

Netrin requires focal adhesion kinase and Src family kinases for axon outgrowth and attraction

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Although netrins are an important family of neuronal guidance proteins, intracellular mechanisms that mediate netrin function are not well understood. Here we show that netrin-1 induces tyrosine phosphorylation of proteins including focal adhesion kinase (FAK) and the Src family kinase Fyn. Blockers of Src family kinases inhibited FAK phosphorylation and axon outgrowth and attraction by netrin. Dominant-negative FAK and Fyn mutants inhibited the attractive turning response to netrin. Axon outgrowth and attraction induced by netrin-1 were significantly reduced in neurons lacking the FAK gene. Our results show the biochemical and functional links between netrin, a prototypical neuronal guidance cue, and FAK, a central player in intracellular signaling that is crucial for cell migration.

Axon outgrowth and pathfinding are crucial for the formation of a normal nervous system. Extracellular cues that control these processes have been discovered in the past two decades. The netrin family of proteins can both promote axon outgrowth and guide the direction of axon pathfinding in many regions of the nervous system including the neocortex and the spinal cord^{1–4}.

In *Caenorhabditis elegans*, the gene *unc-6* encodes the prototypical member of the netrin family^{1,5}. In mammals, studies of commissural axons in the spinal cord showed a diffusible guidance activity in the floorplate⁶. The floorplate was able to promote the outgrowth of commissural axons and to attract them^{7–10}. Biochemical purification of the axon-promoting activity from the chick brain led to the isolation of netrin-1, which was then shown to act as both a factor promoting axon outgrowth and a guidance cue to attract axons^{2,3}. Sequence similarities between UNC-6 and netrin-1 proteins established a remarkably conserved family of molecular cues in invertebrates and vertebrates^{2,3,5,11–15}.

The receptors for the netrins are UNC-40 (refs. 1,16,17) and UNC-5 (ref. 18) in *C. elegans*, and their corresponding mammalian homologs are DCC (deleted in colorectal cancer), neogenin^{19,20} and UNC5A–D (also known as UNC5H-1, -2, -3 and -4; refs. 21–24). DCC/UNC-40 seems to be required for both the attractive and repulsive responses, whereas UNC-5 is required only for repulsion^{17–20,25,26}. Heterodimerization of UNC-40 with UNC-5 may convert UNC-40 from mediating attraction to repulsion²⁷. There is also evidence, however, that DCC/UNC-40 can mediate repulsion in the absence of UNC-5 in specific cells in *C. elegans*²⁸. In contrast to our knowledge of the multiple roles of netrins and their receptors,

much less is known about intracellular signal transduction pathways that mediate netrin responses. A genetic screen in *C. elegans* for mutations that suppress the phenotype caused by ectopic expression of *unc-5* has uncovered *unc-34*, *unc-44*, *unc-129*, *seu-1*, *seu-2* and *seu-3* (ref. 29). A screen for genes controlling motor axon guidance led to the isolation of *max-1*, which genetically interacts with *unc-5* and *unc-6* but not with *unc-40* in *C. elegans*³⁰. In vertebrates, netrin signaling may involve phospholipase C (PLC)³¹, phosphoinositol-3-kinase (PI3K)³¹, MAP kinases^{31–33} and the small GTPases Cdc42 and Rac^{34,35}.

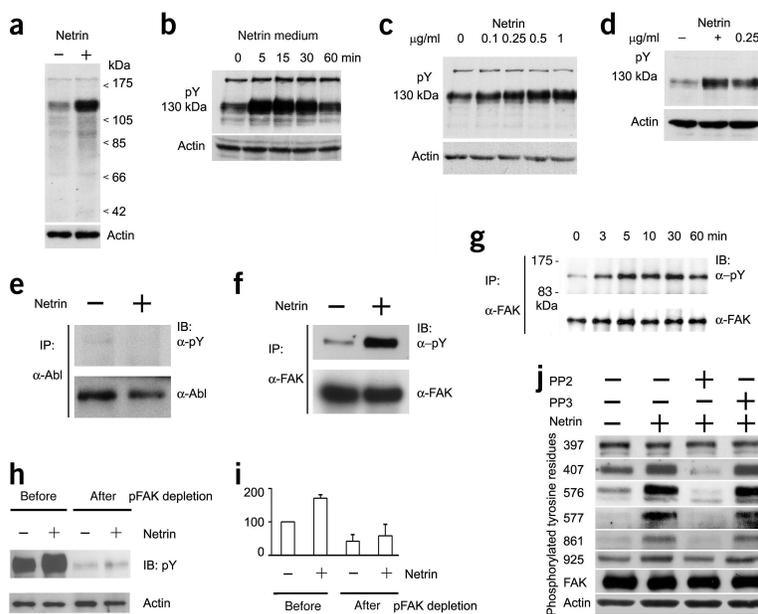
FAK is a cytoplasmic protein tyrosine kinase that was discovered a decade ago^{36–40}. FAK is involved in multiple cellular processes including adhesion, spreading, migration, survival, cell cycle progression and proliferation⁴¹. FAK is localized to sites of integrin–extracellular matrix interaction in fibroblasts. Tyrosine phosphorylation of FAK is implicated in integrin-stimulated cell migration. FAK phosphorylation can also be induced by multiple stimuli such as cytokines, cell adhesion molecules and growth factors. Phosphorylated tyrosine residues in FAK provide specific binding sites for a number of proteins including Src family kinases, the p85 subunit of PI3K, Grb2 and p130CAS, thereby locally activating these molecules^{36–40}. Activated Src can form a complex with FAK that further stimulates FAK activity and acts on downstream substrates. Interactions between FAK and adaptor proteins mediate the cellular response to extracellular molecules⁴¹.

We have been studying signal transduction mechanisms that mediate cellular responses to neuronal guidance cues⁴². We report here that netrin can induce the phosphorylation of FAK and that elimina-

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Figure 1 Regulation of tyrosine phosphorylation by netrin-1. Tyrosine phosphorylation was examined with anti-phosphotyrosine (pY; 4G10). The same samples were stained with anti-actin to control for equal loading of proteins. **(a)** Cortical neurons were treated with netrin-1-conditioned medium for 5 min. **(b)** p130 tyrosine phosphorylation after treatment with netrin medium (for control HEK293 medium, see **Supplementary Fig. 1**). **(c)** Dose dependency of p130 tyrosine phosphorylation in cortical neurons induced by purified chicken netrin-1 for 5 min. **(d)** Tyrosine phosphorylation of p130 in primary cells from the dorsal half of E13 spinal cord after a 5-min treatment with netrin-conditioned medium (two lanes on the left) or purified human netrin-1 (0.25 $\mu\text{g}/\text{ml}$; right lane).

(e) α , anti; IP, immunoprecipitation; IB, immunoblot. **(e,f)** Tyrosine phosphorylation of **(e)** Abl and **(f)** FAK in cortical neurons with and without netrin-1 treatment. **(g)** Time course for netrin induction of FAK phosphorylation. Numbers on the top indicate minutes after netrin-1 treatment. **(h)** Contribution of phospho-FAK to netrin-induced p130 phosphorylation. Antibodies to all six phosphotyrosine residues in FAK were used to immunoprecipitate cortical lysates. Anti-phosphotyrosine was used to probe the blot. **(i)** Quantification of the depletion results. The y axis shows signal density in arbitrary units. The values \pm s.d. are 100 ± 0 , 170.3 ± 10.8 , 40.9 ± 20.4 and 57.4 ± 36.0 ($n = 2$). In addition to the reduction in tyrosine phosphorylation in both netrin-treated and netrin-untreated samples, the fold induction in tyrosine phosphorylation was also reduced. **(j)** Sites of phosphorylation in FAK that are induced by netrin-1. Antibodies to the phosphorylated tyrosine residues in FAK were used to probe western blots of cortical lysates treated with netrin, PP2 (2 μM) or PP3 (5 μM). The bottom panels show equal loading of FAK and actin in different lanes.



tion of FAK inhibits axon outgrowth induced by netrin-1. Fyn, a Src family kinase associated with FAK, is also activated by netrin. We have obtained biochemical and functional evidence demonstrating the importance of the cytoplasmic tyrosine kinases FAK and Fyn as intracellular components that mediate both axon outgrowth and turning responses to the netrins.

RESULTS

Protein tyrosine phosphorylation induced by netrin-1

In our efforts to study the potential role of protein tyrosine phosphorylation in the intracellular pathways for neuronal guidance cues, we observed considerable induction of tyrosine phosphorylation by the secreted protein netrin-1. After primary cells from the cerebral cortex of embryonic day (E) 15 rats were treated with conditioned medium from control HEK293 cells or HEK293 cells that stably express the human netrin-1 protein, extracts were made and analyzed by immunoblotting with the antibody to phosphotyrosine (anti-phosphotyrosine; clone 4G10). Treatment with netrin-1-conditioned medium increased tyrosine phosphorylation of several proteins, especially some with molecular weights of approximately 130 kDa (p130; Fig. 1a). Tyrosine phosphorylation increased within 5 min and returned to baseline within 1 h (Fig. 1b). In contrast, no substantial effect on tyrosine phosphorylation was observed after treatment of cortical neurons by conditioned medium from control HEK293 cells (see **Supplementary Fig. 1** online) or from HEK293 cells that secreted the guidance protein slit (data not shown).

To confirm that netrin-1 was directly responsible for increasing tyrosine phosphorylation of 130-kDa proteins, we further tested purified netrin-1 from two sources: chicken netrin-1 from a commercial source and human netrin-1 purified from a stable HEK293

cell line established in our laboratory. Netrin-1 from both sources increased tyrosine phosphorylation of p130 within 5 min in a dose-dependent manner. Chicken netrin-1 at a concentration of 100 ng/ml induced a modest increase in p130 tyrosine phosphorylation; a concentration of 500 ng/ml produced the maximum response (Fig. 1c). In addition to cortical neurons, commissural neurons in the dorsal spinal cord also respond to netrins^{2,3}. We therefore examined whether netrin-1 induced p130 tyrosine phosphorylation in spinal neurons. Dissociated cells from the dorsal half of E13 spinal cords were cultured and treated with netrin-1. Within 5 min, both the netrin-conditioned medium and 0.25 $\mu\text{g}/\text{ml}$ of purified netrin-1 increased tyrosine phosphorylation of p130 (Fig. 1d).

Induction of FAK phosphorylation by netrin

To identify the molecular nature of proteins whose phosphorylation was induced by netrin, we examined tyrosine phosphorylation of several proteins with molecular weights around 130 kDa. We either used specific antibodies to candidate proteins to carry out immunoprecipitation before probing the western blots with anti-phosphotyrosine, or used anti-phosphotyrosine to carry out immunoprecipitation before probing the western blots with antibodies to specific candidate proteins. The conclusions were the same.

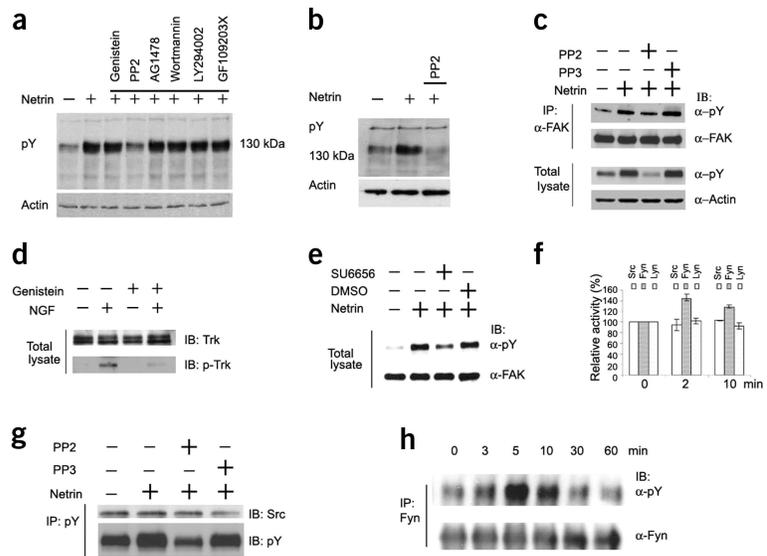
Netrin-1 did not induce tyrosine phosphorylation of the Abelson tyrosine kinase (Abl; Fig. 1e). In contrast, tyrosine phosphorylation of p125 FAK was induced by netrin-1 within 5 min; phosphorylation persisted until 30 min and decreased by 60 min (Fig. 1f,g). These results indicate that netrin can stimulate phosphorylation of FAK.

There are other proteins of around 130 kDa that might be phosphorylated during netrin stimulation. To determine the contribution of FAK to this phosphorylated population, we used antibodies

Figure 2 Activation of the Fyn tyrosine kinase by netrin-1. **(a)** Effects of inhibitors of intracellular enzymes on FAK activation in cortical neurons: genistein (100 μ M), PP2 (2 μ M), AG1478 (5 μ M), wortmannin (400 nM), LY294002 (10 μ M) or GF109203X (10 μ M). Immunoblots were analyzed with anti-phosphotyrosine (4G10; upper) or anti-actin (lower). **(b)** The effect of PP2 (2 μ M) on the induction of p130 tyrosine phosphorylation in E13 dorsal spinal cord cells by netrin-1. **(c)** PP2 inhibited netrin induction of FAK phosphorylation. **(d)** Genistein (100 μ M) inhibited TrkA phosphorylation that was induced by nerve growth factor (NGF) in dorsal root ganglion neurons. **(e)** SU6656 but not DMSO inhibited netrin-induced tyrosine phosphorylation. **(f)** An immune complex kinase assay for Src family kinases showed that Fyn was activated by purified human netrin-1. Cortical cells (6×10^6 plated/well) were stimulated with 250 ng/ml netrin-1. Protein extracts were immunoprecipitated with antibodies to Src, Fyn or Lyn before the kinase reactions were carried out. Relative activities and the s.d. were calculated from three separate experiments.

(g) Phospho-Src was not increased by netrin-1

(upper). In the same immunoprecipitates, phosphoproteins of approximately 130 kDa were increased (lower). **(h)** Time course of Fyn activation by netrin-1. The numbers on the top indicate minutes after netrin-1 treatment. Anti-Fyn was used to immunoprecipitate the extracts, and anti-phosphotyrosine was used to probe the immunoblots. The lower panel shows amount of input Fyn. IP, immunoprecipitation; IB, immunoblot.



to phosphorylated FAK to deplete phospho-FAK from the immuno-precipitation. We found that antibodies to phospho-FAK (anti-phospho-FAK) reduced the basal level of phosphoproteins around 130 kDa and the netrin-induced component of phospho-FAK (Fig. 1h,i). Reduced induction of p130 phosphorylation by netrin (Fig. 1i) supports the idea that phospho-FAK is an important component of the p130 proteins. Control antibodies did not reduce netrin-induced phosphorylation of p130 proteins (Supplementary Fig. 2 online).

There are six tyrosine residues in FAK that can potentially be phosphorylated. To determine which residues are regulated by netrin, we treated primary cortical neurons with netrin and then used specific antibodies to each of the phosphorylated sites. Phosphorylation of residues at 407, 576, 577 and 861 was increased by netrin (Fig. 1j). Phosphorylation of Tyr925 was modestly increased by netrin (Fig. 1j). Phosphorylation of Tyr397 was not, however, affected by netrin (Fig. 1j). The specificity of each phospho-specific antibody was confirmed using FAK mutants that lacked each tyrosine residue (Supplementary Fig. 3 online). These results indicate that netrin induces the phosphorylation of specific tyrosine residues in FAK.

FAK phosphorylation requires Src family kinases

To investigate molecular mechanisms that underlie netrin-induced tyrosine phosphorylation, we tested the effects of pharmacological inhibitors on netrin-induced tyrosine phosphorylation of p130 in cortical neurons. The induction of p130 tyrosine phosphorylation in neocortical neurons was prevented by a Src family kinase inhibitor (PP2) but not by inhibitors of PI3K (wortmannin and LY294002) and protein kinase C (GF109203X) (Fig. 2a). The induction was also not prevented by an epidermal growth factor receptor kinase inhibitor (AG1478), by MAP kinase inhibitors such as PD98059 and SB203580 or by genistein, a general inhibitor of tyrosine kinases (Fig. 2a and data not shown). Genistein could

inhibit induction of phosphorylation by the nerve growth factor (NGF) (Fig. 2d).

Netrin-induced p130 tyrosine phosphorylation in E13 dorsal spinal cord was also inhibited by 2 μ M PP2 (Fig. 2b). FAK phosphorylation that was induced by netrin-1 in cortical neurons was inhibited by PP2 but not by PP3 (an inactive control for PP2; Fig. 2c). Netrin-induced FAK phosphorylation was also inhibited by SU6656, another inhibitor of Src family kinases (Fig. 2e) and herbimycin A (data not shown), but not by DMSO (Fig. 2e).

To determine whether Src family kinases were required for netrin-induced FAK phosphorylation, primary cortical cells were treated with netrin-1 in the presence of PP2 or PP3. Netrin-induced tyrosine phosphorylation of FAK was reduced by PP2 but not by PP3 (Fig. 2c). Netrin-induced phosphorylation of tyrosine residues at 407, 576, 577, 861 and 925 was inhibited by PP2 but not by PP3 (Fig. 1j). These findings indicate that the Src family tyrosine kinases are essential for netrin-dependent FAK phosphorylation. We examined whether netrin could change the activities of Src family kinases by measuring the activities of Src, Fyn and Lyn, three Src family kinases that are abundant in the embryonic brain. After netrin-1 treatment, Src, Fyn and Lyn were immunoprecipitated by specific antibodies. Their activities on a standard peptide substrate were measured. Netrin-1 increased the activity of Fyn but not of Src or Lyn (Fig. 2f). Src phosphorylation was also not stimulated by netrin-1 (Fig. 2g).

To compare the time course of Fyn phosphorylation with that of FAK, we collected cell lysates at different time points after netrin-1 treatment before immunoprecipitation with the antibody to Fyn (anti-Fyn). The blot was probed with anti-phosphotyrosine. Similar to FAK, the phosphorylation of Fyn also increased within 3 min, peaked within 5–10 min and decreased by 60 min (Fig. 2h). The time course of Fyn phosphorylation correlated with that of FAK phosphorylation, and no temporal difference was detected. These results indicate that FAK and Src kinases are coactivated by netrin in cortical neurons.

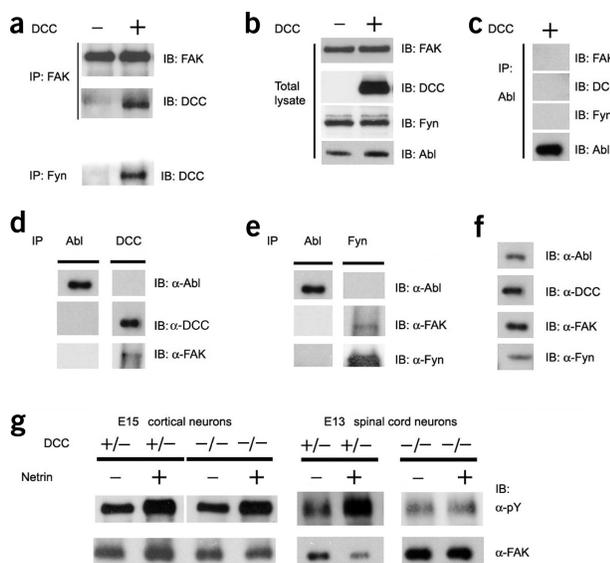


Figure 3 Formation of protein-protein interaction complexes of DCC with FAK and Fyn. (a) DCC forms protein-protein interaction complexes with FAK and Fyn in HEK293 cells (left lane, control cells; right lane, cells expressing DCC). (b) Equivalent amounts of input FAK, Fyn and Abl. (c) In DCC-expressing cells, anti-Abl precipitated Abl but not FAK, DCC or Fyn. (d) Anti-DCC precipitated both DCC and FAK from E16 cortical neurons, whereas anti-Abl precipitated Abl but not FAK or DCC. (e) Anti-Fyn precipitated FAK and Fyn, whereas anti-Abl precipitated Abl but not FAK or Fyn. (f) Amounts of Abl, DCC, FAK and Fyn present in cortical lysates. (g) Netrin induction of p130 tyrosine phosphorylation in cortical and spinal cord neurons from wild-type (heterozygous; +/-) and DCC knockout (-/-) mice.

DCC forms complexes with FAK and Fyn

DCC is a transmembrane receptor that mediates neuronal responses to netrins^{5,22,31,32}. Netrin induction of FAK and Fyn phosphorylation prompted us to test whether DCC forms protein-protein interaction complexes with these two proteins.

We transfected a cDNA encoding DCC into HEK293 cells. When the antibody to FAK (anti-FAK) was used to immunoprecipitate cell lysates, DCC was detected by probing the western blots with antibody to DCC (anti-DCC; Fig. 3a). In cells transfected with DCC and in untransfected controls, amounts of endogenous FAK and Fyn were equivalent, whereas DCC was detected only in the transfected HEK293 cells (Fig. 3b). When anti-Fyn was used to immunoprecipitate the cell lysates, DCC was also detected in the precipitates (Fig. 3a). As a control, the antibody to Abl (anti-Abl) was used to immunoprecipitate proteins from DCC-expressing HEK293 cells and was found to be unable to precipitate DCC, FAK or Fyn (Fig. 3c). These results indicate that DCC can form protein-protein interaction complexes with FAK and Fyn in HEK293 cells.

To examine protein interaction complexes involving DCC, FAK and Fyn in primary neurons, neocortical neurons were isolated from E15 mice. Anti-DCC immunoprecipitated DCC and FAK (Fig. 3d), and anti-Fyn precipitated Fyn and FAK (Fig. 3e). Either anti-FAK or anti-Fyn immunoprecipitated DCC (data not shown). As a control, the presence of the proteins in the neocortical neurons was confirmed by western analysis (Fig. 3f). These results indicate that there is a protein-protein interaction complex involving DCC, FAK and Fyn in primary neurons.

To determine the role of DCC, we isolated neurons from wild-type or DCC knockout mice²⁰. Tyrosine phosphorylation was induced by netrin-1 in spinal cord neurons from E13 wild-type mice but not in spinal cord neurons from DCC knockout mice (Fig. 3g), indicating that netrin-1 requires DCC to induce tyrosine phosphorylation in the spinal cord. Notably, netrin induction of p130 phosphorylation in cortical neurons did not require DCC (Fig. 3g), indicating the presence of other receptors that are functionally redundant with DCC in cortical neurons.

Src family kinases and netrin-induced axon outgrowth

To investigate the role of Src family kinases in netrin function, we tested the effect of Src kinase inhibitors on netrin-1-induced axon outgrowth in cortical explants. After cortical explants from E15 mice were cultured in mixed gels with collagen and Matrigel for 16–18 h, TuJ1, an antibody to neuron-specific β tubulin, was used to show neuronal processes (Fig. 4a–f). Axon bundles from control explants were few (Fig. 4a,q). Netrin-1 stimulated axon outgrowth as indicated by the increased number of axon bundles (Fig. 4b,q). PP2 (0.2 μ M) blocked netrin-1-induced axon outgrowth, resulting in a reduced number of axon bundles (Fig. 4c,q). In contrast, axon outgrowth induced by netrin-1 was not inhibited by the same concentration of PP3 (Fig. 4d,q). SU6656, another inhibitor of Src family kinases, also inhibited axon outgrowth that was induced by netrin-1, whereas the vehicle DMSO did not (Fig. 4e,f). Netrin-induced axon outgrowth was also inhibited by herbimycin A, another Src family kinase inhibitor, but not by genistein or AG1478 (data not shown).

PP2 inhibition of netrin-induced axon outgrowth could be due either to a general requirement for Src family kinases in axon growth or to a specific role for Src family kinases in netrin signaling. To examine whether the Src family kinases are generally required for axon outgrowth, we cultured the cortical explants for 20 or 40 h in PP2 or PP3 without netrin-1 stimulation. There was little basal outgrowth in three-dimensional cultures after 20 h (Fig. 4g–k,r), and it was not inhibited by PP2 (Fig. 4h,r) or SU6656 (Fig. 4k,r). Robust basal outgrowth after 40 h was independent of netrin (Fig. 4l–p,s) and was also not inhibited by PP2 (Fig. 4m,s) or SU6656 (Fig. 4p,s). The effect of the same concentration of PP2 on netrin-induced outgrowth in 20-h cultures therefore indicates that PP2 can inhibit axon outgrowth that is induced by netrin-1, without inhibiting basal axon outgrowth in 20-h cultures or netrin-independent outgrowth in 40-h cultures. Taken together, these results demonstrate a functional role for Src family kinases in netrin-induced axon outgrowth.

Src family kinases and netrin-induced axon attraction

An important function of netrin is to attract axons^{1–4,43}. Attraction of cortical axons by netrin-1 was demonstrated previously with a coculture assay⁴⁴. To investigate whether Src family kinases are required for attraction of axons by netrin, we first cocultured cortical explants with aggregates of either control HEK293 cells or HEK293 cells that were transfected with netrin-1 cDNA and that expressed netrin-1 protein (Supplementary Fig. 4 online).

When cortical explants were cocultured with control HEK293 cells, axon bundles were low in number and were symmetric in their distribution, so that the number of axon bundles in the explant region that was proximal to HEK293 aggregates was similar to that in the region that was distal (Supplementary Fig. 4 online). When cortical explants were cultured with HEK293 cells that secreted netrin-1, the number of axon bundles increased and their distribution

Figure 4 Inhibition of netrin-induced axon outgrowth by Src family kinase inhibitors.

(a–f) Cortical explants from E15 embryos were cultured in mixed collagen Matrigels for 16–18 h in the presence of pharmacological reagents before immunostaining with TuJ1. Scale bars, 100 μ m.

(a) Control explants without netrin-1 (Con) showed fewer bundles (38.14 ± 7.37 ; $n = 36$) than did (b) explants with netrin-1 (135.44 ± 15.11 ; $n = 46$).

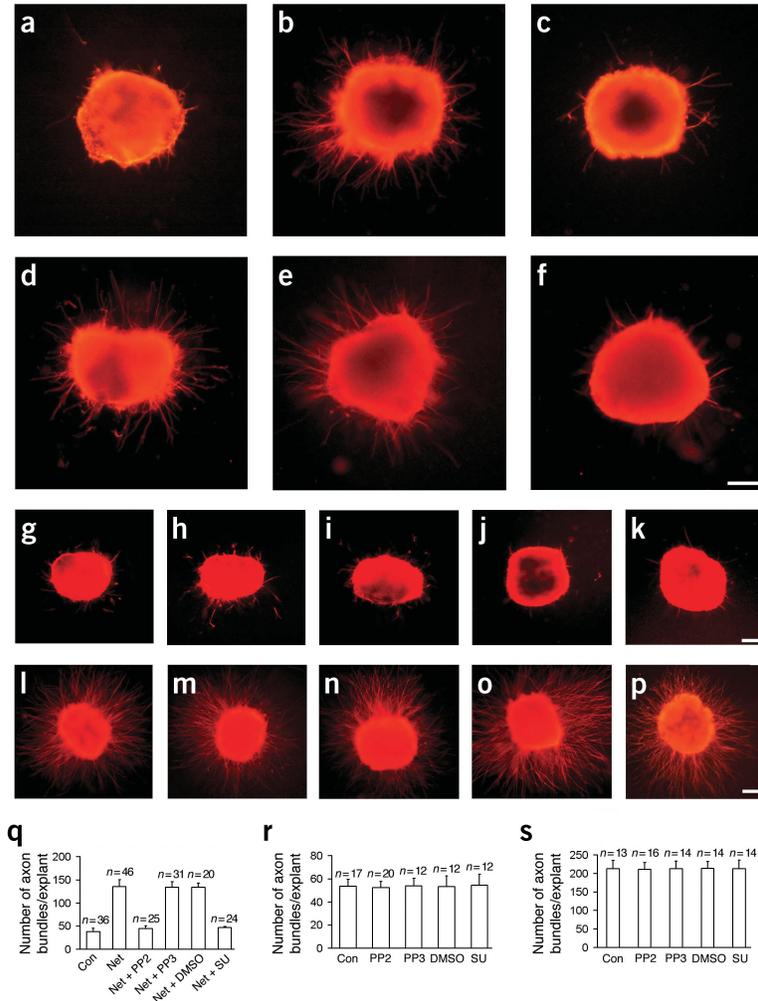
Netrin-induced axon outgrowth was inhibited by (c) PP2 (0.2 μ M; 44.84 ± 5.77 ; $n = 25$) and by (f) SU6656 (2 μ M; 46.63 ± 3.02 ; $n = 24$) but was not inhibited by (d) PP3 (0.2 μ M; 134.10 ± 11.46 ; $n = 31$) and by (e) DMSO (133.45 ± 9.70 ; $n = 20$).

(g–k) Cortical explants from E15 embryos were cultured for 20 h in control DMEM+B27 medium either (g) alone (53.7 ± 5.7 ; $n = 17$) or with (h) 0.2 μ M PP2 (52.3 ± 5.6 ; $n = 20$) (i) PP3 (53.8 ± 6.7 ; $n = 12$) (j) DMSO (53.08 ± 9.24 ; $n = 12$) or (k) SU6656 (54.58 ± 9.48 ; $n = 12$), none of which affected netrin-independent axon outgrowth. Scale bars, 100 μ m.

(l–p) Cortical explants from E15 embryos were cultured for 40 h (l) alone (212.5 ± 22.4 ; $n = 13$) or in the presence of (m) PP2 (211.1 ± 18.1 ; $n = 16$) (n) PP3 (212.6 ± 20.4 ; $n = 14$) (o) DMSO (213.79 ± 18.09 ; $n = 14$) or (p) SU6656 (212.5 ± 6.84 ; $n = 14$). Scale bars, 100 μ m. (q–s) The number of TuJ1-positive axon bundles per explant were counted ($n =$ number of cortical explants).

(q) Quantification of basal axon outgrowth (axon bundles) after culturing for 16–18 h. P values: <0.001 between Con and Net; <0.001 between Net+PP2 and Net+PP3; <0.001 between Net+PP2 and Net+PP3; >0.5 between Net and Net+PP3; <0.001 between Net+DMSO and Net+SU.

(r) Quantification of basal axon outgrowth (axon bundles) after culturing for 20 h. P values: 0.95 between Con and PP2; 0.94 between PP2 and PP3; 1 between Con and PP3; 0.9 between DMSO and SU. (s) Quantification of basal axon outgrowth after culturing for 40 h. P values: 0.8 between Con and PP2; 0.8 between PP2 and PP3; 1 between Con and PP3; 0.95 between DMSO and SU.



changed so that the number of proximal bundles was significantly higher than that of distal bundles ($P < 0.001$ for the difference between netrin and control HEK293 cells; Supplementary Fig. 4 online). When PP2 (0.2 μ M) was added to the culture medium, the number of axon bundles was reduced, but notably, the number of proximal axon bundles was similar to the number of distal bundles (Supplementary Fig. 4 online). By contrast, PP3 did not affect the attractive response of cortical axons to netrin-1 (Supplementary Fig. 4 online). Asymmetric growth toward netrin-1 was also inhibited by SU6656 but not by DMSO (Supplementary Fig. 4 online).

We also used a turning assay with the chick spinal cord to determine the role of Fyn in netrin attraction of commissural axons (Fig. 8a). As will be described later, this assay specifically examines the turning response. A kinase-dead mutant of Fyn was able to inhibit commissural axon turning toward netrin-1 (Fig. 8f,g).

These results indicate that Src family kinases are involved in netrin attraction of cortical and commissural axons.

Involvement of FAK in netrin-induced axon outgrowth

To determine whether FAK was involved in netrin-induced outgrowth of cortical axons, we used a conditional knockout that pro-

duces a forebrain-specific deletion of FAK in offspring when the parental line is crossed to mice expressing Cre recombinase under the control of the *Emx1* promoter⁴⁵.

After E16 cortical explants were cultured for 16–18 h in collagen Matrigels, they were stained with TuJ1 to show axons. Cortical explants from E16 wild-type embryos showed a robust response to netrin-1 (Fig. 5a,b,e,f), whereas netrin-1 induction of axon outgrowth was significantly reduced in explants from mutant embryos (Fig. 5c–f). There is a small but significant reduction of basal axon outgrowth in collagen Matrigels (Fig. 5e). Because the reduction in netrin-induced outgrowth was much bigger, the effect of FAK knockout on netrin-induced axon outgrowth was still detectable when the ratio of netrin-induced axon outgrowth from wild-type explants was compared with that of netrin-induced axon outgrowth from FAK mutant explants (Fig. 5g,h). These results are consistent with the idea that FAK is required for netrin induction of axon outgrowth from cortical neurons.

To further confirm a role for FAK in netrin-induced axon outgrowth, cortical neurons from E15 embryos of wild type and FAK-null mice were cultured on coverslips coated with poly-D-lysine (PDL) or laminin for 18 h, in the absence or presence of netrin-1.

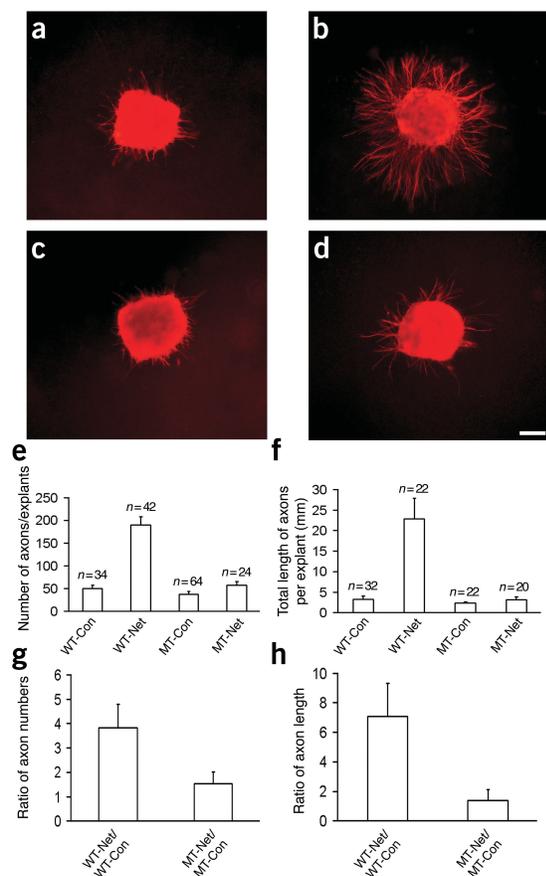


Figure 5 Reduced axonal responses to netrin-1 in FAK mutants. Cortical explants from E16 embryos were cultured in collagen gels for 16–18 h before being immunostained with TuJ1. (**a,b**) Axon outgrowth from wild-type (heterozygous) explants (**a**) without or (**b**) with netrin-1. (**c,d**) Outgrowth from FAK mutant explants (**c**) without and (**d**) with netrin-1. Scale bar, 100 μ m. Note that axons still grew in FAK-null mutants, but their responses to netrin-1 were reduced. (**e**) Axon bundles per explant. WT, wild-type explants; MT, FAK-null mutant explants; Con, control collagen gel; Net, collagen gel with netrin. Average numbers of axons per explant \pm s.d.: WT-Con, 49.51 ± 7.43 ; WT-Net, 190.21 ± 18.66 ; MT-Con, 37.55 ± 6.13 ; MT-Net, 57.04 ± 7.95 . *P* values: <0.001 between WT-Con and WT-Net; <0.001 between MT-Con and MT-Net; 0.003 between WT-Con and MT-Con. (**f**) Total length of axons per explant. Average total length of axons per explant: WT-Con, 3.23 ± 0.84 mm; WT-Net, 22.88 ± 4.97 mm; MT-Con, 2.27 ± 0.36 mm; MT-Net, 3.10 ± 0.72 mm. *P* values: <0.001 between WT-Con and WT-Net; 0.09 between MT-Con and MT-Net; 0.04 between WT-Con and MT-Con. (**g**) Ratio of axon numbers with and without netrin-1 treatment: WT-Net/WT-Con, 3.84 ± 2.51 ; MT-Net/MT-Con, 1.52 ± 1.29 . (**h**) Ratio of the total lengths of axons per explant with and without netrin-1 treatment: WT-Net/WT-Con, 7.08 ± 2.24 ; MT-Net/MT-Con, 1.37 ± 0.75 .

netrin, we carried out two assays, one with cortical neurons from FAK knockout mice (Fig. 7) and another with spinal cord neurons in chick embryos with a construct expressing a dominant-negative FAK mutant protein (Fig. 8).

When cortical explants were isolated from wild-type E15 mice and were cultured with aggregates of control HEK293 cells, the number of axon bundles that were proximal to the aggregates was similar to that of axon bundles that were distal to the aggregates (Fig. 7a,e). As expected⁴⁴, when cortical explants from wild-type mice were cultured with aggregates secreting netrin-1, the number of proximal axon bundles was larger than that of distal axon bundles (Fig. 7b,e). When cortical explants were isolated from E15 FAK-null mice and were cultured with aggregates of netrin-secreting HEK293 cells, however, the number of proximal axon bundles was similar to that of distal axon bundles (Fig. 7d,e). When cortical explants were isolated from E15 FAK-null mice and were cultured with aggregates of control HEK293 cells, the number of proximal axon bundles was also similar to that of distal axon bundles (Fig. 7c,e). For these experiments and those shown in Figure 5, we confirmed the reduction of FAK protein in FAK-mutant neurons into which the Cre recombinant had been introduced (Fig. 6j).

Although results from FAK knockout mice support a role for FAK in axon attraction by netrin, the minimal response of axon outgrowth induced by netrin in FAK knockout neurons could have precluded a direct test of the role of FAK in axon attraction. We thus turned to a very different assay with chick embryos (Fig. 8). In this assay, axon outgrowth is not dependent on netrin-1. The Venus variant of green fluorescent protein (GFP) was introduced into one-half of the chick spinal cord by *in ovo* electroporation. This half of the spinal cord was then isolated and cultured with the outer side of the spinal cord down and the inner side up. Projecting axons were marked by Venus expression (Fig. 8a). An aggregate of control HEK293 cells was placed on one edge of the spinal cord preparation in such a way that axons in the spinal cord would project toward its original floorplate in a direction parallel to the HEK293 aggregate (Fig. 8b). When the same kind of spinal cord explant was cultured with an aggregate of HEK293 cells that secreted netrin-1, some axons projected toward the netrin source (Fig. 8c,e), indicating that these axons were attracted by netrin-1. When FAK-related non-kinase (FRNK), a dominant-negative FAK mutant, was expressed with Venus GFP, it inhibited the turning of spinal cord axons toward netrin (Fig. 8d,e). With the same assay,

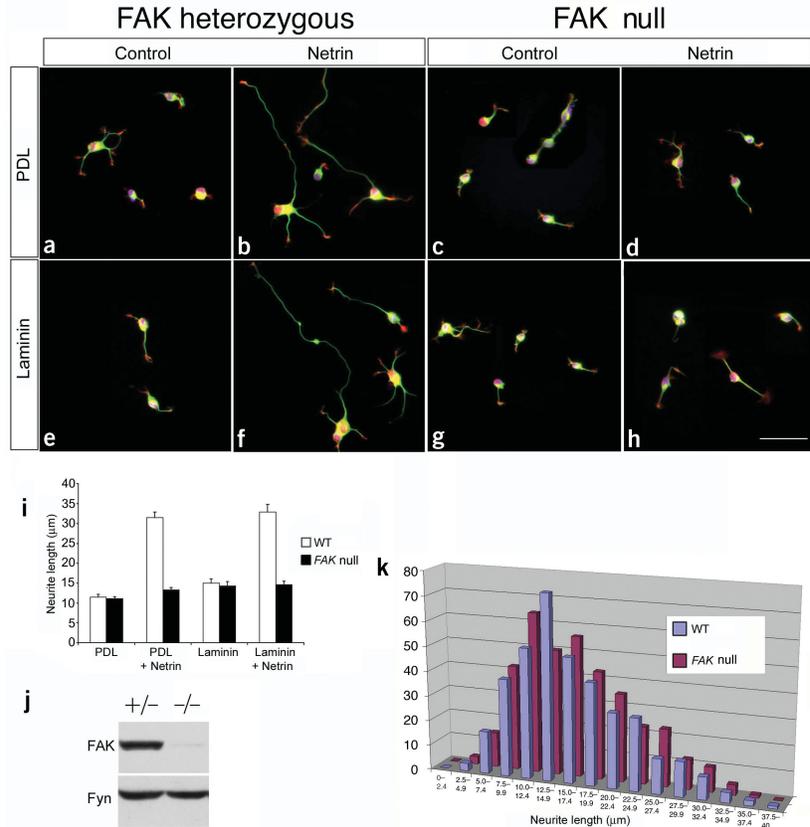
Axon outgrowth was observed from both wild-type (Fig. 6a,b,e,f) and FAK-null neurons (Fig. 6c,d,g,h). Axon outgrowth was not reduced in FAK knockout neurons grown on PDL (Fig. 6a,c,i) or laminin (Fig. 6e,g,i).

When netrin was added to wild-type cortical neurons growing on either PDL or laminin dishes, robust axon outgrowth was stimulated to a much higher degree than for neurons growing on PDL or laminin alone (Fig. 6a,b,e,f). The stimulatory effect of netrin was, however, significantly reduced in FAK-null neurons (Fig. 6c,d,g,h). The amount of axon outgrowth induced by netrin in FAK-null neurons remained above that observed from treatment with PDL and laminin alone, indicating that the observed inhibition in FAK-null neurons was not due to PDL or laminin effects but to an inhibition of netrin-induced outgrowth (Fig. 6i). The average length of neurites grown on PDL and laminin was similar between wild-type and FAK-null neurons. When the distribution of the length of all wild-type axons was compared with the same number of axons from FAK-null neurons, it could be seen that FAK-null neurons were not generally defective in neurite outgrowth (Fig. 6k). Taken together, these results indicate that FAK is required for axon outgrowth that is stimulated by netrin-1.

Involvement of FAK in netrin-induced axon attraction

Although some molecules, such as the neural cell adhesion molecule, can function to promote axon outgrowth but not attraction, netrins are chemoattractants with effects on both axon outgrowth and attraction. To test for a potential role of FAK in axon attraction by

Figure 6 Axonal outgrowth from dissociated neurons of heterozygous and FAK-null embryos. (a–h) Outgrowth of TuJ1-positive axons from dissociated cortical neurons plated on (a–d) PDL or (e–h) laminin in the absence (in a, c, e, g) or presence (in b, d, f, h) of netrin-1 (after culturing for 18 h). Control mice are heterozygous (in a, b, e, f); FAK-null indicates FAK conditional knockout mice (in c, d, g, h). Neurons are labeled with TuJ1 (green), rhodamine-phalloidin (red) and Hoechst (blue). Scale bar, 25 μ m. (i) The length of axons was measured from isolated neurons not in contact with other cells from three heterozygous and two FAK-null mice. Average axon length \pm s.d.: WT (PDL), 11.5 ± 0.6 ($n = 204$); WT (PDL + netrin), 31.5 ± 1.4 ($n = 225$); WT (laminin), 15.0 ± 1.9 ($n = 231$); WT (laminin + netrin), 32.9 ± 1.9 ($n = 246$); FAK-null (PDL), 11.1 ± 0.5 ($n = 238$); FAK-null (PDL + netrin), 13.3 ± 0.6 ($n = 232$); FAK-null (laminin), 14.3 ± 1.1 ($n = 228$); FAK-null (laminin + netrin), 14.6 ± 0.9 ($n = 212$). (j) FAK and Fyn proteins in cortical explants of heterozygous and FAK conditional knockout mice detected by anti-FAK and anti-Fyn. (k) Outgrowth of TuJ1-positive axons from dissociated neurons plated on laminin and PDL. Cortical neurons from E15 embryos were plated on pre-made Biocoat PDL- and laminin-coated 8-well culture slides (BD Biosciences) and cultured for 20 h. Axon length was measured from isolated neurons not in contact with other cells from two wild-type ($n = 381$ neurons) and three FAK-null ($n = 441$ neurons) mice. All neurons were used in the statistical analyses, but equal numbers (381) were used to plot the distribution of axons. The average length of axons from heterozygous mice ($16.68 \pm 0.37 \mu$ m) was not significantly different from that of axons in FAK knockout explants ($16.58 \pm 0.32 \mu$ m).



introduction of a dominant-negative Fyn also inhibited axon turning toward netrin-1 (Fig. 8f,g), indicating that Src family kinases can inhibit attraction by netrin. Thus, results from two distinct functional assays support a role for FAK in axon attraction by netrin-1.

DISCUSSION

Our biochemical and functional studies led to the findings that netrin induces FAK phosphorylation and that FAK is required for both the axon outgrowth-promoting activity and the axon-attracting activity of netrin-1. These results provide strong evidence that FAK is important in netrin signaling.

Our observation that netrin activates Fyn further indicates that proteins associated with FAK function are important for netrin function. Our functional data obtained with Src family kinase inhibitors are consistent with a role for the Src family kinases in FAK activation as well as in axon outgrowth and attraction induced by netrin-1 in mammalian neurons. The accompanying paper by Li *et al.* provides genetic evidence that Src is involved in controlling cell migration that is guided by UNC-5/netrin in *C. elegans*⁴⁶, whereas the accompanying paper by Ren *et al.* shows that the dominant-negative FAK mutant blocks netrin-induced turning of axons in *Xenopus laevis* spinal cord⁴⁷. Taken together, these results support an essential role for FAK and Src in netrin signaling in axon outgrowth, cell migration and axon attraction.

The strongest direct evidence that demonstrates a role for FAK in netrin signaling is the reduced axon outgrowth and axon attraction observed in explants of FAK-null mice in response to netrin-1

(Figs. 5–7). Previous studies have implicated PLC- γ , PI3K, Rac, Cdc42 and the MAP kinases in netrin function^{31–33,35,48}. The involvement of FAK in netrin signaling indicates that FAK may be a central component of the netrin pathway and that it may provide a link to other molecules that are important in cell migration. FAK is associated with Src family kinases and functions through downstream components including PLC- γ , PI3K, Akt, Rac, Cdc42, MAP kinases, paxillin, p130CAS, Crk and Grb^{38–41}. Although MAP kinases have been implicated in netrin-induced axon outgrowth³², we have not been able to detect netrin induction of MAP kinase phosphorylation (data not shown; see also ref. 46).

Because FAK participates in and regulates integrin signaling^{36–41}, our findings indicate that it may be interesting to investigate biochemical and functional relationships between netrin (and other neuronal guidance cues) and the integrins. We found it interesting that the FAK knockout did not inhibit initial axon outgrowth on laminin and PDL, at least at the early time points tested (18 h). This finding indicates that other kinases, such as Pyk2, may be involved in basal neurite outgrowth or that the role of FAK is more specific than previously thought. We should note, however, that our data establish only that netrin-1 requires FAK; they do not exclude the involvement of FAK in other signaling pathways. In fact, it is likely that FAK is involved in multiple pathways, as supported by the *in vivo* phenotype of FAK knockout mice⁴⁵.

Among the neuronal guidance cues, only ephrins are known to functionally require tyrosine phosphorylation, because their Eph

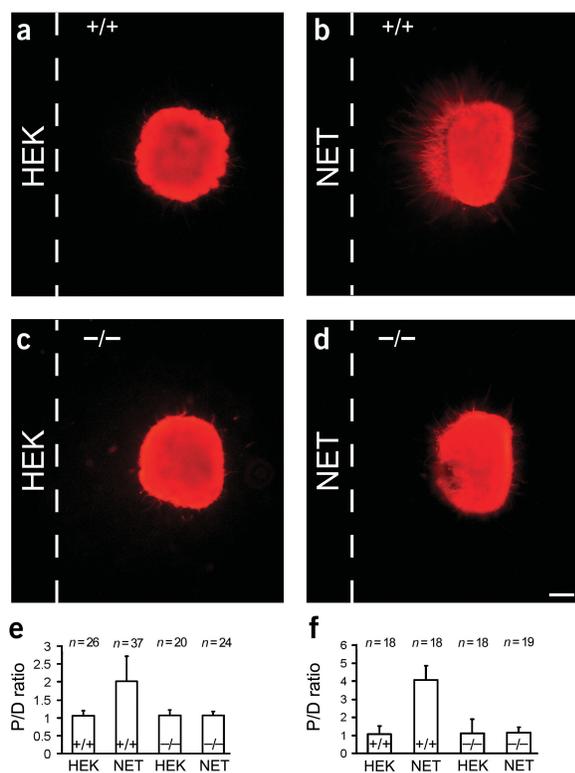


Figure 7 Loss of netrin attraction of cortical axons in FAK mutants. Cortical explants from E15 embryos were cultured with aggregates of control or netrin-1-secreting HEK293 cells for 16–18 h before being immunostained with TuJ1. **(a,b)** Wild-type explants (+/+) cultured with **(a)** control (HEK) or **(b)** netrin-1 (NET) aggregates. **(c,d)** FAK-null explants (-/-) cultured with **(c)** control or **(d)** netrin-1 aggregates (scale bar, 100 μ m). Note that the residual axons in FAK-null mutants did not grow asymmetrically toward the netrin source. **(e)** Proximal/distal (P/D) ratios of the numbers of axon bundles \pm s.d.: WT-HEK, 1.1 \pm 0.2; WT-NET, 2.0 \pm 0.7; FAK null-HEK, 1.1 \pm 0.2; FAK null-NET, 1.1 \pm 0.1 (n = number of explants). P values (Student's t -test): 0.004 between WT-HEK and WT-NET; 1.0 between FAK null-HEK and FAK null-NET; 0.9 between WT-HEK and FAK null-HEK; 0.004 between WT-NET and FAK null-NET. **(f)** P/D ratios of axon bundle length \pm s.d.: WT-HEK, 1.1 \pm 0.4; WT-NET, 4.1 \pm 0.8; FAK null-HEK, 1.1 \pm 0.3; FAK null-NET, 1.2 \pm 0.3. P values (Student's t -test): <0.001 between WT-HEK and WT-NET; 0.9 between FAK null-HEK and FAK null-NET; 0.9 between WT-HEK and FAK null-HEK; <0.001 between WT-NET and FAK null-NET.

receptors are tyrosine kinases. By contrast, all other neuronal guidance cues, including netrins, semaphorins and slits, function through receptors that do not contain known enzymatic motifs. All classes of neuronal guidance cues are now known to regulate the activities of Rho GTPases. There is therefore a possibility that some neuronal guidance receptors may function through the Rho GTPases without regulating protein tyrosine phosphorylation. For example, slit can induce its receptor to directly interact with a GTPase-activating protein⁴², whereas there is no evidence that slit regulates protein tyrosine phosphorylation.

The netrin receptors DCC and UNC-5 are single-transmembrane proteins without sequence similarities to tyrosine kinases or other motifs with known enzymatic activities^{1,16–23}. Because FAK is involved in attraction, it was not surprising to us that it could associate with DCC (Fig. 3 and refs. 46,47). We were, however, surprised to find that whereas DCC was required for netrin induction of FAK phosphorylation in the spinal cord, DCC was not required for netrin induction of FAK phosphorylation in the cortex (Fig. 3g). It is not clear whether this difference results from the presence of differentially spliced forms of neogenin or other membrane proteins. Recently, netrin has been found to induce phosphorylation of UNC-5 in primary neurons, and an activated viral Src can cause multiple phosphorylation of UNC-5 in cultured cells⁴⁹. Furthermore, phosphorylation of Tyr482 in UNC-5 is functionally important in axon guidance and cell migration⁵⁰. It seems that UNC-5 phosphorylation could be involved in both axon attraction and repulsion⁵⁰. In principle, activation of Fyn and FAK could be involved in regulating the activity of UNC-5 and DCC by phosphorylating these receptors or by relaying a signal to downstream components. The physiological relevance of one or both of these possibilities remains to be investigated.

Our results have clearly shown that netrin induces tyrosine phosphorylation of intracellular proteins. Specifically, phosphorylation of FAK and Fyn in primary neurons is induced by netrin-1. Furthermore, neuronal responses to netrin are inhibited in FAK conditional knockout mice (Fig. 5) and by blockers of the Src kinases (Fig. 4). These results establish an essential role for tyrosine kinases in netrin signaling.

Our studies have shown that netrin-1 activates a Src family tyrosine kinase, Fyn, in primary neurons and that specific Src kinase blockers inhibit both biochemical and functional activities of netrin. These results indicate that tyrosine phosphorylation by the Src family of protein tyrosine kinases mediates the function of netrin in promoting axon outgrowth. Because there are multiple Src-related kinases, it is perhaps not surprising that gene targeting of Src family kinases did not cause major defects in axon outgrowth or axon guidance. The Src kinase inhibitors PP2 and herbimycin A provide alternative tools to block multiple Src family kinases and thus uncover phenotypes that could be masked by compensation among different Src kinases. One needs to be cautious in interpreting results with pharmacological inhibitors. Our conclusion that Src family kinases are required for netrin function can only be reached after integrating the data not only from PP2 and herbimycin A inhibition results but also from the correlation of PP2 and herbimycin A inhibition of netrin-induced biochemical changes and their inhibition of netrin-induced axon outgrowth. In addition to the Src family kinases and PI3K, FAK is associated with multiple downstream components for its functions. We believe it will be important to determine which downstream targets of FAK are involved in netrin signaling.

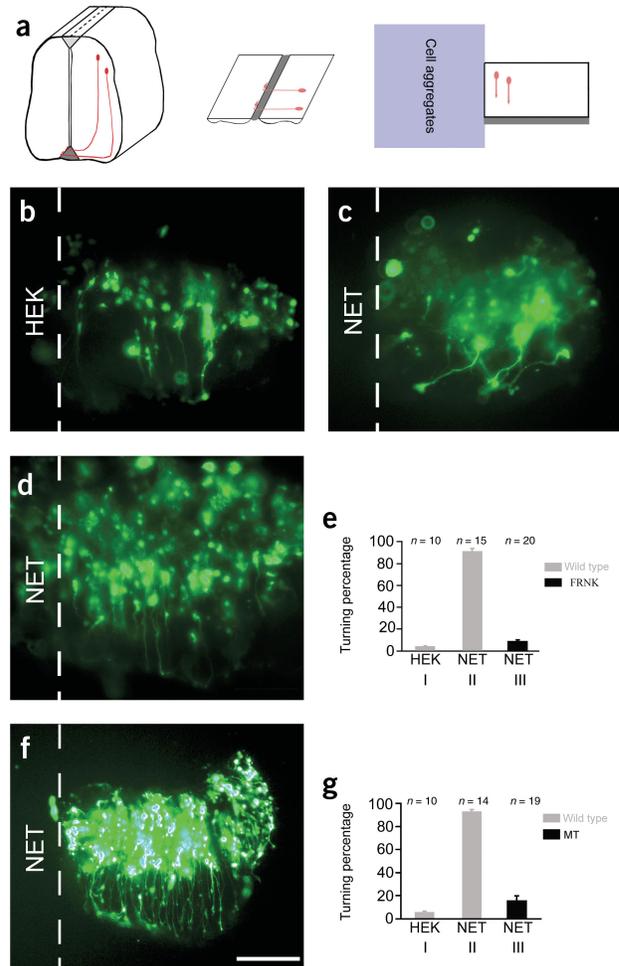
We and others^{46,47} have shown biochemical relationships among the Src family kinases, FAK and DCC: Src kinases and FAK can bind to DCC (Fig. 3a–g) in distinct domains^{46,47} and Src kinase activity is required for netrin induction of FAK phosphorylation (Fig. 2c and refs. 46,47). It remains to be determined whether the downstream components of Src kinases and FAK relay signals through integrins to c focal adhesion or through the Rho GTPases to actin polymerization and membrane protrusion.

METHODS

All experiments involving animals were approved by the Washington University Animal Studies Committee.

Materials. We used the following antibodies: anti-Src (Oncogene Science); anti-Fyn, anti-Lyn, anti-PYK2, anti-Abl, anti-actin, anti-TrkA, anti-Src, anti-FAK, anti-phospho-FAK and anti-netrin-1 (Santa Cruz); anti-FAK

Figure 8 Inhibition of netrin-induced turning of spinal cord axons by a dominant-negative FAK mutant and a kinase-dead Fyn mutant. **(a)** Schematic diagram of axon projection in a coronal section of the spinal cord of chick embryos and the coculture assay with the open book preparation. **(b)** Axons expressing Venus GFP and wild-type FAK projected straight toward the floorplate when cultured with a control aggregate of HEK293 cells (HEK) placed on one side of the preparation. **(c)** Axons expressing Venus GFP and wild-type FAK projected sideways toward an aggregate of HEK293 cells secreting netrin-1 (NET). **(d)** Axons expressing Venus GFP and FRNK projected straight, ignoring the aggregate of HEK293 cells secreting netrin-1. Scale bar, 100 μ m. **(e)** Quantification of axon turning in wild-type FAK and FRNK explants. The turning percentages were $4 \pm 1\%$ for control aggregates cultured with explants expressing wild-type FAK (group I), $90 \pm 3\%$ for netrin-secreting aggregates cultured with explants expressing wild-type FAK (group II) and $9 \pm 2\%$ for netrin-secreting aggregates cultured with explants expressing FRNK (group III; n = number of explants tested). P values (Student's t -test): <0.001 between groups I and II; <0.001 between groups II and III. **(f)** Axons expressing Venus GFP and the kinase-dead Fyn mutant were not attracted by the aggregate of HEK293 cells secreting netrin-1. **(g)** Quantification of axon turning in wild-type and kinase-dead Fyn mutants. The turning percentages were $5 \pm 2\%$ for control aggregates cultured with explants expressing Venus GFP (group I), $92 \pm 2\%$ for netrin-secreting aggregates cultured with explants expressing Venus GFP (group II) and $15 \pm 4\%$ for netrin-secreting aggregates cultured with explants expressing Venus GFP and the kinase-dead Fyn mutant (MT; group III; n = number of explants tested). P values: <0.001 between groups I and II; <0.001 between groups II and III.



(Transduction Labs.); 4G10 (Upstate Biotechnology); anti-myc 9E10 and anti-phospho-Src (Bioscience) and TuJ1 (Babco). Anti-phospho-TrkA antibodies and pharmacological reagents including genistein, PP2, PP3, herbimycin A, PD98059, SB203580 and LY294002 were obtained from Calbiochem. Chicken netrin-1 was from R&D systems. NGE, SU6656 and other reagents were purchased from Sigma. cDNA constructs for FAK and FRNK were generously provided by J. Guan (Cornell University).

Stable HEK293 cell lines were established with human netrin-1 cDNA transfection. Proteins were purified from conditioned medium by heparin affinity chromatography. The purified protein was analyzed by silver staining and immunostaining with anti-netrin-1. More than 90% homogeneity could be achieved as assessed by silver staining. The same procedure was carried out with the conditioned medium from control HEK293 cells, whose products were used as the sham-purification control.

Dissociated cultures. Embryos were removed from anesthetized timed-pregnant rats and mice of appropriate stages. Telencephalic vesicles, spinal cords or dorsal root ganglions were dissected in cold F12-DMEM medium (Gibco), and meninges were removed. The explants were chopped into small pieces with tungsten needles and then trypsinized for 15 min at 37 °C. After trituration, cells were resuspended in DMEM supplemented with heat-inactivated FCS (Gibco) and 20 U/ml of penicillin/streptomycin. Cells were grown on PDL-coated (100 μ g/ml) dishes overnight at 37 °C in a 5% CO₂ incubator. Cells were treated with several drugs or DMSO vehicle control for 30 min before netrin-1 stimulation.

For analysis of axon outgrowth from dissociated neurons, cortical neurons were isolated from heterozygous or FAK-null embryos and were plated on laminin-coated (20 μ g/ml) or PDL-coated (50 μ g/ml) cover slips (Fisher) and cultured for 18 h in 12-well plates at a concentration of 10⁴ cells in 2 ml of medium. Cortical neurons were cultured in Neurobasal medium (Invitrogen) supplemented with B27 (Invitrogen), 5% FCS, 0.5 mM glutamine, 30 mM glucose and penicillin/streptomycin at 37 °C with 5% CO₂. Cells were then fixed with 4% paraformaldehyde for 10 min, preincubated with blocking solution and stained overnight with TuJ1 monoclonal antibody (1:500) and rhodamine-phalloidin (Molecular Probes). Nuclei were visualized with Hoechst dye.

Western analysis. Cells were lysed with boiled 2× SDS sample buffer (375 mM Tris (pH 6.8), 60% glycerol, 12% SDS, 864 mM 2-mercaptoethanol, 0.05%

bromophenol blue) and were boiled for 5 min. Protein extracts were separated with 10% SDS-PAGE. Western blots were visualized with the enhanced chemiluminescence kit (Amersham). Some membranes were stripped with a buffer containing 100 mM mercaptoethanol, 2% SDS and 62.5 mM Tris-HCl (pH 7.6) and then were reprobed. All experiments were repeated at least three times with similar results.

Immunoprecipitation and kinase assays. Cortical cells were lysed with a modified RIPA buffer: 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.5% deoxycholic acid, 0.5% Triton X-100, 1 mM PMSF, 1 mM sodium orthovanadate and 1× protease inhibitor mixture (Roche Molecular Biochemicals). Lysates were immunoprecipitated with specific antibodies and protein A/G-agarose beads for 3 h or overnight at 4 °C. For the immunoblotting, the washed immunoprecipitates were boiled in 1× SDS sample buffer for 5 min. For the kinase assay, the immunoprecipitates were washed twice with kinase reaction buffer: 50 mM Tris-HCl (pH 7.4), 10 mM MgCl₂ and 0.1 mM sodium orthovanadate. The immune complex kinase assay was carried out with a Src assay kit (Amersham). Briefly, immune complexes were incubated in 30 μ l of kinase reaction buffer containing 10 μ Ci of [γ -³²P]ATP and a Src substrate peptide for 10 min at 30 °C. The reaction was terminated by adding 20 μ l of trichloroacetic acid, and the reaction solution was spotted onto P81 phosphocellulose paper. The papers were washed with 0.75% phosphoric acid five times before radioactivity was counted with a scintillation counter.

Explant cultures and analysis of axon outgrowth. Embryos of appropriate stages were removed from anesthetized timed-pregnant mice. Telencephalic

vesicles were dissected in cold F12-DMEM medium, and meninges were removed. Cortical explants were dissected out with thin tungsten needles. Explants comprised the whole thickness of the vesicles, and explants of similar size were selected under a dissecting microscope. Explants were transferred to culture dishes, and 35 μ l of mixed gel (3:2:1 collagen/Matrigel/medium) was layered over the explants. After polymerization, gels were covered with conditioned medium from control HEK293 cells or netrin-1-secreting HEK293 cells in the absence or presence of pharmacological reagents. Overnight cultures were carried out to observe the effect of netrin-1 on axon outgrowth. Cortical explant cultures were fixed with 4% paraformaldehyde in PBS and then were immunostained with TuJ1. The number and length of TuJ1-positive axons were measured from cortical explants with the NIH Image program.

Cocultures of cortical explants with HEK293 cells were similar to those described previously⁴⁴, with the exception that mice were used and that the explant size was smaller. Cortical explants from E15 mice embryos were dissected and trimmed into blocks of 200–300 μ m in thickness. The trimmed explants were cocultured with HEK293 or netrin-1 cell aggregates in mixture gel for 16–18 h.

Chicken spinal cord axon turning assay. White leghorn chicken embryos were collected and staged. For electroporation, 0.2 μ l of plasmid was injected into the neural tube of chicken embryos *in ovo* at stages 12–15. The plasmids were wild-type FAK (4 μ g/ μ l), FRNK (4 μ g/ μ l), the Venus variant of GFP (1 μ g/ μ l) and Kinase-dead Fyn mutant (4 μ g/ μ l). The electroporation program was 25 V, 5 ms, five pulses. At stages 20–21, embryos were dissected out and examined under a fluorescence microscope. This stage was chosen for analysis because the commissural axon has not made contact with the floorplate *in vivo*. Half of the spinal cord was electroporated with the plasmids and showed fluorescence. This half was isolated as an open-book preparation with the thickness of the slices. Each open-book preparation of the spinal cord was cultured with an aggregate of control or netrin-1-secreting HEK293 cells for 40 h⁴⁵. The percentage of turning axons was calculated from the number of fluorescent axons that turned toward the HEK293 cell aggregate divided by the total number of fluorescent axons within 300 μ m of the edge of the aggregate.

Note: Supplementary information is available on the Nature Neuroscience website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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