

Deleted in Colorectal Cancer Binding Netrin-1 Mediates Cell Substrate Adhesion and Recruits Cdc42, Rac1, Pak1, and N-WASP into an Intracellular Signaling Complex That Promotes Growth Cone Expansion

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Extracellular cues direct axon extension by regulating growth cone morphology. The netrin-1 receptor deleted in colorectal cancer (DCC) is required for commissural axon extension to the floor plate in the embryonic spinal cord. Here we demonstrate that challenging embryonic rat spinal commissural neurons with netrin-1, either in solution or as a substrate, causes DCC-dependent increases in growth cone surface area and filopodia number, which we term growth cone expansion. We provide evidence that DCC influences growth cone morphology by at least two mechanisms. First, DCC mediates an adhesive interaction with substrate-bound netrin-1. Second, netrin-1 binding to DCC recruits an intracellular signaling complex that directs the organization of actin. We show that netrin-1-induced growth cone expansion requires Cdc42 (cell division cycle 42), Rac1 (Ras-related C3 botulinum toxin substrate 1), Pak1 (p21-activated kinase), and N-WASP (neuronal Wiskott–Aldrich syndrome protein) and that the application of netrin-1 rapidly activates Cdc42, Rac1, and Pak1. Furthermore, netrin-1 recruits Cdc42, Rac1, Pak1, and N-WASP into a complex with the intracellular domain of DCC and Nck1. These findings suggest that DCC influences growth cone morphology by acting both as a transmembrane bridge that links extracellular netrin-1 to the actin cytoskeleton and as the core of a protein complex that directs the organization of actin.

Key words: embryonic spinal commissural neuron; chemotropism; chemotropic; axon guidance; motility; DCC; netrin

Introduction

Axon guidance is achieved by integrating the response to cues regulating adhesion and to cues that direct the reorganization of the growth cone cytoskeleton. Netrins are a family of secreted proteins that guide migrating cells and axons, including spinal commissural axons, during neural development (Kennedy, 2000). Receptors for netrin-1 in the vertebrate CNS include deleted in colorectal cancer (DCC), neogenin, and the UNC-5 homologs (Dickson, 2002). DCC is a type I transmembrane Ig superfamily member that is expressed by embryonic spinal commissural neurons and is required for their axons to be attracted toward a source of netrin-1 (Keino-Masu et al., 1996; Fazeli et al., 1997).

Lamellipodia and filopodia form at the leading edge of a growth cone by continuous remodeling of the actin cytoskeleton

(Bentley and O'Connor, 1994; Tanaka and Sabry, 1995). DCC is enriched in filopodia, and in response to netrin-1 it exerts a powerful influence on the organization of actin (Shekarabi and Kennedy, 2002). Intracellularly, the organization of the actin cytoskeleton is regulated by Rho-GTPases that act as molecular switches, cycling between active and inactive forms (Hall, 1998). Based on studies initially performed in fibroblasts, RhoA has been implicated in stress fiber formation, Ras-related C3 botulinum toxin substrate 1 (Rac1) has been implicated in lamellipodia formation, and cell division cycle 42 (Cdc42) has been implicated in filopodia formation (Ridley, 2001). These GTPases also play key roles in regulating growth cone morphology and axon outgrowth (Mueller, 1999; Dickson, 2001).

We have reported previously that netrin-1, through DCC, activates Cdc42 and Rac1, causing filopodia formation and cell spreading in human embryonic kidney 293T (HEK293T) and neuroblastoma glioma 108–15 (NG108–15) cell lines (Shekarabi and Kennedy, 2002). Consistent with this, genetic analysis in *Caenorhabditis elegans* indicates that *ced-10*, a Rac-like GTPase, is required for axons to respond to the netrin homolog UNC-6 (Gitai et al., 2003). In addition, we have identified a role for the Src homology 2 (SH2) and SH3 domain-containing adaptor protein Nck1, demonstrating that it binds the intracellular domain (ICD) of DCC and is required for DCC-induced outgrowth of neurite-like processes from neuroblastoma 1E-115 (N1E-115) cells (Li et al., 2002).

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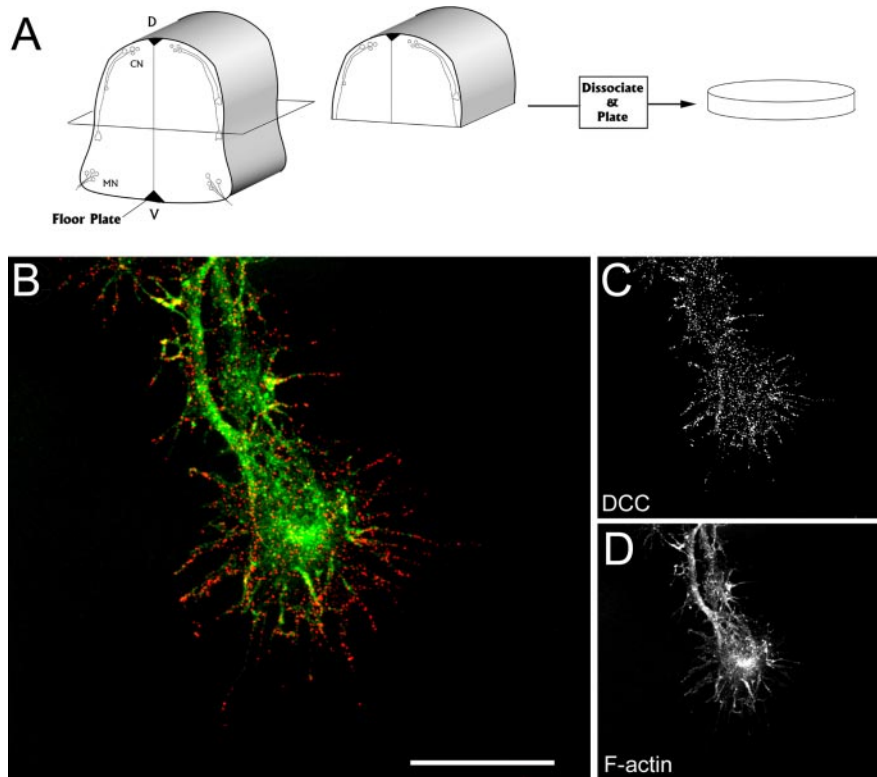


Figure 1. Distribution of DCC in cultured embryonic rat commissural neuron growth cones. **A**, The dorsal halves of E13 rat spinal cords were microdissected, dissociated, and plated (D, dorsal; V, ventral; CN, commissural neurons; MN, motoneurons). **B**, The morphology of commissural neuron growth cones grown for 2 DIV in the presence of netrin-1 and then immunolabeled for DCC (red) and for F-actin with FITC-coupled phalloidin (green). **C**, DCC staining alone. **D**, F-actin staining alone. Scale bar, 10 μ m.

The majority of netrin-1 in the embryonic CNS is associated with either cell membranes or the extracellular matrix (Serafini et al., 1994; Manitt et al., 2001; Manitt and Kennedy, 2002), indicating that, although netrin-1 is a secreted protein, most is not freely diffusible *in vivo*. Here we show that netrin-1, added in solution or as a substrate, causes embryonic rat commissural neuron growth cone expansion and that this response requires DCC. We provide evidence for an adhesive interaction between substrate-bound netrin-1 and cell-surface DCC, suggesting that DCC-mediated adherence contributes to netrin-1-induced changes in growth cone morphology. In addition, we show that netrin-1 recruits Cdc42, Rac1, neuronal Wiskott–Aldrich syndrome protein (N-WASP), and the serine/threonine kinase p21-activated kinase 1 (Pak1) into a complex with the DCC ICD; that netrin-1 activates Cdc42, Rac1, and Pak1 in commissural neurons; and that Cdc42, Rac1, Pak1, and N-WASP are required for netrin-1-induced growth cone expansion. These findings provide evidence that DCC functions as a transmembrane bridge between netrin-1 and the cytoskeleton and identify a signal transduction complex recruited to the DCC ICD that directs the organization of actin in the growth cone.

Materials and Methods

Reagents and cell culture. The following antibodies were used: affinity-purified rabbit polyclonal netrin antibody PN3 (25 μ g/ml) (Manitt et al., 2001), purified nonimmune rabbit IgG (25 μ g/ml; Invitrogen, San Diego, CA), function-blocking DCC mouse monoclonal anti-DCC_{FB} (AF5; Calbiochem, La Jolla, CA), mouse monoclonal anti-DCC_{IN} (G97-449; PharMingen, Mississauga, Ontario, Canada), rabbit polyclonal anti-Cdc42 (SC-87; Santa Cruz Biotechnology, Santa Cruz, CA), mouse monoclonal anti-Rac1 (Transduction Laboratories, Lexington, KY), rab-

bit polyclonal anti-Pak1 (New England Biolabs, Beverly, MA), anti-phospho-specific (Ser¹⁹⁸ and Ser²⁰³) Pak1 (provided by M. Greenberg, Harvard University, Cambridge, MA) (Shamah et al., 2001), goat polyclonal anti-N-WASP D15 and mouse monoclonal anti-glutathione *S*-transferase (anti-GST; Santa Cruz Biotechnology), and anti-Flag epitope tag (Sigma–Aldrich, Oakville, Ontario, Canada). Filamentous actin was visualized using fluorescein-conjugated (FITC) phalloidin (Sigma–Aldrich). Recombinant netrin-1 protein was purified from a 293-Epstein–Barr virus nuclear antigen (EBNA) cell line secreting netrin-1, as described previously (Serafini et al., 1994; Shirasaki et al., 1996).

Dominant-negative (N17) and constitutively active (V12) forms of Cdc42- and Rac1-expressing adenoviruses were provided by Dr. J. Bamberg (Colorado State University, Fort Collins, CO). Expression constructs encoding GST fusion proteins of wild-type Cdc42 and Rac1 were provided by G. Bokoch (The Scripps Research Institute, La Jolla, CA) (Bagrodia et al., 1995). The Pak peptide and control peptide (Kiosses et al., 2002) were provided by M. A. Schwartz (The Scripps Research Institute). An adenovirus expressing a Flag-tagged dominant-negative mutant form of N-WASP was constructed as described previously (He et al., 1998). The N-WASP mutant [Δ cofilin N-WASP (Δ cof N-WASP)] contains a 4 aa deletion in its C-terminal domain that compromises its ability to bind the Arp2/3 complex and therefore does not promote actin polymerization. Recombinant protein was visualized by Flag epitope tag immunoreactivity, and endogenous N-WASP was detected by the use of anti-N-WASP.

Embryonic rat spinal commissural neurons were cultured as described previously (Bouchard et al., 2004). Briefly, dorsal halves of the spinal cord of embryonic day 13 (E13; E0, vaginal plug) rat embryos were microdissected (see Fig. 1A), dissociated for 30 min at 37°C in Ca²⁺/Mg²⁺-free HBSS (Invitrogen), followed by trituration with a flame-polished Pasteur pipette, and cultured in Neurobasal (Invitrogen) plus 10% heat-inactivated fetal bovine serum with 100 U/ml penicillin and 100 U/ml streptomycin. After 24 h, the medium was changed to Neurobasal supplemented with 2% B-27 (Invitrogen), 2 mM glutamine, and penicillin/streptomycin. Tissue culture plastic was coated with 20 μ g/ml poly-D-lysine (PK; Sigma, St. Louis, MO) at 37°C for 2 h. In experiments that used netrin-1 as a substrate, the coverslips were coated with PK, washed, and then coated with 5 μ g/ml netrin-1 protein at 37°C overnight. For biochemical analysis of proteins, the dissociated neurons were cultured at 4×10^6 cells per 60 mm plate. For immunostaining, the neurons were plated on 13 mm glass coverslips (Carolina Biological Supply, Burlington, NC) at 7×10^3 cells per coverslip. At 36 h after plating, the cultures were washed, changed to B-27-free Neurobasal, and incubated for another 6 h before stimulation with 80 ng/ml purified netrin-1 protein. Then the cells were either lysed or fixed and immunostained. Filopodia number and growth cone surface area were quantified as described previously (Shekarabi and Kennedy, 2002). Statistical significance of differences between means was evaluated by a one-way ANOVA with Scheffé's *post hoc* test (Systat, Chicago, IL).

Immunofluorescence. Photomicrographs were taken with an Axiovert microscope (Zeiss, Oberkochen, Germany) and a Magnafire CCD camera (Optronics, Goleta, CA) and analyzed with Northern Eclipse image analysis software (Empix Imaging, Mississauga, Ontario, Canada) by an observer blind to the experimental conditions. Values are expressed as the mean \pm SEM. Statistical significance was evaluated by a one-way ANOVA with a Scheffé's *post hoc* test (Systat).

Cell substrate adhesion assay. To assay cell substrate adhesion, we dried 20 μ l of 0.1% nitrocellulose (Hybond ECL; Amersham Biosciences, Piscataway, NJ) dissolved in methanol (histological grade; Fisher Scientific, Houston, TX) at the bottom of a four-well plate, followed by incubation with either HBSS or 2 μ g/ml netrin-1 in HBSS for 2 h at room temperature. All substrates then were blocked for 1 h with 1% BSA (Fisher Scientific) in HBSS and then again with 1% heparin (Sigma) in HBSS. Substrates were incubated with one of the following (in μ g/ml): 25 anti-netrin PN3 (Manitt et al., 2001), 5 DCC-Fc (R & D Systems, Minneapolis, MN), or 25 nonimmune rabbit IgG (Invitrogen) for 1 h. All substrates were washed once and kept in HBSS before 2.5×10^5 cells from dissociated dorsal spinal cords were plated in Neurobasal supplemented with 2% B-27 and 2 mM glutamine. Cells were cultured for 2 h at 37°C, 5% CO₂, gently washed three times with PBS, and fixed with 4% PFA in PBS. For cell counting, the nuclei were labeled with 0.5 μ g/ml Hoechst 33258 (Sigma) in PBS for 30 min.

GTP γ S loading assay. GTP γ S loading assays were performed as described previously (Knaus et al., 1992). Neurons cultured in 60 mm plates were treated with purified 80 ng/ml netrin-1 protein for 3 min and lysed in ice-cold lysis buffer [LB; containing 150 mM NaCl, 25 mM HEPES, pH 7.5, 25 mM NaF, 1 mM EDTA, 1 mM sodium orthovanadate plus 1% NP-40 and 0.25% sodium deoxycholate] with 10 mM MgCl₂, 5% glycerol, and protease inhibitors (containing 2 μ g/ml leupeptin, 2 μ g/ml aprotinin, 1 μ g/ml pepstatin plus 2 mM PMSF). Lysates were incubated with 100 μ M GTP γ S in the presence of 10 mM EDTA for 12 min at 31°C. GTP-bound Cdc42 and Rac1 were pulled down with 20 μ l of glutathione-coupled Sepharose 4B beads (Amersham Biosciences) that had been loaded with 10 μ g of bacterially expressed GST-Pak1-Cdc42/Rac1 interactive-binding (CRIB) domain fusion protein (amino acids 56–272) (Sander et al., 1999). Components of the protein complex were resolved by SDS-PAGE and Western blot analysis, using anti-Rac1 or anti-Cdc42. Signals were detected using ECL (PerkinElmer, Wellesley, MA). Densitometry and quantification were performed using NIH Image software.

GST-Cdc42 and GST-Rac1 pull-down assays. GST-Cdc42 and GST-Rac1 fusion proteins were expressed in bacteria and isolated as described previously (Sander et al., 1999). Cultured commissural neurons were treated with 80 ng/ml netrin-1 protein for the times indicated. Cell lysates were incubated with GTP γ S, 10 mM EDTA, and either GST-Cdc42 or GST-Rac1 fusion proteins at 31°C for 12 min. The protein complex was isolated using glutathione-coupled Sepharose 4B beads as described above. The pellet was washed with LB, and the bound proteins were resolved by SDS-PAGE.

Coimmunoprecipitation. For coimmunoprecipitation (coIP) analyses the commissural neurons were plated in 60 mm PK-coated cell culture plates at 5×10^6 cells per plate as described above. Cells were then treated with 100 ng/ml netrin-1 for either 5 or 30 min, washed with ice-cold PBS, and then lysed in ice-cold LB with protease inhibitors for 15 min on ice. Cell lysate was collected and centrifuged at $15,000 \times g$ for 10 min at 4°C; the supernatant was used as the input for coIP. Protein G-Sepharose beads (Sigma) were blocked for 1 h at 4°C in LB plus 10% BSA, washed four times, and resuspended in LB. Immunoprecipitations were performed using 1 μ g of anti-DCC_{IN}, 2 μ g of anti-Pak1, or 12 μ l of goat polyclonal anti-N-WASP (D15) on a rocking platform at 4°C. Blocked beads were added after 1 h of incubation with the primary antibody and allowed to incubate for 1 h. Beads and associated proteins were then

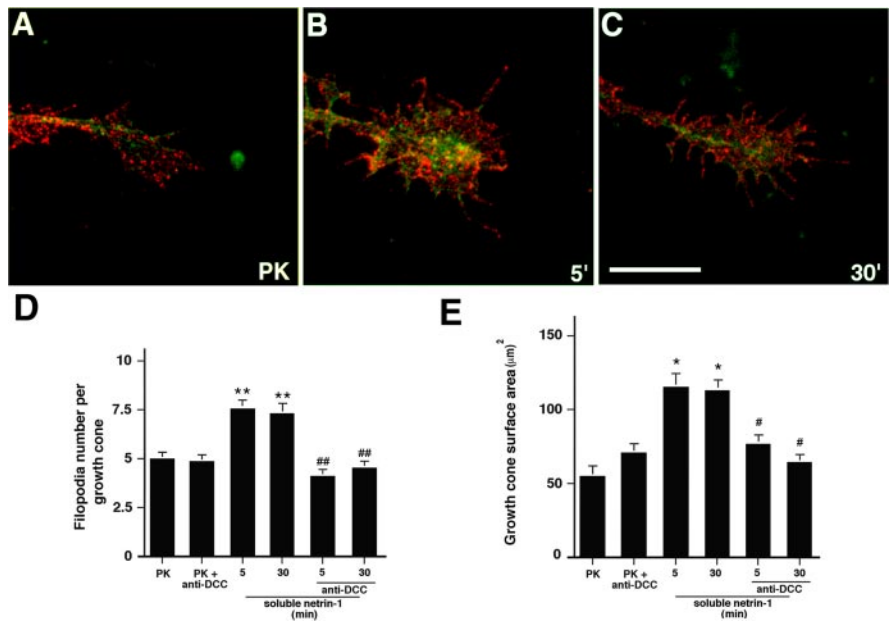


Figure 2. Netrin-1 causes commissural neuron growth cone expansion. **A–C**, Representative examples of commissural neuron growth cone morphologies grown on a PK substrate (**A**) or for 5 min (**B**) and 30 min (**C**) after the addition of 80 ng/ml netrin-1 protein to the culture media. DCC immunoreactivity is shown in red. FITC-phalloidin staining of F-actin is green (100 \times objective). Scale bar, 10 μ m. **D, E**, Quantification of the increase in growth cone filopodia number (**D**) and growth cone surface area (**E**) after application of 80 ng/ml netrin-1 for 5 or 30 min. Netrin-1 produced a significant increase in mean filopodia number per growth cone and growth cone surface area (* $p < 0.05$; ** $p < 0.01$). Anti-DCC_{FB} (5 μ g/ml) blocked netrin-1-induced growth cone expansion (# $p < 0.05$; ## $p < 0.01$), whereas the application of anti-DCC_{FB} alone had no effect ($n = 25$ per condition; error bars indicate SEM).

pelleted and washed three times in LB. Proteins were eluted from the beads using 25 μ l of PAGE loading buffer and characterized by Western blot analysis.

Results

Netrin-1 causes DCC-dependent commissural neuron growth cone expansion

Commissural neurons express *dcc* as they extend an axon toward the floor plate at the ventral midline of the embryonic spinal cord (Keino-Masu et al., 1996). To investigate the morphological and biochemical response of these neurons to netrin-1, we microdissected and dissociated dorsal halves of E13 embryonic rat spinal cords and then cultured these cells (Fig. 1A). More than 90% of the cells in these cultures express TAG-1 (transient axonal glycoprotein-1) and DCC, both markers of embryonic spinal commissural neurons *in vivo* (Dodd et al., 1988; Keino-Masu et al., 1996; Bouchard et al., 2004). DCC immunoreactivity was detected throughout the growth cones of commissural neurons grown *in vitro*, including along filopodia (Fig. 1B–D).

Growth cone turning involves actin-dependent membrane extension on one side of the growth cone, which is coordinated with membrane withdrawal on the other side (Mueller, 1999). Growth cone collapse has been used widely as an assay to study mechanisms underlying the action of repellent guidance cues (Castellani and Rougon, 2002). Netrin-1 is an attractant for embryonic spinal commissural neurons; therefore, we assessed the possibility that it might exert the opposite effect. The addition of netrin-1 (80 ng/ml) to spinal commissural neurons *in vitro* induced a rapid increase in the number of filopodia and growth cone surface area (Fig. 2), an effect that we describe as growth cone expansion.

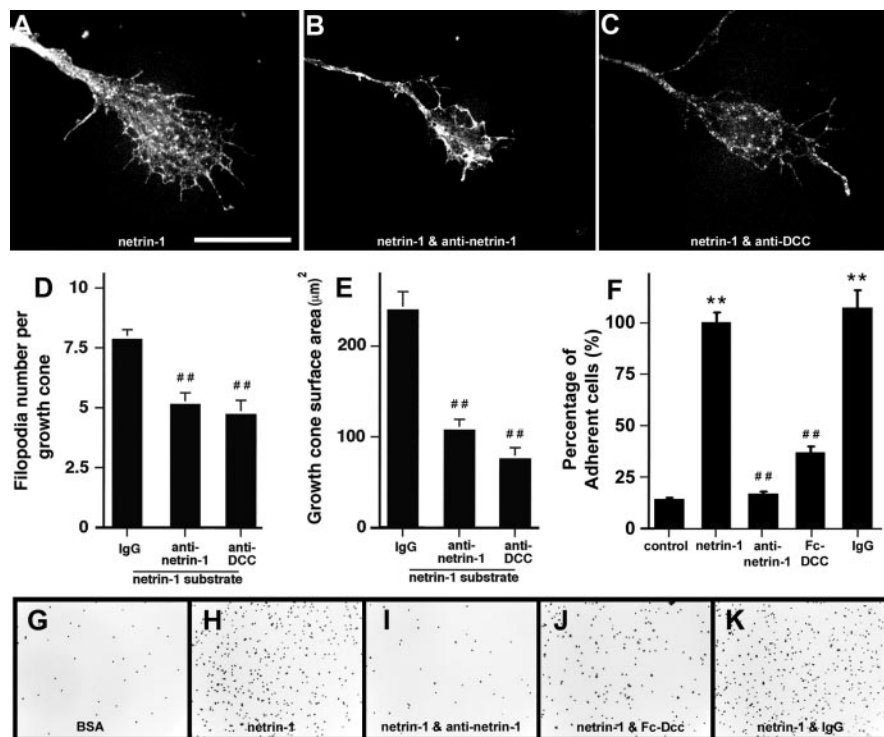


Figure 3. Substrate-bound netrin-1 induces growth cone expansion: evidence for an adhesive interaction between DCC and netrin-1. *A–C*, Commissural neuron growth cones cultured on a substrate of netrin-1 in the absence (*A*) or in the presence (*B*) of 25 $\mu\text{g/ml}$ anti-netrin-1 or in the presence (*C*) of 5 $\mu\text{g/ml}$ anti-DCC. Shown is the quantification of the number of filopodia per growth cone (*D*) and growth cone surface area (*E*) for the conditions shown in *A–C*. *F*, Quantification of cell substrate adhesion of dissociated E13 rat dorsal spinal cord cells. Representative examples of the assay are shown in *G–K* (10 \times objective lens; Hoechst-stained nuclei; grayscale inverted). A netrin-1 substrate generates a more than sevenfold increase in the number of adherent cells compared with a control BSA substrate. Preincubation of the substrates with anti-netrin-1 (*H*; 25 $\mu\text{g/ml}$ PN3) or DCC-Fc (5 $\mu\text{g/ml}$) significantly reduced the number of adherent cells compared with netrin-1. Control IgGs (*K*) did not affect adherence to netrin-1 (ANOVA; ** $p < 0.005$ compared with control; ## $p < 0.005$ compared with netrin-1 or IgG control; mean \pm SEM).

Substrate-bound netrin-1 promotes growth cone expansion and adhesion

We have reported previously that the majority of netrin-1 protein is not freely soluble *in vivo* but is bound to cell surfaces or the extracellular matrix (Manitt et al., 2001; Manitt and Kennedy, 2002). This suggested that netrin-1 might influence growth cone morphology by acting as an extracellular anchor that promotes cell adhesion. Consistent with a functional role for substrate-bound netrin-1, we detected growth cone expansion when the cells were plated on coverslips that had been precoated with a 5 $\mu\text{g/ml}$ solution of netrin-1 (Fig. 3*A–E*). The addition of a function-blocking antibody against DCC (DCC_{FB}) abolished the effect of soluble or substrate-bound netrin-1, indicating that DCC is required for netrin-1-induced commissural neuron growth cone expansion (Figs. 2*D,E*, 3*C–E*). Interestingly, the addition of netrin-1, either soluble or substrate-bound, did not affect the rate of axon outgrowth from these cells significantly, indicating that netrin-1 can influence growth cone morphology without affecting the rate of axon extension (PK, 40.15 \pm 3.99 μm ; PK plus netrin, 44.48 \pm 2.9 μm ; PK plus s-netrin, 42.37 \pm 3.85 μm ; PK plus s-netrin plus anti-netrin, 39.98 \pm 3.5 μm ; “plus netrin” indicates the addition of soluble netrin-1, and “plus s-netrin” indicates netrin-1 substrate). These results are consistent with previous findings indicating that netrin-1 can induce growth cone turning without altering the rate of axon growth (Ming et al., 1997).

To determine whether a netrin-1 substrate promotes adhesion, we plated cells derived from dissociated E13 rat embryonic

spinal cord on substrates of either netrin-1 or BSA and counted the number of adherent cells. A substrate of netrin-1 resulted in a more than sevenfold increase in the number of adherent cells when compared with control BSA substrates (Fig. 3*F–K*). Adhesion was blocked by preincubating the netrin-1 substrate for 1 h with 25 $\mu\text{g/ml}$ anti-netrin-1 (Fig. 3*H*). Control IgG (25 $\mu\text{g/ml}$) had no effect on the number of adherent cells (Fig. 3*K*). Preincubating the netrin-1 substrate with a DCC-Fc recombinant protein chimera encoding the extracellular domain of DCC fused to an antibody Fc domain also blocked adhesion to netrin-1, consistent with DCC mediating an adhesive interaction with substrate-bound netrin-1 (Fig. 3*J*).

Netrin-1-induced growth cone expansion requires Rac1 and Cdc42

We have reported previously that netrin-1 causes cell spreading and filopodia formation in cell lines transfected to express DCC (Shekarabi and Kennedy, 2002). These studies demonstrated that DCC independently activates Cdc42 and Rac1 in HEK293T cells and NG108–15 neuroblastoma glioma cells. To assess the role of Cdc42 and Rac1 in the morphological changes induced by netrin-1 in the growth cones of embryonic rat commissural neurons, we infected cells [20 multiplicity of infection (MOI)] with adenoviruses encoding either dominant-negative (N17Cdc42, N17Rac1) or constitutively active (V12Cdc42, V12Rac1) forms of Cdc42 and Rac1. Adenoviral vectors encoding green fluorescent protein (GFP) served as controls. Recombinant Cdc42, Rac1, and GFP were myc epitope tagged. At 48 h after infection, the neurons were treated with 80 ng/ml netrin-1 for 30 min. Cells expressing recombinant protein were identified immunocytochemically by the myc epitope tag. The distribution of F-actin was visualized using FITC-coupled phalloidin. Expression of dominant-negative Cdc42 (N17Cdc42), but not GFP alone, significantly reduced the effect of netrin-1 on the number of growth cone filopodia and growth cone surface area (Fig. 4*A,B*). Expression of dominant-negative Rac1 (N17Rac1) blocked the netrin-1-induced increase in growth cone surface area (Fig. 4*B*) and significantly reduced the netrin-1-induced increase in the number of filopodia (Fig. 4*A*). Expression of either constitutively active Cdc42 (V12Cdc42) or constitutively active Rac1 (V12Rac1) was sufficient to increase significantly both the growth cone surface area and the number of filopodia in these neurons (Fig. 4*A,B*). Interestingly, the morphological changes induced by constitutively active Cdc42 and Rac1 were significantly less than those induced by the addition of netrin-1 to control GFP-expressing cells, suggesting that activation of either Cdc42 or Rac1 alone is not sufficient to recapitulate the effect of netrin-1 on the growth cone.

Netrin-1 activates Cdc42 and Rac1 in embryonic rat spinal commissural neurons

We then determined whether netrin-1 activates Cdc42 and Rac1 in these neurons. A technical challenge encountered during in-

investigation of the activation of Cdc42 and Rac1 was the relatively small number of commissural neurons obtained by microdissection and the limited amount of endogenous Cdc42 and Rac1. These limitations were overcome via the adaptation of a GTP γ S loading assay. Rho-GTPases are activated by guanine nucleotide exchange factors (GEFs) that catalyze the exchange of GDP for GTP. GTP γ S binds irreversibly to Rho-GTPases, trapping them in the bound state. When we incubated lysates of commissural neurons with GTP γ S and isolated GTP γ S-bound endogenous Cdc42 and Rac1 with the GST-Pak-CRIB fusion protein, it was possible to visualize and quantify the activation of endogenous Rho-GTPases with antibodies specific for Cdc42 and Rac1 (Fig. 4G). An analysis of the time course of Cdc42 and Rac1 binding to GTP γ S indicated that binding was saturated after 30 min of incubation (data not shown). Subsequently, GST-Pak-CRIB binding to GTP γ S was assayed after 12 min of incubation in commissural neuron homogenates, a nonsaturated time point. We detected Cdc42 and Rac1 activation within 3 min of netrin-1 application to the intact cells (Fig. 4C–F). Adding anti-DCC_{FB} (1 or 5 μ g/ml) with netrin-1 blocked the activation of both Cdc42 and Rac1, indicating that netrin-1-induced activation of these GTPases requires DCC (Fig. 4D). Adenoviral-mediated expression of N17Cdc42 blocked the activation of Rac1 (Fig. 4E), whereas N17Rac1 expression did not block the activation of Cdc42 (Fig. 4E), suggesting that Cdc42 activation is upstream of Rac1 in embryonic rat spinal commissural neurons. Furthermore, because this assay measures the accumulation of GTP γ S bound to Cdc42 or Rac1, the increase in GTP γ S binding implicates netrin-1 in the activation of a GEF in commissural neurons.

Netrin-1 activates Pak1 and recruits Cdc42, Rac1, and Pak1 to the DCC ICD

The serine/threonine kinase Pak1 is an effector of Cdc42 and Rac1 and a key component of a well established signal transduction pathway that promotes actin polymerization (Bokoch, 2003). Activated Cdc42 and Rac1 bind directly to Pak1, regulating its activity (Bagrodia and Cerione, 1999). Pak1 activation can be assessed by using antibodies that recognize phospho-Ser¹⁹⁸ and phospho-Ser²⁰³ of Pak1 (Sells et al., 2000; Shamah et al., 2001).

To determine whether netrin-1 promotes an interaction between Pak1 and activated Cdc42 or Rac1, we again used GTP γ S loading. Purified GST-Cdc42 or GST-Rac1 fusion proteins were added to lysates of dissociated commissural neurons in the presence of GTP γ S and interacting proteins identified by Western blotting. The results obtained indicate that the addition of netrin-1 to commis-

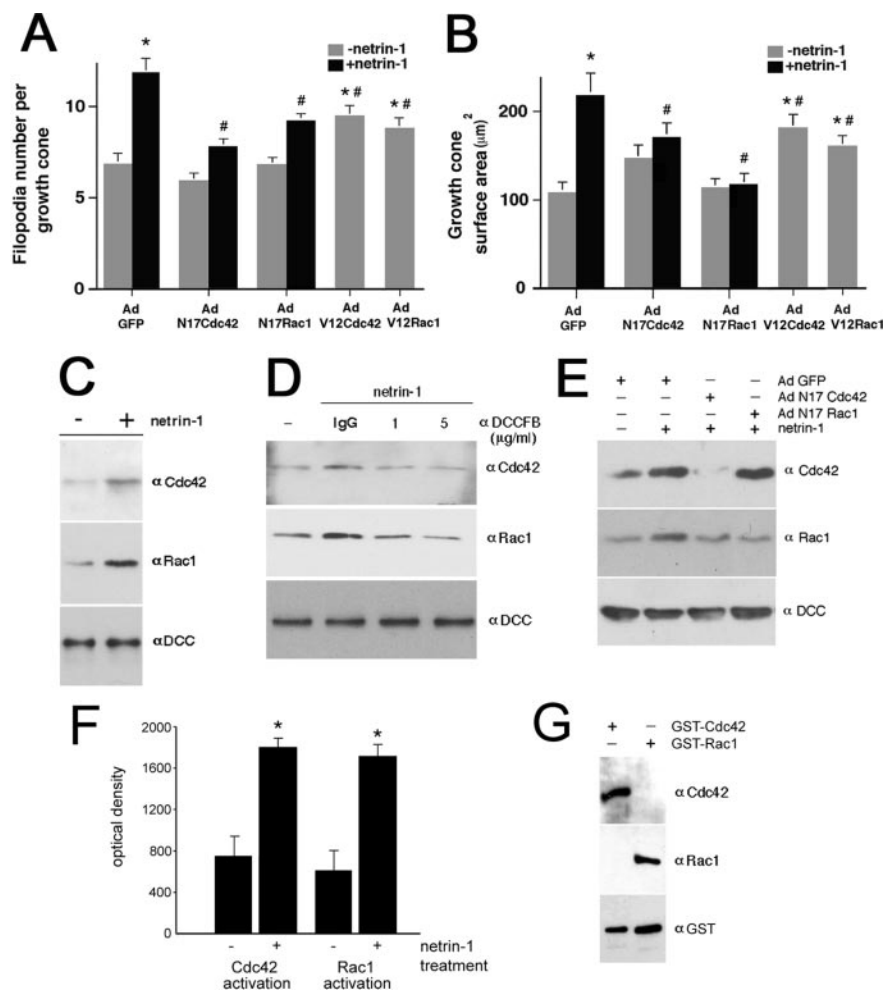


Figure 4. Netrin-1-induced commissural growth cone expansion requires DCC and activated Cdc42 and Rac1. **A, B**, Commissural neurons were infected with adenovirus expressing dominant-negative Cdc42 or Rac1 (N17Cdc42, N17Rac1), constitutively active Cdc42 or Rac1 (V12Cdc42, V12Rac1), or GFP. At 48 h after infection, 80 ng/ml netrin-1 was added, and 30 min later, the cells were fixed and immunostained. Netrin-1 significantly increased filopodia number and growth cone surface area in cells expressing GFP alone (mean \pm SEM; * p < 0.05, significant increase compared with control cells expressing GFP in the absence of netrin-1). Expression of either N17Cdc42 or N17Rac1 significantly decreased the number of filopodia per growth cone and growth cone surface area (n = 25; # p < 0.05, significant decrease compared with control cells expressing GFP in the presence of netrin-1). In the absence of added netrin-1, the expression of V12Cdc42 or V12Rac1 significantly increased both filopodia number and growth cone surface area (n = 15), but to an extent significantly less than netrin-1 applied to adenoviral (Ad) GFP controls. **C–E**, Dissociated embryonic spinal commissural neurons were treated with 80 ng/ml netrin-1 or carrier for 5 min and then lysed and incubated with GTP γ S at 31°C for 12 min. GTP γ S-bound Cdc42 and Rac1 were isolated by using GST-Pak1-CRIB fusion protein and assayed by Western blotting with antibodies against Rac1 or Cdc42. Total cell lysates were probed with anti-DCC to confirm that equal amounts of total protein were loaded per lane. **D**, The addition of anti-DCC_{FB} (1 or 5 μ g/ml) blocked the activation of Cdc42 and Rac1 by netrin-1. **E**, Expression of N17Cdc42 blocked the netrin-1-dependent activation of Rac1. In contrast, expression of N17Rac1 did not block netrin-1-induced activation of Cdc42. **F**, Quantification of netrin-1-induced activation of endogenous Cdc42 and Rac1 in commissural neuron homogenates (after a 3 min application of 80 ng/ml netrin-1; n = 4; mean \pm SEM; * p < 0.05). **G**, Western blot analysis of recombinant GST-Cdc42 and GST-Rac1 (~100 ng) illustrates specificity of the antibodies against Cdc42 and Rac1. Anti-GST immunoreactivity confirms that similar amounts of recombinant protein were loaded in each lane.

sural neurons promotes the association of activated Cdc42 and Rac1 with phospho-Pak1 and DCC (Fig. 5A).

Using coIP of endogenous DCC and Pak1, we then tested the hypothesis that Pak1 might be recruited to a complex with DCC. Increased amounts of Pak1 were determined to coimmunoprecipitate with DCC from homogenates of commissural neurons exposed to netrin-1 (Fig. 5B). Furthermore, increased amounts of DCC were found to coimmunoprecipitate with anti-Pak1 from homogenates of commissural neurons after the application of netrin-1 (Fig. 5C), indicating that netrin-1 promotes the formation of a complex that includes Pak1 and DCC.

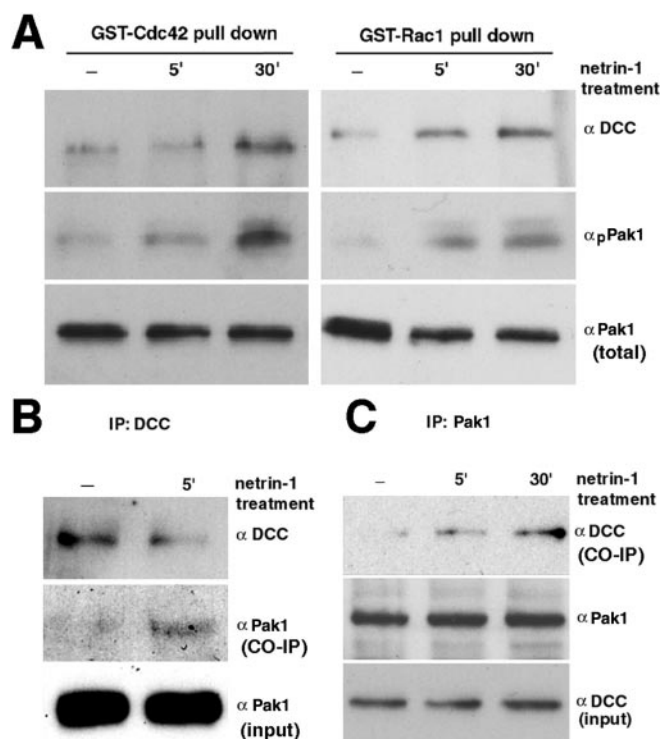


Figure 5. Netrin-1 promotes Cdc42, Rac1, Pak1, and DCC complex formation. **A**, After treatment with netrin-1 (5 or 30 min) commissural neuron cell lysates were incubated with recombinant GST-Cdc42 or GST-Rac1 and GTP γ S. Phospho-Pak1 protein associated with the GST fusion proteins was isolated and analyzed by Western blot. Pak1 immunoreactivity in the whole-cell lysate (total) is shown in the last row. **B**, DCC was immunoprecipitated (1 μ g of anti-DCC_N) from commissural neuron lysates and then analyzed by Western blot with the use of anti-Pak1. Treatment of the cells with 80 ng/ml netrin-1 for 5 min increased the amount of Pak1 protein found to coimmunoprecipitate with DCC. The colIP results are shown above Pak1 immunoreactivity in corresponding whole-cell lysates. **C**, Increased amounts of DCC were detected in a colIP with anti-Pak1 after treatment with 80 ng/ml netrin-1 for 5 min. The colIP results are shown above the Western blots showing immunoreactivity for Pak1 and DCC in corresponding cell lysates.

We then determined whether Pak1 might be activated by netrin-1 in commissural neurons. Immunostaining commissural neuron growth cones revealed a significant increase in phospho-Pak1 (pPak1) within 5 min of the addition of netrin-1 (Fig. 6A–E). Western blot analysis of the relative levels of phospho-Pak1 in commissural neuron lysates indicated that netrin-1 activated Pak1 within 5 min of application (Fig. 6H,I). Phospho-Pak1 levels remained elevated for at least 1 h. Coincident application of netrin-1 and the DCC function-blocking antibody indicated that netrin-1-induced activation of Pak1 requires DCC.

Recruitment of Pak1 is required for netrin-1-induced growth cone expansion

Nck1, an adaptor protein composed of one SH2 and three SH3 domains, binds directly to Pak1 through its second SH3 domain (Li et al., 2001). We have reported that the intracellular domain of DCC binds directly to the first and third SH3 domains of Nck1 (Li et al., 2002). Furthermore, expression of dominant-negative Nck1 inhibited the DCC-induced extension of neurite-like processes from N1E-115 neuroblastoma cells and blocked DCC-dependent activation of Rac1 by netrin-1 in fibroblasts. These findings suggest that the intracellular domain of DCC may form a complex with Nck1 and Pak1.

To determine whether Pak1 binding Nck1 contributes to netrin-1-induced growth cone expansion, we used a cell-

permeable peptide that is a competitive inhibitor of Pak1 binding Nck1. This peptide (Pak peptide) consists of 13 aa corresponding to the first proline-rich domain of Pak1, fused to the polybasic sequence of the human immunodeficiency virus Tat protein, which facilitates entry into cells. The proline-rich domain binds to the second SH3 domain of Nck1 (Hing et al., 1999), inhibiting the interaction between Pak1 and Nck1 (Kiosses et al., 2002). A control peptide mutated at two prolines does not affect Nck1/Pak1 binding. The addition of the Pak peptide (20 μ g/ml) to cultures of embryonic rat spinal commissural neurons 45 min before the addition of netrin-1 (80 ng/ml) blocked the netrin-1-induced increase in growth cone surface area and filopodia number, whereas the application of the control peptide did not (Fig. 6F,G). Together, these results provide evidence that netrin-1 causes a DCC-dependent activation of Cdc42, Rac1, and Pak1 in spinal commissural neurons and recruits Cdc42, Rac1, and Pak1 to a complex with DCC and Nck1.

N-WASP is required for netrin-1-induced growth cone expansion

N-WASP binds directly to Cdc42 and to Nck1 (Millard et al., 2004) and functions as a downstream effector of active Cdc42 that regulates actin polymerization (Mullins, 2000). Immunocytochemical analyses detected N-WASP in commissural neuron growth cones, including filopodia, both when they were cultured on a substrate of PK alone (Fig. 7B,D) and 30 min after the addition of 80 ng/ml netrin-1 (Fig. 7A,C,E). To determine whether netrin-1 promotes an interaction between Cdc42 and N-WASP or Pak1, we isolated proteins binding to GST-Cdc42 in commissural neuron lysates. After treatment of commissural neurons with 80 ng/ml netrin-1 for 5 or 30 min, increased binding of N-WASP and Pak1 was detected (Fig. 8C). Furthermore, increased amounts of DCC were detected to coimmunoprecipitate with N-WASP from commissural neuron lysates after the application of netrin-1 (Fig. 8D).

To investigate a functional role for N-WASP in the response of commissural growth cones to netrin-1, we generated an adenovirus expressing a Flag-tagged dominant-negative mutant form of N-WASP, Δ cof N-WASP. This mutation contains a 4 aa deletion in the N-WASP C-terminal domain, rendering the protein incapable of binding the Arp2/3 complex and unable to promote actin polymerization. It therefore functions as a dominant negative, blocking Cdc42-induced filopodia formation and neurite extension (Banzai et al., 2000; Hufner et al., 2002). Cultured commissural neurons were infected with Δ cof N-WASP or GFP adenoviruses (20 MOI). At 48 h after infection, cells were exposed to 80 ng/ml netrin-1 for 30 min and then fixed and stained with an antibody against the Flag tag to identify the cells expressing Δ cof N-WASP. Quantification of growth cone morphology indicates that interfering with N-WASP function blocks both the netrin-1-dependent increase in filopodia number and growth cone surface area (Fig. 8A,B). These results indicate that netrin-1 recruits N-WASP into a complex with DCC and that N-WASP is required for the growth cone response to netrin-1.

Discussion

Our findings indicate that netrin-1 binding to DCC profoundly affects embryonic rat spinal commissural neuron growth cone morphology, approximately doubling growth cone surface area and filopodia number. These findings are consistent with reports that the application of netrin-1 increases growth cone complexity *in vitro* (de la Torre et al., 1997; Lebrand et al., 2004) and with observations of increased growth cone complexity *in vivo* at

points along axon trajectories where guidance decisions are made (Mason and Wang, 1997). We provide evidence that netrin-1 activates Cdc42, Rac1, and Pak1 in embryonic spinal commissural neurons and that it recruits Cdc42, Rac1, Pak1, and N-WASP into a complex with the DCC ICD. Disruption of Pak1 recruitment or the binding of N-WASP to downstream effectors blocks netrin-1-induced growth cone expansion. We also demonstrate that DCC promotes adhesion to substrate-bound netrin-1. These findings suggest that DCC functions as a transmembrane bridge linking immobilized extracellular netrin-1 to the cytoskeleton and that mechanisms of substrate–cytoskeletal coupling (Suter and Forscher, 2000) may contribute to netrin-1-dependent axon guidance.

Netrin-1 activates Cdc42 and Rac1 in commissural neurons

Here, netrin-1-induced activation of Cdc42 and Rac1 was assayed with a GTP γ S loading assay. Because GTP γ S is not hydrolyzed to GDP, the increases that were detected suggest that a GEF is activated by netrin-1. Our findings do not rule out the possibility that netrin-1 may influence other regulatory mechanisms such as GTPase-activating proteins or guanine nucleotide dissociation inhibitors. The observed activation of Cdc42 in the presence of dominant-negative Rac1 suggests the action of a mechanism that does not require active Rac1. In contrast, dominant-negative Cdc42 blocks Rac1 activation, suggesting that Rac1 is activated either by a GEF having shared specificity for Cdc42 and therefore sequestered by dominant-negative Cdc42 (Feig, 1999) or by a mechanism activated downstream of Cdc42. The latter possibility is consistent with reports that Cdc42 can act upstream to activate Rac1 (Kozma et al., 1995; Nobes and Hall, 1995).

A complex of DCC, Nck1, Pak1, and N-WASP regulates embryonic spinal commissural neuron growth cone morphology

The serine/threonine kinase Pak1 is an important downstream effector of Cdc42 and Rac1 (Bagrodia and Cerione, 1999). The DCC ICD binds the adaptor Nck1 (Li et al., 2002). We show that disrupting Nck1 binding to Pak1 blocks netrin-1-induced growth cone expansion. Pak1 activation plays an essential role in the cytoskeletal changes underlying neurite outgrowth in PC12 (pheochromocytoma) cells (Manser et al., 1998), and Pak1 is recruited rapidly to the leading edge of leukocytes as they respond to extracellular chemoattractants (Dharmawardhane et al., 1999). Pak1 also may exert an influence on motility by regulating neuronal myosins (Lin et al., 1996).

Nck1 and Nck2 are closely related adaptor proteins, both of which are expressed in the early embryonic spinal cord (Bladt et al., 2003). Mice lacking Nck1 and Nck2 exhibit a severe pheno-

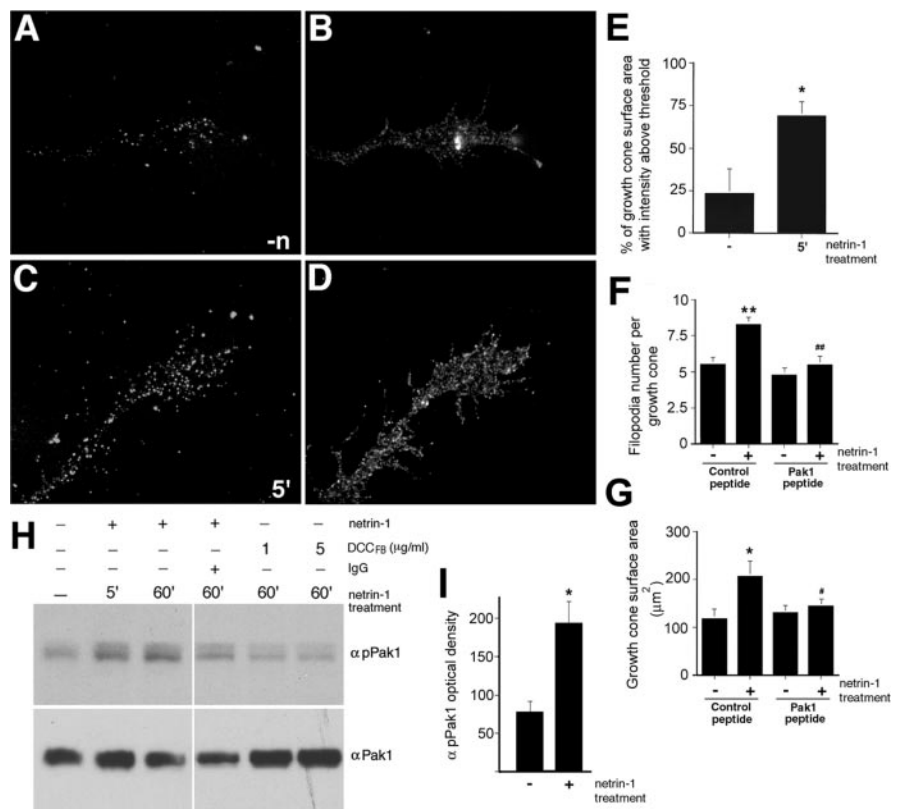


Figure 6. DCC-dependent activation of Pak1 by netrin-1. Commissural neurons double-immunolabeled for phospho-Pak1 (**A**, **C**) and DCC (**B**, **D**) are shown. Increased phospho-Pak1 immunoreactivity was detected in growth cones after 5 min of exposure to netrin-1 (–n in **A** indicates without added netrin-1). **E**, Quantification of phospho-Pak1 immunoreactivity ($n = 28$; mean \pm SEM; * $p < 0.05$). **F**, **G**, The addition of control peptide did not affect the netrin-1-induced increase in growth cone surface area or filopodia number (mean \pm SEM; * $p < 0.05$; ** $p < 0.005$). Adding the Pak peptide 40 min before the addition of netrin-1 blocked the netrin-1-induced increase in filopodia number and growth cone surface area ($n = 25$; mean \pm SEM; * $p < 0.05$; ** $p < 0.005$). **H**, The addition of 80 ng/ml netrin-1 to commissural neurons increased phospho-Pak1 as assayed by Western blot, consistent with the change in immunofluorescence shown in **E**. The same blot was reprobed with anti-Pak1, confirming that comparable amounts of protein are present in each lane. The netrin-1-induced increase in phospho-Pak1 was blocked by anti-DCC_{FB} added to the cultures 1 h before the addition of netrin-1. **I**, Quantification of increased phospho-Pak1 as detected by Western blot analysis (after a 5 min application of 80 ng/ml netrin-1; $n = 3$; mean \pm SEM; * $p < 0.05$).

type, including failure of the embryonic neural tube to close dorsally and embryonic lethality at approximately E9.5, which has prevented the use of these animals to identify roles for Nck1 and Nck2 during axon guidance. Dock, the *Drosophila* ortholog of Nck, is expressed widely by neurons in the fly CNS and enriched in growth cones (Desai et al., 1999). Pak binds Dock, and loss of Dock function generates defects in longitudinal and commissural axon guidance (Desai et al., 1999; Hing et al., 1999). In mammalian cells Nck also associates with activated focal adhesion kinase (FAK) (Schlaepfer et al., 1997), which binds the DCC ICD and is required for axonal chemoattraction to netrin-1 (Li et al., 2004; G. Liu et al., 2004; Ren et al., 2004). These reports also indicate that Fyn, a Src family tyrosine kinase, is activated downstream of DCC and FAK in response to netrin-1. Src family members regulate Rho family GTPase activity (Hoffman and Cerione, 2002), and therefore are possible candidates to activate Cdc42 and N-WASP in response to netrin-1. Supporting this, DCC ICD phosphorylation by Fyn is required for netrin-1-dependent activation of Rac1 (Meriane et al., 2004).

Filopodia and lamellipodia formation is initiated downstream of Cdc42 and Rac1 by members of the WASP family of proteins (Zigmond, 2000). We detect N-WASP, a broadly expressed WASP family member, in the growth cones of embryonic rat

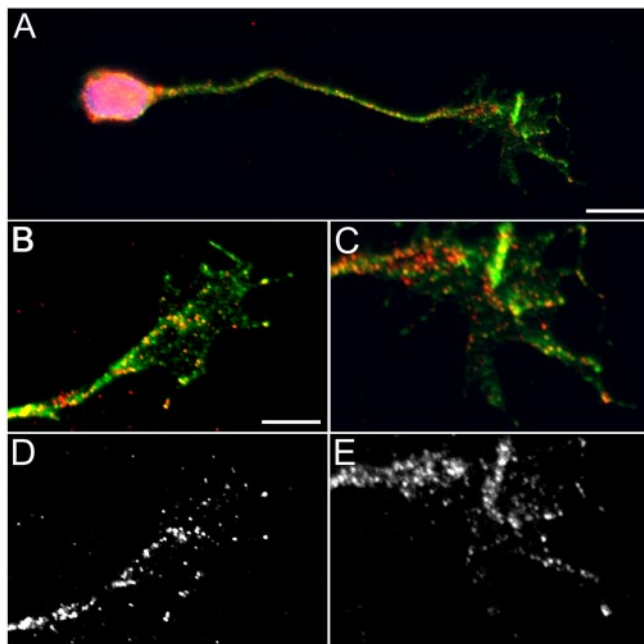


Figure 7. Distribution of N-WASP and DCC in commissural neuron growth cones. Commissural neurons cultured on a control PK substrate (**B**, **D**) or 30 min after the addition of 80 ng/ml netrin-1 (**A**, **C**, **E**) were labeled with anti-DCC (green) and anti-N-WASP (red, **A–C**; white, **D**, **E**). N-WASP immunoreactivity was detected readily along the axon, throughout the growth cone, and along filopodia; **C** and **E** present an enlargement of the growth cone shown in **A**, illustrating the punctate distribution of N-WASP immunoreactivity. Scale bars: **A**, 10 μ m; (in **B**), **B–E**, 5 μ m.

spinal commissural neurons. Furthermore, the addition of netrin-1 recruits N-WASP into a complex with the DCC ICD, and disrupting N-WASP function blocks netrin-1-induced growth cone expansion. The N-terminal domain of N-WASP binds directly to F-actin, potentially providing a link between DCC and the cytoskeleton. The C terminus of N-WASP, a domain conserved in all WASP family members, binds to and activates Arp2/3, a protein complex that catalyzes the formation of actin filaments (Mullins, 2000). A recent model proposes that filopodia are formed by Arp2/3 nucleating a population of barbed ends, generating a dendritic array of F-actin typical of lamellipodia, which then align to form the bundles of F-actin at the core of a filopodium (Svitkina et al., 2003; Vignjevic et al., 2003). Arp2/3 is typically absent from established filopodia (Svitkina et al., 2003), and extension of actin filaments at the tip of a filopodium is promoted by anti-capping proteins, such as the Enabled/vasodilator-stimulated phosphoprotein (Ena/VASP) family (Krause et al., 2003). This model is consistent with our detection of N-WASP in growth cone lamellipodia and proximally along filopodia, where the nucleation of new actin filaments is expected to occur, but rarely at filopodia tips (Fig. 7), where anti-capping proteins regulate extension (Lanier et al., 1999).

The Ena/VASP homolog UNC-34 is required for axon chemoattraction to netrin in *C. elegans* (Gitai et al., 2003), and recent findings indicate that netrin-1-induced filopodia formation requires Ena/VASP function (Lebrand et al., 2004). The model described above suggests that members of the WASP and Ena/VASP families act sequentially and in a spatially segregated manner; however, their actions may be related more closely. Genetic analysis in *C. elegans* indicates that these two protein families play substantially overlapping roles during neural development (Withee et al., 2004). Furthermore, the neuronal scaffold protein Tuba binds to both N-WASP and Ena/VASP proteins (Salazar et

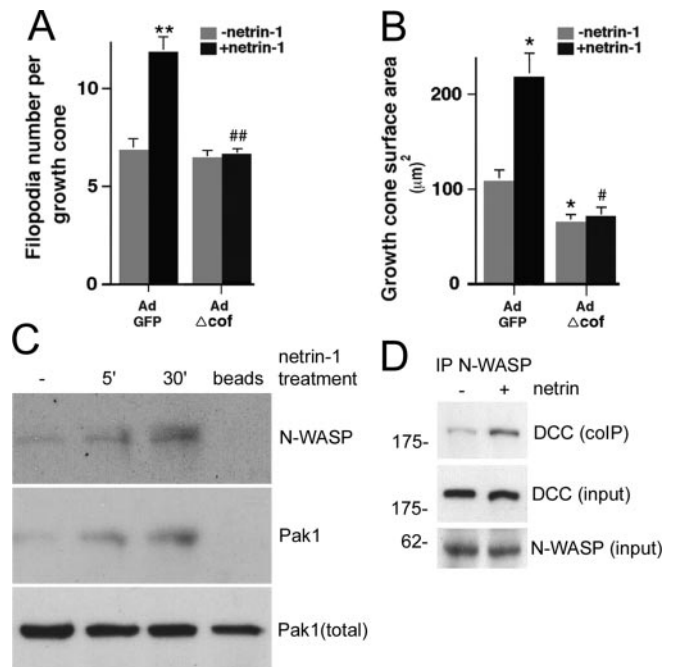


Figure 8. N-WASP is recruited to a complex with DCC and required for netrin-1-induced growth cone expansion. **A**, **B**, After the infection with adenoviral vectors encoding either GFP or Δ cof N-WASP, growth cone morphology was quantified. The addition of netrin-1 (80 ng/ml, 30 min) significantly increased the number of filopodia and growth cone surface area (* $p < 0.05$; ** $p < 0.005$). Expression of Δ cof N-WASP blocked the increase in both the number of filopodia (**A**) and surface area (**B**) of commissural growth cones treated with netrin-1 (# $p < 0.05$; ## $p < 0.005$). Expression of Δ cof N-WASP significantly reduced the growth cone surface area below the level found in the presence of netrin-1 or control ($n = 15$; mean \pm SEM). **C**, After treatment with netrin-1 (5 or 30 min) commissural neuron cell lysates were incubated with recombinant GST-Cdc42. Netrin-1 promotes the association of Pak1 and N-WASP with GST-Cdc42. Pak1 immunoreactivity in corresponding whole-cell lysates is shown in the bottom row. **D**, Increased amounts of DCC were detected in colIPs from commissural neuron lysates by the use of anti-N-WASP. The levels of DCC and N-WASP present in the whole-cell lysates are shown below the blot of the colIP.

al., 2003), and WASP itself binds to VASP (Castellano et al., 2001). It is possible that DCC may activate Ena/VASP activity at the tips of filopodia to promote elongation and also initiate filopodia formation by activating N-WASP more proximally in the growth cone. Alternatively, N-WASP and Ena/VASP may interact more closely downstream of DCC. Additional investigation is required to unravel the specific roles of these two families of proteins in axonal growth cones.

Evidence that DCC and substrate-bound netrin-1 form an adhesive receptor–ligand pair

Cell adhesion molecules (CAMs) regulate growth cone motility by acting as a transmembrane bridge that links an immobilized extracellular cue to the cytoskeleton (Suter and Forscher, 2000). Although netrins often are described as diffusible axon guidance cues, the majority of netrin-1 protein is not freely soluble *in vivo* but is bound to either cell membranes or the extracellular matrix (Serafini et al., 1994; Manitt et al., 2001; Manitt and Kennedy, 2002). Our findings indicate that substrate-bound netrin-1 both influences growth cone morphology and promotes adherence of DCC-expressing cells, suggesting that DCC and immobilized netrin-1 may form an adhesive receptor–ligand pair.

Recent reports implicate netrin-1 in the regulation of cell–cell interactions, including evidence that netrin-1 regulates epithelial morphogenesis in the mammary gland, pancreas, and lung at

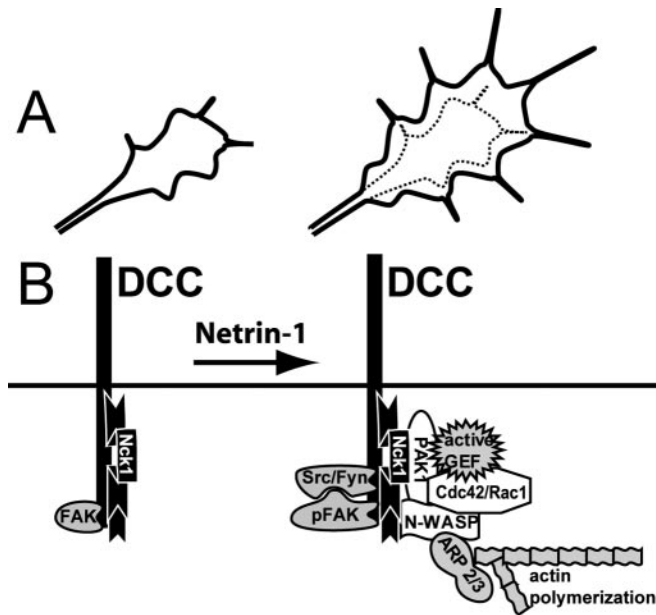


Figure 9. The DCC ICD recruits a complex of signaling proteins to the plasma membrane. **A**, The addition of a uniform concentration of netrin-1 induces growth cone expansion, namely an increase in surface area and in the number of filopodia. **B**, Model of the molecular mechanisms that act downstream of netrin-1 and DCC. Nck1 binds DCC constitutively by its first and third SH3 domains. FAK, bound to the DCC ICD, recruits and activates the tyrosine kinases Src or Fyn in response to netrin-1. Netrin induces the activation of an as-yet-unidentified GEF, leading to the activation of Cdc42, Rac1, and Pak1. We speculate that the activation of a member of the Src family may regulate the activation of Cdc42 by regulating a GEF. Activated Cdc42 activates N-WASP, which promotes the nucleation of F-actin via the Arp2/3 complex.

least in part by influencing cell–cell adhesion (Manitt et al., 2001; Slorach and Werb, 2003; Srinivasan et al., 2003; Yebra et al., 2003; Hebrok and Reichardt, 2004; Y. Liu et al., 2004). In the developing mammary epithelium, netrin-1 and the DCC homolog neogenin mediate an adhesive interaction between cell layers (Srinivasan et al., 2003). It is currently not clear whether neogenin mediates adhesion to netrin-1 directly or induces a netrin-1-independent adhesive mechanism. Binding of $\alpha 6\beta 4$ or $\alpha 3\beta 1$ integrins to the C terminus of netrin-1 regulates the adhesion and migration of embryonic pancreatic epithelial cells (Yebra et al., 2003). Preliminary findings that use peptide inhibitors of this binding suggest that these integrins are not required for the DCC-dependent adhesion to netrin-1 that is described here (data not shown). Our findings suggest that netrin-1 contributes directly to cell adhesion, because the cells must interact with the netrin-1 substrate, and that DCC, an IgG superfamily CAM-like transmembrane protein, either directly mediates an adhesive interaction with substrate-bound netrin-1 or is required to engage an additional netrin-1-dependent adhesive mechanism.

Figure 9 illustrates the intracellular molecular complex recruited to the DCC ICD in response to netrin-1. We have reported previously that Nck1 constitutively binds to the DCC ICD (Li et al., 2002). With netrin-1 binding, Nck1 serves as a scaffold for the recruitment of Pak1, Cdc42, Rac1, and N-WASP. FAK, bound constitutively to the DCC ICD, activates Fyn in response to netrin-1 (Li et al., 2004; G. Liu et al., 2004; Meriane et al., 2004; Ren et al., 2004), which we speculate may be upstream of Cdc42 activation. We hypothesize that DCC promotes filopodia formation and membrane extension via two complementary mechanisms: DCC functions as a transmembrane bridge linking netrin-1 to the actin cytoskeleton and as the core of a protein

complex that directs the organization of F-actin, leading to filopodia formation and membrane extension in response to netrin-1.

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