Development/Plasticity/Repair

Netrin-1 Attracts Axons through FAK-Dependent Mechanotransduction

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The mechanism by which extracellular cues influence intracellular biochemical cascades that guide axons is important, yet poorly understood. Because of the mechanical nature of axon extension, we explored whether the physical interactions of growth cones with their guidance cues might be involved. In the context of mouse spinal commissural neuron axon attraction to netrin-1, we found that mechanical attachment of netrin-1 to the substrate was required for axon outgrowth, growth cone expansion, axon attraction and phosphorylation of focal adhesion kinase (FAK) and Crk-associated substrate (CAS). Myosin II activity was necessary for traction forces >30 pN on netrin-1. Interestingly, while these myosin II-dependent forces on netrin-1 substrates or beads were needed to increase the kinase activity and phosphorylation of FAK, they were not necessary for netrin-1 to increase CAS phosphorylation. When FAK kinase activity was inhibited, the growth cone's ability to recruit additional adhesions and to generate forces >60 pN on netrin-1 was disrupted. Together, these findings demonstrate an important role for mechanotransduction during chemoattraction to netrin-1 and that mechanical activation of FAK reinforces interactions with netrin-1 allowing greater forces to be exerted.

Introduction

Netrin-1 is a secreted protein that has been detected in all organisms with bilateral symmetry studied so far (Moore et al., 2007). It has known functions in axon guidance, tissue morphogenesis and cancer. In the context of axon guidance, netrin-1 is recognized by the transmembrane receptors Deleted in Colorectal Cancer (DCC), UNC5, and Down syndrome cell adhesion molecule (dsCAM) (Lai Wing Sun et al., 2011).

The extension of axons is primarily guided by patterned proteins in the extracellular space (Raper and Mason, 2010). In most cases, these guidance cues are physically tethered to a surface either because they span the membrane (e.g., ephrin-Bs and sema-1, -4, -5 and -6), are glycophosphatidylinositol (GPI)-linked (e.g., ephrin-As and sema-7A) or, if secreted, they associate with extracellular matrix components (e.g., netrins, bone morphogenetic proteins, sema-3s and slits) (Hu, 2001; Manitt and Kennedy, 2002; De Wit et al., 2005; Rider, 2006). Netrin-1 can diffuse a few hundred micrometers from its source before adsorbing to surfaces. Specifically, when dorsal spinal cord explants were separated from netrin-1-expressing cells in a collagen gel, outgrowth was observed at distances of up to ~250 μm (Kennedy et al., 1994). Over a similar distance, a source of netrin-1 deflected the trajectory of spinal commissural axons within the neuroepithelium. However, it was also recognized that the majority of netrin-1 was not present in the soluble fraction, but rather in the membrane-bound fraction and could only be extracted with salt concentrations nearly 10 times higher than physiological (1.2 M) (Serafini et al., 1994). As such there is a strong electrostatic interaction of netrin-1 with the extracellular environment. Further, it has been shown in different contexts that substrate attached netrin-1 is sufficient to reorient axons, including: dissociated hippocampal and commissural neuron axons on polylysine-coated surfaces (Mai et al., 2009; Moore et al., 2009) and for midline crossing of commissural neurons within the developing *Drosophila melanogaster* (Brankatschk and Dickson, 2006).

The growth cone both senses guidance cues and mechanically pulls the axon forward (Lamoureux et al., 1989; Davenport et al., 1993; Huber et al., 2003; Moore and Sheetz, 2011). The mechanical tethering of axon guidance cues raises the possibility that growth cones pull directly on the cues that guide them. Indeed, in the case of netrin-1, we have found that growth cones exert a direct pulling force of >60 pN during chemoattraction (Moore et al., 2009). To affect the trajectory of axons, guidance cues regulate intracellular biochemical signaling pathways (Bashaw and Klein, 2010). The mechanical relationship of the growth cone with its guidance cues could directly impact intracellular signaling events through a process known as mechanotransduction whereby mechanical forces alter the activity of physically linked intracellular proteins. A growing list of proteins behave as mechanotransducers, including: ion channels, intracellular kinases, extracellular matrix components and several adhesion-associated proteins (Moore et al., 2010; Hoffman et al., 2011). Here we report that mechanical forces on netrin-1 are necessary for chemoattraction and for the regulation of two intracellular mechanosensory proteins: focal adhesion kinase (FAK) and Crk-associated substrate (CAS).

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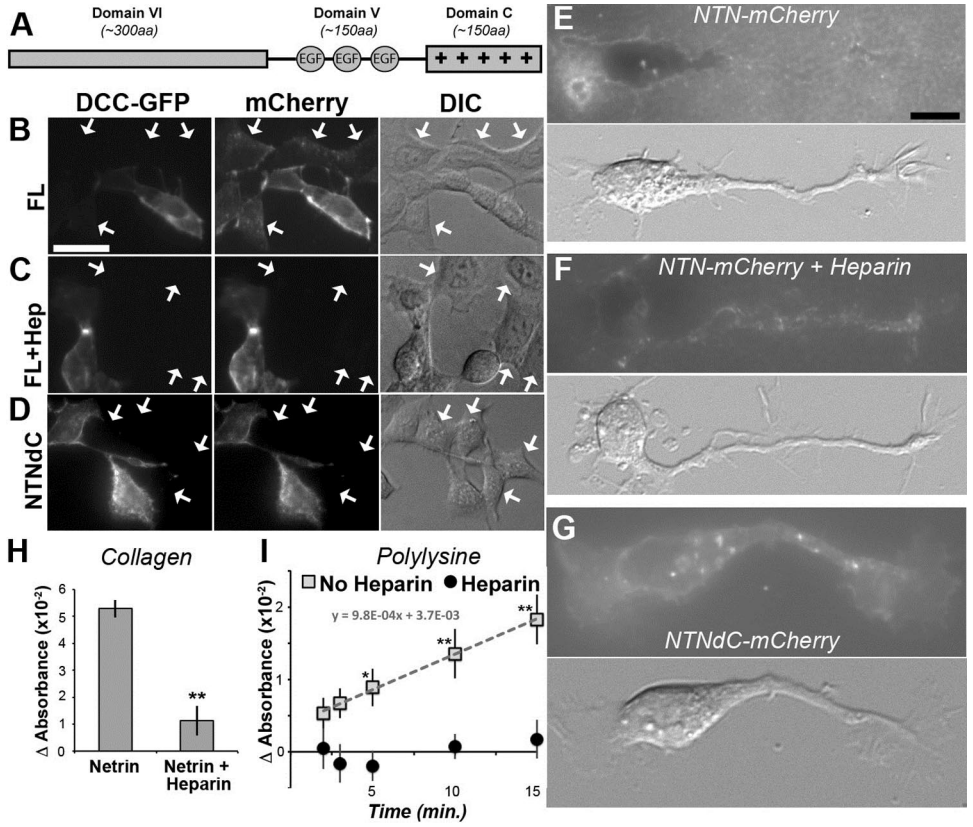


Figure 1. Heparin or deletion of domain C prevents nonspecific adsorption of netrin-1 but preserves DCC binding. ***A***, Domain structure of netrin-1. Domain V and VI bind to netrin-1's receptor DCC, while domain C contributes to substrate adsorption through its many positively charged amino acids. ***B–D***, HEK293 cells were transfected with GFP-tagged DCC (DCC-GFP) and incubated with either 1 μg/ml full-length mCherry-tagged netrin-1 (***B***), 1 μg/ml full-length NTN1-mCherry and 2 μg/ml heparin (***C***), or 1 μg/ml NTN1dC (***D***) for 90 min. As highlighted by the cells that do not express DCC (white arrows), the presence heparin or deletion of domain C restricts binding to only the cells that express DCC. ***E–G***, Differential interference contrast (DIC) images (bottom) and unmodified fluorescent intensity images (top) of spinal commissural neurons incubated for 15 min with, either 1 μg/ml NTN1-mCherry (***E***), 1 μg/ml NTN1-mCherry with 2 μg/ml heparin (***F***), or 1 μg/ml NTNdC-mCherry (***G***). Relative to full-length netrin applied alone, there is a decrease in background binding in the presence of heparin (37%, $p < 0.01$, $n = 40$) or when domain C is deleted (20%, $p < 0.01$, $n = 40$) and there is distinct labeling of both axon and growth cone. ***H***, ***I***, ELISA detecting the myc tag within recombinant, full-length netrin-1 when media containing 200 ng/ml are incubated with either a collagen cushion for 16 h (***H***) or a polylysine-coated tissue culture well (***I***) for time periods of 15 min or less. Significant binding of full-length netrin is seen to collagen gels ($n = 16$, $p < 0.01$) and to polylysine dishes within 5 min ($n > 15$, $p < 0.05$) compared with media alone. Inclusion of 2 μg/ml heparin significantly reduced the association of netrin-1 to collagen by 79% ($n = 16$, $p < 0.01$) and to a polylysine-coated dish by 90% after 15 min. Fluorescent intensity comparison based on average intensities of 10 μm^2 areas from images of equal exposure (5 s). Scale bars: ***B***, 50 μm; ***E***, 10 μm.

Materials and Methods

Plasmids. A C-terminal, mCherry-tagged, full-length chicken netrin-1 (NTN1-mCherry) plasmid was generated through site-directed mutageneis (QuikChange II, Agilent) of the stop codon within pGNET1 (gift from Marc Tessier-Lavigne, Rockefeller University, New York, NY) and subcloning into pmCherry-N1 (Clontech). mCherry-tagged chicken netrin-1 lacking its entire C-terminal domain (amino acids 455–606m NTN1dC-mCherry) was generated through mutagenesis of NTN1-mCherry. For electroporations, wild-type mouse FAK (pRc/CMV-FAK) and the FAK-Y397F mutant (pRc/CMV-FAK-397F; Calalb et al., 1995), chicken FAK C-terminal domain (FRNK; pcDNA3-FRNK; Lin et al., 1997), and pCS2-Venus (Nagai et al., 2002) were used. All plasmids were verified by sequencing.

Reagents. Full-length and truncated netrin-1 proteins were purified from stably transfected HEK 293 cells by liquid chromatography over a heparin column (HiTrap Heparin HP, GE Healthcare Life Sciences) using 0.1 M phosphate-buffered (pH 7.4) 0.5 M NaCl and 2 M NaCl. Polyclonal rabbit antibodies against the heavy chains of NMM-IIA were obtained as a gift from Dr. Robert Adelstein (National Institutes of Health, Bethesda, MD). Monoclonal antibody against NMM II-B (clone CMII 23) was from Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA). Monoclonal anti-β-tubulin (clone 3F3-G2) was purchased from Santa Cruz Biotechnology. PP2, Blebbistatin, Y-27632 and ML-7 were purchased from EMD Biosciences. Antibodies against FAK and CAS were obtained from BD Transduction Laboratories. Phosphospecific antibodies against CAS Y165 and Y410 were obtained from Cell Signaling Technology. Alexa Fluor 546-tagged phalloidin and phosphospecific antibodies against FAK Y397 and Y861 were obtained from Invitrogen. Antibodies against c-myc (9E10) and phosphorylated FAK Y576 were obtained from Santa Cruz Biotechnology. PF-573228 (PF228) was obtained from Tocris Bioscience. Heparin (grade I-A from procine intestinal mucosa, 17–19 kDa) was obtained from Sigma.

ELISA. For netrin-1 binding to type I collagen gels, typical outgrowth conditions were reproduced. Specifically, 10 μl of collagen solution was allowed to gel for 30 min at the bottom in each well of a 96-well plate. Each well was then incubated at 37°C with 5% CO_2 with 100 μl of Neurobasal/FBS media (see below). After 18 h, each well was washed three times for 45 min with PBS with 0.1% BSA (and where appropriate, 2 μg/ml heparin) and then fixed for 1 h in 4% paraformaldehyde/PBS. The wells were then blocked for 3 h in PBS with 0.1% BSA and 1% Triton X-100. Labeling was achieved with sequential overnight incubations with 9E10 and HRP-tagged antibodies in PBS with 0.1% BSA and 1% Triton with at least six 1 h washes in between and after, followed by a final overnight wash.

For netrin-1 binding to polylysine-coated surfaces, typical culturing conditions were reproduced. Each well of a plasma-treated 96-well plate was incubated with 100 μl of 2 μg/ml poly-L-lysine for an hour at room temperature and then washed several times with PBS. Each well was then

incubated for 24 h with 100 μl of Neurobasal/FBS media (see below) at 37°C with 5% CO_2. Media containing 200 ng/ml netrin-1 and with in some cases 2 μg/ml heparin were then included for the indicated times. Each well was then washed with several changes of PBS, and then blocked for an hour at room temperature with PBS with 0.1% BSA, followed by 1 h at room temperature of incubations with 0.2 ng/ml 9E10 and then HRP-tagged antibody with several washes of PBS in between. Colorimetric detection of *o*-phenylenediamine dihydrochloride (Pierce) was achieved by taking the absorbance at 450 nm after 30 min.

Explants and dissociated cultures. Dissections of explants and dissociated spinal cultures were performed as described except that embryonic day 10 (E10; turning) or E12 (dorsal explants and dissociated cultures) CD1 mice embryos of both sexes were used (vaginal plug = E1; Moore and Kennedy, 2008). Culture media consisted of Neurobasal supplemented with 10% FBS, 2 mM GlutaMAX-1, 100 U/ml penicillin, and 100 μg/ml streptomycin (Neurobasal/FBS).

Electroporations. Plasmids were mixed at a 4:1 molar ratio with pCS2-Venus to a final concentration of 0.5 μg/μl. Approximately 2 μl of this DNA solution was injected into E12 mice spinal cords with the dorsal tissue still attached. Five 30 V pulses lasting 50 ms were applied via 3 × 5 mm Genepaddles (Harvard Apparatus) attached to a BTX ECM 830 square wave electroporator (Harvard Apparatus). Following electroporation, the dorsal tissue was removed and spinal commissural neurons were isolated and cultured as described above.

Phosphoprotein analysis. Spinal commissural neurons were pretreated for 1 h with 50 μM blebbistatin, 10 μM PP2, or 10 μM PF-228 and then for 15 min with 200 ng/ml netrin-1. For Western blot analysis, cells were lysed in radioimmunoprecipitation assay buffer (containing, in mM: 10 phosphate, pH 7.5, 150 NaCl, 1% NP-40, 0.1% SDS, 0.5% deoxycholate, 2 μg/ml aprotinin, 5 μg/ml leupeptin, 1 EDTA, and 1 PMSF). For immunofluorescent analysis, cells were fixed with 37°C 4% paraformaldehyde/0.1% glutaraldehyde for 2 min, followed by permeabilization (0.1% Triton), blocking (1% BSA), and antibody labeling in Tris-buffered saline solution. Coverslips were mounted with Slowfade (Invitrogen), immobilized with 2% agarose, and imaged immediately.

Optical trap assays. Proteins were covalently coupled to 2.3-μm-diameter silica beads using cyanogen bromide (Technote #205, Bangs Laboratories). Neurons were plated on PLL (poly-L-lysine, 30–70 kDa, Sigma)-coated 22 × 22 mm coverslips (no. 1.5, Corning) in 6-well tissue culture dishes. Before assaying, neurons were preincubated for 1 h with 2 μg/ml heparin and, where indicated, 50 μM blebbistatin, 10 μM Y-27632, 10 μM ML-7, or 10 μM PF-228. The optical trap was constructed using a 2W diode pumped 1064 nm laser (CrystalLaser) and calibrated using viscous drag (Dai and Sheetz, 1998). Cultured neurons on coverslips were enclosed into a 0.75-mm-thick sandwich, with media equilibrated to 37°C and 5% CO_2, that was sealed with vacuum grease and VALAP (McGee-Russell and Allen, 1971). Assays were performed at 37°C and within 1 h of mounting. The analysis of the dynamic motion of the bead within the trap was performed by a home-written correlation plug-in for ImageJ (Nanotrack).

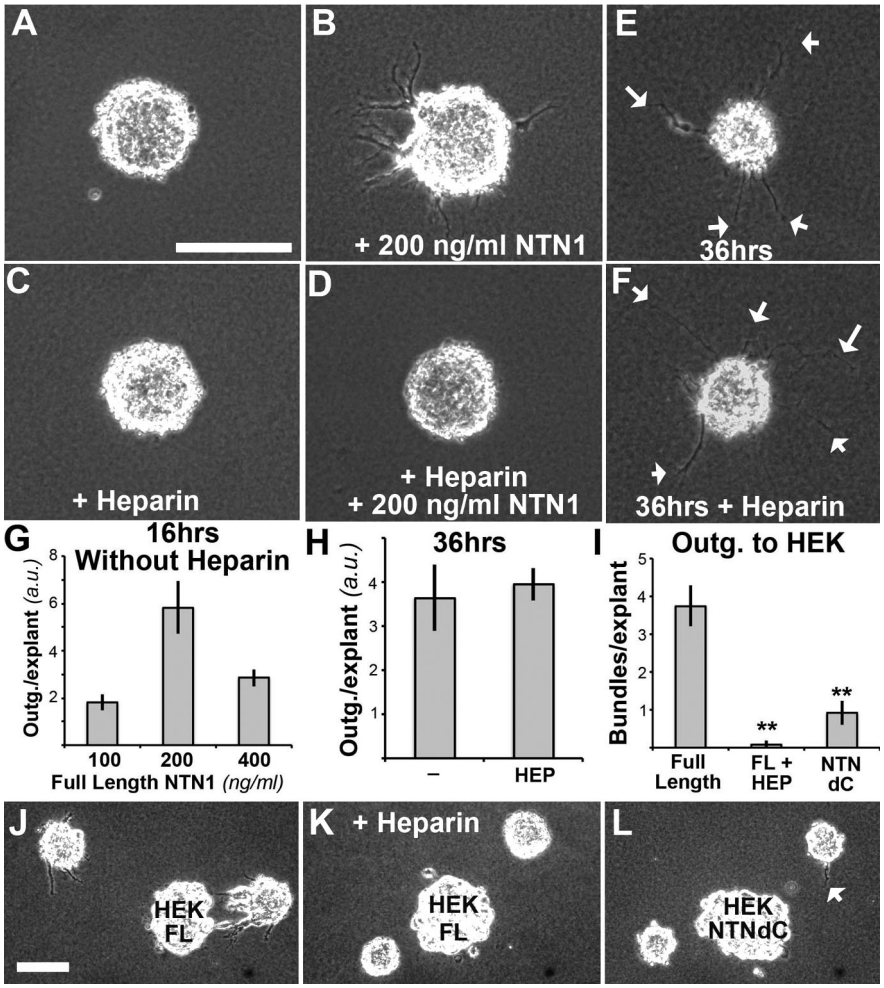


Figure 2. Substrate adsorption of netrin-1 is required for outgrowth from dorsal spinal cord explants. ***A–D***, Dorsal spinal cord explants cultured for 16 h in a collagen gel. Netrin-1 (NTN1) elicits maximum outgrowth at 200 ng/ml (***B***, ***G***). However, when 2 μg/ml heparin was coapplied to prevent substrate adsorption of netrin-1, all detectable outgrowth to netrin-1 was eliminated at 100, 200, and 400 ng/ml netrin-1 ($n > 10$ explants, ***D***). ***E***, ***F***, ***H***, The presence of heparin in the media had no effect on this netrin-independent outgrowth after 36 h (see white arrows in ***E*** and ***F***, $n = 4$ explants). ***I–L***, Transiently transfected HEK293 aggregates expressing either full-length netrin-1 (HEK FL) or netrin-1 lacking the substrate-binding, C-terminal domain (HEK NTNdC) were cultured in close proximity to two dorsal explants for 16 h. ***I***, Full-length netrin-1 triggered robust outgrowth (3.9 bundles per explants, $n = 12$). However, the presence of heparin within the media largely eliminated this outgrowth (0.1 bundles per explants, $n = 11$, ***K***) and expression of domain C lacking netrin-1 severely reduced outgrowth (0.9 bundles per explant, $n = 11$, ***L***). Outgrowth in G and H was quantified at the total length of bundles from each explant. Outgrowth to in ***J–L*** was quantified as the number of bundles per explant. Scale bars, 100 μm.

Results

Coapplication of heparin or deletion of domain C reduces netrin-1 binding to substrates

Netrin-1 is a secreted protein of ~600 aa whose sequence can be divided into three domains: VI, V, and C (Fig. 1*A*). Domains VI and V are homologous to the γ-chain of laminin and mediate interaction with the netrin receptors DCC, UNC5, and dsCAM (Keino-Masu et al., 1996; Leonardo et al., 1997; Geisbrecht et al., 2003; Ly et al., 2008). Domain C has many basic amino acids and a predicted α-helical secondary structure, and is homologous to domains found in the complement C3, -4, and -5 protein family, secreted frizzled-related proteins, type I C-proteinase enhancer proteins, and tissue inhibitors of metalloproteinases. In contrast to domains VI and V, domain C is not involved in binding the netrin receptors but, rather, electrostatically binds negatively charged glycosaminoglycans within the extracellular environment (Kappler et al., 2000; Yebra et al., 2003). As such, domain C

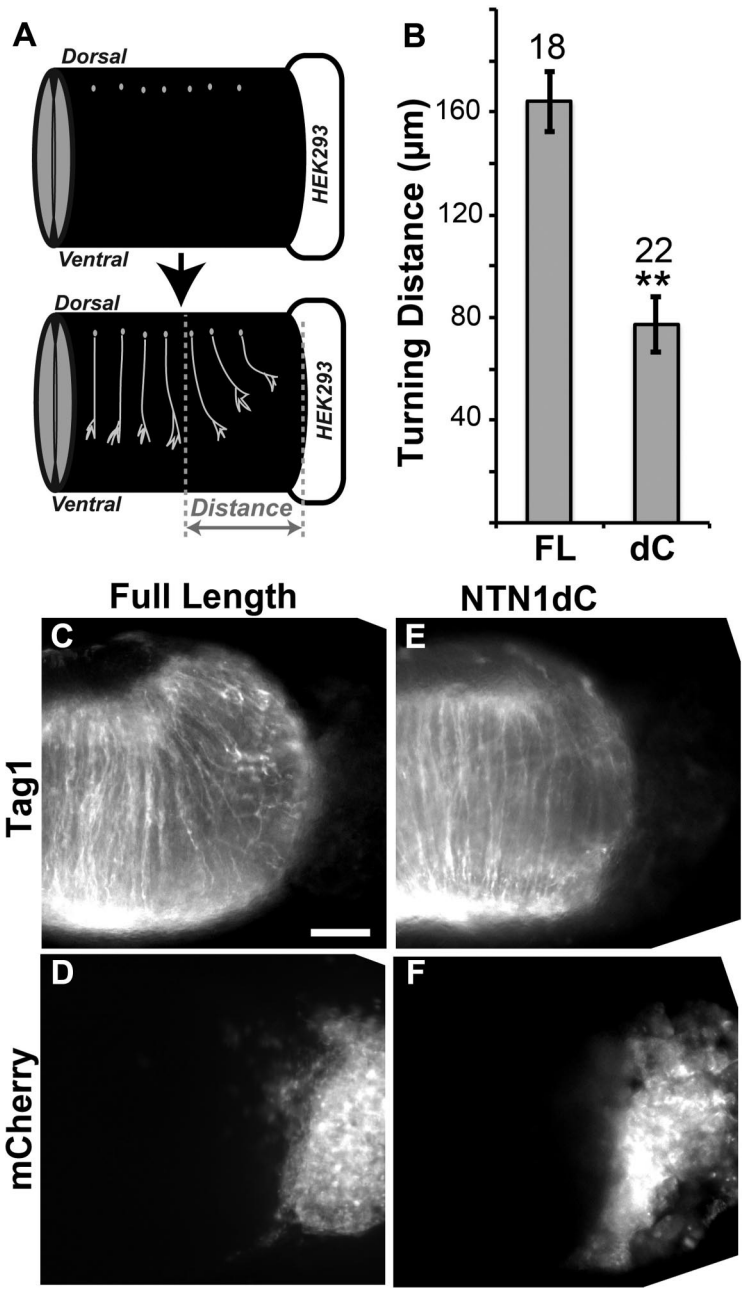


Figure 3. Substrate adsorption of netrin-1 is important for attracting spinal commissural neuron axons within the developing spinal cord. ***A***, Schematic of the turning assay whereby mouse E10 dorsal cord were dissected and cultured alongside aggregates of HEK293 cells expression either full-length or NTN1dC. ***B–D***, HEK293-expressing full-length netrin-1 deflected axons over an average distance of 164 μm ($n = 18$). ***B***, ***E***, ***F***, However, when cellular aggregates expressed NTN1dC, the average distance decreased by 53% (mean of 77 μm, $n = 22$). Scale bar, 100 μm. $^{**}p < 0.01$ (least significant difference, LSD).

appears primarily responsible for nonspecific immobilization of netrin-1 to the extracellular environment.

To test the importance of this immobilization, we explored strategies that disrupted the binding of netrin-1 to surfaces, while leaving its interaction with known receptors unaffected. One approach was to coapply the negatively charged, heavily sulfated glycosaminoglycan heparin. Heparin inhibited nonspecific adsorption of netrin-1 to surfaces, but not its specific interactions with the DCC, UNC5, and dsCAM receptors (Keino-Masu et al., 1996; Leonardo et al., 1997; Geisbrecht et al., 2003; Ly et al., 2008). Importantly, when netrin-1 was covalently attached to a surface, the presence of soluble heparin was not disruptive to either DCC-mediated traction forces >60 pN or the attractive turning responses of spinal commissural neurons (Moore et al., 2009). Here we found that heparin did not disrupt binding of mCherry-tagged netrin-1 to HEK293 cells expressing GFP-tagged DCC, but did reduce the nonspecific binding to cells that do not express DCC (Fig. 1*B*,*C*).

To further rule out the possibility that coapplication of heparin had effects in addition to maintaining netrin-1 in a diffusible state, we developed mCherry-tagged netrin-1 (NTN1dC-mCherry) lacking the entire domain C (amino acids 455–606) based on similar constructs that had reduced substrate adsorption (Mirzayan, 1997; Geisbrecht et al., 2003). Consistent with a lower nonspecific binding affinity, the NTN1dC-mCherry construct eluted from a heparin column at 0.7 M NaCl, which was considerably lower than the 1.2 M NaCl needed to elute full-length netrin-1. To determine whether domain C was necessary to support traction forces >60 pN, we covalently attached NTN1dC-mCherry to beads that were applied to spinal commissural neuron growth cones using optical trapping. Traction forces of >60 pN were generated that were indistinguishable from those generated when full-length netrin-1 was used to coat beads ($n = 6$). Similar to coapplication of heparin with full-length netrin-1, we observed that NTN1dC-mCherry bound specifically to cells expressing DCC and not to cells lacking DCC (Fig. 1*D*). To examine whether coapplication of heparin or deletion of domain C preserved binding to DCC while reducing nonspecific binding to the substrate within spinal commissural neuron cultures, we fluorescently observed the binding of either full-length netrin-1 (NTN1-mCherry) or NTN1dC-mCherry after 15 min. We found that while full-length NTN1-mCherry bound to cell culture surfaces and neurons, coapplication of heparin or deletion of domain C biased binding to neurons (Fig. 1*E–G*). This indicated that binding to DCC expressed by spinal commissural neurons was preserved in the presence of heparin or upon deletion of domain C. Moreover, compared with when full-length netrin was applied alone, the fluorescent intensity of 10 μm^2 areas where no cells were present was significantly reduced in the presence of heparin (37%, $p < 0.01$, $n = 40$) or upon deletion of domain C (20%, $p < 0.01$, $n = 40$).

Reduced substrate absorption was also detected through an ELISA against the myc tag within recombinant netrin-1. We tested adsorption of full-length netrin-1 to type I collagen gels after a 16 h incubation, as well as upon acute addition to polylysine-coated cell culture surfaces that had been incubated for 24 h in culture media. These paradigms replicate commonly used assay conditions that test for netrin-1-induced outgrowth and intracellular biochemical events within dissociated neurons, re-

spectively. Consistent with the ability of netrin-1 to adsorb to these surfaces, we found significant binding to type I collagen gels and a linear increase from 5 to 15 min in binding to polylysine-coated tissue culture surfaces (Fig. 1*H*,*I*). We then examined the ability of heparin to disrupt these nonspecific interactions. Indeed, we measured a 79% decrease in netrin-1 adsorption to collagen ($n = 16$, $p < 0.01$) and a 90% decrease in the binding to polylysine-coated tissue culture plastic ($n > 10$, $p < 0.01$, Fig. 1*H*,*I*). Therefore, coapplication of 2 μg/ml heparin or deletion of netrin-1 domain C effectively reduced nonspecific substrate adsorption of netrin-1 while preserving binding to its receptor DCC.

Netrin-1 immobilization is important for outgrowth and chemoattraction

We then tested the importance of netrin-1 substrate adsorption in prototypical assays for netrin-1 function–axon outgrowth from dorsal spinal cord explants embedded in a collagen gel and turning of spinal commissural neuron axons within the developing spinal cord (Serafini et al., 1994). In the outgrowth assay, netrin-1 diffuses though collagen matrix to stimulate the explant; however, as shown in Figure 1*H*, netrin-1 also adsorbs to collagen (Yebra et al., 2003). If a soluble form of netrin-1 was causing outgrowth, then blocking the binding of netrin-1 to collagen should not affect outgrowth. Normally, netrin-1 induces outgrowth when present at concentrations of 100, 200 and 400 ng/ml (Fig. 2*G*; Serafini et al., 1994). However, when adsorption of netrin-1 was prevented with coapplication of heparin, all detectable growth at each of these concentrations was eliminated ($n > 10$ explants from three independent experiments, Fig. 2*A–D*). Given the complete inhibition of outgrowth, it was important to verify that the general outgrowth potential of these explants was unaffected. Normal outgrowth occurs in the absence of netrin-1 by 36 h in culture. When explants were cultured in the absence of netrin-1 for 36 h, heparin had no effect on this outgrowth (Fig. 2*E*,*F*,*H*). As a further test, we examined outgrowth evoked by HEK293 explants expressing full-length or netrin-1 lacking its substrate-binding domain (NTNdC). Normally, aggregates expressing full-length netrin-1 evoke robust outgrowth (3.9 bundles/explant). However, consistent with an important role for mechanical restraint of netrin-1, outgrowth was largely eliminated in the presence of 2 μg/ml heparin (0.1 bundles/

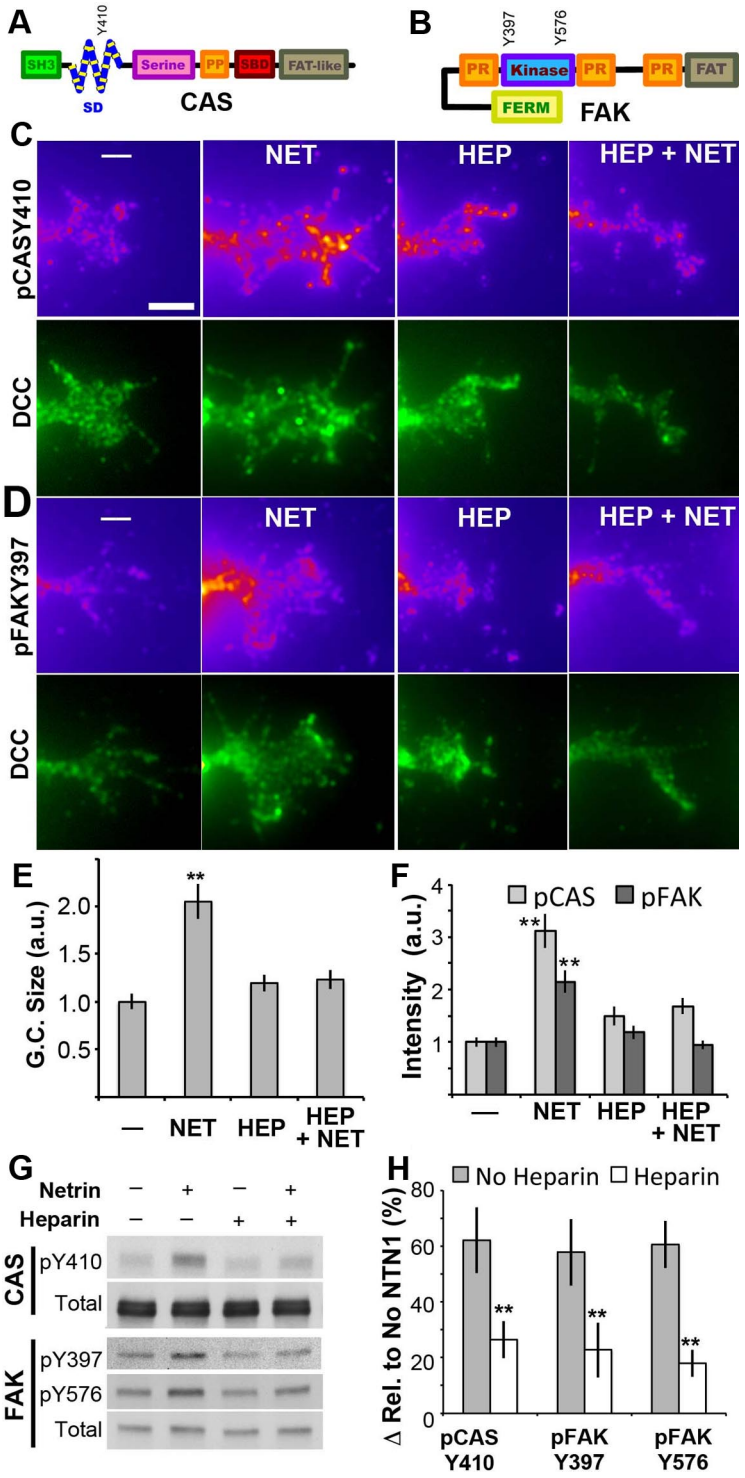


Figure 4. Netrin-1 Substrate adsorption is important for the phosphorylation of CAS and FAK. ***A***, ***B***, Diagrams of the domain structure of CAS and FAK showing the examined tyrosine phosphorylation sites within the substrate domain (SD) of CAS and the kinase domain of FAK. ***C***, ***D***, Top panels are immunofluorescent images of phosphorylated tyrosine 410 of CAS (pCASY410) or tyrosine 397 of FAK (pFAKY397) within spinal commissural growth cones. Brightness and contrast values are unmodified, but to highlight intensity differences, gray scale images were converted to the "Fire" LUT (Look Up Table) of ImageJ. Bottom panels show DCC labeling. ***E***, Normally, netrin-1 increases average growth cones area approximately doubles (2.1-fold, $n = 60$, $p < 0.01$) after 15 min with 200 ng/ml netrin-1. However, reducing substrate adsorption of netrin with 2 μg/ml heparin disrupts this expansion ($n = 60$) as measured based on DCC staining. ***F***, Addition of netrin-1 increases the integrated density of pCASY410 (3.1-fold, $n = 50$) and pFAK397 (2.2-fold $n = 50$) labeling. Coapplication of heparin significantly reduced the pCASY410 by 54% (1.7-fold of baseline), $n = 49$) and pFAKY397 by 44% (1.0-fold of baseline, $n = 47$). ***G***, Western blot images from spinal commissural neuron (SCN) cultures for FAK and CAS phosphorylation following 15 min stimulation with 200 ng/ml netrin-1. ***H***, Quantification of the change in netrin-induced phosphorylation relative to the absence of netrin. The presence of heparin significantly reduced the amount of CAS-Y410 (57%, $n = 6$), FAK-Y397 (61%, $n = 8$) and FAK-Y576 (71%, $n = 6$) phosphorylation. $^{**}p < 0.01$ (LSD). Scale bar, 5 μm.

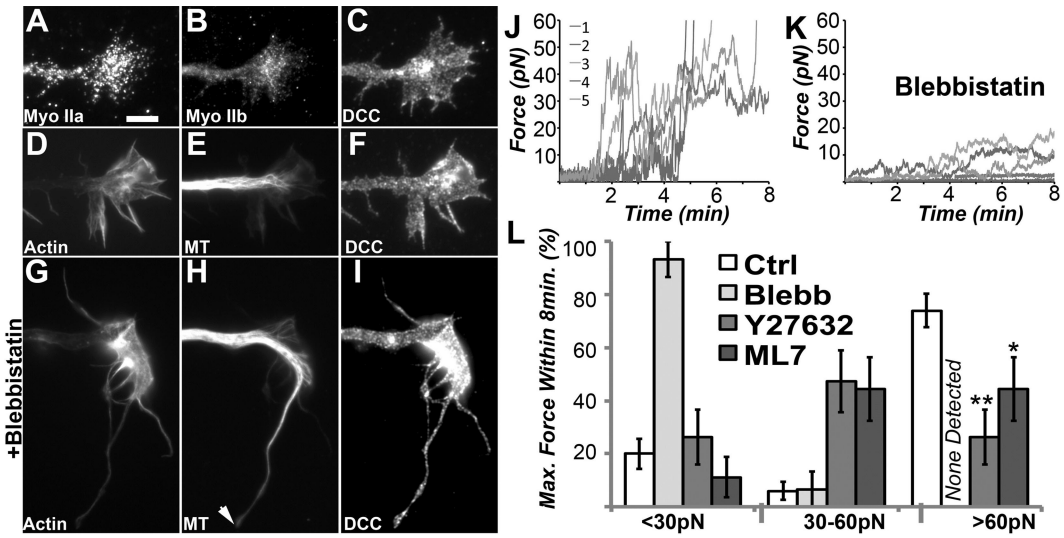
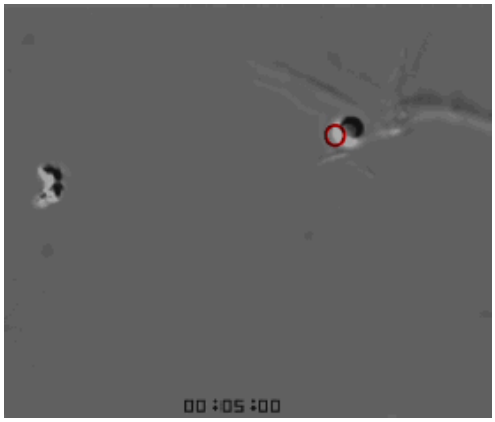
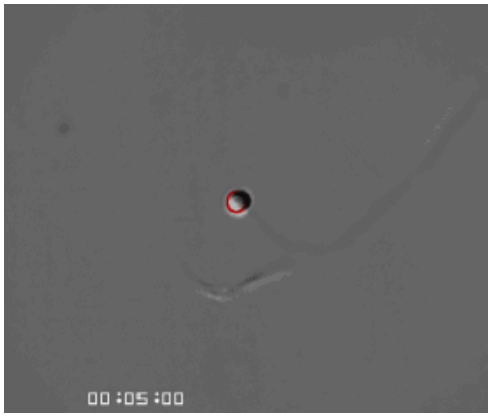


Figure 5. Myosin II is required for traction on Netrin-1. ***A–C***, Immunofluorescent labeling of myosin IIa and IIb shows enrichment in the central region of the growth cone. ***D–F***, Normally microtubule filaments are concentrated in the central region of normal growth cones, while actin filaments are found throughout. ***G–I***, However, inhibition of myosin II with 50 μM blebbistatin for 1 h resulted in growth cones with dramatically longer filopodia and dense microtubule arrays that penetrate deep into the peripheral region of the growth cone (arrow). ***J***, ***K***, Five representative plots of the amount of force over time exerted on netrin-1-coated beads normally (***J***) and in the presence of 50 μM blebbistatin (***K***). ***L***, Growth cone pulling forces on netrin-1 within 8 min of initial contact were categorized into three responses: $<$30 pN, between 30 and 60 pN, and $>$60 pN. Typically, approximately three quarters of spinal commissural neuron growth cones exert $>$60 pN of force on netrin-1 within 8 min (74%, $n = 50$). However, in the presence of the myosin II inhibitor blebbistatin (Blebb) not a single growth cone was able to generate $>$60 pN. Instead the vast majority generated $<$30 pN (93%, $n = 15$). Similarly, significantly less growth cones exerted $>$60 pN in the presence of Y-27632 (47%, $n = 19$) and ML7 (44%, $n = 18$). $^*p < 0.05$, $^{**}p < 0.01$ relative to Ctrl (LSD). Scale bar, 5 μm.



Movie 1. Normal pulling of netrin-1. Normal pulling of an optically trapped 2.3 μm bead coated with netrin-1 by a spinal commissural neuron growth cone. The red circle denotes the center of the trap. Note the recruitment of additional contacts and expansion of the growth cone after 3 min that leads to extraction of the bead from the trap after ~5 min. Time stamp = hh:mm:ss.



Movie 2. Inhibition of myosin II disrupts forces on netrin-1. Pulling of an optically trapped 2.3 μm bead coated with netrin-1 by a spinal commissural neuron growth cone in the presence of the myosin II inhibitor blebbistatin. The red circle denotes the center of the trap. Note the long filopodia that move laterally but not retrogradely, as well as, the much smaller forces generated on the bead (as evidenced by the much smaller displacement of the bead from the center of the trap). Time stamp = hh:mm:ss.

explant) or when the aggregates expressed NTNdC (0.9 bundles/explant, Fig. 2*I–L*).

We then examined the importance of substrate adsorption on the chemoattractive ability of netrin-1 within the developing spinal cord. To test this, we used an assay whereby the spinal cord was dissected at a time before spinal commissural neurons extended. When explants of either the floor plate or aggregates of cells expressing chemotactic cues are cultured on the rostral or caudal edge, commissural neurons are deflected from their normally parallel dorsal-ventral trajectory (Fig. 3*A*; Moore and Kennedy, 2008). Consistent with an important role for netrin-1 adsorption during chemoattraction to netrin-1 within the developing spinal cord, we observed a 53% reduction in the distance over which spinal commissural axons were deflected to a source of netrin-1 lacking the substrate-binding domain C ($n \geq 18$, Fig. 3*B–F*). Together, these observations demonstrate that netrin-1 binding to surfaces is important for both its outgrowth-promoting and chemoattractive effects on spinal commissural neuron axons.

Substrate binding of netrin-1 is important for the activation of CAS and FAK

The importance of substrate adsorption was consistent with a role for mechanotransduction in the response to netrin-1. If this was indeed the case, then the intracellular signaling pathways linked to netrin-1 chemoattraction should also be dependent on the mechanical pulling forces on netrin-1. In axon chemoattraction settings, netrin-1 regulates numerous intracellular pathways (Moore et al., 2007; Lai Wing Sun et al., 2011). In those experi-

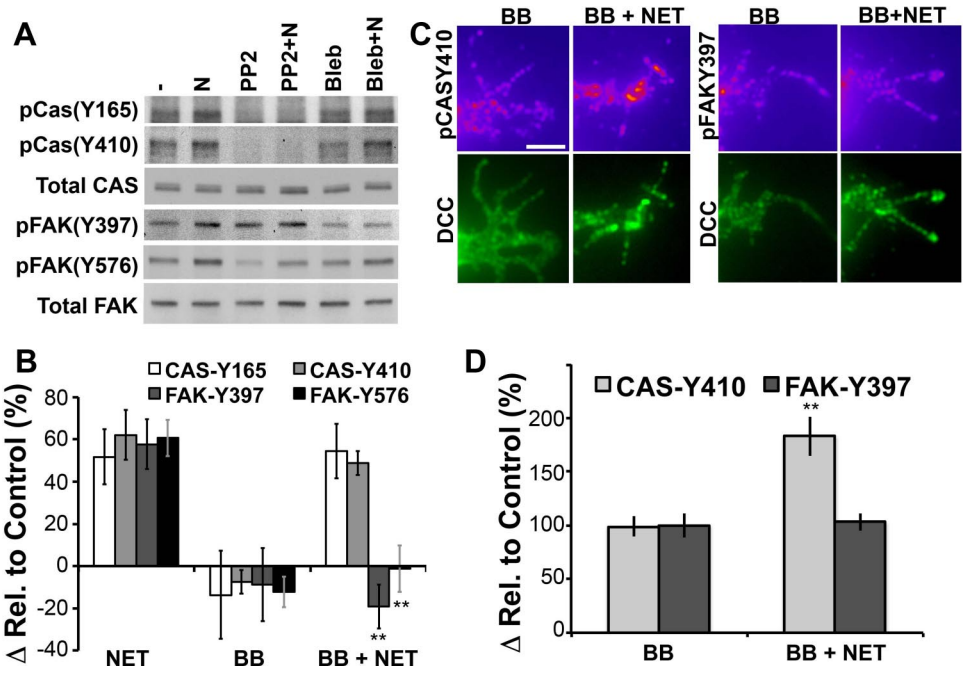


Figure 6. Myosin II contractions underlie netrin-1-induced FAK Phosphorylation. ***A***, Western blot images of tyrosine phosphorylation within the substrate domain of CAS (Y165 and Y410) or the kinase domain of FAK (Y397 and Y576) following 15 min netrin stimulations (200 ng/ml) in the absence of inhibitors or following preincubation for 1 h with the Src Family Kinase inhibitor PP2 (10 μM) or the myosin II inhibitor blebbistatin (50 μM, blebb). ***B***, Quantification of netrin-1 stimulated phosphorylation intensity changes relative to control cells (in the absence of both netrin and blebbistatin). Inhibition of myosin II reduced netrin-1-induced phosphorylation of FAK on the Y397 and Y576 sites by 77% ($n = 8$) and 62% ($n = 5$), respectively. No significant effects were seen on the netrin-1-induced phosphorylation within the substrate domain of CAS (Y165 and Y410). ***C***, Top panels are immunofluorescent images of phosphorylated tyrosine 410 of CAS (pCASY410) and tyrosine 397 of FAK (pFAKY397) within spinal commissural growth cones. Brightness and contrast values are unmodified, but to highlight intensity differences, gray scale images were converted to the Fire LUT of ImageJ. Bottom panels show DCC labeling. ***D***, Inhibition of myosin prevented an increase in the integrated density of FAK-Y397, but not of CAS-Y410, labeling ($n = 50$ for each condition) relative to control (in the absence of both netrin and blebbistatin). $^{**}p < 0.01$(LSD). Scale bar, 5 μm.

ments, soluble netrin-1 was added to the culture media and the biochemical consequences were typically analyzed within 15 min. This acute application of netrin-1 to the media could indicate a soluble, unrestrained role. However, as shown in Figure 1*I*, detectable netrin-1 adsorption occurred to these cell culture surfaces within 5 min. To determine the importance of mechanically restrained netrin-1 under these acute conditions, we examined the consequences of preventing absorption on netrin-1's ability to expand of spinal commissural neuron growth cones within 15 min (Shekarabi et al., 2005). Consistent with an important role for restrained netrin-1, we found that disrupting adsorption with heparin prevented the expansion of growth cones (Fig. 4*C–E*).

We then explored the possibility that the intracellular signaling cascades activated by netrin-1 were dependent on mechanically restrained netrin-1. Previous studies had found that the phosphorylation of CAS (p130Cas, BCAR1) and FAK (Ptk2) were increased upon acute (15 min) addition of netrin-1 (Li et al., 2004; Liu et al., 2004, 2007; Ren et al., 2004). In the case of CAS, netrin-1 addition promoted phosphorylation of tyrosine 410 within CAS's substrate domain (pY410, Fig. 4*A*). Phosphorylation of tyrosines within this substrate domain underlie CAS's ability to function as a mechanosensor (Sawada et al., 2006). In terms of FAK, netrin-1 increased phosphorylation on tyrosines 397 (Y397) and 576 (Y576) (Fig. 4*B*). The Y397 site is an autophosphorylated site that upon phosphorylation is recognized by the SH2 domains of several Src family kinases (SFKs) (Schaller et al., 1994), while the Y576 site is phosphorylated by SFKs and is thought to promote FAK kinase activity (Calalb et al., 1995).

We confirmed that netrin induced phosphorylation of these sites within CAS and FAK in dissociated spinal commissural neurons. Using Western blot analysis, we found that netrin-1 increased the phosphorylation within the substrate domain of CAS (Y410) by 62% ($n = 18$), and FAK phosphorylation by 58% on Y397 ($n = 18$) and by 61% on Y576 ($n = 14$; Fig. 4*G*,*H*). Similarly, immunofluorescent detection of their growth cones demonstrated a 3.1-fold increase in the phosphorylation of CAS-Y410 ($n = 50$) and a 2.2-fold increase in FAK-Y397 ($n = 50$; Fig. 4*C*,*D*,F). When the adsorption of netrin-1 to the surface was then blocked by coapplication of heparin, the level of phosphorylation of each of the sites on CAS and FAK was dramatically decreased. By Western blot, the relative increase in the presence of heparin was reduced by 57% ($n = 6$) on CAS-Y410, by 61% ($n = 8$) on FAK-Y397, and by 71% ($n = 6$) on FAK-Y576. Immunofluorescent detection of their growth cones revealed a 54% ($n = 49$) reduction of CAS-Y410 phosphorylation and a 44% ($n = 47$) reduction of FAK autophosphorylation (Y397). Thus, mechanical restraint of netrin-1 has an important role in the activation of the major signaling pathways of CAS and FAK.

Traction on netrin-1 requires myosin II activity

If the phosphorylation of CAS and FAK is indeed a mechanosensory process, traction forces on netrin-1 could be triggering their phosphorylation. If so, inhibition of force on netrin-1 should inhibit phosphorylation. According to the "substrate to cytoskeleton" model of axon extension, traction forces are generated when liganded extracellular matrix receptors are bridged to retrogradely flowing actin (Suter and Forscher, 2000). In neuronal growth cones, non-muscle myosin II transports actin rearward (Brown and Bridgman, 2003). We found an enrichment of myosin IIa and IIb within the central domain of the growth cones in spinal commissural neurons (Fig. 5*A–C*). When blebbistatin, a small molecule inhibitor that locked the myosin II head in a

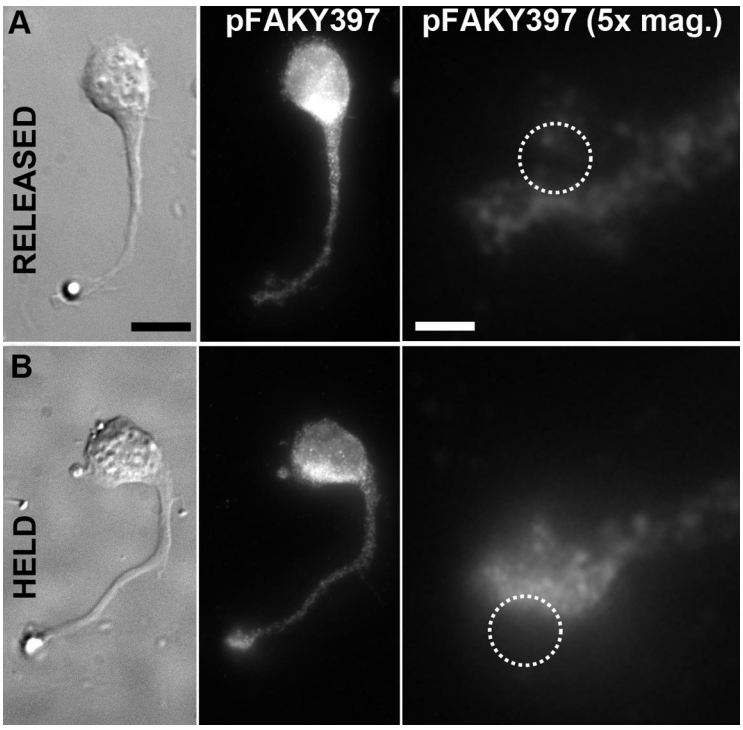


Figure 7. ***A***, ***B***, Mechanical force on netrin-1 triggers FAK autophosphorylation. DIC and immunofluorescent images of spinal commissural neurons that were presented with netrin-1-coated beads, then fixed and immunofluorescently processed for FAK autophosphorylation (pFAKY397). In one scenario the bead was released from the optical trap 15–30 s after its initial contact with the growth cone and then processed 5′ later (***A***). In the other scenario, the bead was held for the entire 5′ period thus allowing the growth cone to build >30 pN of force on the bead (***B***). The average immunofluorescent signal within each growth cone was normalized to the average intensity of a 5 μm^2 area of the axon segment immediately adjacent to the growth cone. When Netrin-1-coated beads were held (***B***) there was a 63% ($n = 13$, $p < 0.05$) increase in FAK Y397 autophosphorylation within the growth cone compared with when it is released (***A***). Brightness levels of immunofluorescent images are unmodified. Dotted circle denotes the location of the bead. Scale bars are 10 μm (left) and 2 μm (right).

low actin affinity conformation (Kovács et al., 2004), was added to growth cones, waves of actin stopped moving rearward and the average length of growth cone filopodia grew from 2.2 μm to 7.9 μm ($n > 40$, $p < 0.01$). Further, microtubules extended into the growth cone's periphery (Fig. 5*D–I*). Thus, inhibition of myosin II contraction should inhibit mechanical force generation on adhesion sites coupled to actin flow.

To determine whether the traction forces on netrin-1 were dependent on myosin II activity, we presented spinal commissural neurons with optically trapped beads coated with netrin-1. Normally, growth cones both progressively increase the number of contacts with the bead and pull in excess of 60 pN within 8 min (Fig. 5*J*; Movie 1; Moore et al., 2009). When myosin II activity was inhibited with blebbistatin, filopodia were still capable of lateral movement (Movie 2). However, none of the growth cones examined generated forces >60 pN on netrin-1 beads and the vast majority (93%) generated forces <30 pN (Fig. 5*K*,*L*). When myosin activity was indirectly inhibited through inhibition of myosin light chain kinase (with ML-7) or Rho-associated kinase (with Y-27632), pulling of netrin-1 was also inhibited, but less efficiently (Fig. 5*L*). Thus, inhibition of myosin II through blebbistatin treatment is an effective strategy to eliminate traction forces on netrin-1 >30 pN.

Traction forces are required for netrin-induced phosphorylation of FAK, but not of CAS

If forces >30 pN on netrin-1 are involved in the phosphorylation of CAS and FAK, then inhibition of myosin II activity should reduce the phosphorylation levels. We found that inhibition of myosin II reduced the ability of netrin-1 to elevate FAK phosphorylation. Specifically, by Western blot analysis, we found that blebbistatin reduced netrin-1-triggered FAK autophosphorylation (Y397) by 77% ($n = 8$) and phosphorylation on Y576 by 62% ($n = 5$; Fig. 6*A*,*B*). Similarly, immunofluorescence for FAK-Y397 phosphorylation within the growth cones revealed no significant difference upon netrin-1 stimulation when myosin II was inhibited (Fig. 6*C*,*D*). In contrast to FAK, phosphorylation of CAS's substrate domain was unaffected by myosin II inhibition. We observed that inhibiting myosin II had no significant effect on the phosphorylation state of either CAS Y165 or Y410 by either Western blot analysis or immunofluorescence within the growth cone (Fig. 6*A–D*).

Because myosin II inhibition disrupted traction forces >30 pN, the above Western blotting and immunofluorescence data indicated that traction forces activated FAK phosphorylation. To further test this hypothesis, netrin-1-coated beads were presented to growth cones for 5 min with either a brief restraint (15–30 s) or with restraint throughout. We then used immunofluorescence techniques to detect FAK autophosphorylation within the growth cones (Fig. 7). We found a 63% increase ($n = 13$, $p < 0.05$) in the amount of FAK autophosphorylation within the growth cones when beads were restrained for 5′ and growth cones were allowed to build larger forces (>30 pN) on the beads. Thus, myosin II contractile force on netrin-1 has an important role in the activation of FAK at the site of attachment.

FAK's kinase activity builds traction forces on netrin

In fibronectin adhesions, force production on fibronectin stabilizes adhesions and results in greater force production (Choquet et al., 1997). Similarly, activation of the FAK kinase by force on substrate-bound netrin-1 may cause strengthening of the netrin-1 adhesion, resulting in greater force generation on netrin-1. When the FAK-specific inhibitor PF-228 (Slack-Davis et al., 2007) was added, both endogenous and netrin-1-stimulated autophosphorylation of FAK was inhibited (Fig. 8*A*). Further, PF-228 addition caused a dramatic (86%) reduction in the number of growth cones that exerted traction forces >60 pN on netrin-1 (Fig. 8*B*). Because PF-228 may have affected other enzymes, we used the expression of FRNK or FAK-Y397F to inhibit FAK function in spinal commissural neurons. FRNK is a variant of FAK that lacks the FERM and kinase domains, but has the proline-rich and focal adhesion targeting domains responsible for binding p130Cas and DCC (Fig. 8*C*; Harte et al., 1996; Ren et al., 2004). FAK-Y397F is a mutant of FAK with its Y397 autophosphorylation site mutated to phenylalanine, thus impairing the recruitment of numerous proteins including Src family kinases (Schaller et al., 1994). Although transient expression of wild-type FAK had no effect on traction forces on netrin-1 beads,

expression of FRNK and FAK-Y397F reduced the fraction of growth cones that exerted >60 pN of force on netrin-1 by 56 and 69%, respectively (Fig. 8*D*). Thus, the activation of the FAK kinase is an important step in the generation of high forces (>60 pN) on netrin-1.

To gain further insight into the underlying cause of the reduced pulling force, we carefully examined the growth cone's interactions with an optically trapped netrin-1-coated bead. Normally, initial filopodial contacts with netrin-1 beads grew in area through the recruitment of additional filopodia and/or engulfment by the growth cone's lamellipodium within 8 min (Movie 1). When the activity of the growth cone filopodia was monitored before and after netrin-1 bead binding, there was a clear increase in the number of filopodia that contact and are stabilized onto the bead. Pharmacological and genetic inhibition of FAK disrupted that activation and recruitment process in a manner that mirrored the reduction in pulling forces (Fig. 8*E*,*F*). Therefore, disrupting FAK function reduced the expansion of adhesions between the growth cone and netrin-1 beads.

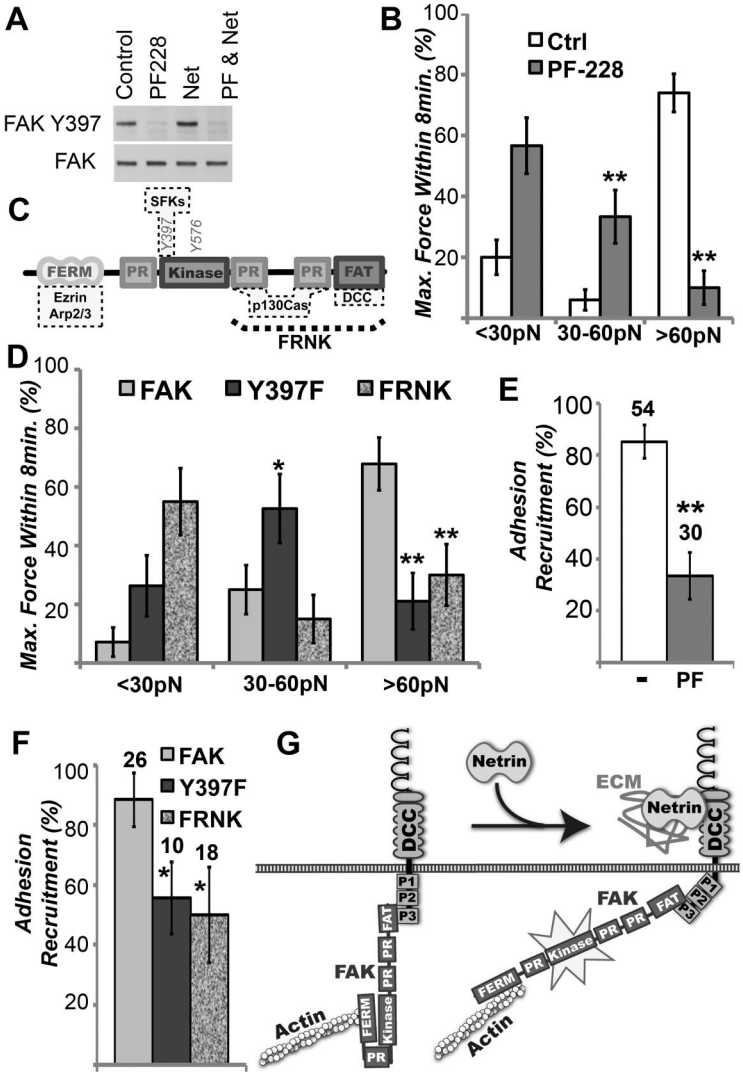


Figure 8. Inhibition of FAK disrupts strong traction forces on netrin-1. ***A***, Western blot images of the autophosphorylation site within FAK (Y397) and total FAK. One hour preincubations with the FAK kinase inhibitor PF-228 (10 μM) efficiently eliminated both endogenous and netrin-induced (15 min, 200 ng/ml) phosphorylation. ***B***, Inhibition of FAK with 10 μM PF-228 reduced the number of growth cones capable of pulling on netrin-1 with >60 pN (10%, $n = 30$). ***C***, Diagram of FAK domain structure indicating selected protein association regions. FRNK lacks the FERM, the first proline-rich (PR) and kinase domains. DCC binds to FAK's FAT domain. ***D***, Overexpression of FAK had no significant effect on the ability of growth cones to pull on netrin-1 with >60 pN (70%, $n = 23$). However, fewer axons generated >60 pN on netrin-1 when expressing FAK-Y397F (22%, $n = 18$) or FRNK (35%, $n = 17$). ***E***, ***F***, Inhibition of FAK kinase activity or expression of FAKY397F and FRNK reduced the percentage of growth cones that expanded their initial contacts on the netrin-1 beads. Adhesion expansion was quantified as the number of filopodia whose contacts are stabilized on the bead. Numbers above each bar represent the number of growth cones examined for each condition. ***G***, A possible mechanism whereby traction force on netrin activates FAK's kinase activity through separation of FAK's FERM domain from the kinase domain. The C-terminal FAT domain of FAK associates with the P3 intracellular domain of DCC, while its N-terminal FERM domain indirectly associates with actin filaments. *$p < 0.05$, **$p < 0.01$ relative to Ctrl (***B***, ***E***) or FAK (***D***, ***F***) (LSD).

Discussion

These findings define several important steps in the process of axon guidance to netrin-1. First, in contrast to unrestrained netrin-1, substrate-bound netrin-1 supports axon outgrowth, growth cone expansion, and chemoattractive signaling through FAK and CAS. Second, myosin II-dependent traction forces on netrin-1 activate FAK's kinase activity. Third, FAK kinase activity produces increased traction force on netrin-1 through the expansion of filopodial contacts. Thus, we suggest that guidance to netrin-1 is mediated by a gradient of substrate-attached netrin-1 and not a soluble gradient.

Substrate attachment of netrin-1 in axon guidance

The ability of pulsatile release of netrin-1 from a pipette to guide axons could be regarded as evidence of a guidance activity of freely diffusible netrin-1 (de la Torre et al., 1997; Ming et al., 1997). However, there are several lines of evidence that it is substrate-adsorbed netrin-1 that is guiding axons in these assays. First, there is rapid adsorption of netrin-1 (within 5 min) to polylysine-coated surfaces (Fig. 1*I*). We have also directly observed binding of netrin-1 using fluorescently coupled netrin-1 that was pulsed for 30 min from a pipette onto glass surfaces that were incubated for 12 h with *Xenopus* spinal neuron culture media (49% L15, 50% Ringer's, 1% fetal bovine serum; S. W. Moore and M. P. Sheetz, unpublished observations). Finally, our evidence that disrupting substrate adsorption of netrin-1 reduces outgrowth (Fig. 2), turning (Fig. 3), and intracellular activation of FAK and CAS (Figs. 4–7) suggests that it is substrate-bound netrin-1 that guides axons in these pipette axon-turning assays.

The dependence upon substrate binding raises the question of what netrin-1 adsorbs to *in vivo*. In the developing retina, netrin-1 is closely associated with the glial cells that produce it (Deiner et al., 1997). In the developing spinal cord, there is an exponential decrease in density of netrin-1 more than ~250 μm from its source (Kennedy et al., 2006). Prime binding sites for netrin-1 are the glycosaminoglycans found on both extracellular matrix and cell surface proteins. They are emerging as key regu-

lators of axon guidance cue function (de Wit and Verhaagen, 2007). The heparan sulfate polymerizing enzyme Ext1, like netrin-1 and DCC, is required for proper formation of major commissural tracts (Serafini et al., 1996; Fazeli et al., 1997; Inatani and Yamaguchi, 2003). When heparan sulfate proteoglycan (HSPG) expression is disrupted within spinal commissural neurons, there is a disruption of axon fasciculation (Matsumoto et al., 2007). As such, HSPGs both on netrin-attractive axons and along their trajectory are likely to provide substrate attachment sites for netrin *in vivo*.

FAK as a mechanosensor in a variety of contexts

Mechanical force induces tyrosine phosphorylation of FAK in aortic endothelial cells, tracheal smooth muscle, and fibroblasts (Yano et al., 1996; Tang et al., 1999; Zhang et al., 2008), and blocking FAK Y397 phosphorylation using antibodies disrupts flow-induced dilation of coronary arteries (Koshida et al., 2005). In the absence of FAK, fibroblasts have reduced migration rates and altered responses to applied force, as well as impaired rigidity sensing and traction forces on collagen substrates (Wang et al., 2001). Therefore, FAK appears to function as a mechanosensor in a variety of cellular contexts. In terms of axon guidance, FAK has also been implicated in the guidance of axons to a growing number of cues, including: ephrins, Sema-3s, and brain-derived neurotrophic factor (BDNF) (Ren et al., 2004; Falk et al., 2005; Woo and Gomez, 2006). Notably, each of these cues are physically restrained; Ephrins span the plasma membrane or are GPI-linked, while Sema-3s and BDNF are secreted but are known to bind to extracellular matrix components (De Wit et al., 2005; Liang et al., 2010; Martino and Hubbell, 2010). As such, FAK may have a ubiquitous function as a mechanosensor in the response to these guidance cues.

Mechanism of FAK activation

There is mounting evidence that the activation of FAK is through mechanical extraction of the FERM domain from its kinase domain. The FERM domain has been shown to interact with and inhibit its kinase domain (Cooper et al., 2003). Using fluorescent resonance energy transfer-based sensors in fibroblasts, conformational changes of the FERM domain have been detected in growing integrin adhesions (Cai et al., 2008; Papusheva et al., 2009). As mentioned above, FAK tyrosine phosphorylation is mechanically induced in a variety of cellular contexts (Yano et al., 1996; Tang et al., 1999; Zhang et al., 2008; Pasapera et al., 2010), and crystal structures have demonstrated that when FAK is phosphorylated, the FERM domain no longer blocks access to the catalytic site (Lietha et al., 2007). The N-terminal FERM domain of FAK interacts with the f-actin binding proteins Ezrin and the Arp2/3 complex (Poullet et al., 2001; Serrels et al., 2007), while the C-terminal focal adhesion targeting (FAT) domain interacts with netrin-1's receptor DCC (Ren et al., 2004). As such, one possible mechanism of FAK activation is that it experiences traction forces that extract the FERM domain from its kinase domain (Fig. 8*G*).

FAK in both adhesion assembly and disassembly

Deletion of FAK leads to a greater number of focal adhesions in fibroblasts (Ilić et al., 1995) and reintroduction reduces steady-state traction forces (Dumbauld et al., 2010). On the other hand, several reports have shown that FAK activity is required for early events in adhesion formation in both fibroblasts and neuronal growth cones (Robles and Gomez, 2006; Michael et al., 2009). Therefore, FAK is implicated in both the assembly and disassembly of adhesions. Similarly, the guidance of growth cones is a multistep process that involves both adhesion assembly and disassembly. While FAK has a significant role in the guidance of axons to netrin-1 (Liu et al., 2004), it is not clear from those studies how FAK is involved. Based upon these studies, it is clear that myosin II mechanical forces are critical in activating FAK and FAK activity is important for increasing the force on netrin-1 through the recruitment of additional contacts.

CAS as a myosin II-independent mechanosensor

The precise role of CAS in cell migration and axonal extension remains largely unclear. CAS deficient fibroblasts have reduced spreading and migration rates on a variety of substrates (Honda et al., 1999; Carter et al., 2002; Sanders and Basson, 2005; Tazaki et al., 2008). In terms of axon extension and guidance, disruption of CAS slows extension rates and reduces the ability of axons to chemoattract to netrin-1 (Huang et al., 2006; Liu et al., 2007). Interestingly, while there are CAS homologues in vertebrates and *Drosophila*, no homolog exists in *Caenorhabditis elegans*, where netrin is known to have an evolutionary conserved axon guidance function (Hedgecock et al., 1990; Singh et al., 2008). Thus, the role of CAS in axon attraction to netrin-1 may not be ubiquitous but, rather, may indicate there are different control or effector systems.

CAS and FAK appear to cooperate in certain cellular events—they bind each other and their ligand-induced phosphorylation requires proper actin organization in a variety of contexts (Nojima et al., 1995; Vuori and Ruoslahti, 1995; Ojaniemi and Vuori, 1997). However, we found that, while inhibition of myosin II reduces phosphorylation of FAK (on both Y397 and Y576), it does not significantly affect CAS phosphorylation (Fig. 6). Conversely, inhibition of SFKs reduces CAS phosphorylation but does not significantly affect FAK Y397 phosphorylation. As such there are independent pathways that activate FAK kinase activity and CAS phosphorylation. Importantly, both require substrate-bound netrin-1, which is consistent with both being downstream of mechanosensory activation (Fig. 4). Determining the cellular events that trigger CAS phosphorylation is therefore an important topic to address.

Conclusion

Guidance of growth cones to netrin-1 involves a mechanosensing process in the link between substrate-bound netrin-1 and the actomyosin contractile network. FAK and CAS have important roles in the steps of guidance that follow initial binding and involve further recruitment of receptors to areas with substrate bound netrin-1. In such a multistep process, there must be a coordination of many motile systems and a rigid netrin-1 cue provides a simple way to guide the process.

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