Netrin 1 and Dcc regulate oligodendrocyte process branching and membrane extension via Fyn and RhoA

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The molecular mechanisms underlying the elaboration of branched processes during the later stages of oligodendrocyte maturation are not well understood. Here we describe a novel role for the chemotropic guidance cue netrin 1 and its receptor deleted in colorectal carcinoma (Dcc) in the remodeling of oligodendrocyte processes. Postmigratory, premyelinating oligodendrocytes express Dcc but not netrin 1, whereas mature myelinating oligodendrocytes express both. We demonstrate that netrin 1 promotes process extension by premyelinating oligodendrocytes in vitro and in vivo. Addition of netrin 1 to mature oligodendrocytes in vitro evoked a Dcc-dependent increase in process branching. Furthermore, expression of netrin 1 and Dcc by mature oligodendrocytes was required for the elaboration of myelin-like membrane sheets. Maturation of oligodendrocyte processes requires intracellular signaling mechanisms involving Fyn, focal adhesion kinase (FAK), neuronal Wiscott-Aldrich syndrome protein (N-WASP) and RhoA; however, the extracellular cues upstream of these proteins in oligodendrocytes are poorly defined. We identify a requirement for Src family kinase activity downstream of netrin-1-dependent process extension and branching. Using oligodendrocytes derived from Fyn knockout mice, we demonstrate that Fyn is essential for netrin-1-induced increases in process branching. Netrin 1 binding to Dcc on mature oligodendrocytes recruits Fyn to a complex with the Dcc intracellular domain that includes FAK and N-WASP, resulting in the inhibition of RhoA and inducing process remodeling. These findings support a novel role for netrin 1 in promoting oligodendrocyte process branching and myelin-like membrane sheet formation. These essential steps in oligodendroglial maturation facilitate the detection of target axons, a key step towards myelination.

KEY WORDS: Oligodendroglia, Myelination, Myelin, Netrin, Integrin, Laminin, Autocrine, Mouse, Rat, FAK (Ptk2), N-WASP (Wasl)

INTRODUCTION

To form a myelin sheath, an oligodendrocyte initially extends multiple branching processes that survey the local environment for suitable axons (Hardy and Friedrich, 1996; Kirby et al., 2006). Upon contact with a target axon, oligodendrocyte processes coalesce to form a spreading sheet of membrane that begins to wrap the axon. The extracellular cues that govern this process and the intracellular mechanisms involved are poorly understood.

Laminin 2, which promotes oligodendrocyte maturation by signaling through α6β1 integrins (Baron et al., 2005), has been suggested to regulate process elongation. Laminin-2-deficient mice have dysmyelinated and hypomyelinated axons (Chun et al., 2003). However, laminin 2 is not ubiquitous in myelinated CNS axon tracts (Colognato et al., 2002) and transgenic mice lacking β1 integrin expression in oligodendrocytes exhibit no defects in CNS myelination (Benninger et al., 2006). Thus, other ligand-receptor interactions must also direct the changes in oligodendrocyte morphology required for myelination.

The axon-guidance cue netrin 1 (Ntn1) is a chemorepellent for migrating oligodendrocyte precursors (OPCs) in the embryonic spinal cord (Jarjour et al., 2003; Tsai et al., 2003). These cells express the netrin 1 receptors Dcc, Unc5a and Unc5b, but not netrin 1 itself. In the adult CNS, netrin 1 is expressed by myelinating oligodendrocytes and is associated with non-compact oligodendroglial membranes (Manitt et al., 2001). We therefore investigated the possibility that netrin 1, expressed in the developing CNS and later by the oligodendrocytes themselves, might influence late stages of oligodendrocyte differentiation.

The lamella elaborated by the tip of an extending oligodendrocyte process has been compared to a neuronal growth cone (Fox et al., 2006; Jarjour and Kennedy, 2004; Sloane and Vartanian, 2007). Interestingly, a number of the same intracellular signaling proteins have been implicated in netrin-1-mediated axon guidance and the development of oligodendrocyte processes required for myelination (Fox et al., 2006; Jarjour and Kennedy, 2004). In both cases, the reorganization of the actin cytoskeleton requires activation of the Src family kinase (SKF) Fyn (Meriane et al., 2004; Osterhout et al., 1999; Umemori et al., 1994) and involves Wiscott-Aldrich syndrome protein (N-WASP; Wasl – Mouse Genome Informatics) and Rho GTPases (Bacon et al., 2007; Liang et al., 2004; Shekarabi et al., 2005).

Here we provide evidence that netrin 1 and Dcc promote the extension of oligodendrocyte processes in vivo. We used in vitro assays to demonstrate that netrin 1 increases oligodendrocyte process branching. Furthermore, expression of Dcc and netrin 1 by oligodendrocytes promotes the formation of myelin-like membrane sheets. Addressing the signaling mechanisms involved, we show that the SKF Fyn is required for netrin-1-induced process branching, that netrin 1 recruits Fyn to Dcc in oligodendrocytes, increases SKF activity, and promotes process extension and branching associated with a decrease in RhoA activity. Our findings reveal a novel role for netrin 1 and Dcc in activating a signaling cascade in oligodendrocytes that directs process remodeling.

MATERIALS AND METHODS

Animals and oligodendrocyte cultures

Sprague Dawley rat pups and pregnant BALB/c mice were obtained from Charles River Canada (Montreal, Quebec, Canada). Ntn1<sup>−/−</sup> and Dcc<sup>−/−</sup> mice were obtained from Marc Tessier-Lavigne (Genentech, South San Francisco, Murray Research Laboratories, Berkeley, CA). Sprague Dawley rats, Sprague Dawley rats, and transgenic mice were housed and maintained in a 12-hour light/dark cycle under specific pathogen-free conditions. All animal procedures were approved by the Animal Care Committee of the University of Toronto and conducted in accordance with the Canadian Council on Animal Care guidelines.

Animals were killed by dislocation and were divided into two groups: untreated (controls) and netrin 1-treated. The netrin 1 group was treated with netrin 1 (100 μg/ml) for 24 hours, while the control group was treated with vehicle (Dulbecco’s modified Eagle’s medium) for 24 hours. Netrin 1 was purchased from R&D Systems (Minneapolis, MN). Cells were harvested and processed for further analysis.

Assessment of oligodendrocyte process branching and membrane extension

Oligodendrocyte processes were imaged using a confocal microscope (Olympus Fluoview 300, Tokyo, Japan) equipped with a 63× objective. Images were acquired from multiple fields of view and analyzed using ImageJ software (Wayne Rasband, NIH, USA).

Analysis of intracellular signaling proteins

Oligodendrocytes were lysed in lysis buffer containing protease inhibitors. Equal amounts of protein were loaded onto SDS-PAGE gels and transferred to PVDF membranes. Membranes were probed with antibodies specific for Fyn, N-WASP, and RhoA. Blots were incubated with horseradish peroxidase-conjugated secondary antibodies and developed using an enhanced chemiluminescence system (Pierce, Rockford, IL).

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CA, USA) and Robert Weinberg (Harvard University, Cambridge, MA, USA), respectively. Fyn KO (Fyn<sup>−/−</sup>) and control F2 hybrid (B6129SF2/J) breeding pairs were obtained from Jackson Laboratories (Bar Harbor, ME, USA). All procedures were performed in accordance with the Canadian Council on Animal Care guidelines for the use of animals in research.

**Cell culture**

OPCs were derived from mixed glial cultures from the cerebral cortices of postnatal day 0 (P0) rat pups and grown in oligodendrocyte defined medium (OLDEM) as described previously (Armstrong, 1998; Jarjour et al., 2003), with 0.1% fetal bovine serum (FBS) to initiate differentiation. Cells were seeded at 1.5×10<sup>6</sup> cells/chamber in 8-well chamber slides coated with 10 μg/ml poly-1-lysine (Nalgene Nunc, Rochester, NY, USA). For immunoprecipitation, western blots and GST-rhotekin pulldowns, cells were plated at 1.5×10<sup>6</sup> cells/well in 6-well tissue culture dishes.

**Mouse oligodendrocyte cultures**

Culturing of mouse OPCs was similar to that of rat OPCs, but 10% horse serum was used instead of 10% FBS in mixed glial culture media. Each T75 flask of mixed glial culture required two to three pups. Newborn Ntn<sup>+/−</sup>- and Dcc<sup>−/−</sup>- mice were identified by distinct behaviors. The genotype of individual pups was confirmed by PCR.

**Antibodies**

Primary antibodies used in this study were: rabbit polyclonal anti-netrin 1 PN3 (Manitt et al., 2001), mouse monoclonal anti-Dcc (G97-449; BD Biosciences Pharmingen, San Jose, CA, USA), goat polyclonal anti-Dcc (Santa Cruz Biotech, Santa Cruz, CA, USA), rabbit polyclonal anti-myelin basic protein (Mbp, Chemicon, Temecula, CA, USA), mouse monoclonal anti-Mbp (Chemicon), mouse monoclonal RIP antibody (Chemicon), mouse monoclonal anti-2',3'-cyclic nucleotide 3' phosphodiesterase (Cnp, Sternberger Monoclonals, Lutherville, MD, USA), rabbit polyclonal anti-Mag (Chemicon), rabbit polyclonal anti-NfM (Nefm – Mouse Genome Informatics) (Chemicon), rabbit polyclonal anti-Fyn (Upstate Cell Signaling, Charlottesville, VA, USA) used for western blots, rabbit polyclonal anti-Fyn (gift of Dr Andre Veillette and described previously (Davidson et al., 1992)) used for immunoprecipitation and western blots, mouse monoclonal anti-FAK (BD Biosciences), rabbit polyclonal anti-N-WASP (Santa Cruz), and rabbit polyclonal anti-phospho-Src (Cell Signaling), which recognizes the pY416 epitope in all SFK members.

For analyses of oligodendrocyte morphology in vivo, we crossed Ntn1<sup>−/−</sup> or Dcc<sup>−/−</sup> heterozygous mice, obtained embryonic day 18 (E18) embryos (plug date taken as E1), fixed them in 4% paraformaldehyde and cut 18 μm sections of the spinal brachial enlargement with a cryostat. Sections were then stained with anti-Cnp (1:100), visualized using Alexa 546- or Alexa 488-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA, USA), and nuclei stained with Hoechst. Images were obtained with a Magnafire CCD camera (Optronics, Goleta, CA, USA) and a Zeiss Axiovert 100 microscope (Toronto, Ontario, Canada).

**Analysis of oligodendrocyte morphology**

Analyses of immature oligodendrocytes in vitro and in vivo were performed using the Neuron plugin for ImageJ (NIH, Bethesda, MD, USA). The length of the longest process was measured from the base of the process to its tip (Fig. 3D; Fig. 4B). For Sholl analysis, the grid function in Northern Eclipse (Empix) was used to draw concentric circles 15 μm apart around the cell body of mature RIP-positive oligodendrocytes. The number of intersections made by processes with each successive circle was counted. For studies using the β1 integrin subunit function-blocking antibody and mouse oligodendrocyte cultures, a Sholl analysis plugin was used with ImageJ (starting radius, 1.02 cm; step size, 1.02 cm; end, 5.08 cm; thickness, 0.02 cm). The Mbp-positive myelin-like membrane sheets were outlined in ImageJ and the surface area reported in arbitrary units.

The pharmacological inhibitors PP2 and PP3 (Calbiochem) were used at 2 μM to inhibit SFK activity. Purified hamster anti-rat CD29 (β1 integrin) monoclonal and purified hamster anti-IgM monoclonal antibodies were used at 2 μM to investigate β1 integrin function.

**Analysis of phospho-Src puncta**

The number of phospho-Src-positive puncta was measured using ImageJ. The brightness and contrast of the images of individual oligodendrocytes were modified to enhance puncta associated with extending processes; modifications made were consistent across all images. Images were converted to a binary format and then the number of particles counted automatically. Staining in the cell body and major processes was not punctate and thus counted as one particle, making variations under different conditions a direct indicator of changes in the number of distally located puncta. The number of puncta was divided by oligodendrocyte area to control for variance in oligodendrocyte size.

**Quantification and statistical analyses**

Statistical significance was calculated by ANOVA followed by a post-hoc Tukey test using Systat Software (San Jose, CA, USA). Analyses of oligodendrocyte morphology in vitro used three independent experiments with a minimum of 30 cells per condition. In vivo analysis of process extension and purification of oligodendrocytes from transgenic mice were performed using at least four pups of each genotype, derived from at least two different litters.

**Immunoprecipitation**

Cells in 6-well dishes were treated with netrin 1 for 5 minutes, then lysed in RIPA lysis buffer (10 mM sodium phosphate pH 7.2, 150 mM NaCl, 1% NP40, 0.1% SDS, 0.5% deoxycholate) and centrifuged at 13,000 rpm (13,800 g) for 7 minutes. Supernatant was pre-cleared with 30 μl protein A/G beads (Santa Cruz) for 30 minutes, incubated with 1 μg/ml anti-Dcc (monoclonal) or anti-Fyn (rabbit polyclonal) for 1 hour, followed by addition of 30 μl protein A/G beads for 45 minutes.

**GST pulldown assays**

Fusion proteins comprising the RhoA-binding domain of hrotekin (a downstream substrate of RhoA) or Pak-1-CRIB and glutathione-S-transferase (GST) were purified as described (Reid et al., 1996). Oligodendrocytes were treated with netrin 1 for 24 hours. Cells were then lysed and protein purified as described (Ren and Schwartz, 2000; Shekarabi et al., 2005).

**RESULTS**

**Oligodendrocytes express netrin 1 during myelination in the developing spinal cord**

Netrin 1, expressed by floor plate and neuroepithelial cells in the early embryonic spinal cord, directly migrates OPCs away from the ventral midline towards axons in the nascent white matter (Jarjour et al., 2003; Kennedy et al., 1994; Tsai et al., 2006; Tsai et al., 2003). Although not expressed by OPCs, netrin 1 is widely expressed by spinal interneurons, motoneurons and by most, if not all, mature myelinating oligodendrocytes in dorsal and ventral white matter in the adult rat and mouse CNS (Manitt et al., 2001).

To determine when netrin 1 is expressed by differentiating oligodendrocytes, we performed a time-course analysis. Prenyelinating, postmigratory oligodendrocytes within the corticospinal tract at brachial, thoracic and lumbar levels were examined. Netrin 1 expression was not detected in oligodendrocytes at embryonic stages of development in mice (data not shown). For postnatal stages, netrin-1-expressing cells were identified immunohistochemically. Oligodendrocytes in the developing dorsolateral white matter tracts were identified by expression of 2',3'-cyclic nucleotide 3' phosphodiesterase (Cnp), a marker that labels oligodendrocyte cell bodies and processes in vivo, and by expression of the mature oligodendrocyte marker myelin basic protein (Mbp). At P8, before myelination begins in the corticospinal tract, netrin 1 was not expressed by premyelinating oligodendrocytes (Fig. 1A-C). Netrin-1-expressing neuronal cell bodies and neuronal-epithelial cells (Fig. 1D,E), but not astrocytes (Fig. 1I), were detected immediately adjacent to differentiating oligodendrocytes.
At P12, following the initiation of myelination in the rat corticospinal tract (Schwab and Schnell, 1989), netrin 1 was detected in the cell bodies of a subset of oligodendrocytes double labeled with Cnp (Fig. 1F,G). At this stage of development, netrin 1 immunoreactivity was associated with axons (Fig. 1I). By P22, large numbers of mature myelinating oligodendrocytes expressing netrin 1 were readily detectable throughout the nascent white matter of the spinal cord (Fig. 1J,K). These findings indicate that netrin 1 begins to be expressed by oligodendrocytes during early stages of myelination.

Oligodendroglial expression of netrin 1 and Dcc does not require neuronal contact

The timing of netrin 1 expression by oligodendrocytes suggested that expression might be regulated by axonal signals. To test this, purified rat oligodendrocyte progenitors were cultured in the absence of neurons in conditions that promote differentiation. Immature oligodendrocytes in these cultures [4 days in vitro (DIV)] were defined as multipolar cells expressing Cnp and are the equivalent of the premyelinating cells in vivo. These cells did not express netrin 1. More mature oligodendrocytes, cultured for 6-8 DIV and identified by expression of Mbp and Cnp (Watanabe et al., 2006), extend highly branched processes that coalesce to form cytoplasmic sheets (Fox et al., 2006). These sheets, which resemble unwrapped non-compacted myelin membrane (Knapp et al., 1987), were immunopositive for netrin 1 (Fig. 2A). This staining was present on cells that were not permeabilized, consistent with netrin 1 protein associated with the extracellular face of the plasma membrane. To verify that the cells were not permeabilized, the absence of Mbp staining was used as a negative control (not shown). We conclude that in the absence of neuronal contact in vitro, oligodendrocytes express netrin 1, and that netrin 1 protein is associated with the surface of myelin-like membrane sheets.

The netrin 1 receptor Dcc was detected along the processes of immature and mature oligodendrocytes. In mature oligodendrocytes, Dcc was associated with major branches and present in small puncta at the leading edge of myelin-like membrane sheets (Fig. 2B). Addition of recombinant Myc-tagged netrin 1 to mature oligodendrocytes revealed a preferential localization of exogenous ligand at the branches and edges of sheets formed by the cells, similar to the distribution of Dcc (Fig. 2A′,B′).

Fig. 1. Expression of netrin 1 by oligodendrocytes in vivo. (A,A′) Longitudinal section through a P8 rat spinal cord showing Mbp-immunopositive oligodendrocytes (red) that do not express netrin 1, intermingled with netrin-1-expressing cells (green). (B,B′) At P8, netrin 1 immunoreactivity (green) was detected in the immediate environment surrounding the cell bodies of Cnp-positive oligodendrocytes (red), but netrin 1 was not expressed by the oligodendrocytes themselves. (C,C′) An Mbp-positive netrin-1-negative process, surrounded by netrin-1-expressing neuroepithelial cells (arrows) in P8 spinal cord. (D,D′) Longitudinal section through a P8 rat spinal cord showing axons positive for neurofilament medium polypeptide (Nfm), surrounded by netrin-1-immunopositive neurons. (E,E′) Cell bodies of netrin-1-immunopositive neurons. (F) Cross-section of a P12 rat dorsal spinal cord showing Cnp-positive, myelinating oligodendrocytes (red) expressing netrin 1 (green). (G-G′) Oligodendrocytes adjacent to the central canal (arrow) express netrin 1 in P12 spinal cord. (H,H′) Netrin 1 immunoreactivity (green) associated with a Cnp-immunopositive (red) myelinating oligodendrocyte in the P12 rat spinal cord. (I-I′) By P12, netrin 1 expression (green) colocalizes with Nfm-immunoreactive axons (blue), but is not detected in Gfap-positive astrocytes (red). (J,J′) Transverse section of a P22 thoracic spinal cord showing widespread expression of netrin 1 (green) by Cnp-positive, myelinating oligodendrocytes (red). (K-K′) Magnification of the boxed region from J showing Cnp-positive netrin-1-expressing cell bodies. A-F, 20×0.5 n.a. objective; G-I, 40×0.75 n.a. objective; D,E,I,K, confocal microscopy, 40×0.75 n.a. objective; B,C,H, confocal microscopy, 100×1.4 n.a. objective. Scale bars: 10 μm in B′,C′,I; 20 μm in A′,D,E,G,K; 40 μm in F,I′,J.
Netrin 1 and Dcc promote process extension by premyelinating oligodendrocytes in vivo

In the developing spinal cord, as premyelinating oligodendrocytes mature into myelinating oligodendrocytes, netrin 1 is widely expressed by neurons and neuroepithelial cells, but not by oligodendrocytes (Fig. 1D,I; Fig. 3C). Mice lacking either netrin 1 or Dcc die within hours of birth (Fazeli et al., 1997; Serafini et al., 1996). We therefore used E18 littermates to compare the morphology of postmigratory, premyelinating oligodendrocytes in tissue sections from the spinal cords of wild-type and Ntn1–/– and Dcc heterozygotes and knockout mouse embryos (Fig. 3A-C). At E18, Cnp-immunoreactive premyelinating oligodendrocytes in the dorsolateral spinal cord typically extend one or more processes (Fig. 3D, dashed lines). We found the length of these processes in both Ntn1+/− and Dcc−/− mice to be significantly shorter than in wild-type littermates (Fig. 3E,F). These findings provide evidence that at the end of precursor migration and at the initiation of oligodendrocyte differentiation, netrin 1 in the local environment of a postmigratory, premyelinating oligodendrocyte promotes Dcc-dependent process extension in vivo, a phenomenon closely associated with the capacity of an oligodendrocyte to contact target axons (Hardy and Friedrich, 1996; Kirby et al., 2006).

Interpretation of these findings could be confounded by two factors. First, a subset of neurons in the spinal cords of Ntn1−/− and Dcc−/− mice exhibits defects in axon guidance. However, this is unlikely to exert a profound influence on the differentiation of individual oligodendrocytes as the majority of axons extend normally in the absence of netrin 1 function and the nascent white matter is well populated with axons. Second, a developmental delay in OPC dispersal in the Ntn1−/− and Dcc−/− mutants might delay process extension. In order to directly address the mechanisms underlying the apparent influence of netrin 1 on oligodendrocyte differentiation in vivo, we determined whether the putative aberrant process extension detected in Ntn1−/− and Dcc−/− mice could be replicated in vitro in the absence of axonal influences or migration deficits.

Netrin 1 does not affect the differentiation of immature or mature oligodendrocytes in vitro

Distinct changes in oligodendrocyte morphology accompany the differentiation of oligodendrocytes in vitro. Immature oligodendrocytes are characteristically multipolar cells that express Cnp but not Mbp (Fig. 4A,B). These cells differentiate into mature oligodendrocytes that elaborate myelin-like membrane sheets and express both Mbp and Cnp (Fig. 4A,D), and eventually myelin-associated glycoprotein (Mag). Immature cells (4-5 DIV) grown in the presence of netrin 1 (100 ng/ml) for 24 hours showed a modest (4%) decrease in the ratio of Cnp- to Mbp-positive cells. In more-mature Mbp/Mag-positive cultures (6-8 DIV), the ratio of Mbp-positive cells to Mag-positive cells was not affected by the addition of netrin 1 (Fig. 4A). We conclude that the addition of netrin 1 in vitro does not alter the acquisition of a mature phenotype, as assessed by the expression of standard markers.

Netrin 1 induces Dcc-dependent process extension by immature oligodendrocytes in vitro

The effect of netrin 1 on process extension, as assayed by measuring the length of the longest process, was investigated in Cnp-immunopositive immature oligodendrocytes in culture (Fig. 4B). Application of 100 ng/ml netrin 1 for 24 hours to immature oligodendrocytes increased process length compared with control cells (Fig. 4C). To determine whether Dcc is required for netrin-1-induced extension of oligodendrocyte processes in vitro, a Dcc function-blocking antibody (Dccfb) was added for 24 hours to immature oligodendrocytes treated with netrin 1. Consistent with the findings obtained in vivo and described above (Fig. 3F), disruption of Dcc function blocked the netrin-1-induced increase in process length, but did not significantly alter process extension when added alone (Fig. 4C). We conclude that the addition of netrin 1 promotes Dcc-dependent oligodendrocyte process extension, and that these changes occur independently of defects in migration and axon growth.

Netrin 1 promotes Dcc-dependent increases in oligodendrocyte branching and myelin-like sheet formation

To investigate roles for netrin 1 during later stages of oligodendrocyte development, we characterized changes in oligodendrocyte process branching and in the capacity of these cells to elaborate myelin-like membrane sheets in vitro. Mature oligodendrocytes cultured for 6-8 DIV were double labeled with RIP antibody (against Cnp) to identify major processes, and Mbp antibody to visualize extended myelin-like membrane sheets (Fig. 4D, left). Oligodendrocytes grown in the presence of 100 ng/ml netrin 1 for 24 hours exhibited a significant increase in the area of Mbp-positive sheets (Fig. 4E). The morphological complexity of oligodendrocyte processes was quantified using Sholl analysis (Richards et al., 2001) (Fig. 4D). Cells treated with 100 ng/ml netrin 1 for 24 hours exhibited a significant increase in branching compared with the control (Fig. 4F,G). A dose-response analysis determined 100 ng/ml netrin 1 to be optimal (Fig. 4F).

As described above, immunocytochemical analyses detected Dcc, but little, if any, netrin 1 associated with the branches of oligodendrocyte processes. Both Nfb (netrin function-blocking...
antibody) and Dccfb antibodies blocked the increase in myelin-like membrane sheet formation and process branching induced by the addition of exogenous netrin 1 (Fig. 4E,G), indicating that the netrin-1-induced changes in oligodendrocyte morphology are Dcc-dependent. Substantial netrin 1, but not Dcc, immunoreactivity was detected in association with the myelin-like membrane sheets (Fig. 2A); however, function-blocking antibodies applied in the absence of netrin 1 did not alter sheet formation or process branching (Fig. 4E,G). We therefore tested the hypotheses that netrin 1 made by oligodendrocytes does not exert an autocrine influence on the formation of myelin-like membrane sheets, and, alternatively, that the relatively short-term (24 hours) loss-of-function assays described above are too brief to reveal a role for endogenous netrin 1.

Autocrine netrin 1 regulates the formation of myelin-like membrane sheets, but not process branching, in a Dcc-dependent manner

To investigate an autocrine role for netrin 1, oligodendrocytes were isolated from mixed glial cultures derived from P0 Ntn1–/– or Dcc–/– mice (Fig. 5A). Application of netrin 1 for 24 hours to mature oligodendrocytes lacking Dcc did not increase process branching (Fig. 5B), indicating that oligodendrocyte processes require Dcc to respond to exogenous netrin 1. Furthermore, in agreement with the function-blocking antibody data described above (Fig. 4G), baseline levels of process branching were not altered in oligodendrocytes lacking either Dcc or netrin 1 (Fig. 5B,C). We conclude that netrin 1 does not exert an autocrine effect on branching, and hypothesize that this is a consequence of netrin 1 being sequestered to the myelin-like membrane sheets (Fig. 2A), thereby exposing the leading edges of processes to little, if any, endogenous netrin 1.

By contrast, oligodendrocytes lacking either netrin 1 or Dcc exhibited a significantly reduced surface area of the myelin-like membrane sheets compared with cells derived from wild-type or heterozygote littermates. *P<0.05, **P<0.0001 versus control. The number of cells analyzed in each condition is indicated in parentheses. Error bars indicate s.e.m. A, 20×0.5 n.a. objective; B-D, 40×0.75 n.a. objective. Scale bars: 40 μm in A; 20 μm in B-D.

Process elaboration induced by netrin 1 does not require β1 subunit-containing integrins

Oligodendrocytes express αvβ1, αvβ3, αvβ5 and α6β1 integrins. Engagement of integrins, specifically α6β1, by extracellular matrix (ECM) components activates SFKs to regulate changes in oligodendrocyte morphology (Baron et al., 2005). Netrins are members of the laminin family of ECM proteins, and netrin 1 has been proposed to function as a ligand for α3β1 and α6β4 integrins...
in pancreatic cells (Yebra et al., 2003). We therefore investigated whether netrin-1-mediated changes in the elaboration of oligodendrocyte processes involve an interaction between netrin 1 and integrins.

Application of a β1 integrin function-blocking antibody, which has been demonstrated to block fibronectin-induced changes in oligodendrocyte morphology (Liang et al., 2004), did not significantly alter the netrin-1-induced increase in

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**Fig. 4.** Netrin 1 induces Dcc-dependent process extension by immature oligodendrocytes, and Dcc-dependent process branching and myelin-like membrane sheet extension by mature oligodendrocytes, in vitro. (A) Addition of netrin 1 (100 ng/ml, 24 hours) to cultured rat oligodendrocytes results in a 4% decrease in the ratio of immature cells expressing Cnp only (arrowheads) as compared with more mature cells expressing Cnp and Mbp. Netrin 1 did not change the ratio of cells expressing Mbp only versus Mbp and Mag. (B) Cnp-positive immature oligodendrocytes are multipolar cells with one major process. Dashed red lines indicate examples of the processes measured. (C) The length of the major process increased following the addition of netrin 1 (100 ng/ml) for 24 hours. Application of Dcc function-blocking antibody (Dccfb) to immature oligodendrocytes blocks the netrin-1-induced increase in process extension, whereas Dccfb alone does not have a significant effect on process extension. (D) A mature Mbp- and RIP-positive oligodendrocyte extending both myelin-like membrane sheets and branched processes. Sheet area was quantified by tracing the Mbp-positive myelin-like membrane sheets (left, red outlines). Branching was quantified by measuring the number of intersections that processes made with concentric circles (right, red), which are numbered 1-5 to reflect increasing distance from the cell body (and as labeled on the x-axis of bar charts displaying branching complexity). (E) Netrin 1 (100 ng/ml, 24 hours) increased the area of Mbp-positive sheets compared with the control. This effect was blocked by Dccfb. (F) Processes of mature oligodendrocytes exposed to netrin 1 for 24 hours exhibited an increase in branching. Dose-response analysis indicated maximal branching at 100 ng/ml netrin 1. (G) Addition of Dcc function-blocking antibody together with netrin 1 prevented the netrin-1-dependent increase in branching, and decreased branching compared with controls. A, 20×0.5 n.a. objective; B,D,F, 40×0.75 n.a. objective. Scale bars: 40 μm in A; 20 μm in B,D,F. A.U., arbitrary units. *P<0.05, **P<0.005, versus control. Error bars indicate s.e.m.
oligodendrocyte process branching (Fig. 6A). By contrast, this antibody blocked HEK293T cell spreading on a fibronectin substrate (Fig. 6B), verifying its efficacy. Yebra and colleagues identified a 25 amino acid region within the C-terminus of netrin 1 that binds α6β4 and α3β1 integrins, and hypothesized that potential interactions between netrin 1 and other integrins might also occur through this region (Yebra et al., 2003). To determine whether such an interaction might contribute to netrin-1-induced changes in oligodendrogial morphology, we incubated cells with a peptide comprising the putative integrin-binding sequence that functions as a competitive inhibitor of integrins binding netrin 1 (Yebra et al., 2003). The netrin 1 peptide (20 μg/ml, a gift from Dr V. Cirulli, UCSD, CA, USA) did not affect process complexity (Fig. 6A), nor did it disrupt the netrin-1-dependent increase in branching (Fig. 6A). We conclude that the novel role for netrin 1 in regulating oligodendrocyte morphology occurs through Dcc independently of β1-containing integrins and the integrin-binding region at the C-terminus of netrin 1.

**Netrin 1 binding to Dcc recruits Fyn to a complex containing FAK**

We next assayed signaling proteins implicated as regulators of cytoskeletal organization downstream of netrin 1 and Dcc in axonal growth cones to determine whether similar signaling complexes might be engaged in oligodendrogliocytes. Oligodendrocytes express the SFKs Src, Fyn and Lyn (Colognato et al., 2004). Of these, only mice lacking Fyn show defects in myelination (Sperber et al., 2001). Furthermore, Fyn activation has been implicated in the regulation of process branching during the morphological maturation of oligodendrocytes (Osterhout et al., 1999; Umemori et al., 1994). In axonal growth cones, application of netrin 1 recruits Fyn to the Dcc intracellular domain, where it is activated by focal adhesion kinase (FAK; Ptk2 – Mouse Genome Informatics) (Liu et al., 2004). To determine whether Fyn might function downstream of Dcc in oligodendrocytes, cells derived from newborn (P0) rats were allowed to mature in culture for 3–4 days or for 5–6 days until they expressed Mbp. Co-immunoprecipitation studies carried out using
two different antibodies against Fyn revealed an interaction between Fyn and Dcc in both immature (4 DIV) and mature (6 DIV) oligodendrocytes (Fig. 7A). Five minutes following application of netrin 1, an increased amount of Fyn co-immunoprecipitated with Dcc (Fig. 7A,D). A relatively minor Fyn-immunoreactive band of slightly higher molecular weight was consistently detected following immunoprecipitation and might reflect Fyn phosphorylation as a result of activation by netrin 1. Our findings also revealed a constitutive interaction between Dcc and FAK (Fig. 7A), suggesting that application of netrin 1 to oligodendrocytes recruits Fyn into a complex with FAK that is bound to the intracellular domain of Dcc, as has been reported for neurons (Li et al., 2004; Ren et al., 2004). Application of netrin 1 led to increased phosphorylation of SFK tyrosine 416 (Y416), an event associated with kinase activation (Smart et al., 1981), in the SFK associated with Dcc (Fig. 7B,D). Analysis of SFK phosphorylation in whole-cell lysates did not detect a global change in phospho-Y416, indicating that the netrin-1-induced change is specific to SFK recruited into a complex with Dcc (Fig. 7C). Using an antibody specific for the SFK Src, an interaction with Dcc was not detected (not shown). This provides evidence for specific recruitment of Fyn to Dcc; however, we do not rule out that other SFKs might be involved in netrin 1 signaling in oligodendrocytes.

**The netrin-1-induced increase in oligodendrocyte branching requires Fyn**

To determine whether Fyn is required downstream of netrin 1 in oligodendrocytes, we isolated cells from mice lacking Fyn \([Fyn^{tm1Sor}]\), Fyn knockout (KO) and from wild types of a matched genetic background (B6129SF2/J, F2 hybrid) (Fig. 8A). Treatment of Fyn KO cells with netrin 1 did not result in increased branching, in contrast to its effect upon cells isolated from control mice (Fig. 8A,B). We conclude that Fyn is essential for these changes to occur. We therefore determined whether activation of SFKs is required to promote the morphological changes induced by netrin 1.

**SFK activity is required for the netrin-1-induced increase in oligodendrocyte process length and branching**

Immunocytochemistry revealed SFK phospho-Y416 immunoreactivity distributed within the oligodendrocyte cell body and proximal branches, and punctate staining within the distal branches (Fig. 8C,D). Colocalization with Dcc was observed, consistent with our immunoprecipitation results (Fig. 7B). To determine whether changes in SFK phosphorylation occurred upon netrin 1 stimulation, the relative number of SFK phospho-Y416-immunoreactive puncta was measured per unit area of the cell (Fig. 8E). Treatment of mature oligodendrocytes with netrin 1 (100 ng/ml) for 24 hours significantly increased the number of SFK phospho-Y416-positive puncta per unit area, consistent with an association between netrin 1 stimulation and increased SFK activity (Fig. 8F). Treatment with the SFK inhibitor PP2 (2 μM) (Hanke et al., 1996) blocked the netrin-1-induced increase in SFK phospho-Y416-positive puncta (Fig. 8F), whereas the inactive SFK inhibitor analog PP3 (2 μM) had no significant effect on the number of puncta per unit area (Fig. 8F). Application of PP2 alone led to a decrease in the relative number of puncta per unit area, consistent with constitutive SFK activity contributing to the basal level of puncta observed (Fig. 8F). To exclude the possibility that the increase in SFK phospho-Y416-positive puncta was secondary to increased branching formation, the same quantification was performed with cells immunostained for Fyn. No difference was found in the number of Fyn-immunopositive puncta per unit area in control and netrin-1-treated cells (Fig. 8G), consistent with the increase in SFK phospho-Y416-positive puncta resulting from an increase in SFK activity and not a netrin-1-induced increase in the number of branches.

Our findings indicate that netrin 1 recruits Fyn to a complex with Dcc, increasing SFK activity in oligodendrocytes. We then tested the hypothesis that SFK activation is required for the morphological changes induced by netrin 1. SFK activity was assessed in both immature (4 DIV, Cnp-positive) and mature (6 DIV, Mbp-positive) oligodendrocytes. Treatment of oligodendrocytes with PP2 blocked netrin-1-induced process extension in Cnp-immunoreactive immature cells and the netrin-1-dependent increase in branching in Mbp-expressing mature oligodendrocytes, whereas the inactive analog PP3 had no effect (Fig. 8H,I). Immature oligodendrocytes treated with PP2 alone showed a small but significant decrease in process length (Fig. 8H), which was not seen with PP3 treatment. Mature oligodendrocytes appeared less sensitive to the inhibition of basal SFK activity than immature cells, as PP2 alone did not affect branching (Fig. 8I). We conclude that netrin 1 binding to Dcc results in the recruitment of the SFK Fyn, and that subsequent activation of Fyn is required for the netrin-1-induced changes in oligodendrocyte morphology.
Netrin 1 inhibits RhoA but does not affect Cdc42 or Rac1 in oligodendrocytes

We next asked what signals might act downstream of SFKs to trigger the morphological changes induced by netrin 1 in oligodendrocytes. Members of the Rho family of small GTPases, including RhoA, Rac1 and Cdc42, regulate the elaboration and branching of oligodendrocyte processes (Liang et al., 2004). The effect of netrin 1 on Cdc42 and Rac1 activity was investigated using a GST-Pak-CRIB pulldown assay (Sander et al., 1998). Cell lysates of cultured mature oligodendrocytes were incubated with GST-Pak-CRIB fusion protein to quantify the levels of GTP-bound Cdc42 and Rac1. No significant change in the levels of GTP-bound Rac1 or Cdc42 was detected in oligodendrocytes following application of netrin 1 (Fig. 9B,C). We have reported that the Cdc42 effector protein N-WASP is recruited into a protein complex with the intracellular domain of Dcc following the addition of netrin 1 to embryonic spinal commissural neurons (Shekarabi et al., 2005). Unlike in oligodendrocytes, netrin 1 activates Cdc42 and Rac1 in commissural neurons. In mature oligodendrocytes, N-WASP also constitutively co-immunoprecipitated with Dcc; however, addition of netrin 1 did not significantly alter the amount of N-WASP associated with Dcc (Fig. 9D), which is consistent with netrin 1 not increasing the activation of Cdc42 in oligodendrocytes.

Inhibiting RhoA, or its downstream effector Rho kinase (Rock1/2), in oligodendrocytes increases process extension (Liang et al., 2004; Miron et al., 2007; Wolf et al., 2001). Interestingly, activation of Fyn in oligodendrocytes leads to inactivation of RhoA (Wolf et al., 2001), and we therefore investigated the possibility that netrin 1 might regulate RhoA activity in oligodendrocytes. Levels of GTP-bound RhoA in mature Mb-positive oligodendrocytes were assessed using a GST-rhotekin pulldown assay (Reid et al., 1996). Following a 5 minute treatment of mature oligodendrocytes with netrin 1, a significant decrease in RhoA-GTP was detected compared with controls (Fig. 9A). We conclude that netrin-1-induced inhibition of RhoA, in the absence of altered Rac1 or Cdc42 activity, promotes process elaboration by oligodendrocytes.

DISCUSSION

Developing oligodendrocytes extend and retract their processes over substantial distances, sampling the local environment to locate unmyelinated axons (Hardy and Friedrich, 1996; Kirby et al., 2006). As the cell matures, these processes branch and eventually form lamellae that ensheath target axons. Our findings indicate that netrin 1 and Dcc regulate oligodendrocyte process extension, branching and myelin-like membrane sheet formation, which are all essential events for initiating myelination.

Exogenous and autocrine netrin 1 contribute to oligodendrocyte maturation

Our results support the conclusion that netrin 1, as expressed by neurons and neuroepithelial cells, when encountered by a premyelinating oligodendrocyte evokes Dcc-dependent extension of the motile processes of the cell. In more mature cells, exogenous netrin 1 promotes Dcc-dependent process branching and myelin-like membrane sheet formation. Based on our findings in vitro and in vivo, we hypothesize that by promoting the morphological maturation of oligodendrocytes, netrin 1 facilitates the search for appropriate axonal targets. Interestingly, although exogenous netrin 1 promotes changes in oligodendrocyte morphology, at later stages of maturation oligodendrocytes themselves begin to express netrin 1. We identify a selective, Dcc-dependent autocrine role for netrin 1 in promoting the formation of myelin-like membrane sheets. Oligodendrocytes only begin to express netrin 1 in the developing spinal cord after myelination has begun. Our findings indicate that
contact with axons is not essential for the initiation of netrin 1 expression by these cells, but that it occurs coincident with myelin-like membrane sheet formation. We hypothesize that autocrine expression of netrin 1 specifically promotes later stages of maturation, facilitating the formation of large myelin-like membrane sheets by these cells. Netrin 1 expression by oligodendrocytes might also facilitate axon remodeling or prevent aberrant axonal sprouting at later stages of development.

**Common signaling mechanisms in the regulation of oligodendrocyte processes and axonal growth cones by netrin 1**

The extending tips of oligodendrocyte processes in some ways resemble motile axonal growth cones (Fox et al., 2006; Jarjour and Kennedy, 2004), and increasing evidence suggests that common regulators of actin nucleation function in neuronal growth cones and at the leading edge of oligodendrogial...
Regulation of oligodendrocyte maturation

**Netrin 1 activates a canonical signaling mechanism required for oligodendrocyte maturation**

To date, the candidate extracellular signals that might regulate the elaboration of oligodendroglial processes immediately preceding myelination have been limited to ECM proteins, of which laminin 2 and its receptor integrin α6β1 have been well characterized. Studies in vitro have shown that integrin-dependent activation of Fyn activates Cdc42 and Rac1 and deactivates RhoA, leading to process outgrowth (Liang et al., 2004; Osterhout et al., 1999). However, oligodendrocytes lacking the β1 integrin subunit mature and myelinate normally, demonstrating that this pathway is not essential in vivo (Benninger et al., 2006). Furthermore, laminin 2 is not ubiquitously present in myelinating axon tracts in the CNS, indicating that other ligand-receptor complexes must trigger these signaling mechanisms independently of β1 integrin function. Our results show that netrin 1, acting through Dcc, activates an intracellular signaling pathway that is required for the morphological maturation of oligodendrocytes. Crucially, the effects of netrin 1 do not require β1 integrin function and do not appear to act through netrin-binding integrins.

Our findings identify a novel mechanism regulating oligodendrocyte morphology during later stages of differentiation. Interestingly, many oligodendroglial cells detected in multiple sclerosis lesions appear to have differentiated, but remain unable to elaborate myelin (Chang et al., 2002). A better understanding of the mechanisms that promote myelination will advance the development of therapeutics that aim to promote the recovery of nervous system function.

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**Fig. 9. Treatment of mature oligodendrocytes with netrin 1 inhibits RhoA activity** (A) Levels of GTP-bound RhoA in rat oligodendrocytes were assessed using GST-rhotekin pulldown. Treatment of mature oligodendrocytes with netrin 1 (100 ng/ml, 5 minutes) caused a significant decrease in RhoA-GTP compared with controls. (B,C) Similar treatment of cells resulted in no significant change in the levels of GTP-bound Cdc42 or Rac1, as assessed by GST-Pak-CRIB pulldown. (D) Addition of netrin 1 to mature oligodendrocytes (100 ng/ml, 5 minutes) did not alter the amount of N-WASP recruited to Dcc. *P<0.05 versus control. Error bars indicate s.e.m.

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**References**


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**Methods**

We have implicated Fyn, Rac1, Cdc42, RhoA and N-WASP in the intracellular signaling proteins downstream of netrin 1: Fyn, FAK, N-WASP and Rho GTPases (Li et al., 2004; Liu et al., 2004; Ren et al., 2004; Shekarabi and Kennedy, 2002). We demonstrate that Fyn is required for the netrin-1-dependent increase in oligodendrocyte process branching. Netrin 1 recruits Fyn to a complex that includes Dcc and FAK, resulting in SFK phosphorylation and activation. Importantly, previous studies have implicated Fyn, Rac1, Cdc42, RhoA and N-WASP in the mechanism governing the cytoskeletal changes in oligodendrocytes that lead to myelination (Simons and Trotter, 2007).

Activating Fyn results in RhoA inactivation in oligodendrocytes (Wolf et al., 2001). We demonstrate reduced levels of GTP-bound RhoA upon netrin 1 stimulation, but found no evidence for the activation of Cdc42 or Rac1. Interestingly, transgenic mice specifically lacking Cdc42 and Rac1 in oligodendrocytes exhibit normal oligodendroglial differentiation, but show eventual defects in myelin compaction (Thurnherr et al., 2006). Our findings support the hypothesis that in a background of unchanging Rac1 and Cdc42 activity, modulation of RhoA function plays a key role in regulating the morphological differentiation of oligodendrocyte processes.

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**Fig. 9. Treatment of mature oligodendrocytes with netrin 1 inhibits RhoA activity** (A) Levels of GTP-bound RhoA in rat oligodendrocytes were assessed using GST-rhotekin pulldown. Treatment of mature oligodendrocytes with netrin 1 (100 ng/ml, 5 minutes) caused a significant decrease in RhoA-GTP compared with controls. (B,C) Similar treatment of cells resulted in no significant change in the levels of GTP-bound Cdc42 or Rac1, as assessed by GST-Pak-CRIB pulldown. (D) Addition of netrin 1 to mature oligodendrocytes (100 ng/ml, 5 minutes) did not alter the amount of N-WASP recruited to Dcc. *P<0.05 versus control. Error bars indicate s.e.m.


