Deleted in colorectal cancer (DCC) encodes a netrin receptor

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This thesis is based on the following articles

1) Keino-Masu, K., Masu, M., Hinck, L., Leonardo, E. D., Chan, S.-S.-Y., Culotti, J. G., and Tessier-Lavigne, M. (1996). *Deleted in Colorectal Cancer (DCC)* encodes a netrin receptor. Cell *87*, 175-185.

2) Fazeli, A., Dickinson, S. L., Hermiston, M. L., Tighe, R., Steen, R.G., Small, C. G. III, Stoeckli, E. T., Keino-Masu, K., Masu, M., Rayburn, H., Simons, J., Bronson, R. T., Gordon, J. I., Tessier-Lavigne, M., and Weinberg, R. A. (1997). Defective axon guidance but no colorectal tumors in mice lacking functional *Deleted in Colorectal Cancer (Dcc)* gene. Nature in press.

Contents

General introduction

The function of the nervous system is based on the accurate wiring of billions of neurons. Making precise neuronal connections requires developing neurons to select the correct pathway toward their targets and to find their correct targets for making synaptic connections. Pathfinding and target selection processes are believed to be guided by molecular cues in the environment, while fine synaptic refinement is generated in an activity-dependent manner after specific connections are established (Goodman and Shatz, 1993; Garrity and Zipursky, 1995; Keynes and Cook, 1995; Tessier-Lavigne and Goodman, 1996). In the developing brain, growth cones, the1 leading edges of axons, appear to be guided by long-ranged (diffusible) and short-ranged (contact-mediated) cues, each of which includes both attractive and repulsive molecules. Short-ranged, contact-mediated cues consist of cell-surface and extracellular matrix molecules such as laminin, collagen, NCAM, cadherins as attractive cues, and tenascin, Eph-ligands as repulsive cues. Accumulating evidence from a lot of studies now suggests some insights into the mechanisms on how these molecules work for guiding growing axons (Tessier-Lavigne and Goodman, 1996). On the other hand, the molecular mechanisms of long-ranged, chemotropic guidance remain to be elucidated, although previous studies provided the evidence that there are diffusible chemoattractants secreted by the targets which attract axons at a distance and there are diffusible chemorepellants secreted by the non-target cells which make the exclusion zones that axons avoid (Goodman, 1996; Tessier-Lavigne and Goodman, 1996).

The netrins comprise a phylogenetically-conserved family of long-range guidance

cues related to the extracellular matrix molecule laminin. The members are implicated in attraction and repulsion of axons in *Caenorhabditis elegans* (UNC-6; Ishii et al., 1992), in vertebrates (netrin-1 and netrin-2; Serafini et al., 1994; Kennedy et al., 1994; Colamarino and Tessier-Lavigne, 1995; Shirasaki et al., 1995, Shirasaki et al.,1996) and in *Drosophila melanogaster* (netrin-A and netrin-B; Mitchell et al., 1996; Harris 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 brains 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). *In vitro* studies in rodents showed that netrin-1 attracts not only spinal commissural axons but also midline-crossing axons in the midbrain and hindbrain (cerebellar plate neurons), suggesting that the same mechanism also operates in other axial levels in the nervous system (Shirasaki et al.,1995 and 1996). These hypotheses were verified by the studies of the *netrin-1* knockout mouse, which showed that spinal commissural axons deviate from the normal trajectories and extend in aberrant directions and that as a result, the number of the commissural axons in the ventral commissure is greatly reduced. Defects in the brain commissures (corpus callosum, anterior commissure and hippocampal commissure) are also observed in the *netrin-1*- deficient mice (Serafini et al., 1996). These results indicate that netrin-1 is important for guiding spinal commissural axons as well as the axons in the brain *in*

vivo. Netrin-1 also can act as a chemorepellant *in vitro* for some classes of axons like trochlear motor axons, which migrate away from the ventral midline (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). In *Drosophila*, both *netrin-A* and *netrin-B* are expressed by glial cells of the CNS midline. In the fly mutant embryos that are deficient of both *netrin-A* and *netrin-B* genes, commissures are thin or absent. And this phenotype is rescued by expression of cDNA for either gene at midline (Mitchell et al., 1996; Harris et al., 1996). These results establish netrins as important axon guidance cues that are conserved in nematodes, flies, and vertebrates.

Although the structure and function of netrins are well studied and shown to be conserved among species, the signal transduction mechanisms of netrins are poorly understood in any species, except that two *C. elegans* genes (*unc-5* and *unc-40*) were reported to be involved in the same pathway as *unc-6* (Hedgecock et al., 1990). Several lines of evidence have indicated that the *unc-5* gene product is part of a receptor mechanism that mediates dorsal migrations away from sources of UNC-6. First, loss of *unc-5* function results in selective disruption of dorsal migrations (Hedgecock et al., 1990). Second, *unc-5* gene product is a transmembrane protein (Leung-Hagesteijn et al., 1992). Third, ectopic *unc-5* expression in neurons that ordinarily project longitudinally makes them project dorsally (Hamelin et al., 1993). On the other hand, 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 *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). In addition, *unc-40* encodes a membrane protein and appears to act cell-autonomously (Chan et al., 1996). These results suggest *unc-40* gene product is a candidate receptor that mediates attractive effects of UNC-6. However, in *unc-40* mutant, some dorsal migrations are also affected as are several other patterning events in the nematode and some axonal migrations which are normal in *unc-6* mutants (Hedgecock et al., 1990). Thus the precise function of *unc-40* gene product in mediating responses to UNC-6 were not fully elucidated from previous studies.

To investigate signal transduction mechanisms of netrins, I attempted to identify a receptor which transduces netrin-mediated signals into neurons or axons. In chapter 1, I identified a netrin receptor in *in vitro* experiments: I isolated two rat homologues of *unc-40* gene and showed the evidence that one of them, *Deleted in Colorectal Cancer (DCC)*, encodes a netrin receptor or a component of a receptor that mediates the attractive effects of netrin-1 on commissural axons. In chapter 2, to examine whether DCC functions as a netrin receptor *in vivo*, I analyzed the neural phenotype of the *Dcc* knockout mouse. In these mutant animals, defects in the spinal commissural axon projections, as well as some forebrain commissures were observed. The phenotypes observed in *Dcc*-deficient mice are strikingly similar to those of *netrin-1*-deficient mice. These results indicate, together with my *in vitro* experiments, that DCC is in the response pathway for netrin-1 effects and functions as a netrin receptor *in vivo*.

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Chapter 1

Deleted in colorectal cancer (DCC) encodes a netrin receptor

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 complement genetic evidence for interactions between DCC and netrin homologues 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 which attract axons to their targets, and diffusible chemorepellents secreted by non-target 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; Serafini et al., 1994; Kennedy et al., 1994; Colamarino and Tessier-Lavigne, 1995) and in *Drosophila melanogaster* (netrin-A and netrin-B; Mitchell et al., 1996; Harris 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), 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 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* homologue 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) through analysis of a DCC homologue, Frazzled, in *Drosophila*.

Results

DCC and *neogenin* are expressed in the developing rat spinal cord

The finding that UNC-40 is a homologue 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 homologues 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 observations).

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

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

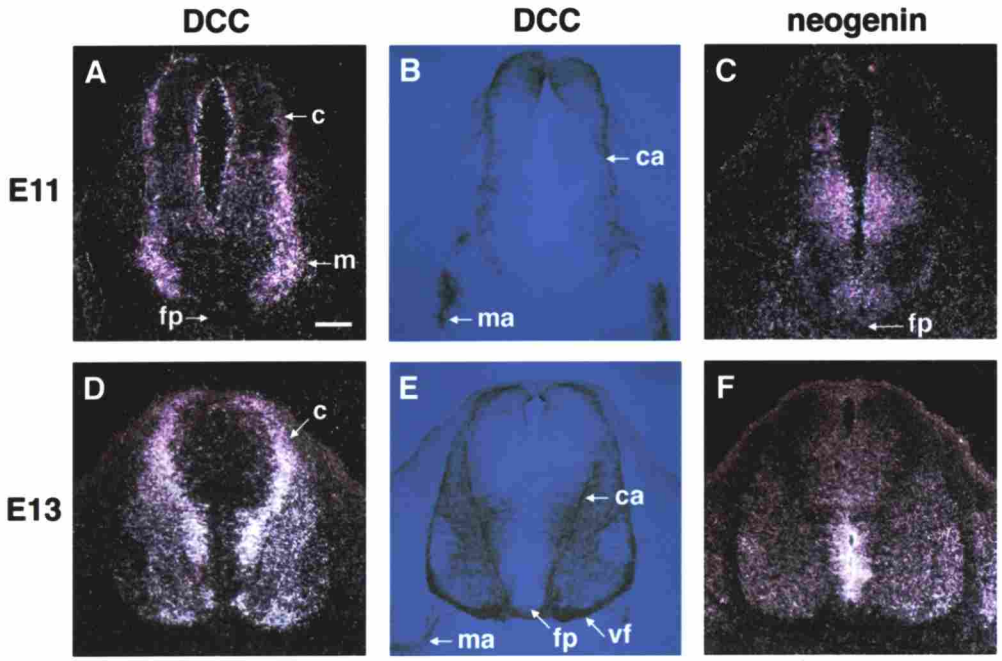


Figure 1. Expression of DCC and neogenin in the developing rat spinal cord

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). Semi-adjacent 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 bars are 50 μm in (A)-(C) and 100 μm in (D)-(F).

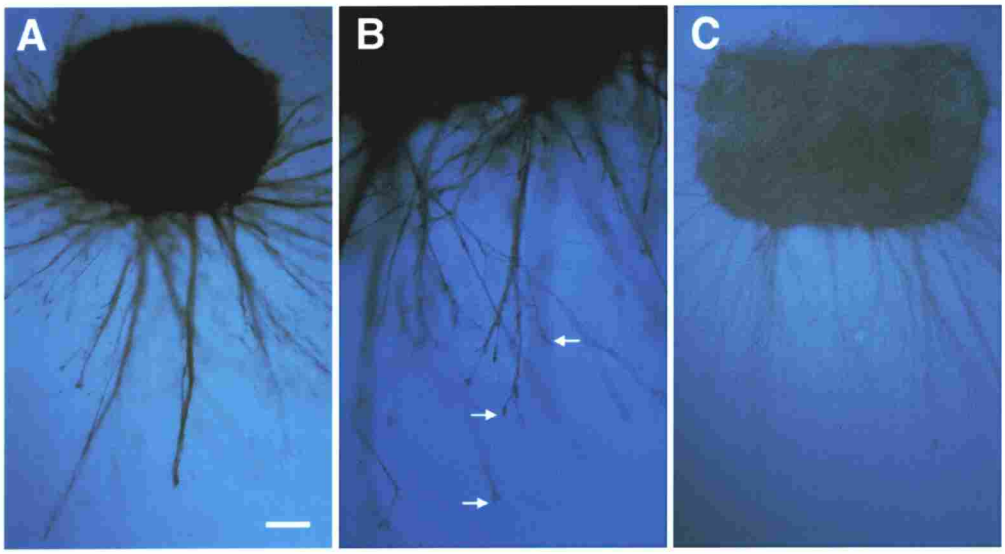


Figure 2. DCC is expressed by commissural axons and their growth cones
E11 dorsal spinal cord explants were cultured for 40 hr in collagen matrices with netrin-1 to evoke commissural axon outgrowth (Serafini et al., 1994), and immunostained with an antibody to DCC and an HRP-conjugated secondary antibody (A, B) or with secondary antibody alone (C). DCC immunoreactivity is detected on commissural axons (A) and their growth cones (B, arrows). Scale bars are 100 μm in (A) and (C), and 25 μm in (B).

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 netrin-1 binding was detected on non-expressing 293 cells (Figure 3E), consistent with the fact that netrins are heparin-binding proteins that can associate non-specifically 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 observations), specific binding of netrin-1 to DCC-expressing cells was maintained, but non-specific 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.

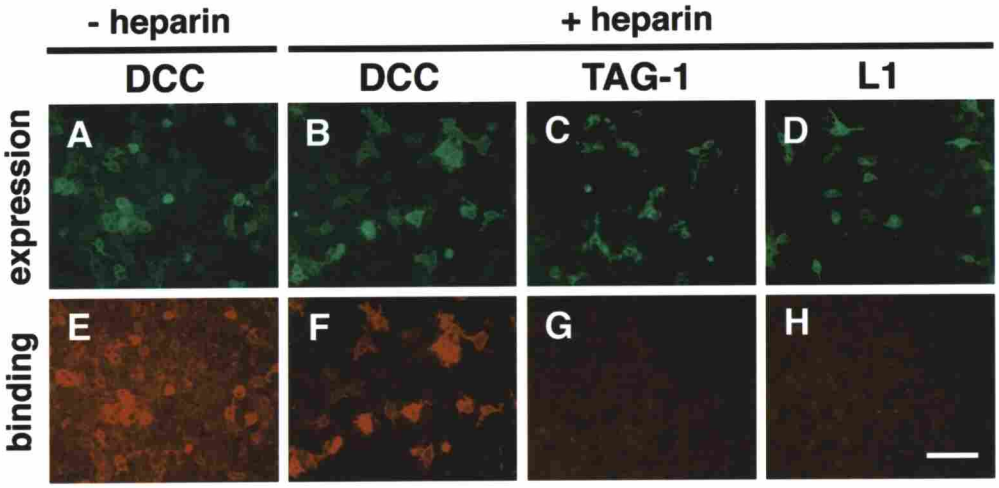


Figure 3. Netrin-1 binds 293 cells expressing DCC but not TAG-1 or L1
Fluorescense photomicrographs of 293 cells transfected with DCC (A, B, E, and F), TAG-1 (C and G), or L1(D and H) expression constructs. (A-D) Protein expression was detected using antibodies specific to each protein and FITC-conjugated 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). Panels (A) and (E) show the same field of cells, as do panels (B) and (F), and (C) and (G), respectively. Scale bar is 100 μm.

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 +/- 1.6 nM and n_H = 1.5 +/- 0.1 (mean +/- S. E. M.), 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 observations), 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., manuscript in preparation). 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 +/- 0.2 nM and n_H = 1.2 +/- 0.01 (mean +/- S. E. M., n = 2). Taken together our results suggest an order of magnitude for the K_d of ~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 interaction of the α2ß1 integrin with laminin-1 (Pfaff et al., 1994).

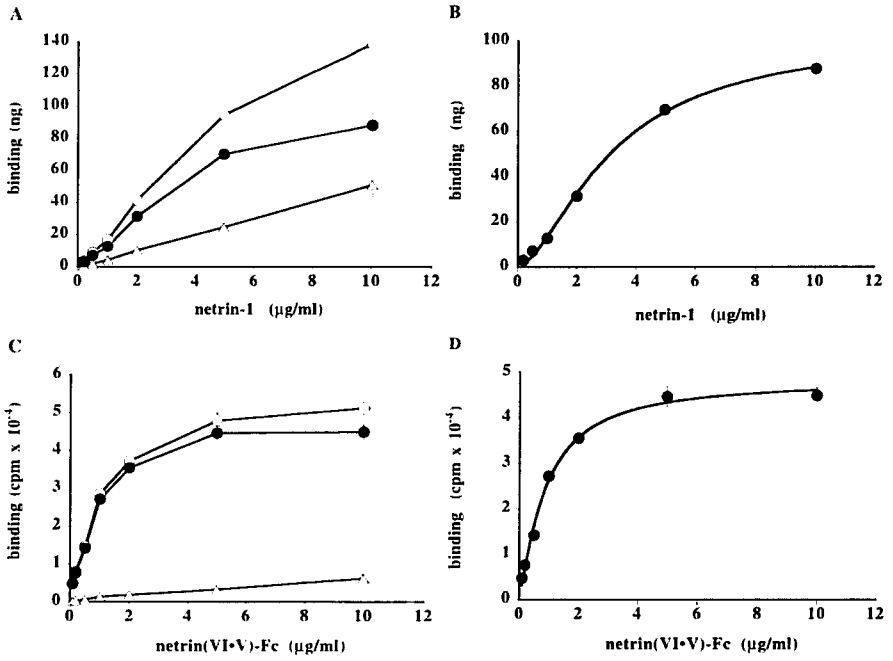


Figure 4. Equilibrium binding of netrin-1 and a netrin(VI•V)-Fc fusion protein to DCC-expressing cells

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 non-expressing cells; closed circles: specific binding (defined as the difference between these two values at each concentration). Bars indicate S. E. M. 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 Kd 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).

DCC function is required for axon outgrowth evoked by netrin-1

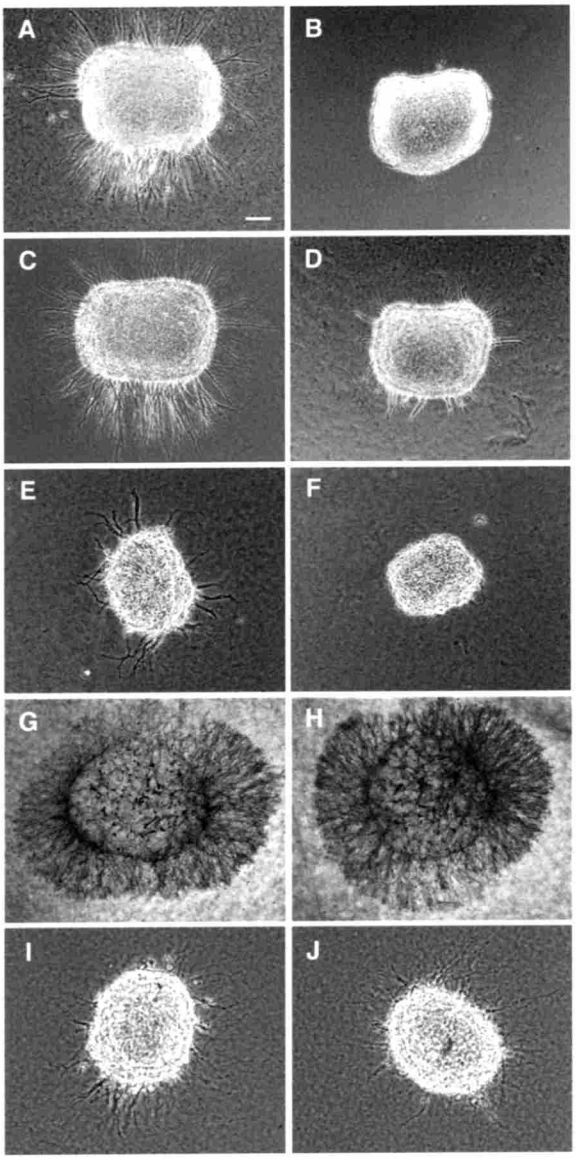
To test directly whether DCC is involved in mediating the effects of netrin-1, we examined the effect of perturbing DCC function on commissural axon outgrowth evoked by netrin-1 from explants of E11 or E13 rat dorsal spinal cord (Figures 5A and 5E). Addition of 0.1-10 μg/ml of a monoclonal antibody against 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, 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 less than 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 which appears to be due to netrin-1 secreted by these cells (Kennedy et al., 1994; Serafini et al., 1996). 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 (Figure5I). 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 quantify 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 +/- S. E. M., 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 dorso-ventral 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 towards 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.



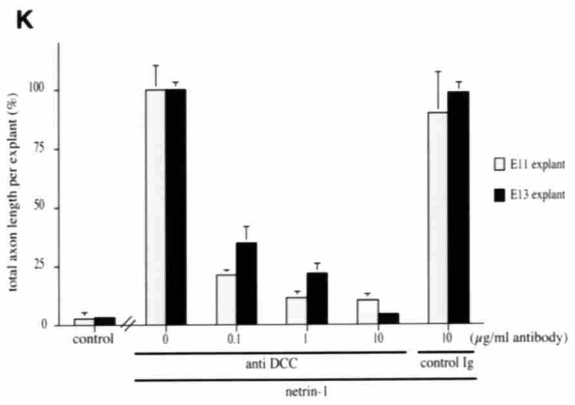


Figure 5. DCC function is required for axon outgrowth evoked by netrin-1

(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 + S. E. M. (n=4).

Scale bars are 100 μm in (A)-(D) and (G)-(H), and 50 μm in (E)-(F) and (I)-(J).

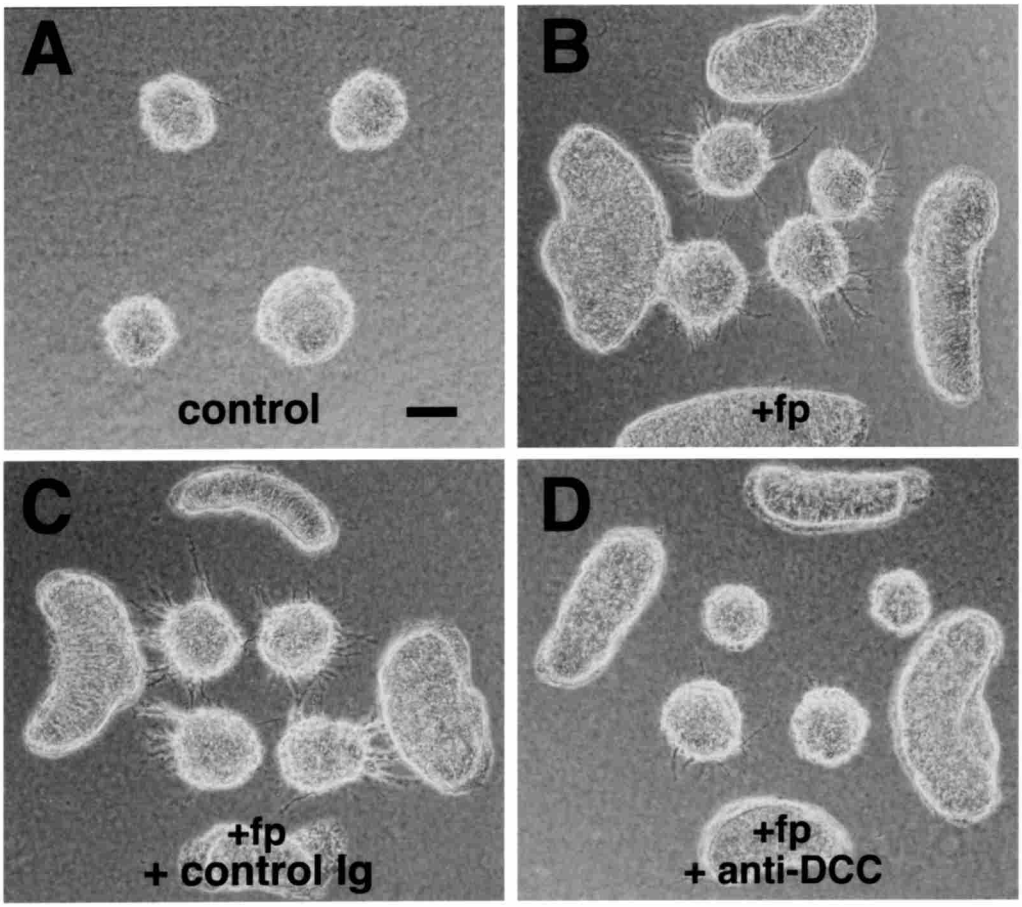


Figure 6. DCC function is required for axon outgrowth evoked by floor plate cells
Each panel shows four E13 dorsal spinal cord explants cultured for 14 hr in collagen matrices either alone (A) or surrounded by four explants of E13 rat floor plate (B-D). Explants were cultured either without antibody (A, 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 is 100 μm.

Other sites of DCC expression

Taken 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; Serafini et al., 1996), and other axonal populations have been shown to respond to netrin-1 at other axial levels in the nervous system (Shirasaki et al., 1996). 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 (Figure7 and data not shown). DCC was in particular observed on the axons 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).

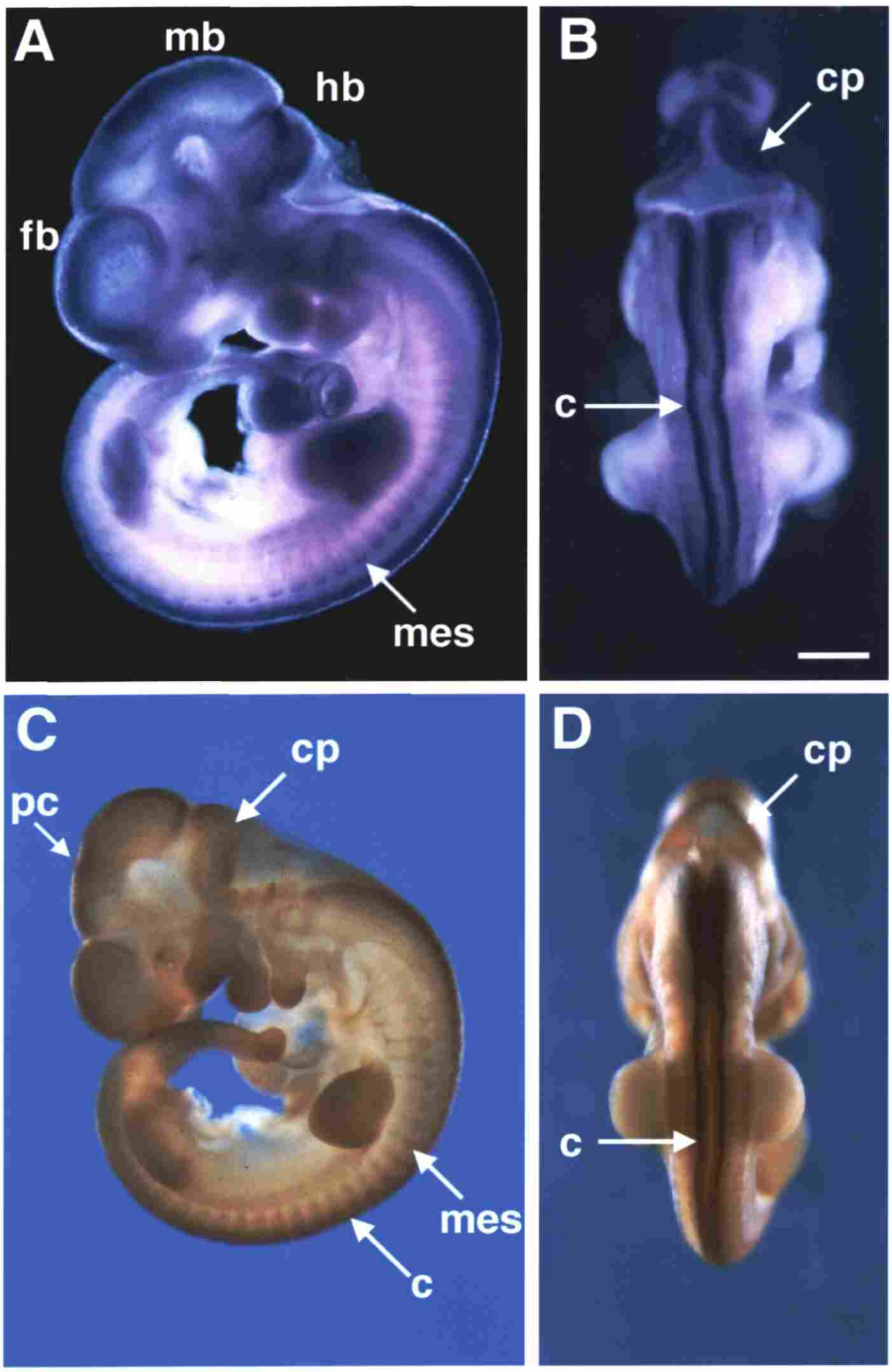


Figure 7. Sites of DCC expression at midgestation
Lateral views (A, C) and dorsal (back) views (B, D) of E12 rat embryos are shown. (A, B) *DCC* mRNA expression visualized by whole-mount in situ hybridization. Intense hybridization is seen to spinal commissural neurons (c), and to cells in the hindbrain (hb), midbrain (mb), forebrain (fb), and paraxial mesoderm (mes).
(C, 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 are 1.1 mm in (A) and (B), 1.3 mm in (C) and (D)

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 (Serafini et al., 1996), 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 homologue UNC-40 is required for a subset of axon guidance events that are directed

by UNC-6, a netrin-1 homologue. 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 homologue 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 homologues 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 observations; Shirasaki et al., 1996), 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 non-responsive 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. Thus, our test of whether DCC is required to mediate turning of commissural axons towards 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 non-netrin guidance cues. DCC could in principle interact with different ligands through different domains, as shown for the receptor-type protein tyrosine phosphatase ß (RPTPß) 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.,1995). 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 homologue 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 homologue 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 kind 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.

In situ hybridization

10 μm cryostat sections 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 non-overlapping 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 (Shirasaki et al., 1996; C. Mirzayan and M. T.-L., manuscript in preparation). 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). 48 hrs 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 1:1,000 dilution). Staining of L1 protein and bound netrin-1 for the L1-transfected cells were 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 x 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 x 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 an ^{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, 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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Chapter 2

Phenotype of mice lacking functional *Deleted in Colorectal Cancer (Dcc)* gene

Summary

The *Deleted in Colorectal Cancer (DCC)* gene was first identified as a candidate for a tumor suppressor gene on human chromosome 18q. More recently, *in vitro* studies in rodents have provided evidence that DCC might function as a receptor for the axonal chemoattractant netrin-1. Inactivation of the murine *Dcc* gene caused defects in axonal projections that are strikingly similar to those observed in *netrin-1*-deficient mice but did not affect growth, differentiation, morphogenesis ortumorigenesis in mouse intestine. These observations fail to support a tumor suppressor function for *Dcc*, but are consistent with the hypothesis that DCC is a component of a receptor for netrin-1.

Introduction

Deleted in Colorectal Cancer (DCC) encodes a group of structurally similar 175-190 Kilodalton (kDa) transmembrane proteins with four immunoglobulin and six fibronectin type III repeats in their extracellular domains (Fearon et al., 1990). *DCC* was first identified as a candidate for a tumor suppressor gene on human chromosome 18q that is frequently subject to loss of heterozygosity (LOH) in colorectal tumors (Fearon et al., 1990). LOH is a process often used by developing tumor cell clones to eliminate both copies of a tumor suppressor gene (Cavenee et al., 1983). Initially, one gene copy is inactivated by mutation; thereafter the second, intact gene copy is discarded and often replaced by a duplicated copy of the mutant allele. During the development of about 30% of colorectal tumors, LOH of 18q occurs in a relatively early step during which small adenomas (polyps) progress to more dysplastic, larger, intermediate adenomas (Boland et al., 1995). In the advanced adenomas - the immediate precursors of carcinomas - the incidence of 18q LOH approaches 65% (Fearon et al., 1990; Boland et al., 1995; Vogelstein et al., 1988).

Consistent with the possibility that 18q carries a colonic tumor suppressor gene are chromosome transfer experiments demonstrating that one normal copy of chromosome 18 is sufficient to suppress the tumorigenicity of human colon carcinoma cell lines (Tanaka et al., 1991; Goyette et al., 1992). However, the accumulated evidence has failed to implicate *DCC* conclusively as the colorectal tumor suppressor gene on chromosome 18q. For example, in one study analysis of the *DCC* alleles retained in human colonic tumors led to the discovery of mutant alleles in only 2 of 60

colonic tumors affected by 18q LOH (Cho et al., 1994). An additional 12% of the tumors carry insertions in one of the *DCC* introns (Fearon et al., 1990), but the effect of these insertions on *DCC* function is not known. Some have suggested, however, that mutations of *DCC* are not necessary to eliminate *DCC* function because *DCC* RNA, which is at the threshold of detection in normal colonic tissue through use of the polymerase chain reaction (PCR) (Fearon et al., 1990), is either reduced or completely lost in colon cancer cell lines, presumably by some means other than intragenic mutation (Fearon et al., 1990; Kikuchi-Yanoshita et al., 1992; Thiagalingam et al., 1996).

In vitro experiments have also failed to provide conclusive proof that *DCC* is a tumor suppressor gene. One study showed that high levels of anti-sense *DCC* RNA could promote the tumorigenicity of Rat-1 cells (Narayanan et al., 1996), but did not control for the possibility that the long term culture of the transfected cells inadvertently selected for those with increased tumorigenicity. A second study showed that DCC-overexpression in immortalized human keratinocytes caused poor growth *in vitro* and reduced tumor growth *in vivo* (Klingelhutz et al., 1996) but did not exclude the possibility that high levels of DCC protein, which is not normally expressed in these cells may, as many ectopically expressed proteins do, exert non-specific cytostatic effects on cells.

An unrelated line of investigation has suggested that DCC may function as a component of a receptor complex that mediates the effects of the axonal chemoattractant netrin-1 on commissural axons of the spinal cord of vertebrates (Keino-Masu et al., 1996). Commissural neurons differentiate in the dorsal portion of the spinal cord and their axons pioneer a ventrally-directed circumferential pathway that leads them to a

specialized group of cells at the ventral midline of the spinal cord termed floor plate cells (Colamarino and Tessier-Lavigne, 1995). During development of the nervous system, these axons are guided to the floor plate at least partly by the chemoattractant netrin-1, secreted by floor plate cells (Tessier-Lavigne et al., 1988; Placzek et al., 1990; Serafini et al., 1994; Kennedy et al., 1994; Serafini et al., 1996). Netrin-1 is a member of a phylogenetically conserved family of laminin-related molecules that has been implicated in axonal guidance in *Caenorhabditis elegans*, *Drosophila melanogaster* and vertebrates (Serafini et al., 1994; Kennedy et al., 1994; Ishii et al., 1992; Wadsworth et al., 1996; Mitchell et al., 1996; Harris et al., 1996). Loss-of-function phenotypes and genetic interactions in *C.elegans* and *Drosophila* have suggested that homologues of the DCC protein function in the response pathway of netrin family members (Chan et al., 1996; Kolodziej et al., 1996). In vertebrates, a biochemical interaction between netrin-1 and DCC has been demonstrated, and DCC has been implicated in mediating netrin-1 effects on spinal commissural axons *in vitro* through antibody perturbation studies (Keino-Masu et al., 1996), but the significance of this interaction for guidance of axons by netrin-1 *in vivo* has not been addressed.

To help elucidate the functions of the *DCC* gene, we have inactivated its homologue (*Dcc*) in the mouse genome through use of homologous recombination and have examined the effects of this inactivation on both the intestine and the developing nervous system.

Results

Inactivation of the mouse homologue of *DCC*

The Dcc^{X3} targeting vector (data not shown) was introduced into 129/Sv D3 embryonic stem (ES) cells (Gossler et al., 1986). Southern blot analysis demonstrated that three of the 200 gancyclovir- and G418-resistant ES cell clones screened had acquired a copy of the neomycin transferase (*neo*) gene in the mouse *Dcc* locus by homologous recombination (data not shown). We term this mutant allele Dcc^{-}. Mouse chimeras generated from two of these $Dcc^{+/-}$ ES cell clones transmitted the Dcc^{-} allele through their germline. All neonatal mice homozygous for the Dcc^{-} allele appeared to be grossly normal at birth but died within 24 hours. The $Dcc^{-/-}$ neonatal mice exhibited striking behavioral phenotypes, including the inability to suckle, labored respiration, abnormal body posture, and abnormal limb flexion in response to pinch stimuli. Immunoblots of proteins extracted from the brains of the $Dcc^{-/-}$ pups confirmed that the Dcc^{X3} mutation caused complete loss of DCC protein expression in the homozygous mutant mice (data not shown).

No increase in tumor predisposition or progression in $Dcc^{+/-}$ mice

To determine if and when $Dcc^{+/-}$ mice develop tumors, we followed a cohort of nearly two-hundred 129Sv/C57BL6 (129Sv/B6) F1 $Dcc^{+/-}$ mice for two years. $Dcc^{+/-}$ animals were monitored closely and screened at different ages for the presence of tumors in

their gut and extra-intestinal tissues. Only three *Dcc*$^{+/-}$ mice displayed signs of sickness, each being observed at ~18 months of age. One of these animals had six adenomas in its duodenum. The other two mice had brain neoplasms, one an astrocytoma, the other a meningioma.

To determine whether aged *Dcc*$^{+/-}$ mice have an increased tumor predisposition compared to the *Dcc*$^{+/+}$ mice, we performed autopsy on ninety-eight 129Sv/C57BL6 (129Sv/B6) F1 and F2 *Dcc*$^{+/-}$ mice and thirty-six *Dcc*$^{+/+}$ littermates, aged 1.5 to 2 years. In addition, the entire intestine of each animal was examined for the presence of adenomas. *Dcc*$^{+/-}$ mice showed no significant increase in tumor predisposition when compared to the control *Dcc*$^{+/+}$ mice (data not shown). Most of the neoplastic lesions found in both cohorts were detectable only by histological examination of tissues removed at the time of autopsy. No brain tumors were found in either the *Dcc*$^{+/-}$ or the *Dcc*$^{+/+}$ cohort. Southern blot analysis of genomic DNA prepared from tumors removed from 18 month old heterozygotes indicated that both the wild-type and the mutant *Dcc* alleles had been retained (data not shown; our analytical procedures were able to readily detect loss of the wild-type *Dcc* alleles in adenomas of comparable size as described below). In addition, over the course of last four years, we monitored cumulatively an additional three-hundred *Dcc*$^{+/-}$ mice for signs of brain tumors or intestinal polyposis but none were observed.

Although germline inactivation of a *Dcc* gene copy did not result in an increased tumor predisposition, it seemed possible that loss of both of gene copies would affect the progression of intestinal adenomas initiated by prior inactivation of the Adenomatous polyposis coli (*Apc*) gene. Indeed, LOH of 18q in human colonic

adenomas correlates with an increase in their size and morphological changes such as acquisition of a more dysplastic phenotype (Boland et al., 1995; Vogelstein et al., 1988; Fearon and Vogelstein, 1990), and some studies have linked the loss of *Dcc* expression to the appearance of adenomas that are of a more villus rather than tubular appearance (Fearon, 1995). To test effects on tumor progression, we introduced the null *Dcc* allele into the germline of the Min mouse (Moser et al., 1990) that carries a mutant *Apc* allele (Apc^{Min}) in its germline that predisposes it to develop small intestinal and colonic adenomas (Moser et al., 1990; Su et al., 1992; Moser et al., 1992).

Molecular genetic linkage and syntenic analyses have mapped the *Dcc* gene to mouse chromosome 18 within thirty centimorgans from the *Apc* gene (Justice et al., 1992; Luongo et al., 1993). Prior studies had demonstrated that nearly all adenomas in intestines of $Apc^{Min/+}$ mice lose not only the wild type allele of *Apc* but also the entire chromosome 18 harboring the wild type *Apc* allele (Luongo et al., 1994). This loss affects any heterozygosity that may exist at the *Dcc* locus, which happens to be located on chromosome 18 (Luongo et al., 1993). We took advantage of this linkage by introducing the mutant *Dcc* allele into the chromosome carrying the Apc^{Min} allele. We then bred mice having a genotype in which this doubly mutant chromosome was present opposite a fully wild-type chromosome 18. LOH analysis on the DNA of adenomas from Apc^{Min} Dcc^{-}/Apc^{+} Dcc^{+} mice followed by densitometric analysis showed that at least 8/10 of the adenomas in the cis compound heterozygous mice had lost the wildtype *Dcc* allele but had retained the mutant (null) Dcc^{-} allele (data not shown), as is seen for the linked wild-type *Apc* in virtually all adenomas in the Min mice (Luongo et al., 1994; Levy et al., 1994; Laird et al., 1995).

Germline heterozygosity for the mutant *Dcc* allele linked to the Apc^{Min} allele did not affect polyp initiation or the average size of the adenomas (data not shown). Moreover, histological analysis failed to reveal a change in the morphology of the adenomas (data not shown). An equal proportion (approximately 1/20) of the adenomas in Apc^{Min} Dcc^{+}/Apc^{+} Dcc^{+} mice and in Apc^{Min} Dcc^{-}/Apc^{+} Dcc^{+} mice exhibited local invasion, with extension of cyst- and finger-like projections of adenomatous cells deep into muscularis mucosa (data not shown). Thus, loss of the wild type allele of the *Dcc* gene occurred in the large majority of intestinal adenomas in the cis compound heterozygous mice but it could not be correlated with tumor progression (data not shown).

Gastrointestinal epithelial proliferation and differentiation in newborn $Dcc^{-/-}$ mice

To determine whether DCC has a role in regulating the proliferation and differentiation of the intestinal epithelium, we compared the intestinal tissue from five inbred 129/Sv newborn (P1) $Dcc^{-/-}$ mice with that from three $Dcc^{+/+}$ and five $Dcc^{+/-}$ littermates. Inspection of hematoxylin- and eosin-stained sections prepared along the length of the duodenal-ileal axis revealed no differences in villus height or cellular architecture (data not shown). S-phase cells were confined to the intervillus epithelium in all animals, and similar numbers and distribution of BrdU-positive cells within the intervillus epithelium were observed in all animals (data not shown). Expression of the CCAAT enhancer-binding protein (C/EBPa) provides a sensitive index of the proliferative state of enterocytes, the principal intestinal epithelial cell lineage (Chandrasekaran et al.,

1993). C/EBPa was present at highest concentrations in epithelial cells located in the intervillus epithelium and the lower half of duodenal and jejunal villi in a pattern that was identical in the epithelia of all three sets of mice (data not shown). Thus, loss of *Dcc* function does not affect intestinal epithelial proliferation on the first day after birth.

Epithelial differentiation was also examined using a panel of nine lectins and eleven antisera. Glycoconjugate production, which can be monitored with lectins, is a sensitive reporter of perturbations in intestinal epithelial differentiation (Falk et al., 1994). Loss of *Dcc* produced no apparent changes in the pattern of lectin binding to the enterocytic lineage (data not shown). Substance P- and serotonin-producing enteroendocrine subpopulations are good markers of the spatial complexity of this lineage's differentiation program along the crypt-villus and duodenal-ileal axes (Roth and Gordon, 1990). Loss of *Dcc* did not have any detectable effects on either of these subpopulations (data not shown).

Previous studies have suggested that DCC is most prominently represented in goblet cells (Hedrick et al., 1994), but loss of *Dcc* did not cause any spatial or developmental changes in goblet cell differentiation, as assessed by the pattern of lectin binding (Falk et al., 1994) (data not shown). Periodic Acid Schiff (PAS) and Alcian Blue together stain essentially all goblet cells in the P1 mouse intestine. There was no statistically significant difference ($p>0.05$) in the number of PAS/Alcian Blue-positive goblet cells in the proximal, mid, or distal small intestine or in the colon of $Dcc^{-/-}$ mice compared to their $Dcc^{+/-}$ or $Dcc^{+/+}$ littermates.

The Paneth cell lineage is confined to the base of small intestinal crypts and undergoes a characteristic developmental stage-specific sequence of expression of

marker gene products from E15 to P28 (Bry et al., 1994). Loss of *Dcc* did not produce any precocious induction of these markers at P1 (data not shown). The basement membrane underlying the intestinal epithelium receives contributions from both epithelial and mesenchymal cell populations (Louvard et al., 1992). Laminin and collagen IV are prominent components of this basement membrane. Loss of *Dcc* did not affect the amount or distribution of these proteins along the nascent crypt-villus or duodenal-colonic axis (data not shown). Thus, these studies failed to detect any change in the development of the intestinal epithelium of newborn mice as a result of loss of *Dcc*.

Finally, as it was the case with the small intestine and the colon, loss of DCC did not affect histochemical stainings (hematoxylin and eosin, Alcian Blue, and PAS), *in situ* binding to lectins or incorporation of BrdU into S-phase cells in the epithelial cells of the stomach (data not shown).

Proliferation, migration and differentiation in adult *Dcc*$^{-/-}$ chimeric intestine

To assess the function of *Dcc* in the adult intestine, we generated chimeras composed partly of wild type B6 cells and partly of 129/Sv *Dcc*$^{-/-}$ cells. In the intestine of a B6<->129/Sv chimeric mouse, the crypts are known to be populated by either B6 or 129/Sv cells but each villus is populated by cells originating in several adjacent crypts (Schmidt et al., 1988; Gordon and Hermiston, 1994). These can be readily distinguished because 129/Sv but not B6 enterocytes bind the lectin Ulex europeaus agglutinin type 1

(UEA-1) (Hermiston et al., 1993; Hermiston and Gordon, 1995) (data not shown).

A portion of the B6 *Dcc*$^{+/+}$<->129/Sv *Dcc*$^{-/-}$ chimeras appeared normal at birth and lived to adulthood. In these mice, some of the villi encountered at the border of ES cell- and host blastocyst-derived epithelium were polyclonal, being composed of cells originating from both 129/Sv-*Dcc*$^{-/-}$ and B6-*Dcc*$^{+/+}$ crypts. These polyclonal villi contained coherent vertical columns of wholly UEA1-positive 129/Sv (*Dcc*$^{-/-}$) enterocytes and adjacent columns of wholly UEA-1-negative B6 (*Dcc*$^{+/+}$) enterocytes (data not shown).

The presence of these two easily distinguished cell populations on a single villus provided the opportunity to assess the effects of *Dcc* loss on morphogenesis and cell migration in adult intestinal villi. The ES-derived *Dcc*$^{-}$/ UEA1^{+} cellular columns extended from the base to the tip of each polyclonal villus. The border between ES and B6 *Dcc*$^{+/+}$ columns was well defined (data not shown), indicating that the absence of *Dcc* function did not affect the normal orderly migration of cells from the crypt to the villus tip. Moreover, the two sides of such polyclonal villi were indistinguishable morphologically. Villi that were composed exclusively of UEA-1^{-} *Dcc*$^{+/+}$ B6 cells had the same height as adjacent villi composed of wholly UEA-1^{+}*Dcc*$^{-/-}$ 129/Sv cells (data not shown), indicating that the absence of *Dcc* did not perturb the rate of cell production in crypts and/or the rate of cell loss from villi. Finally, no intestinal or gastric adenomas or tumors of other tissues were found in a cohort of ten two-year-old chimeric mice at the time of their sacrifice. In these chimeric mice, which sired only *Dcc*$^{+/-}$ progeny when mated with wild type mice, the 129/Sv *Dcc*$^{-/-}$ cells contributed to at least 90% of the coat color.

Hence, by all experimental measures tested, the absence of DCC function did not affect either the normal or the neoplastic mouse gastrointestinal tissue.

Defects in spinal commissural axon projections in *Dcc*$^{-/-}$ mice

Based on *in vitro* experiments, DCC has been proposed to function as a receptor that mediates the guidance of commissural axons by netrin-1 to the floor plate of the spinal cord (Keino-Masu et al., 1996). To assess the trajectories of commissural axons at E9.5-E11.5 in *Dcc*$^{-/-}$ mice, we labeled these axons in transverse sections of spinal cord at forelimb levels (8 -/-, 6 +/- and 5 +/+ embryos) using an antibody to TAG-1, a surface protein expressed by commissural axons as they project to the floor plate (Dodd et al., 1988). Within the dorsal spinal cord of E9.5-E11.5 embryos homozygous for the null *Dcc* allele, we noted a reduction in the number of commissural axons, though those that did extend appeared to adopt a normal dorsal-to-ventral trajectory (Fig. 8 and data not shown). In certain sections (see Fig. 8B and 8D), some axons were observed that projected all the way to the floor plate. In other sections, however, no axons were seen reaching the floor plate (data not shown). This heterogeneity along the rostrocaudal axis was evident in sagittal views of the spinal cord (Fig. 9; n=10 -/-, 6 +/- and 5 +/+ embryos). In addition, there appeared to be a misrouting of many of the axons that did project into the ventral spinal cord, with some projecting more medially and others more laterally (Fig. 8D).

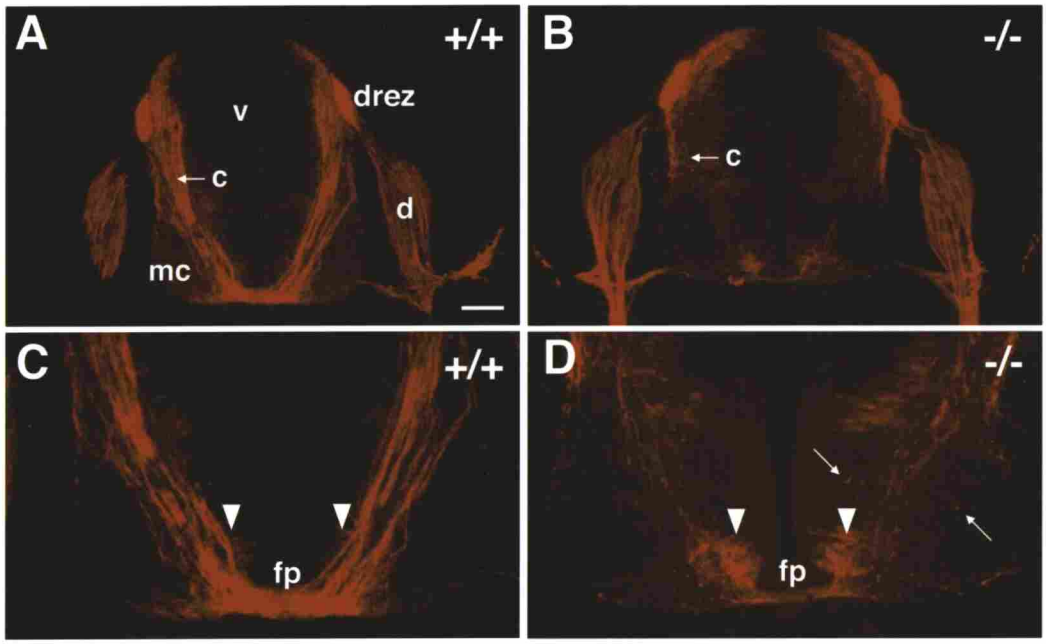

Figure 8. Defects in commissural axon projections in *Dcc*-/- embryos
Trajectories of commissural axons are visualized using an antibody to TAG-1 in sections of wildtype (A, C) and *Dcc*-/- (B, D) E11.5 embryos. In *Dcc*-/- embryos, TAG-1+ commissural neurons are present but few axons extend into the ventral spinal cord (B) and those that do, project along aberrant trajectories (arrows in D). Projections of sensory axons and motor axons in the ventral roots appear largely normal (B). Arrowheads in (C) and (D) indicate a population of TAG-1+ cells adjacent to the floor plate. Additional abbreviations: d, dorsal root ganglion; drez, dorsal root entry zone; mc, motor column; v, ventricle; c, commissural axons; fp, floor plate. Scale bar, 100μm in (A, B), 50 μm in (C, D).

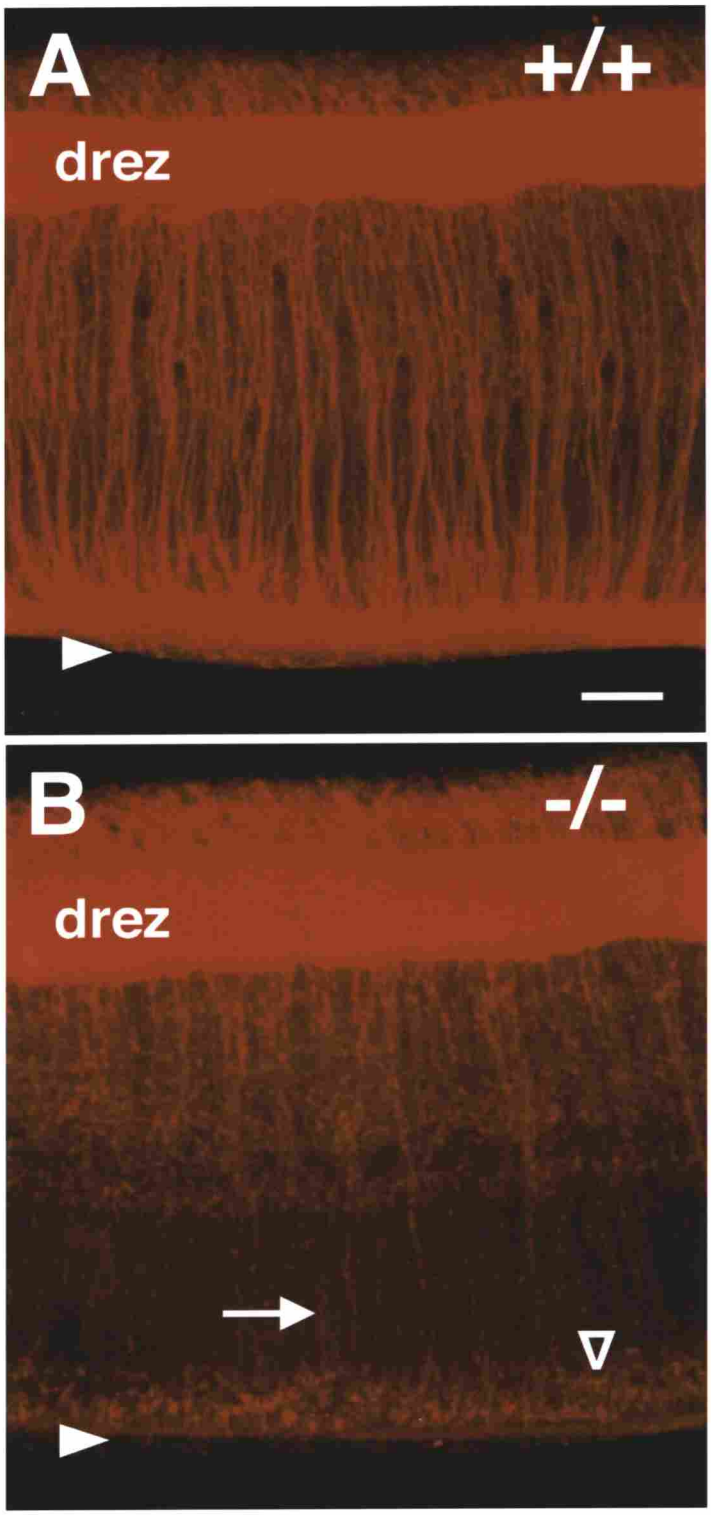

Figure 9.
Few commissural axons reach the floor plate in *Dcc*-/- embryos

Sagittal views (side views) of spinal cords from a wildtype (A) and a *Dcc*-/- (B) E11.5 embryo showing commissural axons visualized by TAG-1 immunohistochemistry. Dorsal is up, ventral is down (arrowhead indicates floor plate) (drez indicates sensory axons in the dorsal root entry zone). The characteristic microsegmented projections of commissural axons along a dorso-ventral trajectory to the floor plate are visible in the wildtype embryo (A). In the mutant (B) many fewer axons extend within the dorsal spinal cord, and only a few reach the floor plate (arrow). Open arrowhead indicates TAG-1+ cells adjacent to floor plate. Scale bar, 100 μm.

This impression was confirmed by examining the trajectory of commissural axons using the fluorescent lipophilic dye-I (DiI) injected into the cell bodies of these neurons in the dorsal spinal cord, which diffused down their axons. The pattern of axonal projections visualized in this way at E11.5 was very similar to that observed by TAG-1 immunoreactivity both in wildtype embryos (compare Fig. 8A, 8C and Fig. 10A, 10B), and in 9/9 *Dcc*$^{-/-}$ embryos (compare Fig. 8B, 8D with Fig. 10D-F), and confirmed the misrouting of commissural axons in the ventral spinal cords of *Dcc*$^{-/-}$ embryos. The defects in axonal projections observed in and around the spinal cord appeared specific to commissural axons, inasmuch as the trajectories of sensory and motor axons (the latter of which express DCC (Keino-Masu et al., 1996)) appeared normal, as visualized using the TAG-1 marker (compare Fig. 8A and 8B) and using an antibody to neurofilament protein (data not shown). Thus, *Dcc*$^{-/-}$ embryos show defects in commissural axon projections that are similar to those seen in *netrin-1*-deficient mice (Serafini et al., 1996) but are more severe (see Discussion).

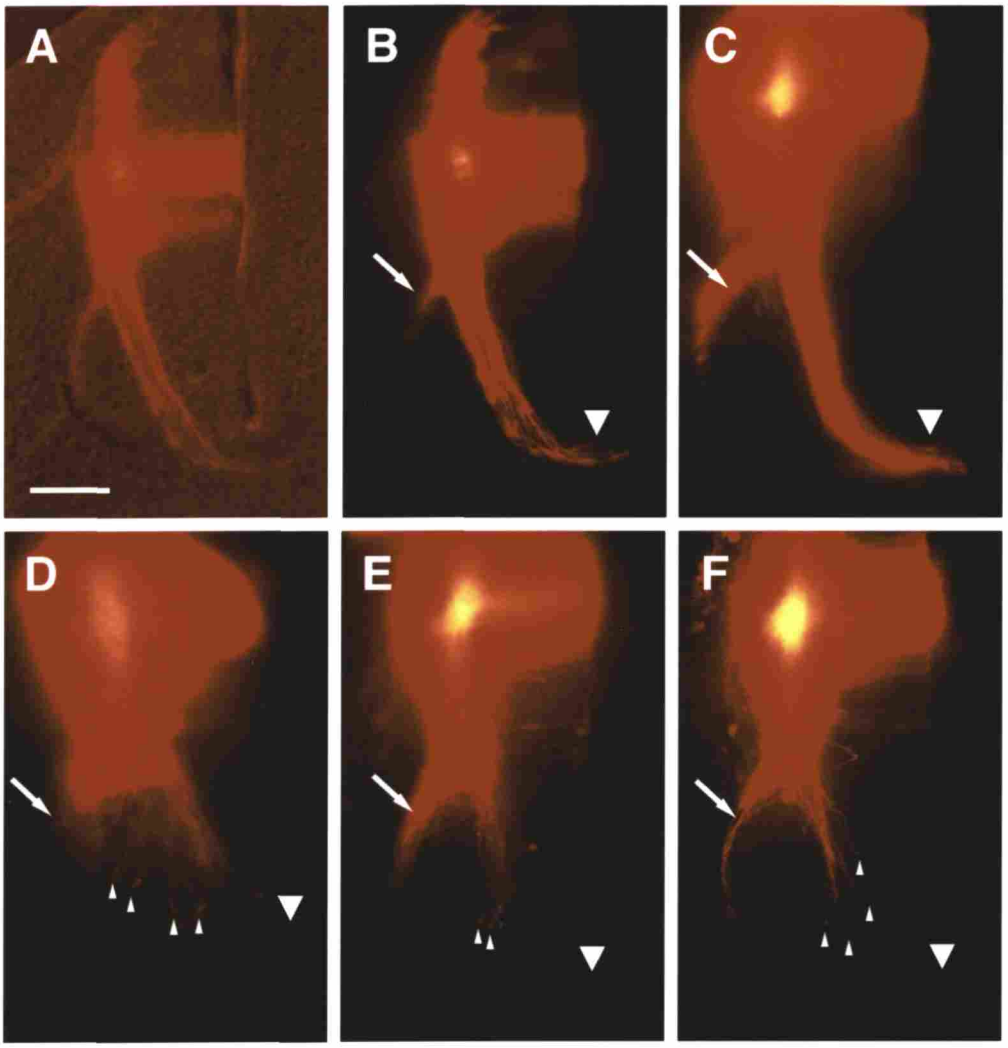

Figure 10. Defects in commissural axon projections revealed by dye-tracing
DiI was injected into the dorsal spinal cord of wildtype (A, B), heterozygous (C), and homozygous mutant *Dcc*-/- E11.5 embryos (D-F) and allowed to diffuse down commissural axons. Axonal projections were visualized in transverse vibratome sections. In (B-F) large arrowheads indicate floor plate and arrows indicate axons projecting along the lateral edge of the motor column (presumptive ipsilaterally-projecting axons). Note that less DiI was injected in the wildtype embryo (A, B) than in the other embryos (C-F). (A, B), Combined differential interference contrast and fluorescence micrograph (A), and fluorescence micrograph (B), of a wildtype embryo, showing the normal trajectory of commissural axons to the floor plate. Note that ipsilaterally-projecting axons originating in the dorsal spinal cord do not express TAG-1 (compare to Fig. 8A and C). (C), A similar pattern of projection is observed in *Dcc*+/- embryos (n = 12/12). (D-F), Trajectory of axons labeled from the dorsal spinal cord in 3 different *Dcc*-/- embryos. The trajectory of presumptive commissural axons revealed by dye-tracing in *Dcc*-/- embryos is similar to that seen by TAG-1 labeling (comapre to Fig. 8B and D): many fewer axons project into the ventral spinal cord; some axons project ventromedially but are not particularly directed towards the floor plate (E, F), and many axons wander within the motor column (D). Small vertical arrowheads in D-F indicate a sampling of commissural growth cones. Few axons reach the floor plate (large arrowheads in D-F). Similar results were observed in 9/9 *Dcc*-/- embryos.

Defects in brain development in *Dcc*$^{-/-}$ mice

In addition to defects in spinal cord development, *netrin-1*-deficient animals show multiple defects in brain development (Serafini et al., 1996). Similar defects were observed in the brains of *Dcc*$^{-/-}$ mice. The corpus callosum and hippocampal commissure appeared to be completely absent (compare Fig. 11A and 11B). The axons that normally form these commissures appeared to be present but failed to cross the midline and remained ipsilateral, projecting to aberrant locations and forming tangles ("Probst bundles"). The anterior commissure in *Dcc*$^{-/-}$ mice was also severely reduced: the large anterior and posterior limbs of the anterior commissure (Fig. 11C) did not form and join near the midline; Fig. 11D shows the largest remnant of this commissure that was detected in *Dcc*$^{-/-}$ mice. As in *netrin-1*-deficient animals, these defects did not reflect a generalized defect in commissure formation, as the habenular and posterior commissures both appeared to be intact (Fig. 11E and 11F). In the brainstem of *Dcc*$^{-/-}$ mice, a commissure that is not normally present was observed in the junctional region between hindbrain and midbrain (compare Fig. 11G and 11H). In addition, the pontine nuclei at the base of the rostral hindbrain appeared to be absent in the mutant animals (compare Fig. 11I and 11J). Thus, the defects seen in the brain of *Dcc*$^{-/-}$ animals were similar to those observed in *netrin-1*-deficient animals (Serafini et al., 1996).

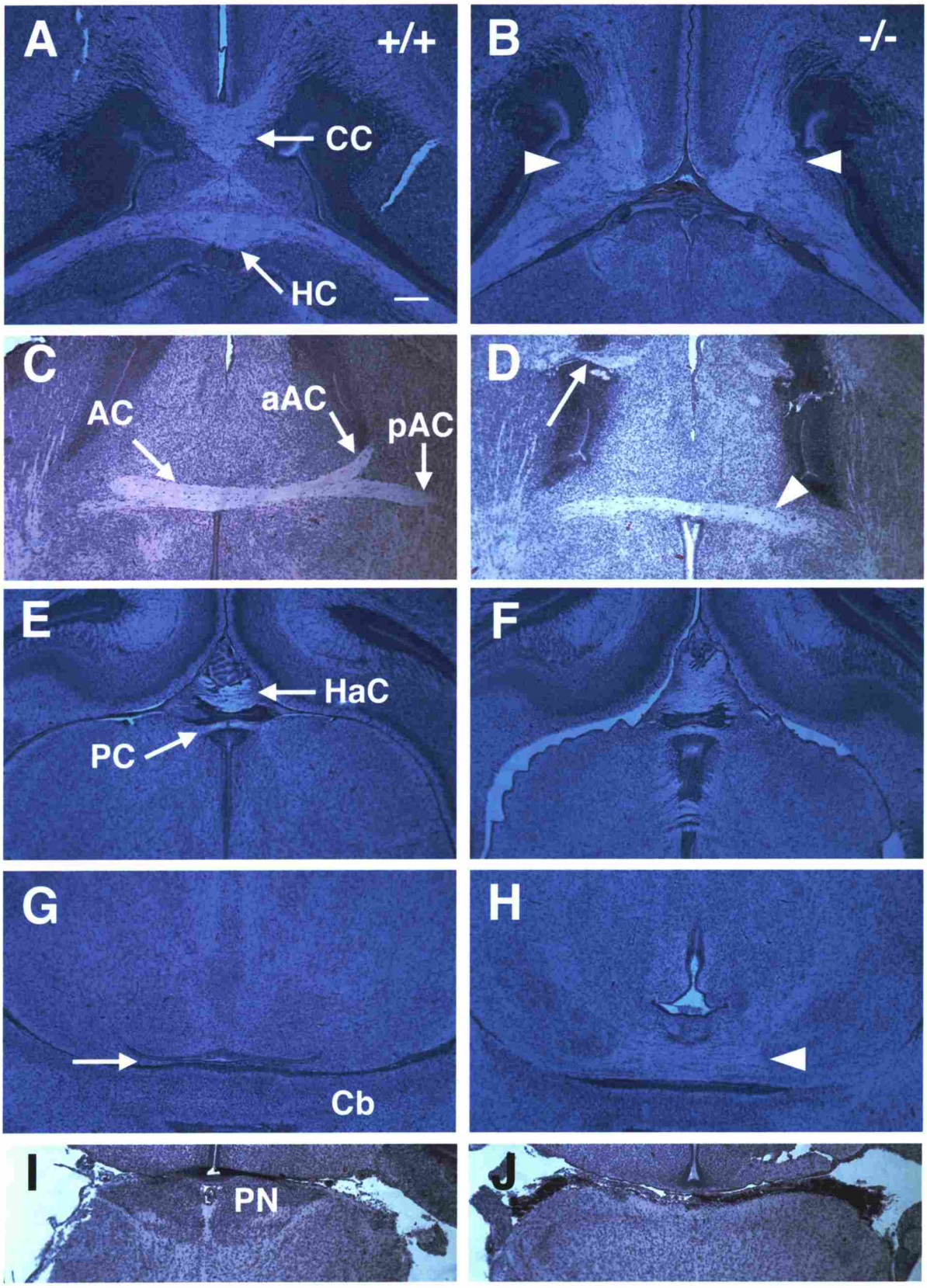
A
+/+
CC
HC
B
-/-
C
AC
aAC
pAC
D
E
HaC
PC
F
G
Cb
H
I
PN
J

Figure 11. Defects in brain commissures and absence of pontine nuclei in *Dcc*$^{-/-}$ embryos

Horizontal sections from brains of wildtype or heterozygous neonatal pups (A, C, E, G, I), and homozygous mutant pups (B, D, F, H, J) stained with hematoxylin and eosin (rostral is up in each). The phenotype of wild-type and heterozygous pups were indistinguishable. (A, B). Corpus callosum (CC) and hippocampal commissure (HC) of a wildtype (A) and *Dcc*$^{-/-}$ (B) pup (arrowheads, Probst bundles). (C, D) The anterior commissure (AC) in a wildtype (C) and a homozygous *Dcc*$^{-/-}$mutant (D) pup. (aAC, anterior limb; pAC, posterior limb). In homozygous mutant brains an aberrant bundle of axons (arrow in D) was observed approaching the midline at more rostral levels, which may consist of misrouted axons that normally form the anterior limb. The posterior limbs were much reduced but not, apparently, completely absent, and in most mutants a reduced commissure was observed (arrowhead). (E, F) The habenular commissure (HaC) and posterior commissure (PC) are present in wildtype (data not shown), heterozygous mutant (E), and homozygous mutant (F) pups. (G, H) In *Dcc*$^{-/-}$pups (H), a thick commissure (arrowhead) is observed in the roof of the fourth ventricle in the region of the hindbrain-midbrain junction, that is not observed in wildtype pups (arrow in G) or heterozygous pups (data not shown). Cb, cerebellum. (I, J) The pontine nuclei (PN) at the base of the rostral hindbrain in wildtype (data not shown) and heterozygous (I) pups are not detected in *Dcc*$^{-/-}$pups (J). Scale bars: 175μm in (A, B, E, F), 135μm in (C, D), 150μm in (G, H), 200 μm in (I, J).

Discussion

Candidacy of *DCC* as a tumor suppressor gene in colon cancer

The experiments described here were initially designed to assess the role of the human *DCC* gene in colonic tumorigenesis through manipulation of its mouse homologue. While we observed a small number of tumors in several tissues in aged $Dcc^{+/-}$ mice, this number was no greater than that observed in wild-type mice (data not shown), and those lesions examined genetically gave no evidence of loss of the remaining wild type *Dcc* allele. A potentially more sensitive test of a tumor suppressor role of *DCC* involved inactivation of both alleles of *Dcc* in mouse intestinal adenomas. Since *DCC* has been described as a tumor suppressor gene whose expression is eliminated during colonic tumor progression following *APC* inactivation (Boland et al., 1995; Vogelstein et al., 1988; Fearon and Vogelstein, 1990; Fearon, 1995), we expected that its loss in the mice would accelerate the progression of, or modify the phenotype of polyps initiated in the $Apc^{Min/+}$ mice. We observed no such effect (data not shown). A third line of investigation addressed any part played by the *Dcc* gene in morphogenesis of colonic crypts and villi, and proliferation and development of the associated epithelial cell populations in the mouse. The absence of *Dcc* had no effect on proliferation or differentiation of intestinal epithelial cells or intestinal morphogenesis (data not shown). Taken together, these three lines of investigation argue that *Dcc* plays little if any part in the physiology of the intestinal epithelium in the mouse. It should also be emphasized, however, that although these results weaken the candidacy of the *DCC* gene as an important regulator of intestinal epithelial cell proliferation in the mouse, they do not

rule out definitively its role in human colon cancer pathogenesis.

The strongest evidence in support of the role of *DCC* as a tumor suppressor stems from observations of the reduction or loss of *DCC* RNA in cell lines or xenografts derived from human colon carcinomas (Fearon et al., 1990; Kikuchi-Yanoshita et al., 1992; Thiagalingam et al., 1996). However, a similar LOH of 18q and loss of *DCC* RNA expression has been observed in many pancreatic tumors (Hohne et al., 1992), in which another gene on chromosome 18q21, termed *DPC4*, has recently been identified as the tumor suppressor gene that is the target of genetic inactivation (Hahn et al., 1996). The importance of *DPC4* in pancreatic tumorigenesis is strongly supported by the discovery of a number of inactivating mutations affecting this gene in a series of tumor samples (Hahn et al., 1996). Thus, in human colon cancer, as is seemingly the case in pancreatic cancer, the loss of *DCC* expression may be a consequence of events affecting a linked gene. According to this scenario, the observed LOH of 18q21 affects not only the *DCC* gene but other neighboring genes as well, one or more of which is the bonafide target of inactivation during colon tumor progression. The definitive proof of this possibility can only come from the identification of a gene on 18q that undergoes LOH and whose retained allele is also mutated in the majority (up to 65%) of colonic tumors. This linked gene is unlikely to be either *DPC4* or *JV18* because their retained alleles appear to be mutated in only a fraction of human colon tumors that suffers 18q LOH (Thiagalingam et al., 1996).

Requirement of DCC for commissural axon projections in the spinal cord

The present results indicate profound effects of loss of DCC function on the development of axonal projections in the central nervous system. These defects are observed in the same classes of axons that are affected in *netrin-1*-deficient animals (Serafini et al., 1996). Our observations are therefore consistent with the hypothesis that DCC is a component of an axonal receptor for netrin-1 (Keino-Masu et al., 1996).

Although the overall similarity in phenotypes in the *DCC*$^{-/-}$ and *netrin -1*-deficient mice is striking, spinal commissural axons appear more foreshortened in the dorsal spinal cord in *Dcc*$^{-/-}$ mice (compare this study to Serafini et al., 1996). This difference could be due to the possibility that the studied *netrin-1* allele was not a complete null allele, and that some residual netrin-1 function was present in those animals (Serafini et al., 1996). An alternative possibility is that DCC is required not only to mediate responses to netrin-1 but also to mediate the responses of commissural axons to other cues that collaborate with netrin-1 to guide these axons.

We also note that some commissural axons do in fact succeed in reaching the floor plate in the *Dcc*$^{-/-}$ mice (Fig. 8 & 9). These observations point to the existence of a DCC-independent mechanism for guidance of commissural axons to the floor plate. It is possible that netrin-1 itself can function through a DCC-independent mechanism to guide commissural axons in the *Dcc*$^{-/-}$ mice. Alternatively, cues distinct from netrin-1 might be present that guide commissural axons toward the floor plate (see Serafini et al., 1996) and function in a DCC-independent mechanism.

DCC as a mediator of netrin-1 effects in the brain

The finding of defects in brain morphogenesis in *Dcc*$^{-/-}$ animals that are similar to those observed in *netrin-1*-deficient animals suggests that DCC is in the response pathway for netrin-1 effects in the brain. It is not known how the defects in the brain arise in either the *netrin-1*-deficient or the *Dcc*$^{-/-}$ animals, although the distribution of *netrin-1* mRNA has suggested that netrin-1 might function as a guidance cue for the cells and axons that are affected (Serafini et al., 1996). This is true not just for the defects in axonal projections that are observed, but also for the pontine nuclei, the absence of which might reflect a failure of migration of postmitotic pontine neurons (discussed in Serafini et al., 1996). If so, the absence of pontine nuclei would indicate that DCC functions to guide migrating cells in addition to axons.

Although the precise role of netrin-1 and DCC in guiding migrations in the brain remains to be elucidated, our finding of similar defects in *Dcc*$^{-/-}$ and *netrin-1*-deficient animals parallels the observation, in *C. elegans* and *Drosophila*, that mutations in homologues of *Dcc* and *netrin-1* give similar neural phenotypes (Chan et al., 1996; Kolodziej et al., 1996), and provides strong support for the hypothesis that the wiring of the nervous system involves rules and mechanisms that are highly conserved among nematodes, insects and vertebrates (Goodman, 1994).

Experimental procedures

Generation and analysis of mice with Dcc^{X3} mutation.

The Dcc^{X3} targeting vector was constructed using a fragment of the *Dcc* gene that extends from intron two to intron three. This DNA fragment was isolated from a 129/SV mouse genomic library using a probe containing human exon 3 (gift of Dr. Bert Vogelstein) which encodes most of the protein's second immunoglobulin-like domain. Sequencing of the isolated fragment identified a stretch of 283 nucleotides that was 100% identical to the reported sequence of exon 3 (codons 138-232) of Mus musculus *Dcc* gene (Cooper et al., 1995). EST data bank searches also revealed that the predicted amino acid sequence encoded by the isolated exon is 41% and 40% identical to a stretch of amino acids in rat and human neogenin, respectively, and 93% identical to codons 138-232 in human DCC. Embryonic stem cell culture, generation of chimeric mice, southern and western blot analyses were performed according to standard procedures.

C57BL/6 $Apc^{Min/+}$ (Jackson Laboratory, Bar Harbor, Maine) and 129/Sv $Dcc^{+/-}$ animals were crossed to generate 129Sv/B6 F1 $Apc^{Min/+}$ $Dcc^{+/-}$ mice. These F1 mice were then backcrossed to 129/Sv mice; the compound heterozygote progeny of this latter cross had the mutant allele of both the *Apc* and the *Dcc* gene in the cis configuration. Homozygous mutant $Dcc^{-/-}$ ES cells were generated from 129/Sv $Dcc^{+/-}$ ES cells using a previously described method (Mortensen et al., 1992).

Immunohistochemical analysis of intestines.

Single and multilabel immunohistochemical analyses of postnatal day 1 (P1) 129/Sv *Dcc*$^{-/-}$, *Dcc*$^{+/-}$ and *Dcc*$^{+/+}$ mice were conducted in a single blinded fashion and as described previously (Hermiston et al., 1993; Hermiston and Gordon, 1995). A panel of 11 antisera were used: (i) goat anti-BrdU (S-phase cells), (ii) rabbit anti-rat liver fatty acid binding protein (villus enterocytes) ; (iii) rabbit anti-intestinal fatty acid binding protein (enterocytes); (iv) rabbit anti-C/EBPa (nonproliferating epithelial cells); (v) rabbit anti-pan-cadherin (epithelial cadherins), (vi) rabbit anti-laminin, (vii) rabbit anti-collagen IV; (viii) rabbit anti-serotonin; (ix) rabbit anti-substance P; (x) rabbit anti-cryptdin (Paneth cells); and (xi) rabbit anti-enhancing factor (Paneth cells). Nine lectins were used to examine differentiation of intestinal epithelial cells: (i) Jacalin (Artrocarpus integrifolia agglutinin); (ii) Peanut agglutinin; (iii) Tirchosantes kirilowii; (iv) Dolichos biflorus agglutinin; (v) Helix pomentia agglutinin; (vi) Griffonia simplifolica; (vii)Ulex europeaus agglutinin type I; (viii) Cholera toxin B subunit; and (ix) Sambucus nigra agglutinin. The number of goblet cells was defined in the proximal, mid and distal thirds of the small intestine and in the colon. PAS/Alcian Blue-stained goblet cells in 20 well-oriented, nascent crypt-villus units were counted/region/mouse (n = 2 animals/genotype). Paired Student's T test was performed to assess whether any statistically significant differences existed in the total number of goblet cells/region of *Dcc*$^{-/-}$, *Dcc*$^{+/-}$ and *Dcc*$^{+/+}$ intestines.

Spinal cord histochemistry and brain histology.

Immunohistochemistry on transverse sections of spinal cord was performed with antibodies to TAG-1 (4D7, gift of M. Yamamoto) and NF-M (gift of V. Lee) as previously described (Serafini et al., 1996) except that 20 μm-thick sections were used

and 0.2% fish skin gelatin was added to PHT buffer. Whole-mount immunohistochemistry was performed essentially as described previously (Serafini et al., 1996) (protocol available on request). Dye-tracing experiments were performed as described previously (Stoeckli and Landmesser, 1995).

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General discussion

I have shown that DCC is expressed on the axons of the spinal commissural neurons in rat embryos when they grow to and across the ventral midline, and that netrin-1 binds to the cells expressing recombinant rat DCC. Moreover, my antibody perturbation experiments showed that the function of DCC is required to mediate the axon outgrowth-promoting effect by netrin-1 on the commissural axons *in vitro*. These results (discussed in Chapter 1) led me to propose that DCC encodes a receptor or a component of a receptor that is required for the outgrowth of the spinal commissural axons. The significance of the DCC functions in guiding axons *in vivo* was addressed by the analysis of the *Dcc* knockout mice (discussed in Chapter 2). *Dcc*-deficient mice show selective defects in the projections of spinal commissural axons, inasmuch as the trajectories of the motor and sensory axons appear normal. The commissural axons project along their ventral trajectory in the dorsal spinal cord, but they deviate from the normal trajectories toward the floor plate and extend in aberrant directions as they approach the developing motor columns. This defect is very similar to, though severer than, that of *netrin-1*-deficient mouse. DCC, therefore, appears to function as a netrin receptor in guiding the spinal commissural axons *in vivo* as proposed from my *in vitro* studies.

The commissural axons of the *Dcc*-deficient mice seem to be more foreshortened and reduced in number in the dorsal spinal cord than those in *netrin-1*-deficient mice (Chapter 2). Three explanations are possible. First, *netrin-1* mutant allele that was studied is not a complete null allele and small amount of residual netrin-1 protein may be sufficient to guide these axons (discussed in Serafini et al.,1996).

Second, DCC might be required, in general way, to extend axons independent of the interaction with its ligands, although it dose not seem very likely because the spinal motor axons and the axons that form the posterior commissure express DCC but appear to be intact in the *Dcc*-deficient mice. Third, DCC might respond to different ligands that collaborate with netrin-1 to guide these axons to the ventral spinal cord.

Although the overall projections of spinal commissural axons are greatly affected, a small number of these axons appear to project along a roughly normal trajectory and reach the ventral midline in *Dcc*-deficient mice (Chapter 2). These observations point to the existence of a DCC-independent mechanism for guidance of commissural axons to the ventral midline. It is possible that netrin-1 can guide axons via a DCC-independent mechanism. Alternatively, the cues distinct from netrin-1 might be present and guide axons via a DCC-independent mechanism. Similar partial defect in the spinal commissural axon projection was also observed in *netrin-1*-deficient mice (Serafini et al.,1996), raising the possibility that there might be other guidance cues for these axons.

It is also possible that DCC has distinct functions (e.g. acts as a receptor for unknown ligands) on the spinal motor axon guidance, because the motor axons express DCC but do not respond to netrin-1 (Chapter 1). However, no obvious defects in motor axon projections are observed in *Dcc*- deficient mice (Chapter 2), suggesting that the role of DCC as itself or as a receptor for unknown ligands is not crucial, if any, for guiding motor axons. Alternatively, neogenin, another rat homologue of UNC-40, could compensate the loss of DCC on the motor axons in the *Dcc*-deficient mouse, because *neogenin* is normally expressed by the motor neurons at lower level and its

gene product also can bind netrin-1 (Chapter 1).

In addition to the defects in the spinal cord, the *Dcc*-deficient mice also show severe defects in the formation of several commissures in the brain; corpus callosum, anterior limb of anterior commissure, hippocampal commissure. Mutant animals also completely lack pontine nuclei, which are formed by a long-range migration of postmitotic pontine precursor cells. These defects are strikingly similar to those observed in the *netrin-1*-deficient mouse (Serafini et al.,1996; Chapter 2). These results suggest that netrin and DCC, most likely as a ligand and its receptor, also play a crucial role in the axon guidance in the developing brain region and possibly also in the cell migration.

My finding of similar defects in *Dcc*- and *netrin-1*-deficient mice parallels the observation in *Drosophila* and in *C. elegans*, which showed that mutations in the homolgues of *Dcc* and *netrin-1* give similar phenotypes (Kolodziej et al., 1996; Chan et al., 1996). In *C. elegans*, the DCC homologue UNC-40 is required for a subset of axon guidance events that are directed by UNC-6, a netrin-1 homologue. 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). These studies have led to the proposal 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 homologue 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. Although it remains to be shown directly in each species that these DCC and netrin homologues can interact biochemically, it is very likely that this netrin-DCC system is highly conserved among different species to make up the nervous system. Thus, parallel studies in different species appear to be a powerful approach to elucidate common fundamental mechanisms in axon guidance, as was proved in the previous studies of other signaling pathways.

This study raises an interesting question in terms of the signal transduction pathway involved in axon guidance. The developing spinal motor axons and axons that form the posterior commissure express DCC (discussed in Chapter 1), although they do not appear to show either attraction or repulsion responses to netrin-1 *in vitro* (K. Brose and M.T.-L., personal communication; Shirasaki et al., 1996) and their projections are normal in *netrin-1*- and *Dcc*- deficient mice (Serafini et al.,1996; Chapter 2). This means that DCC is necessary, but 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 non-responsive axons might express cell-surface or intracellular components that block netrin-responsiveness of these axons despite their expression of DCC.

The identification of DCC as a functional netrin receptor in this study will help to elucidate the insights of the netrin-mediating signal transduction pathways. Further studies are required to identify proteins that interact with DCC, such as other component(s) of a netrin receptor complex, or intracellular signaling component(s). This study will provide important clues to understand how chemotropic signals are

transduced and how neurons or axons respond to these guidance cues.

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