BSA on ice for 3 hr, centrifuged for 5 min, and briefly rinsed with PBS after removing the supernatant. The remaining radioactivity was measured using a gamma counter. For binding studies with netrin(VI•V)-Fc, cells (2.5×10^5) were seeded onto poly-L-lysine-coated dishes (24-well). The next day, cells were incubated in triplicate with different concentrations of netrin(VI•V)-Fc in the same buffer on ice for 3 hr, rinsed with PBS three times, incubated with a ^{125}I-conjugated anti-human IgG antibody (1 μCi/ml, NEN/Dupont) in PBS containing 10% horse serum for 30 min, rinsed again with PBS three times, and solubilized, and the radioactivity bound on the cells was counted. Results were analyzed and plotted using the Kaleidagraph program (Synergy software).

Explant Cultures

Explants of E11 and E13 rat dorsal spinal cord were isolated and cultured in collagen gels as described (Tessier-Lavigne et al., 1988; Serafini et al., 1994). Outgrowth of commissural axons was elicited by addition of 300 ng/ml (for E13 explants) or 1.2 μg/ml (for E11 explants) of purified netrin-1. E15 rat DRG were embedded in collagen gels and cultured for 16 hr in F12/N3 medium with 25 ng/ml 2.5S NGF. For blocking experiments, anti-DCC antibody (Oncogene Sciences, Inc.) and control mouse immunoglobulin solutions were dialyzed against F12 medium before adding to the culture. A measure of axon outgrowth from explants was obtained by adding the lengths of all axons from each explant (regardless of bundle thickness), providing the "total axon length" for each explant. Dorsal spinal cord and DRG explants were stained with the anti-TAG-1 or anti-DCC antibodies, and an antibody directed to the p75 NGF receptor (gift of L. Reichardt), respectively, by whole-mount immunohistochemistry as described (Kennedy et al., 1994; Colamarino and Tessier-Lavigne, 1995).

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GenBank Accession Numbers

GenBank database accession numbers for rat DCC and rat neogenin are U68725 and U68726, respectively.

Note Added in Proof

The work cited as R. Shirasaki et al., submitted, is now in press: Shirasaki, R., Mirzayan, C., Tessier-Lavigne, M., and Murakami, F. (1996). Guidance of circumferentially growing axons by netrin-dependent and -independent floor plate chemotropism in the vertebrate brain. Neuron, in press.