Netrin 1 mediates spinal cord oligodendrocyte precursor dispersal

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SUMMARY

In spinal cord, oligodendrocyte precursors that give rise to myelin-forming cells originate in a restricted domain of the ventral ventricular zone. During development, these cells migrate widely throughout the spinal cord. Netrin 1 is expressed at the ventral ventricular zone during oligodendrocyte precursors emigration, and, in vitro, netrin 1 acts as chemorepellent and antagonizes platelet-derived growth factor (PDGF) chemoattraction. Oligodendrocyte precursors express the netrin receptors DCC and UNC5 and function-blocking anti-DCC antibody inhibits chemorepulsion of ventral spinal cord explants and netrin-secreting cells. In spinal cord slice preparations, addition of function-blocking anti-DCC antibody or netrin 1 dramatically inhibits oligodendrocyte precursor migration from the ventral ventricular zone. These data indicate the initial dispersal of oligodendrocyte precursors from their localized origin is guided by a chemorepellent response to netrin 1.

Key words: Oligodendrocyte precursor, Migration, Netrin, Chick

INTRODUCTION

Development of the vertebrate central nervous system is characterized by extensive cell migrations during which cells initially generated in discrete regions of the neural tube migrate to their appropriate destination using a variety of molecular cues (Hatten, 2002; Tessier-Lavigne and Goodman, 1996). In the mature spinal cord, oligodendrocytes are ubiquitously distributed throughout gray and white matter; however, the founder cells of the oligodendrocyte lineage arise in a specific domain of the ventral ventricular zone (Miller, 1996) that they share with motoneurons (Rowitch et al., 2002). The expression of distinct transcription factors, growth factors and cell-surface antigens characterizes stages in the development of oligodendrocyte precursors. The earliest well-characterized oligodendrocyte precursors expressing Olig1/2, (Lu et al., 2000; Zhou et al., 2000) are bipolar, bind the monoclonal antibody (mAb) A2B5 (Miller, 1996), express the α receptor for platelet derived growth factor (PDGFαR) and proliferate in response to PDGF (Pringle et al., 1989; Pringle et al., 1992). As they mature, rodent oligodendrocyte precursors become multiprocessed, label with mAb O4 (Miller, 1996) and express galactocerebroside a major myelin lipid (Raff et al., 1978). Avian oligodendrocyte development is similar, although immature oligodendrocyte precursors label with mAb O4, which is retained during differentiation (Ono et al., 1995). In vitro and after transplantation, immature precursors are the most dispersive and migratory cells of the oligodendrocyte lineage (Noble et al., 1988; Warrington et al., 1993) and this migration is stimulated by the growth factors PDGF and FGF (fibroblast growth factor) (Armstrong et al., 1990).

Similar mechanisms may mediate oligodendrocyte precursor

and immature neuron migration. For example, radial neuronal migration occurs by association with radial glial cells (Rakic, 1972). Likewise, in forebrain, subventricular-derived glial precursors undergo radial migration (Kakita and Goldman, 1999) and spinal cord oligodendrocyte precursors are found in close association with radial glia (Hirano and Goldman, 1988). Non-radial dispersal or tangential migration is influenced by both chemoattractive and chemorepulsive cues (Hatten, 2002; Tessier-Lavigne and Goodman, 1996) such as netrins (Kennedy et al., 1994). Netrins can act as either chemoattractants or chemorepellents for distinct populations of developing neurons (Alcantara et al., 2000; Bloch-Gallego et al., 1999) depending on the specific combinations of receptors expressed (Hong et al., 1999; Keleman and Dickson, 2001). Two distinct receptor families have been implicated in orchestrating cellular responses to netrins (Ackerman et al., 1997; Chan et al., 1996; Leonardo et al., 1997), including DCC and UNC5. The migration of oligodendrocyte precursors along the developing optic nerve from their origin in the floor of the third ventricle is well established (Ono et al., 1997; Small et al., 1987) and has been proposed to be mediated in part by chemorepulsion to netrin 1 secreted by cells at the optic chiasm (Sugimoto et al., 2001), although netrin has also been suggested to be chemoattractive for optic nerve oligodendrocyte precursors (Spassky et al., 2002). In the spinal cord, netrin 1 is expressed during early embryogenesis in the floor plate and ventral spinal cord (Kennedy et al., 1994). This localization is close to the origin of oligodendrocyte precursors and suggests that netrin 1 might mediate spinal cord oligodendrocyte precursor migration.

In the current study, we demonstrate that netrin 1 is expressed in the ventral spinal cord during the period of initial

oligodendrocyte precursor emigration from their origin. In vitro, ventral spinal cord explants and netrin 1-secreting cells exert a chemorepulsive effect on migratory oligodendrocyte precursors and netrin 1 antagonizes PDGF chemoattraction on purified oligodendrocyte precursors in a chemotaxis assay. We further demonstrate that oligodendrocyte precursors express both DCC and UNC5, and inhibiting DCC signaling blocks the effects of netrin and ventral spinal cord explants. In slice preparations of embryonic chick spinal cord, migration of oligodendrocyte precursors to dorsal regions is inhibited by treatment with anti-DCC antibodies or recombinant netrin 1. These data suggest that netrin 1 provides a chemorepellent signal that results in the initial dispersal of oligodendrocyte precursors from their localized origin in the ventral spinal cord.

MATERIALS AND METHODS

Explant and cell cultures

Explant cultures of chick spinal cord (Fig. 1A) were established from appropriately staged embryos (Hamburger and Hamilton, 1951). For co-cultures, explants were positioned 200-500 μm apart. HEK 293 cells transfected with chick netrin 1 were maintained as described (Serafini et al., 1996) and aggregates were formed from 20 μl of cell suspension containing ~1×10^5 cells. Explants and aggregates were covered with growth factor-reduced matrigel matrix (BD biosciences) in F12 medium containing 10 ng/ml bFGF and 10 ng/ml PDGF and N2 supplement. After 72 hours, cultures were fixed with 4% paraformaldehyde (PFA) for 15 minutes and labeled with mAb O4. For antibody blocking experiments, anti-DCC antibodies in phosphate-buffered saline (PBS) without sodium azide (Oncogene) were added at concentration of 0.5 μg/ml. In controls, normal mouse IgG (R&D systems) was added at the same concentration.

Cell identification

Purified populations of oligodendrocyte precursors (>90%) from chick or rat spinal cords were prepared by immunopanning using mAbs O4 and A2B5 as previously described (Barres et al., 1992; Tsai et al., 2002). Purified oligodendrocyte precursors were double labeled with A2B5 and anti-DCC antibodies (R&D systems) as previously described (Tsai et al., 2002). For chick oligodendrocyte precursors, cells were fixed in 4% PFA and anti-DCC antibodies were diluted in 10% normal goat serum/PBS with 0.1% Triton X-100. Anti-neogenin antibodies (Santa Cruz) were diluted in 10% normal rabbit serum/PBS. All incubations were for 30 minutes at room temperature. Slides were viewed under a fluorescent microscope (Leica DMR) and images were taken by Hamamatsu CCD camera using Simple PCI software (Compix).

Chemotaxis assay

Chemotaxis assays were performed using a 48-well Boyden chamber (Neuro probe, AP 48) (Tsai et al., 2002) (Fig. 4D). The lower chamber was flooded with PDGF (Sigma) and/or chick netrin 1 (R&D systems) in F12 medium with N2 supplement (Gibco). Polycarbonate membranes (Osmonics, 8 μM) were coated with poly-L-lysine and pan-purified oligodendrocyte precursors from E7 chick spinal cords added to the upper chamber with PDGF and/or netrin 1. After incubation for 20 hours at 37°C, the membrane was fixed in methanol for 15 minutes and stained with ethidium bromide (Molecular probes, 4 μM in PBS) for 8 minutes. Non-migrating cells on the upper side of the membrane were removed and the number of migrating cells counted. Experiments were performed in triplicate at least three times. Three to four fields were counted under 20× magnification for each well, and data are compared between groups by the Student's two-tailed *t*-test (Microsoft Excel).

Quantification of oligodendrocyte precursor migration

Directional migration was analyzed by Metamorph (Universal imaging corporation). Migration distances of oligodendrocyte precursors were measured between the center of the explants and the top 10% of the fastest migrating cells. The area covered by oligodendrocyte precursors was measured from the same images by subtracting the area of the explants from the total area covered by O4+ cells. The results from at least four different explants of each type from three independent preparations were pooled and the results compared by the Student's two-tailed *t*-test. In co-cultures, the relative distribution of oligodendrocyte precursors was determined by bisecting the explant into proximal and distal domains relative to the putative guidance cue and the number of cells in each domain compared. Only O4-positive cells from intermediate explants with bipolar or unipolar morphology pointing toward ventral explants were counted in proximal regions (Fig. 6D). Explant culture results were also analyzed by the Student's two-tailed *t*-test (Microsoft Excel).

Slice culture and statistical analysis

Slice cultures were prepared from stage 28 or 31-32 chick thoracic spinal cords. Spinal cords were embedded in 4% low-temperature melting agarose gel (Seaplaque, FMC bioproducts). Sections (250 μM) were cut on a vibratome (Leica, VT1000S) and transferred to culture medium (DMEM with 10 ng/ml PDGF, 10 ng/ml FGF, N2 supplement, 27 mM glucose and 1% BSA). Blocking antibodies and ligands were added at following concentrations: anti-DCC antibody and control mouse IgG at 10 μg/ml, chick netrin 1 (R&D) at 500 ng/ml. Anti-NCAM antibody (clone 5e, Developmental Studies Hybridoma Bank, The University of Iowa) specific to the chick extracellular region of the protein (Frelinger and Rutishauser, 1986; Watanabe et al., 1986) was added at 10 μg/ml. After appropriate culture intervals, preparations were fixed with 4% PFA and labeled with mAb O4 as previously described (Ono et al., 1995). For statistical analysis, O4-positive cells were counted in dorsal, intermediate and ventral regions of each slice. Because the controls were not expected to approach a normal distribution, the proportions of O4-positive cells in each region were compared between control and experimental slices by a Mann-Whitney nonparametric test (SPSS version 11.0).

RT-PCR

Total RNA was extracted by RNeasy Mini kit (Qiagen) and was subjected to reverse transcription for cDNA by Superscript first-strand synthesis system (Gibco). The primers for DCC were 5′-TCA(C/T)-CCTTCCAACCT(C/G)TATGC and 5′-TC(G/T)(A/G)AAGGT(A/G)-TACATGGCTTC. The primers for UNC5-1 were 5′-GAGTCAC-CTTCCCACCTCTAC and 5′-AGACTGGCGATGATCTTTTG. The primers for UNC5-2 were 5′-GTCTCAGGGTCTACTGTCTGG and 5′-GTGGTATCTTGAAGGCATAGG. The primers for neogenin were from published results (Vielmetter et al., 1994). The primers for chick netrin 1 were 5′-TACTGCAAGGAAGGCTTCTAC and 5′-TCAT-GTTGATCTTCAGCTTCC. The PCR programs were run on DNA Engine thermocycler (MJ Research) using the following program: 93°C for 30 seconds; 30 cycles of 93°C for 30 seconds, 56°C for 1 minute, 70°C for 1 minute; and the last step of 70°C for 10 minutes. The DCC receptor products were digested with *Bsa*HI (NEB) for 1 hour at 37°C after RT-PCR amplification. The products were analyzed on 1.2% agarose gel.

RESULTS

Oligodendrocyte precursors are repelled by ventral spinal cord

Spinal cord oligodendrocyte precursors originate in the ventral ventricular zone and subsequently populate both gray and white matter (Miller et al., 1997). In chick spinal cord,

oligodendrocyte precursors can be identified by mAb O4 labeling at stage 28 where they form an integral component of the ventral ventricular lining (Ono et al., 1995). Subsequently, these cells or their progeny migrate to populate the entire spinal cord. To examine whether this dispersal is a result of repulsion from the ventral ventricular zone, three regions of chick spinal cord were cultured in isolation, and their ability to generate oligodendrocyte precursors and direct oligodendrocyte precursor migration assessed.

Separation of stage 29 chick spinal cord into dorsal (D), intermediate (I) and ventral (V) regions (Fig. 1A) provided explants with different oligodendrogenic characteristics. Dorsal explants contained virtually no oligodendrocyte precursors or oligodendrocytes after 3 days in culture (Warf et al., 1991) (Fig. 1B,C). Intermediate explants contained migratory oligodendrocyte precursors but no floor plate or ventricular source. By contrast, ventral explants contained migratory oligodendrocyte precursor cells as well as floor plate and a ventricular source of new cells resulting in substantially more O4-positive cells associated with ventral than intermediate explants (Fig. 1D,E). The extent and pattern of oligodendrocyte precursor migration differed between ventral and intermediate explants. Ventral explants displayed more extensive but not uniformly radial migration, while intermediate explants displayed more uniformly radial migration. Ventrally derived O4-positive cells migrated further (Fig. 1F) and covered a larger area (Fig. 1G) than intermediate derived cells.

The extensive migration of oligodendrocyte precursors from ventral explants may reflect ventral chemorepulsive activity. To test this, stage 29 intermediate explants were co-cultured with ventral explants to screen for directional cues. Intermediate-derived migrating oligodendrocyte precursors were repelled by ventral explants. In isolation, O4-positive cell migration from intermediate explants was uniformly radial (Fig. 2A). By contrast, when co-cultured in close proximity to ventral explants, the distribution of intermediate-derived migratory oligodendrocyte precursors was no longer uniformly radial (Fig. 2B), although the total number of O4-positive oligodendrocyte precursors was not significantly different, suggesting that co-culture did not influence oligodendrocyte precursor survival or proliferation. A greater number of oligodendrocyte precursors were present in regions of intermediate explants distal to than proximal to ventral explants (Fig. 2B). Quantification of the relative distribution of O4-positive cells revealed that 61±3% were distal to ventral explants while only 39±3% of O4-positive cells were proximal to ventral explants. This differential distribution of O4 cells was seen in greater than 70% of ventral-intermediate co-cultures (see Fig. 6E,F), although the magnitude varied depending on the proximity of the explants with a maximal effect at distances of less than 500 μm. The morphology of O4-positive cells proximal and distal to ventral explants was slightly different. Distally the majority of cells were unipolar with a leading process (Fig. 2D); however, cells oriented towards ventral explants had shorter or multiple processes (Fig. 2C). The observation that oligodendrocyte precursors oriented towards ventral explants did not demonstrate morphological characteristics of oriented growth may reflect two populations of oligodendrocyte precursors that differ in response to ventral-derived repulsive cues.

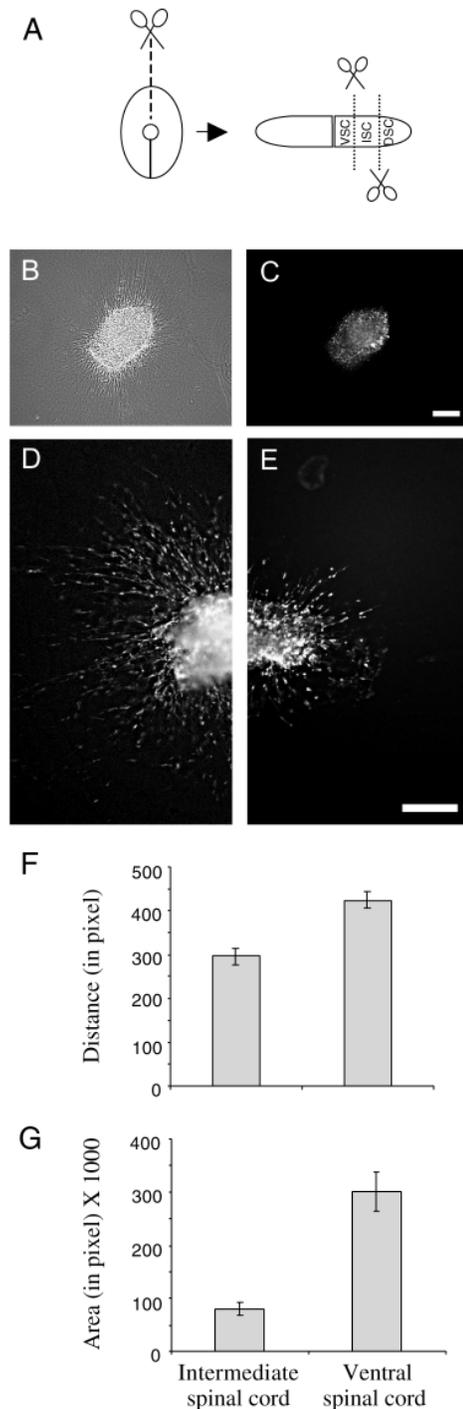Directed migration of oligodendrocyte precursors was only

Fig. 1. Generation and migration of oligodendrocyte precursor depends on the spinal cord region. (A) The spinal cord shows three distinct domains used in this study. The spinal cord was opened at the roof plate and cutting at the sulcus limitians generates dorsal explants. Ventral regions were then bisected into intermediate and ventral explants. (B,C) Dorsal explants from stage 29 chick embryos did not give rise to oligodendrocyte precursors after 3 days in culture, consistent with previous findings (Warf et al., 1991), although numerous axon bundles were seen. (D,E) Ventral and intermediate explants generated O4-positive oligodendrocyte precursors that differed in number and migrational distances. (F,G) Ventral explants generated higher numbers of oligodendrocyte precursors that migrated farther than those from intermediate explants. $P<0.001$. Scale bar: 250 μm.
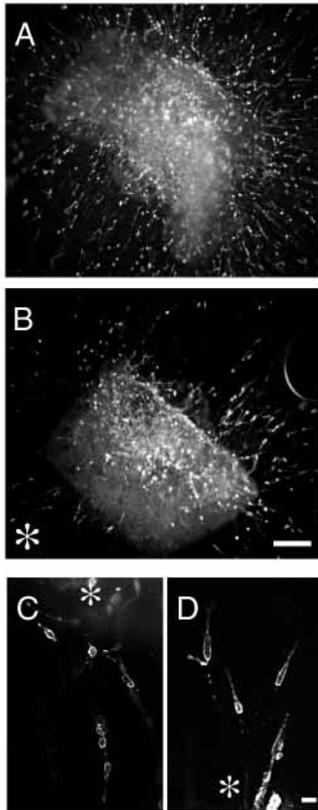
Fig. 2. Ventral explants provide a chemorepulsive cue for intermediate explant derived migratory oligodendrocyte precursors. (A) The migration pattern of O4-positive cells from isolated stage 29 intermediate explants was uniformly radial after 3 days in culture. (B) Co-culture of ventral and intermediate explants resulted in non-radial migration of O4-positive cells. More cells migrated away from than toward to the ventral explant. Asterisk indicates position of the ventral explant. (C,D) The morphology of O4-positive cells proximal and distal from the ventral explant was subtly different. Distally (D), O4-positive cells had longer processes, whereas proximally (C) they had shorter and frequently multiple processes. Asterisks in C,D indicate location of intermediate explants. Scale bars: in A, 100 μm for A,B; in D, 20 μm for C,D.

seen with ventral/intermediate explant combinations. Co-culture of two ventral explants did not result in preferential migration away from adjacent explants, suggesting that directional repulsive cues established by one explant may be negated by those from the other. Likewise, co-culture of two intermediate explants did not result in preferential migration away from adjacent explants. Furthermore, co-culture of dorsal and intermediate explants did not provide evidence for attractive or repulsive cues from dorsal explants (data not shown). These observations are consistent with the hypothesis that a ventrally derived repulsive cue mediates the initial dispersal of migratory spinal cord oligodendrocyte precursors.

Netrin 1 is expressed at the spinal cord ventral midline during oligodendrocyte precursor migration

Candidate guidance molecules for oligodendrocyte precursor dispersal include netrin 1 that is expressed in the floor plate and ventral ventricular zone earlier in development (Kennedy

et al., 1994). To determine whether netrin 1 expression was maintained during the period of initial oligodendrocyte precursor emigration, the presence of netrin 1 mRNA in stage 29 ventral spinal cords was examined by RT-PCR. Using primers specific for chick netrin 1, mRNA was detected in samples of ventral spinal cord and rostral CNS that was retained until E9 (Fig. 3). Control preparations without reverse transcriptase did not amplify any detectable products (data not shown). These data demonstrate the continued expression of netrin 1 between the stages 29 (E6) and E9 in the chick ventral spinal cord, a developmental period that correlates with the initial migration of oligodendrocyte precursors from the ventral ventricular domain.

Netrin 1 repels purified oligodendrocyte precursors in a chemotaxis assay

The preferential migration of intermediate-derived oligodendrocyte precursors away from ventral explants in co-culture assays, combined with the expression of netrin 1 in ventral spinal cord suggests that netrin 1 may provide a chemorepulsive signal to oligodendrocyte precursors. However, such a signal may act directly on oligodendrocyte precursors or indirectly through other netrin responsive neural cells (Varela-Echavarria et al., 1997). To determine unambiguously whether netrin 1 was chemorepulsive for, and acted directly on chick spinal cord oligodendrocyte precursors a chemotactic Boyden chamber (Harvath et al., 1980) assay was employed. Purified oligodendrocyte precursors placed on the filter surface migrate through the filter in response to putative chemotactic molecules added to the upper or lower chamber (Fig. 4D). Addition of netrin 1 to the lower chamber significantly reduced the number of cells that migrated through the filter compared with controls (Fig. 4A,B,E; control 13.1±0.6 cells/field versus netrin 1 treated (100 ng/ml) 7.7±1.0). By contrast, addition of PDGF (20 ng/ml) to the lower chamber increased the number of cells that migrated through the filter (Fig. 4C; 23.4±0.8 cells/field), indicating that PDGF but not netrin 1 is a chemoattractant for chick oligodendrocyte precursors, as it is for rodent cells (Armstrong et al., 1990). The reduction in migrating cells with netrin 1 in the lower chamber might reflect either chemorepulsion or an
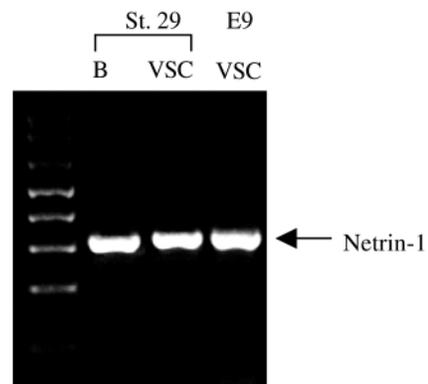

Fig. 3. Netrin 1 mRNA is present in the ventral spinal cord during the period of oligodendrocyte precursor migration. At stage 29 (E6), RT-PCR analyses revealed a band of the expected size (300 bp) in brain (B) and ventral spinal cord (VSC). Expression was retained until E9.

inhibition in cell translocation (Tsai et al., 2002). To distinguish between these possibilities, netrin 1 was added to the upper chamber along with purified oligodendrocyte precursors and the number of cells that migrated through the filter assayed. Although low concentrations of netrin (20 ng/ml) had no effect (13.0±1.3, P=0.9), at higher concentrations the number of migrating cells increased in a dose-dependent manner in response to netrin 1 (18.6±1.0 at 100 ng/ml, 20.1±1.1 at 400 ng/ml) (Fig. 4F). These data indicate that netrin 1 acts as a chemorepellent rather than a stop signal for oligodendrocyte precursor migration.

Netrin chemorepulsion antagonizes the chemoattractive activity of PDGF. When PDGF and 100 ng/ml netrin 1 were added to the lower chamber, the numbers of migrating cells were significantly reduced compared with those seen with PDGF alone (Fig. 4E, 16.4±0.9 cells/field). In the presence of 400 ng/ml netrin 1, PDGF chemoattractive activity was almost totally abolished (Fig. 4E, 14.0±1.0) and the number of migrating cells was similar to that in controls (P=0.44). These observations indicate that netrin 1 is a bona fide chemorepellent for oligodendrocyte precursors and can antagonize PDGF chemoattraction.

Chick spinal cord oligodendrocyte precursors express netrin receptors

Responses to netrin signaling are mediated through receptors including DCC, UNC5 (Ackerman et al., 1997; Chan et al., 1996; Leonardo et al., 1997) and neogenin. Chick spinal cord oligodendrocyte precursors express neogenin. Two bands of appropriate sizes for neogenin were amplified by RT-PCR analyses from pan-purified E10 and E12 O4-positive cells (Fig. 5A) consistent with alternative splicing (Vielmetter et al., 1994). To determine whether netrin receptors were expressed on the surface of oligodendrocyte precursors, purified chick spinal cord O4-positive cells (Fig. 5B) were labeled with anti-neogenin (Fig. 5C) and anti-DCC (Fig. 5D) antibodies. Greater than 70% of cells were labeled with either antibody. To confirm netrin receptor expression on migratory oligodendrocyte precursors the expression of DCC and UNC5 was examined on A2B5 pan-purified immature rat oligodendrocyte precursors. Immature A2B5-positive oligodendrocyte precursors express all three netrin receptors detected by RT-PCR (Fig. 5E). The products amplified from DCC-specific primers were further analyzed by restriction enzyme digestion (Fig. 5E) resulting in two bands of predicted sizes. Consistent with these observations, the majority of A2B5-positive cells purified from newborn rat spinal cord expressed detectable cell surface DCC (Fig. 5F-H).

Netrin secreting cells provide a chemorepulsive guidance cue to migrating oligodendrocyte precursors

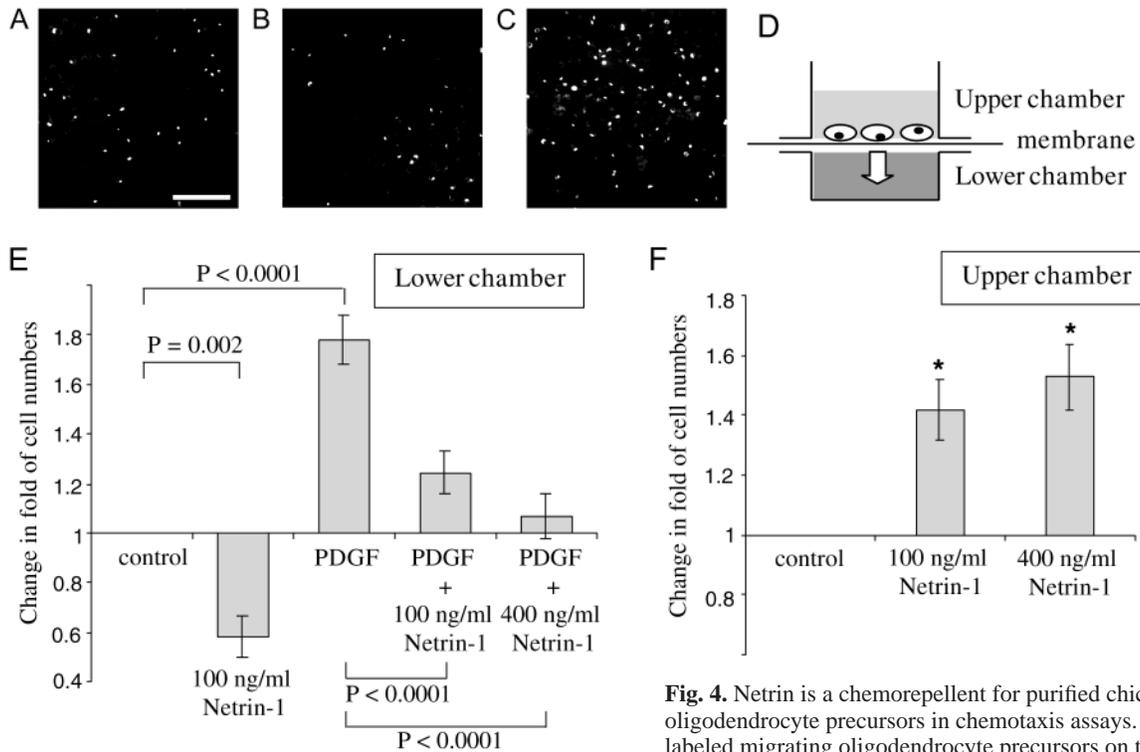To determine whether netrin 1 provided a chemorepulsive

Fig. 4. Netrin is a chemorepellent for purified chick spinal cord oligodendrocyte precursors in chemotaxis assays. (A) Ethidium bromide labeled migrating oligodendrocyte precursors on the lower surface of the filter after 20 hours in control conditions. (B) The number of migrating cells decreased when netrin 1 was added to the lower well, suggesting that cells are repelled by netrin 1. (C) The number of migrating cells increased when PDGF was added to the lower well, suggesting that cells are attracted by PDGF. (D) Boyden chamber assays. Two chambers are separated by a filter, cells are placed in the top chamber and putative chemotactic molecules placed in the upper or lower chamber. Migrating cells are counted on the lower surface of the filter. (E) Quantitation of the migration. Addition of 100 ng/ml netrin 1 to the lower chamber inhibited oligodendrocyte precursor migration, whereas addition of 20 ng/ml PDGF promoted migration (Armstrong et al., 1990). The chemoattraction of PDGF was blocked by addition of netrin 1 to the lower chamber in a dose-dependent manner. (F) Addition of netrin 1 to the upper chamber increased the number of oligodendrocyte precursors migrating through the filter. Scale bar in A: 100 μm for A-C.
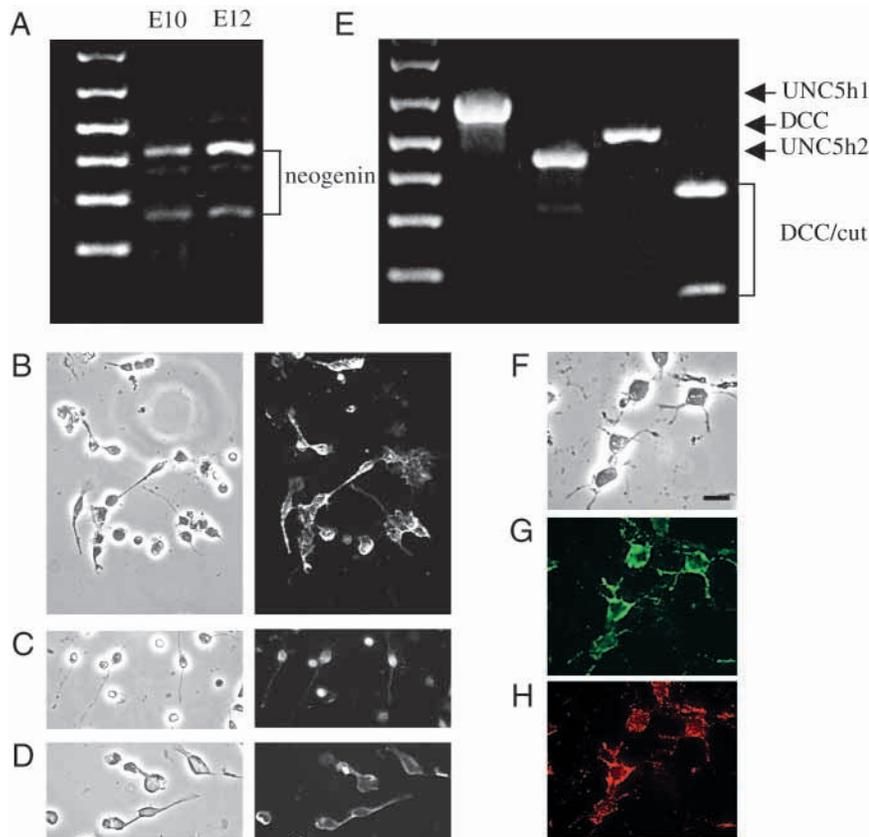
Fig. 5. Chick and rodent oligodendrocyte precursors express netrin receptors. (A-D) Pan-purified O4-positive chick oligodendrocyte precursors. (A) Oligodendrocyte precursors from E10 or E12 chick spinal cords showed alternatively spliced forms (427 and 266 bp) of neogenin by RT-PCR. (B) The majority of O4-positive purified oligodendrocyte precursors (>90%) were labeled with anti-neogenin antibody, showing punctate labeling on the cell processes and brighter labeling over the cell body (C). A similar proportion of O4-positive cells were labeled with anti-DCC antibody (D). (E-H) Pan-purified A2B5-positive rat spinal cord oligodendrocyte precursors. (E) RT-PCR analyses reveal bands with sizes correlating with UNC5h1 (643 bp), UNC5h2 (446 bp) and DCC (498 bp). The amplified DCC product was further confirmed by predicted internal restriction enzyme site (DCC/cut). Most A2B5-positive (G) rat oligodendrocyte precursors were double labeled with anti-DCC antibody (H). (F) Phase contrast view. Scale bar: 20 μm.

signal to migratory oligodendrocyte precursors in the multicelllular context of an explant, aggregates of netrin 1-secreting cells were co-cultured with intermediate explants. Explants grown in isolation or in co-culture with control 293 cells demonstrated uniformly radial migration of O4-positive cells (Fig. 2A, Fig. 6E) such that similar proportions of O4-positive cells were present proximally and distally to cell aggregates (proximal 51±3%, distal 49±3%, n=9, P=0.7). By contrast, in co-culture with netrin 1-positive cells, intermediate explants demonstrated non-uniform radial migration of O4-positive cells (Fig. 6A) with a significantly larger proportion of cells located distal to netrin 1-positive cells (proximal 39±2%, distal 61±2%) (Fig. 6E). More than 70% of intermediate explants showed biased oligodendrocyte precursor migration away from netrin 1-positive cells (Fig. 6F). Taken together with the results from the chemotaxis assays, these data indicate that netrin 1 provides a chemorepulsive cue for migrating spinal cord oligodendrocyte precursors and provide further support for the hypothesis that netrin 1 contributes to the dispersal of spinal cord oligodendrocyte precursors from the ventral ventricular zone.

The chemorepulsive effect of netrin 1 on oligodendrocyte precursors signals through a DCC-like receptor

To determine whether signaling through netrin receptors expressed by oligodendrocyte precursors mediated netrin 1-stimulated chemorepulsion, function-blocking anti-DCC and control antibodies were added to co-cultures. The addition of anti-DCC antibodies neutralized the preferential migration of oligodendrocyte precursors away from the netrin 1-positive cells and re-established a uniformly radial pattern of migration. For example, in the presence of anti-DCC antibodies, 49±3% of oligodendrocyte precursors were located distal to the netrin 1-positive cells compared with 51±3% of precursors located proximal to the netrin 1-positive cells (Fig. 6B,E, P=0.2). By contrast, addition of control IgG had little or no effect on the influence of the netrin 1-positive cells on biasing oligodendrocyte precursor migration and 58% of the cells were located distally to the netrin 1-positive cells (Fig. 6E, n=21). These observations implicate a DCC-like receptor in mediating netrin 1 guidance of migrating oligodendrocyte precursors.

To determine whether the chemorepulsive effect of ventral explants on oligodendrocyte precursors was dependent on netrin 1 signaling through DCC receptors, co-cultures of ventral and intermediate explants were grown in the presence of function blocking anti-DCC and control antibodies. Addition of anti-DCC antibodies neutralized the chemorepulsive cues from ventral explants and re-established a uniformly radial migration from intermediate explants. For example in the presence of anti-DCC, a similar distribution of oligodendrocyte precursors was seen on either side with 48±2% of O4-positive cells located distal and 52±2% located proximal to the ventral explants (Fig. 6C,E). By contrast, chemorepulsive cues from ventral explants were not inhibited by control antibodies (Fig. 6E) with 60±5% of oligodendrocyte precursors located distal and 40±5% located proximal (n=12) to the ventral explants. Taken together, these data demonstrates the ventral spinal cord-derived chemorepulsion is dependent on DCC signaling and is consistent with netrin 1 guiding the initial dispersal of spinal
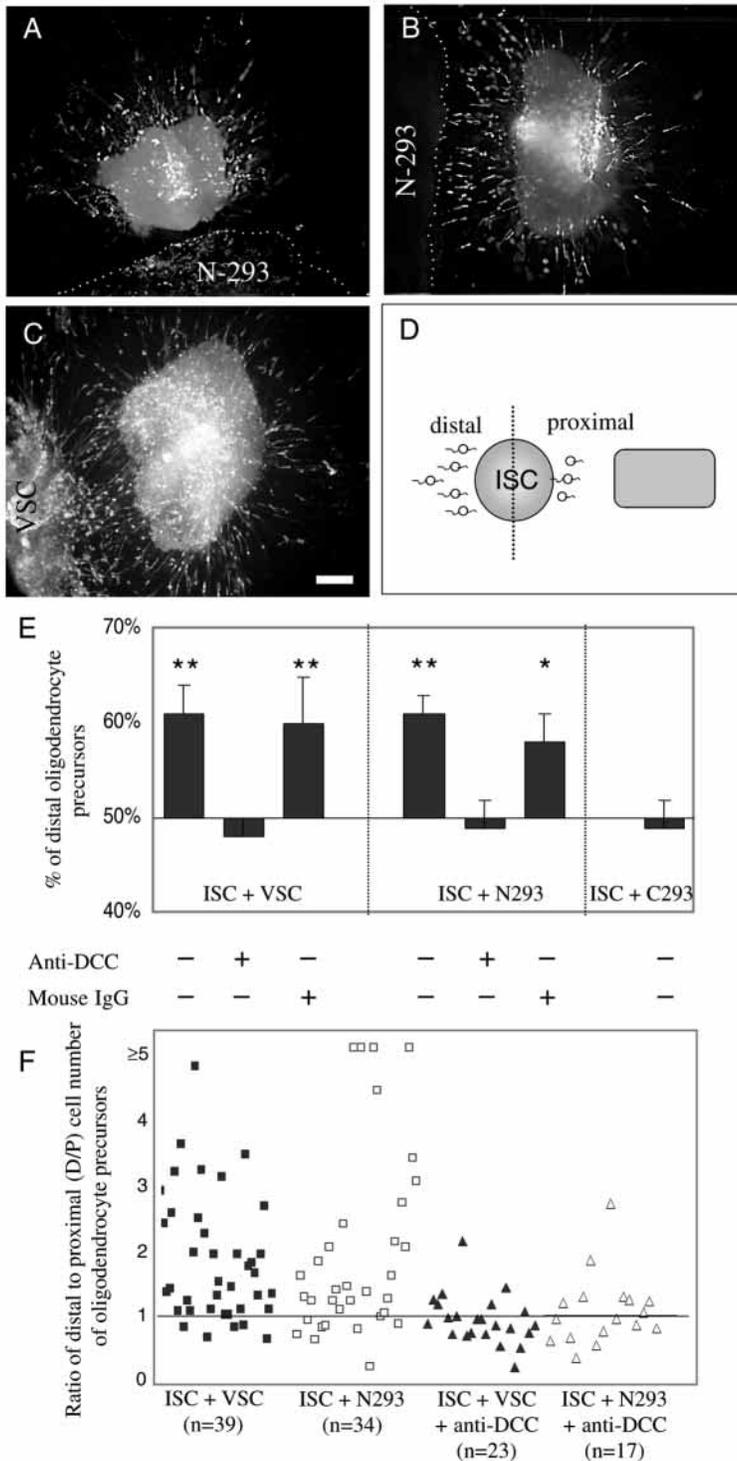
Fig. 6. Netrin 1-induced chemorepulsion of oligodendrocyte precursors is dependent on DCC-like signaling. (A) Netrin 1-positive cells mimic the repulsive activity exerted by ventral explant cells. The majority of intermediate explant-derived oligodendrocyte precursors migrated away from netrin 1-positive cells. The biased migration of oligodendrocyte precursors from intermediate explants was blocked by anti-DCC antibodies in co-cultures with (B) netrin 1-positive cells or (C) ventral explants, demonstrating that a DCC-like receptor mediates netrin 1 signaling. Cell aggregates and ventral explants are outlined. (D) Schematic representation of the quantitative analyses of oligodendrocyte precursors from intermediate explants. The counting criteria are described in the Materials and Methods. (E) Quantitative analysis of cell distribution in migration assays. In isolation, equal proportions of cells migrate in all directions. In co-culture with ventral explants or netrin-secreting cells, a higher proportion of cells are distal to the chemorepulsive cue and this is negated by anti-DCC but not control antibodies. (F) Scatter plot demonstrates the ratio of oligodendrocyte precursors migrating out from the distal to proximal side. Each dot represents one co-culture pair. A ratio larger than 1 represents repulsion, whereas a ratio smaller than 1 is attractive. VSC, ventral spinal cord; ISC, intermediate spinal cord; N293, netrin-producing 293 cells; C293, control 293 cells. *P=0.003, **P<0.001. Scale bar: 100 μm.

DCC antibody on the dispersal of oligodendrocyte precursors in slice preparations of chick spinal cord were assessed. The normal ventral to dorsal migration of spinal cord oligodendrocyte precursors is conserved in embryonic slice preparations. Oligodendrocyte precursors labeled with mAb O4 are first detected around stage 28/29 and stage 28 slices labeled with mAb O4 one hour after dissection showed trace labeling in the ventral ventricular region (Fig. 7A). After 24 hours in culture, the distribution of oligodendrocyte precursors was predominately ventral (Fig. 7B), whereas after 48 hours many O4-positive cells demonstrated extensive migration to the dorsal spinal cord (Fig. 7C) where they had a characteristic unipolar morphology (Fig. 7D). In slice cultures grown in the presence of anti-DCC antibody (10 μg/ml) for 48 hours, the ventral to dorsal migration of oligodendrocyte precursors was significantly inhibited and the majority of oligodendrocyte precursors had smaller cell bodies localized close to the ventral ventricular zone or ventral lateral pial surface (Fig. 7E,F) (Table 1). For example, comparison of the relative number of O4-positive cells in dorsal, intermediate and ventral spinal cord showed that in controls 19±2% of cells were located in dorsal regions, whereas in anti-DCC treated slices this was reduced to 9±2%. A similar perturbation in the pattern of oligodendrocyte precursor migration was seen when slices were grown in the presence of exogenously added netrin 1 (Fig. 7G). For example, the proportion of cells in dorsal spinal cord was reduced to 11±2% in the presence of 500 ng/ml netrin 1. The total number of O4-positive oligodendrocyte precursor cells in slices cultured with anti-DCC antibody (123.0±13.8 cells/slice) or netrin 1 (125.0±12.8) were similar to the control (160.6±14.0). The effects on oligodendrocyte precursor migration were specific for netrin signaling. Non-immune mouse IgG did not significantly affect the distribution

cord oligodendrocyte precursors from the ventral ventricular zone.

Inhibition of netrin signaling blocks the dispersal of oligodendrocyte precursors in chick spinal cord slices

To determine whether netrin 1 signaling mediates the dispersal of oligodendrocyte precursors through the neuropil of the developing spinal cord, the effects of function-blocking anti-
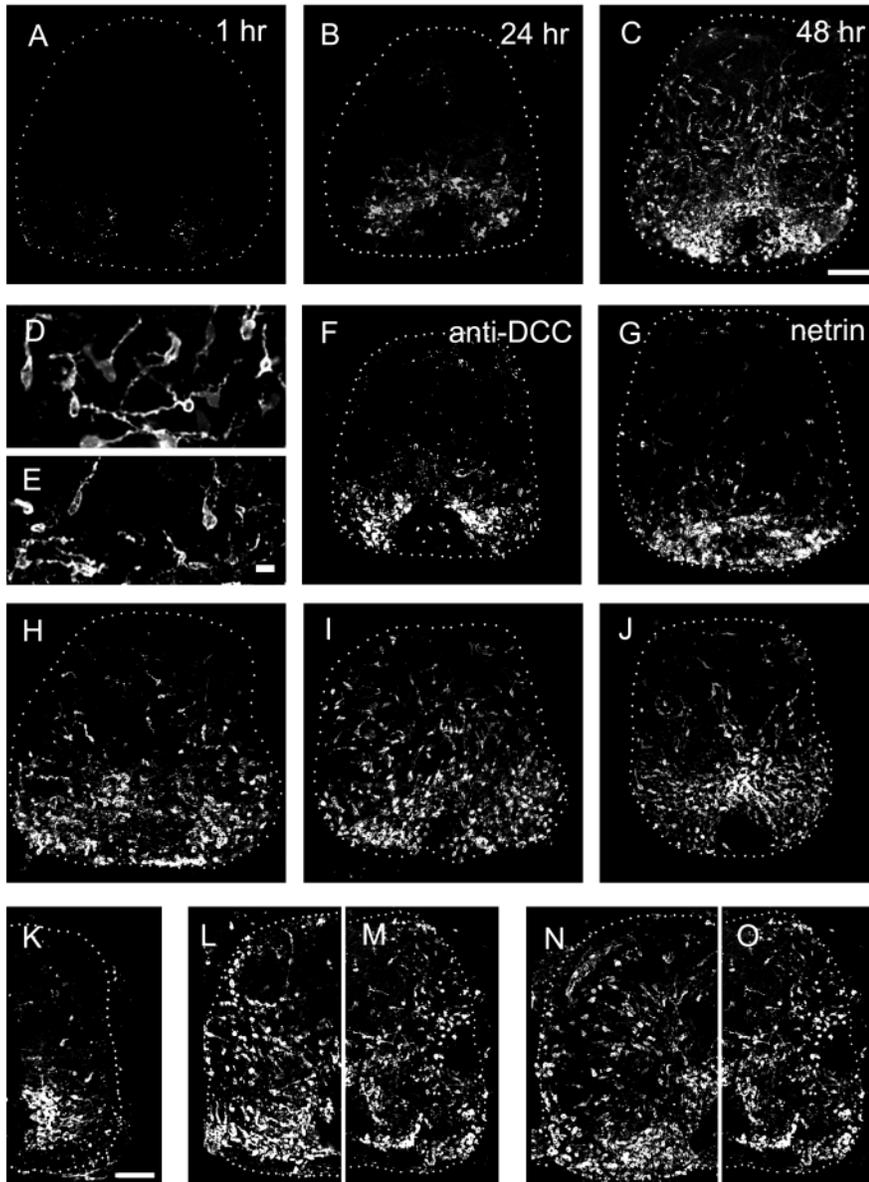
Fig. 7. The dispersal of oligodendrocyte precursors from the ventral ventricular zone is dependent on netrin. (A) Stage 28 chick spinal cord slice labeled with O4 antibody 1 hour after dissection showed minimal staining in the ventral ventricular zone. (B) After 24 hours O4-positive oligodendrocyte precursors showed ventral radial migration, which increased over the next 24 hours (C) (48 hours total). (D) The majority of migrating cells have immature unipolar or bipolar cell morphology. (E) In the presence of anti-DCC antibodies, O4-positive cells have a smaller cell body and more processes. (F) The migration of O4-positive oligodendrocyte precursors is inhibited in the presence of anti-DCC antibody with the majority of O4-positive cells remaining in the ventral spinal cord. (G) Addition of exogenous chick netrin 1 to the slices also inhibits oligodendrocyte precursor migration. (H) Normal mouse IgG did not disrupt oligodendrocyte precursor migration, neither did anti-prion antibody (I). (J) Anti-NCAM antibody altered the pattern of migration, but did not mimic the changes seen with anti-DCC or netrin 1. (K-O) Stage 31 chick spinal cord slices. (K) Stage 31 spinal cord slice after dissection showed ventral radial migration. (L) Spinal cord slices grown in the presence of anti-DCC antibody or chick netrin 1 ligand (N) did not show an altered migration pattern compared with controls (M,O) suggesting that more mature oligodendrocyte precursors are less sensitive to netrin 1. Scale bars: in C, 100 μm for A-C,F-J; in E, 10 μm for D,E; in K, 100 μm for K-O.

of oligodendrocyte precursors (Fig. 7H) nor did addition of anti-prion antibodies (Fig. 7I) that labeled the majority of chick O4-positive cells (data not shown). Antibodies to neural cell-

Table 1. Distribution of O4+ cells in different spinal cord regions is dependent on netrin signaling

	Dorsal (%)	Intermediate (%)	Ventral (%)
Control (n=19)	19±2	38±1	43±2
Anti-DCC (10 μg/ml) (n=13)	9±2*	29±2*	61±4*
Netrin (500 ng/ml) (n=14)	11±2*	29±2*	60±3*
Anti-Prion (10 μg/ml) (n=7)	20±3	38±1	42±3
Mouse IgG (10 μg/ml) (n=11)	16±2	36±2	48±4

Spinal cord slices were divided into dorsal, intermediate and ventral regions. The total numbers of O4+ cells were counted in each region from slices cultured for 48 hours, and the significance of the difference in the relative proportion of cells in each region in control and treated slices tested by Mann-Whitney non-parametric test.

*P<0.001.

adhesion molecule (NCAM) did result in a perturbation of the pattern of migration resulting in a more radial-lateral pattern (Fig. 7J) but did not mimic the migration inhibiting effects of anti-DCC or netrin 1. The influence of anti-NCAM antibodies is consistent with in vitro data, suggesting that NCAM-associated polysialic acid (PSA) is important in oligodendrocyte precursor migration (Decker et al., 2000; Hughson et al., 1998). The dependence of oligodendrocyte precursor dispersal on netrin was transient. When slices were prepared from stage 31 embryos O4-positive cells were more dispersed (Fig. 7K) than at stage 28 after 24 hours (compare Fig. 7B with 7K). After 48 hours in vitro, neither anti-DCC antibody (Fig. 7L,M) nor addition of netrin 1 (Fig. 7N,O) had a significant effect on the migration of oligodendrocyte precursors.

Together these data provide compelling evidence to support the hypothesis that netrin 1 is a chemorepulsive cue that mediates the initial dispersal of chick spinal cord

oligodendrocyte precursors from their localized origin in the ventral ventricular zone, a critical step in positioning these cells to subsequently myelinate axons in presumptive spinal cord white matter.

DISCUSSION

Developing spinal cord oligodendrocyte precursors are highly migratory and disperse rapidly from their origin in the ventral ventricular zone to populate presumptive gray and white matter. We show that the initial dispersal of these cells is mediated by a chemorepulsive response to netrin 1. Netrin 1 is expressed in the ventral spinal cord during the period of initial oligodendrocyte precursor emigration from the ventral ventricular zone; both ventral explants and netrin 1-positive cells repel migratory spinal cord oligodendrocyte precursors. Purified oligodendrocyte precursors express the netrin receptors DCC, UNC5 and neogenin and signaling through a DCC-like receptor mediates both the chemorepulsive cues of netrin 1 and ventral spinal cord explants. PDGF is chemotactic for oligodendrocyte precursors (Armstrong et al., 1990) and in chemotaxis assays, netrin 1 antagonized the attractive effects of PDGF. The initial dispersal of oligodendrocyte precursors occurs in slice preparations of developing chick spinal cord and addition of anti-DCC antibody or exogenous netrin 1 severely compromised oligodendrocyte precursor migration from their source at the ventral ventricular zone.

Myelination of the spinal cord is crucially dependent on long-distance migration of oligodendrocyte precursors. The founder cells of the lineage arise in a ventrally located domain as a result of local sonic hedgehog signaling (Orentas and Miller, 1996; Pringle et al., 1996), although their progenies are dispersed throughout the entire spinal cord and eventually concentrated in peripheral white matter. While classical contact inhibition of motility (Heaysman and Pegrum, 1973) would tend to dissipate cells from a high density source, it is unlikely to contribute to the spread of spinal cord oligodendrocyte precursors because it would be relatively slow, not provide direction for isolated cells and is not demonstrated by oligodendrocyte precursors in vitro (Tsai and Miller, unpublished). By analogy with neuronal cell migration, radial glia may facilitate later migration of oligodendrocyte precursors in white matter where they are closely aligned with radial glia (Hirano and Goldman, 1988). The pattern of initial dispersal and ventral to dorsal oligodendrocyte precursor migration is not, however, consistent with the distribution of radial glia in the spinal cord (Noll and Miller, 1993; Ono et al., 1995), suggesting these cells do not influence initial oligodendrocyte precursor dispersal. Rather, the pattern of oligodendrocyte precursor dispersal suggests a repulsive cue regardless of cellular substrate.

Netrin 1 is essential for the initial dispersal of spinal cord oligodendrocyte precursors. In the spinal cord, the netrins are pivotal guidance molecules for dorsoventral patterning mediating both attraction and repulsion in neurons (Wadsworth, 2002) and netrin 1 appears to selectively affect the dorsal and lateral migration of spinal cord oligodendrocyte precursors. In slice preparations grown in the presence of anti-DCC or exogenous netrin 1, ventrally migrating oligodendrocyte precursors appear to be unaffected, while cells migrating laterally and dorsally are severely inhibited. This differential sensitivity to netrin signaling may reflect the expression of different receptor combinations (Hong et al., 1999; Keleman and Dickson, 2001) on subsets of oligodendrocyte precursors, different modes of migration or the use of alternative substrate. It seems likely, however, that there is heterogeneity among ventrally derived spinal cord oligodendrocyte precursors. For example, although the majority of oligodendrocyte precursors emerge from the pMN domain are Nkx2.2 and Olig2 positive (Zhou et al., 2001), a subset are proposed to derive from Nkx2.2-positive/Olig2-negative cells (Soula et al., 2001). It is possible that cells destined to the ventral regions of the spinal cord are derived from a similar subset of ventricular cells. Alternatively, spinal cord oligodendrocyte precursors may intrinsically be a homogenous cell population whose responses to guidance molecules such as netrin 1 are modulated by local environmental signals, thereby allowing a subset of cells to escape netrin 1 repulsion.

The response of oligodendrocyte precursors to netrin 1 is temporally regulated. The initial dispersal from the ventricular zone is blocked by addition of anti-DCC or netrin 1. By contrast, later in development, similar treatments appear to have little effect. This lack of effect may reflect either a maturation event in oligodendrocyte precursors or an alteration in guidance cues. It seems likely that the guidance of oligodendrocyte precursors to appropriate domains of the spinal cord will involve multiple guidance cues such as semaphorin 3A (Sema3A) and slit. The chemorepulsive cue slit is expressed in ventral spinal cord (Brose et al., 1999; Li et al., 1999; Yuan et al., 1999), as is Sema3A (Luo et al., 1995; Puschel et al., 1995). Non-migratory mature oligodendrocytes express the Sema3A receptor neuropilin 1 and retract established processes in response to Sema3A (Ricard et al., 2001), while some optic nerve glial precursors are repelled by Sema3A (Spassky et al., 2002; Sugimoto et al., 2001). Neuropilin, the Sema3A receptor, is functionally associated with adhesion molecules, including NCAM (Castellani et al., 2000). The embryonic form of NCAM (PSA-NCAM) modulates oligodendrocyte precursor migration in vitro (Decker et al., 2000) and alters the pattern of oligodendrocyte precursor migration in spinal cord slices. This altered migratory pattern may reflect disruption of Sema3A guidance. Neither Sema3A nor slit appears to mediate initial oligodendrocyte precursor dispersal; however, as this is inhibited by blocking netrin 1.

The guidance of migratory oligodendrocyte precursors by diffusible cues is not restricted to the spinal cord. In the optic nerve, glial precursors migrate from the chiasm to the retina (Ono et al., 1997; Small et al., 1987) in response to chemorepulsive cues from the chiasm (Spassky et al., 2002; Sugimoto et al., 2001). In postnatal rat optic nerve, netrin 1 provided chemorepulsion to NG2-positive cells, considered to be oligodendrocyte precursors (Nishiyama et al., 1996), while Sema3A was chemorepulsive to an unidentified class of cells (Sugimoto et al., 2001). By contrast, in embryonic mouse optic nerve cultures, netrin 1 was chemoattractive for A2B5-positive cells while Sema3A was chemorepulsive (Spassky et al., 2002). The differential responses in the two systems might represent species differences, analyses of different cell populations or experimental conditions because directionality

is dependent on receptor usage and signal transduction pathways (Hong et al., 1999; Ming et al., 1997). The present study indicates that netrin 1 exerts a direct chemorepulsive effect on unambiguously identified purified chick spinal cord oligodendrocyte precursors. Not only is netrin 1 chemorepulsive, but it can also negate PDGF chemoattraction.

Functional studies indicate that a DCC-like molecule is crucial in mediating the chemorepulsion of oligodendrocyte precursors by netrin 1. In rodent neurons, both DCC and UNC5 are required for netrin 1 to exert repulsive activity (Hong et al., 1999). Consistent with this notion, oligodendrocyte precursors from the rat spinal cord express both UNC5 and DCC receptors and at least in the optic nerve these cells are repelled by netrin 1 (Sugimoto et al., 2001). The expression of UNC5 on chick oligodendrocyte precursors is unclear; however, they do express neogenin another member of the DCC family. It seems likely that netrin-induced chemorepulsion in oligodendrocyte precursors is mediated by similar modular receptors such as DCC and/or neogenin and an UNC5-like receptor. At high netrin concentrations, UNC5 receptors alone may mediate chemorepulsion with DCC required at lower concentrations over a longer distance (Keleman and Dickson, 2001). It is possible that expression of DCC on oligodendrocyte precursors allows them to respond to a broad spectrum of netrin concentrations and thereby ensure their widespread dissemination throughout the cord. In contrast to other systems where signaling through DCC promotes cell survival (Llambi et al., 2001; Mehlen et al., 1998) and in the absence of ligand, DCC receptors trigger the apoptosis pathway (Bloch-Gallego et al., 1999), oligodendrocyte precursors do not appear to depend on DCC for survival. In the developing mouse spinal cord, the normal development of oligodendrocytes appear to be at least partially dependent on netrin and DCC. Recent studies (Jarjour et al., 2003) demonstrate that the normal ventral-to-dorsal migration of oligodendrocyte precursor is impaired in the spinal cord of netrin 1 and DCC mutants, while the number of newly differentiated oligodendrocytes is dramatically reduced in the spinal cord of netrin 1 mutant animals (Tsai and Miller, 2002) suggesting that netrin-mediated precursor dispersal is critical for spinal cord oligodendrogenesis. In addition, the finding that netrin 1 directs the migration of oligodendrocyte precursors from the ventral ventricular zone provides an attractive explanation for that aberrant patterning of oligodendrocytes in the spinal cord of CXCR2-null animals in which white matter is present as a thin layer with oligodendrocytes residing close to the pial surface. The chemokine CXCL1, the ligand for CXCR2 acts as a rapid and reversible stop signal for migrating spinal cord oligodendrocyte precursors (Tsai et al., 2002). In developing spinal cord, CXCL1 is expressed by white matter astrocytes in a tightly regulated pattern. Oligodendrocyte precursors lacking CXCR2 are refractory to CXCL1 and thus continue to migrate away from the ventricular zone under the influence of netrin 1 until they reach the outer limits or pial surface of the spinal cord. Such a combinatorial signaling system is likely to be responsible for patterning oligodendrocyte localization throughout the developing vertebrate CNS.

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