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Anne Briançon-Marjollet[1], Atefeh Ghogha[2], Homaira Nawabi[3], Ibtissem Triki[2], Camille Auziol[1], Sylvie Fromont[1], Chantal Piché[2], Hervé Enslen[6], Karim Chebli[4], Jean-François Cloutier[7], Valérie Castellani[3], Anne Debant*[1,5] and Nathalie Lamarche-Vane*[2,5]

*These authors contributed equally to this work

[1]CRBM , UMR- CNRS 5237, Université Montpellier I et II, IFR 122, Montpellier 34293, France

[2]McGill University, Department of Anatomy and Cell Biology, Montreal, Quebec, Canada H3A 2B2

[3]CGMC UMR-CNRS 5534, Université Claude Bernard Lyon1, 69622 Villeurbanne, France

[4]IGMM UMR-CNRS, Université Montpellier II, Montpellier 34293, France

[6]Unité mixte de recherche Santé UMR-S536, INSERM, Institut du Fer à Moulin, 75005 Paris, France

[7]Montreal Neurological Institute, McGill University, Dept. of Neurology and Neurosurgery, Montreal, Quebec, Canada H3A 2B4

[5]corresponding authors: Mailing address: Anne Debant, CRBM-CNRS, UMR5237, 1919 Route de Mende, 34293 Montpellier Cédex 05, France. Phone (33)-467-613-357; Fax (33)-467-521-559. Emails: anne.debant@crbm.cnrs.fr. Mailing address: Nathalie Lamarche, McGill University, Department of Anatomy and Cell Biology, 3540 University street, Montreal, Quebec, Canada H3A 2B2. Phone: 514-398-1784; Fax: 514-398-5047; email: nathalie.lamarche@mcgill.ca

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Abstract

The chemotropic guidance cue netrin-1 promotes neurite outgrowth through its receptor DCC (Deleted in Colorectal Cancer) via activation of Rac1. The guanine nucleotide exchange factor (GEF) linking netrin-1/DCC to Rac1 activation has not yet been identified. Here we show that the RhoGEF Trio mediates Rac1 activation in netrin-1 signaling. We found that Trio interacts with the netrin-1 receptor DCC in mouse embryonic brains, and that netrin-1-induced Rac1 activation in brain is impaired in the absence of Trio. Trio -/- cortical neurons fail to extend neurites in response to netrin-1, while they are able to respond to glutamate. Accordingly, netrin-1-induced commissural axon outgrowth is reduced in Trio -/- spinal cord explants and the guidance of commissural axons towards the floor plate is affected by the absence of Trio. The anterior commissure is absent in Trio-null embryos, and netrin-1/DCC-dependent axonal projections that form the internal capsule and the corpus callosum are defective in the mutants. Taken together, these findings establish Trio as a GEF that mediates netrin-1 signaling in axon outgrowth and guidance through its ability to activate Rac1.

Introduction

During the development of the central nervous system (CNS), axons are guided to their targets in response to molecular cues that can be either membrane-bound factors or secreted molecules, acting over short or long distances. The neuronal growth cone is a specialized structure found at the tip of the axon that integrates attractive and repulsive signals elicited by these extracellular cues and respond to them by triggering signaling pathways that regulate growth cone motility (16, 18). Netrins are a family of secreted proteins that control axon outgrowth and guidance in multiple vertebrate and invertebrate species (3). Netrin-1 is a bi-functional molecule that attracts and repels different classes of axons. In vertebrates, netrin-1 was first shown to attract commissural axons of the developing spinal cord towards the ventral midline (21, 44). Since then, netrin-1 has been shown to promote outgrowth of a wide variety of axons, including growing cortical axons (30, 41). Two families of netrin-1 receptors in mammals have been identified; the DCC (Deleted in Colorectal Cancer) family, comprising DCC and neogenin, and the UNC5 family of proteins (1, 20, 25). DCC mediates growth cone attraction induced by netrin-1 (1, 20, 25, 43) whereas the repulsive effect of netrin-1 is mediated by the UNC-5 family of netrin receptors, alone or in combination with DCC (17, 22, 35).

DCC is a transmembrane protein without any obvious catalytic activity in its intracellular domain, and for this reason, it was unclear until recently how the intracellular signaling machinery leading to axon outgrowth was initiated. This process has begun to be elucidated with the identification in cortical and commissural neurons of different DCC-binding proteins, including the protein tyrosine kinases FAK, Src and Fyn, the Nck adaptor protein, and PITP-α (26, 27, 30, 40, 48). DCC acts as a tyrosine kinase-associated receptor. It is phosphorylated by Fyn on tyrosine 1418 and this phosphorylation event is required for netrin-1-induced axon outgrowth (26, 34). In addition, various signaling cascades are believed to be important for netrin-1-induced axon outgrowth and guidance including the mitogen activated protein kinase (MAPK) and the

phosphatidylinositol pathways (3). Numerous lines of evidence have established that guidance cues also influence the motility of the growth cone by remodeling the actin cytoskeleton, through activation of the Rho family of GTPases (15). Small GTPases are molecular switches that oscillate between an inactive GDP-bound state and an active GTP-bound state, and are activated by guanine nucleotide exchange factors (GEFs) that accelerate the GDP/GTP exchange (42). Cellular and genetic studies have shown that Rac, Cdc42 and RhoG promote neurite extension and growth cone motility in response to guidance cues, while RhoA mediates neurite retraction through growth cone collapse (10). We and others have shown that the binding of netrin-1 to DCC activates the small GTPase Rac1 (28, 45), and that the adaptor protein Nck-1 is required for this activation (27). Rac1 activation is required for netrin-1-induced neurite outgrowth, but the GEF responsible for this activation has not yet been identified.

The multidomain protein Trio is the founding member of an intriguing family of GEFs that contains two GEF domains, with the first GEF domain (GEFD1) activating Rac1 and RhoG, and GEFD2 acting on RhoA (5, 6, 9). Genetic analysis of Trio orthologs in *C. elegans* (UNC-73) and in *D.melanogaster* (D-Trio) have established Trio as a key component in the regulation of axon guidance and cell migration (2, 4, 29, 36, 46). Functional analysis indicates that the role of Trio in all organisms mainly depends on the catalytic activity of GEFD1. Moreover, D-Trio, the kinase Abl, the Abl substrate Ena, and the netrin receptor Frazzled have been shown to regulate axon guidance at the CNS midline in *Drosophila* (14). In mammals, we have shown that human Trio is a component of the NGF pathway leading to RhoG and Rac1 activation and neurite outgrowth in PC12 cells (11). Moreover, targeted disruption of Trio in mouse resulted in embryonic lethality between E15.5 and birth, suggesting that Trio is required for late embryonic development, probably by playing essential roles in neural tissue and fetal skeletal muscle formation (37). However, the function of mammalian Trio in axon guidance remains unknown. In addition, the upstream signaling pathways leading to Trio activation in mammals are still unclear.

Here we provide evidence that Trio is a key component of netrin-1 signaling in growth cone guidance. We show that Trio and DCC interact in embryonic brain lysates and that this association is probably mediated through the interaction with the kinase PAK1. Netrin-1-induced Rac1 activation is abolished in Trio -/- embryonic brains. Cortical neurons are defective in extending neurites in response to netrin-1, while they respond to glutamate stimulation. Likewise, netrin-1-induced axon outgrowth is also reduced in Trio -/- spinal cord explants. Finally, netrin-1 and DCC-dependent neuronal projections in the developing spinal cord and in the brain, such as the anterior commissure, the internal capsule and the corpus callosum, are impaired in Trio-deficient mouse embryos.

Materials and Methods

DNA constructs

The GFP Trio constructs, GFP-RhoGA37, pRK5-DCC, pGEX4T2-DCC-C, pGEX2T-PAK1 and pRK5myc-PAK1 have been previously described (11, 24, 27, 28). The Nck-1 constructs have been generously provided by Dr. Louise Larose (McGill University, Montreal, Canada).

Genotyping of Trio-null mice

Ablation of the Trio gene in mice has been previously described (37). Trio heterozygous mice have been kindly provided by Dr. Michel Streuli. To obtain Trio-null embryos, female of Trio +/- Balb/C mice were crossed with Trio +/- males. Midday on the day after coitus was considered as embryonic day 0.5 (E0.5). Genomic DNA from embryo tails was prepared for genotyping using PCR method with specific oligonucleotides to detect the wt or the Trio-null allele as previously described (37).

Cell culture and transfection

Cortical neurons from E14.5 embryos were dissociated mechanically and plated on poly-L-lysine (25 µg/ml) treated coverslips at a density of 250,000 cells/well in 24-well dishes. Neurons were cultured for the indicated times in Neurobasal Medium (Invitrogen) supplemented with 1% B27 (Invitrogen). Neurons were transfected with GFP or GFP-Trio constructs using LipofectAMINE 2000 reagent (Invitrogen) according to manufacturer's instructions. Cells were treated as indicated with the following reagents: recombinant netrin-1 (250ng/ml, Sigma), glutamate (50µM, Sigma), blocking anti-DCC antibody (4µg/ml, Calbiochem). N1E-115 neuroblastoma cells, COS-7 cells, and HEK-293 cells were cultured and transfected as previously described (28).

Immunoprecipitation

Immunoprecipitation of proteins expressed in HEK-293 cells: HEK-293 cells expressing GFP-Trio and DCC were lysed in buffer containing 20 mM Hepes, pH 7.5, 100 mM NaCl, 10% glycerol, 1% Triton X-100, 20 mM NaF, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride

(PMSF), and complete protease inhibitor cocktail 1X (Roche Diagnostics). 1 mg of protein lysates were precleared with protein G–Sepharose beads at 4°C overnight. Then, the supernatants were incubated overnight at 4°C with 20 µl of protein G–Sepharose beads and 2.5 µg anti-DCC antibodies (BD Biosciences) or normal mouse IgGs. Beads were washed 3 times with ice cold lysis buffer, boiled in SDS sample buffer, and the protein samples were resolved by SDS-PAGE.

Immunoprecipitation of endogenous Trio and DCC proteins from mouse embryonic brains: Total brains of E18.5 mouse embryos were dissociated mechanically as described elsewhere (12, 31) before lysis in buffer containing 10mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% NP-40, 1 mM PMSF, 10 µg/ml aprotinin, and 10 µg/ml leupeptin. After centrifugation at 10 000g for 1 minute, 1 mg of the cleared protein lysates were incubated 3 hours with 2µg of normal rabbit IgGs or 2µg of anti-Trio antibody (H120, Santa Cruz Inc.), and with 20 µl of protein G–Sepharose beads. The samples were washed 3 times with ice cold lysis buffer, boiled in SDS sample buffer, and the protein samples were resolved by SDS-PAGE. The presence of Trio and DCC in the immunoprecipitates was revealed by western-blotting using the appropriate antibodies anti-DCC (BD Biosciences Inc.) and polyclonal anti-Trio (39).

GST Pull-Down

COS-7 transfected cells were lysed in buffer A (25 mM Hepes pH 7.5, 1% NP40, 10 mM $MgCl_2$, 100 mM NaCl, 5% Glycerol, 1 mM PMSF, protease inhibitor cocktail 1X). Lysates were then incubated for 2h at 4°C with 30µg of either GST, GST-PAK1, or GST-Nck-1 fusion proteins coupled to glutathione-sepharose beads. Total cell lysates and GST pull-down associated proteins were resolved by SDS-PAGE and transferred on nitrocellulose. Membranes were immunoblotted with the following antibodies: polyclonal anti-Trio (39), anti-Nck (BD Transduction Laboratories Inc.), anti-DCC (BD Biosciences Inc.), anti-GFP antibodies (Molecular Probes Inc), and anti-PAK1 antibodies (Santa Cruz).

Rac1 activation assay

Total brains of E14.5 mouse embryos were dissociated mechanically and one half of the brain remained untreated whereas the other half was treated with netrin-1 (500 ng/ml) for the indicated times similarly to the method described elsewhere (12, 31). When indicated, they were treated with blocking anti-DCC antibody (4 µg/ml, Calbiochem) for 10 minutes prior to netrin-1 stimulation. They were lysed in buffer A (see GST pull-down section) and lysates were then subjected to a 10000g centrifugation 30 seconds at 4°C, to remove insoluble materials. Active Rac1 was pulled-down by incubating the supernatants for 1h at 4°C with GST-PAK-PBD beads (Cytoskeleton Inc.). The beads were washed with 25mM Hepes pH 7.5, 1% NP40, 30 mM $MgCl_2$, 40 mM NaCl, 1 mM DTT, and resuspended in loading buffer. Protein samples from total cell lysates and from the GST pull-downs were resolved by SDS-PAGE and transferred on nitrocellulose. Membranes were immunoblotted with anti-Rac1 antibody (BD Transduction Laboratories Inc).

Immunofluorescence

Neurons were fixed and permeabilized as previously described (11). GFP-expressing cortical neurons were visualized using a DMR Leica microscope and a 40x PL APO lens. N1E-115 cells were fixed and permeabilized as already described (28). The cells were examined with a Axiovert 135 Carl Zeiss microscope using a 63X Plan-neofluor objective lens. Images were recorded with a digital camera (DVC) and analyzed with Northern Eclipse software (Empix Imaging Inc.).

Neurite outgrowth analysis

More than 100 cortical neurons were analyzed for each condition. For each neuron, the number of neurites was counted manually, and the length of the neurites was measured using MetaMorph and NeuronJ softwares (33) modified by Volker Backer, Montpellier RIO-Imaging (unpublished data). In N1E-115 cells, a neurite was defined as a process that measured at least the length of one cell body.

Explant assays

Mouse dorsal spinal cord explants from wt, Trio +/- or -/- embryonic day 11.5 (E11.5) embryos were dissected and cultured in three-dimensional collagen type I (BD Biosciences) gels as described previously (47). Recombinant chick netrin-1 protein was produced and purified as described (44). Netrin-1 (500 ng/ml) was added to the culture medium at the beginning of the culture period. Images were captured after 35 h with a digital camera on a Carl Zeiss axiovert 135 microscope using a 10X phase-contrast objective lens. The total length of the axon bundles and number of axons growing out of the explants were quantified using Northern Eclipse Software (Empix imaging). The experiments were performed in a blinded fashion.

Spinal cord immunohistochemistry and brain histology

Eosin staining and immunohistochemistry with antibodies to Nrp2 (1:100, R&D) and to DCC (1:100, BD Biosciences) were performed on horizontal or coronal 70 µm thick vibratome sections from E17 or E18.5 brains as previously described (12). Commissural axon projections were detected by immunohistochemistry using anti-DCC antibodies (1: 200, BD Biosciences) on 20µm thick cryostat transverse sections from E11.5 embryos as described previously (38). Quantification analysis of axon defasciculation in Trio -/- embryos was performed on sections positioned at the forelimb of wild type and Trio -/- embryos.

Results

A Trio mutant defective in Rac1 activation inhibits DCC-induced neurite outgrowth in N1E-115 neuroblastoma cells

The chemotropic guidance cue netrin-1 attracts different types of neurons and activates the GTPase Rac1 through its receptor DCC. However, the GEF responsible for Rac1 activation remains unknown. We have previously demonstrated that netrin-1 but not DCC is constitutively expressed in N1E-115 neuroblastoma cells (28). The expression of DCC in these cells induces neurite outgrowth in a netrin-1- and Rac1-dependent manner. To determine whether Trio mediates netrin-1/DCC-induced neurite outgrowth, N1E-115 cells were co-transfected with DCC, and either Trio, Trio 1-2308 lacking the kinase domain, or TrioAEP, a dominant negative form of Trio containing triple point mutations in the GEFD1 and the adjacent SH3 domain, which drastically reduces its *in vitro* exchange activity towards RhoG/Rac1 (11) (Fig. 1A). Trio and Trio1-2308 were able to induce neurite outgrowth in N1E-115 cells, either alone or together with DCC. However, the expression of TrioAEP with DCC blocked the ability of DCC to induce neurite extension (Fig. 1B), suggesting that Trio mediates netrin-1/DCC-induced neurite outgrowth in N1E-115 cells. The GEFD1 domain of Trio has been previously shown to be active on both Rac1 and RhoG small GTPases (6). To determine whether RhoG is involved in netrin-1/DCC-induced neurite outgrowth, dominant negative RhoGA37 was expressed together with DCC in N1E-115 cells. RhoGA37 did not inhibit DCC-induced neurite outgrowth (Fig. 1B), in contrast to RacN17, which blocked DCC effect on neurite extension (28). Therefore, these data strongly suggest that Trio plays a role in netrin-1/DCC-regulated neurite outgrowth via Rac1 and not RhoG.

Interaction of DCC and Trio

To determine whether Trio interacts with DCC, GFP-Trio was co-expressed with DCC in HEK-293 cells that do not express endogenous DCC (28, 45). We found that Trio was able to

interact with DCC, with no band corresponding to Trio in immunoprecipitates using normal mouse IgGs (Fig. 2A). We next determined whether endogenous Trio and DCC proteins associate in mouse embryonic brains. DCC was detectable in Trio immunoprecipitates from embryonic brain lysates, which was not the case when using normal rabbit IgGs for the immunoprecipitation (Fig. 2B). Stimulation of embryonic brains with netrin-1 did not significantly enhance Trio/DCC association (data not shown), suggesting that Trio binds constitutively to DCC.

To characterize the interaction between Trio and DCC, we examined whether Trio associates with DCC via the adaptor protein Nck-1 and the serine/threonine protein kinase PAK1. Indeed, Rac, PAK, and DOCK, the *Drosophila* ortholog of Nck, interact genetically with D-Trio during axon guidance of *Drosophila* photoreceptors (36). Furthermore, PAK1 is known to interact with the second SH3 domain of Nck-1 (8) whereas the cytoplasmic domain of DCC interacts with the first and third SH3 domains of Nck-1 (27). We performed GST-pull down experiments using either recombinant GST-Nck-1 (Fig. 2C) or GST-PAK1 (Fig. 2D) and lysates from COS-7 cells expressing GFP-Trio, DCC, Nck-1, PAK1, alone or together. Interestingly, we found that Trio alone interacted with PAK1 but not with Nck-1, whereas DCC was able to interact with both proteins (Fig. 2C and D). When PAK1 or DCC were co-expressed with Trio, Trio was now detected in the GST-Nck-1 pull-down (Fig. 2C). Finally, when DCC, Trio, PAK1 and Nck-1 were expressed together, they were all detected in the GST-Nck-1 or GST-PAK1 pull-downs (Fig. 2C and D), suggesting that Trio may associate with DCC and Nck-1 indirectly via its interaction with PAK1.

To characterize further the interaction of Trio with PAK1, GST-PAK1 pull-down were performed with COS-7 cells expressing various deletion mutants of Trio (Fig. 1A). As shown in Fig. 2E, Trio (1-1813) and Trio (1-1203) were able to interact with GST-PAK1, whereas Trio (1203-1813) showed no interaction. Trio (1813-3038) was also detected in the GST-PAK1 pull-down although the interaction appeared much weaker than with Trio (1-1813). These results

suggest that the N-terminus extremity comprising the Sec-14 and the spectrin domains of Trio (1-1203) but not the GEFD1 or the first SH3 domains of Trio (1203-1813) mediates the interaction with PAK1. Additionally, a second region of Trio that contains the GEFD2, the second SH3, and the kinase domains of Trio (1813-3038) is capable of interacting with PAK1, although to a lesser extent. Thus it seems that Trio, which is a large protein of 334 kDa with multiple signaling domains has at least two separate regions that are able to mediate the interaction with PAK1.

Netrin-1-induced Rac1 activation is abolished in Trio -/- embryonic brains

Netrin-1 binding to its receptor DCC has been shown in different cellular systems to induce a rapid and robust Rac1 activation, leading to neurite outgrowth (28, 45), but this has never been tested on endogenous Rac1 expressed in brain. We found that netrin-1 addition to wild type (wt) embryonic mouse brains induced a rapid Rac1 activation with a peak at 5 minutes of stimulation (Fig. 3A). Netrin-1 activation of Rac1 occurred through DCC, as a blocking DCC antibody was able to suppress netrin-1-induced Rac1 activation in embryonic brains (Fig. 3B). We then tested whether the absence of Trio affects netrin-1-induced Rac1 activation, by measuring netrin-1 effect in embryonic brains from Trio -/- mice. As shown in Fig. 3A, netrin-1 failed to activate Rac1 in Trio-null embryos, consistent with Trio being the GEF responsible for netrin-1-induced Rac1 activation through its receptor DCC.

Trio is necessary for netrin-1-induced axon outgrowth in cortical neurons

Netrin-1 has been described as an attractant for axons of cortical explants and dissociated cortical neurons, and DCC has been shown to be expressed in developing cortical neurons (30, 40, 41). To address whether Trio is required for netrin-1 to induce axon outgrowth of cortical neurons, we tested the effect of adding netrin-1 to wt or Trio-null dissociated cortical neurons. As shown in Fig. 4A, netrin-1 stimulated axon outgrowth of wt cortical neurons after 24 hours in culture. This

effect was mediated by the netrin-1 receptor DCC since a blocking DCC antibody completely abrogated netrin-1 effect (Fig. 4B). In contrast, netrin-1 failed to stimulate axon outgrowth of Trio-null cortical neurons (Fig. 4A and B). Consistently, analysis of the distribution of axon length in cortical neurons showed that netrin-1 significantly increased the percentage of long axons (> 48µm) in wt but not in mutant neurons (Fig. 4C). The slight increase in the percentage of intermediary axons (16-32 µm) observed for the mutant neurons treated with netrin-1 was not significant (Fig. 4C). The lack of netrin-1 response of Trio-deficient neurons was not due to a defect in DCC expression as both wt and mutant cortical neurons expressed similar levels of DCC proteins (data not shown). We also observed that the average axon length of Trio-null neurons in the absence of netrin-1 was reduced by 20% compared to wt neurons (Fig. 4B). Nevertheless, Trio -/- neurons were able to extend neurites in response to glutamate (Fig. 4A and B), which has been shown to stimulate growth cone motility by different pathways including Ca^{2+}-dependent activation of Rho GTPases (19, 23, 49). Eventhough the average axon length of Trio-null neurons was lower than the one of wt neurons (Fig. 4B), the ratio of glutamate-induced axon outgrowth versus control was similar in both types of neurons (wt =1.25, -/- = 1.4), while this was not the case when comparing the ratio of netrin-1-induced outgrowth versus control between wt and mutant neurons (wt =1.48, -/-=1). Analysis of the distribution of the axon length showed that glutamate stimulation of wt and mutant neurons induced in both cases the growth of long axons and significantly reduced the percentage of very short axons (Fig. 4C). These data show that Trio-null cortical neurons are able to induce axon extension in response to glutamate, while they are specifically defective in netrin-1-induced axon outgrowth.

Netrin-1-induced axon outgrowth is reduced in Trio -/- dorsal spinal cord explants

To further demonstrate the involvement of Trio in netrin-1/DCC-induced axon outgrowth, we added netrin-1 to dorsal spinal cord explants dissected from E11.5 Trio -/- embryos. As shown

in Fig. 4D, explants from wt dorsal spinal cords, treated for 35h with netrin-1, showed a robust axon outgrowth compared to untreated controls. In contrast, when explants from Trio -/- dorsal spinal cords were cultured in the presence of netrin-1, commissural axon outgrowth showed a 56% reduction compared to wt explants (Fig. 4D and E). To determine that the reduced response of Trio -/- spinal cord explants was not due to a general defect in axon outgrowth, wt or Trio -/- explants were cultured for 70h in the absence of netrin-1. As shown in Fig. 4E, both wt and Trio -/- explants were able to produce axon outgrowth in a netrin-1-independent manner. These findings demonstrate that Trio is required for netrin-1 to promote commissural axon outgrowth.

Trio-deficient mouse embryos show defects in spinal cord and brain development

To determine the role of Trio in netrin-1 function *in vivo*, we next examined the axonal projections of the commissural neurons in the spinal cord of wt and Trio-null littermates by immunostaining with anti-DCC antibodies (Fig. 5A). In wt embryos, commissural axons are directed ventrally towards the floor plate of the developing spinal cord, which secretes the chemoattractant netrin-1 (Fig. 5A and B) (21) . In Trio -/- embryos, commissural axons could reach the floor plate but they appeared defasciculated in the ventral spinal cord (Fig. 5 A-F), suggesting that Trio plays a role in the guidance of these axons. In addition to defects in the developing spinal cord, the netrin-1 and DCC-null mice also present defects in several projections of the brain, namely the anterior commissure, the hippocampal commissure, the corpus callosum and the thalamo-cortical reciprocal projections in the internal capsule (13, 43). Therefore, we examined the anterior commissure in sections of Trio-null brains (Fig. 6). Serial horizontal sections of the whole brain were analyzed, and while anterior and posterior branches forming the anterior commissure were present in the wt sections, they were totally absent in the homozygous mutant embryos in all sections tested (Fig. 6A, compare a to c). Interestingly, the heterozygous mutant embryos presented an intermediate phenotype, as the anterior branch of the commissure could form but was highly

defasciculated, with several roots exiting the cortex at lateral positions (Fig. 6A, b). To confirm that the anterior commissure was absent in Trio -/- embryos, we analyzed different coronal sections of Trio +/- and -/- embryonic brains. As shown in Fig. 6B, defasciculated fibers were present in the heterozygous embryos but were completely absent in the Trio-null embryos (compare 6B a, b to d, e). Thus, similar to DCC and netrin-1, Trio is required for the formation of the anterior commissure.

We next examined the corpus callosum in Trio-null brains. We observed subtle disorganizations of the Trio -/- corpus callosum in horizontal sections, with a few defasciculated fibers being visible (Fig. 7A). However, the reconstruction of the tract of the corpus callosum with horizontal sections revealed that Trio -/- corpus callosum thickness was decreased by 35% compared to wt corpus callosum in the dorso-ventral axis (Fig. 7A). Therefore, the corpus callosum is abnormal in Trio-deficient mice. Finally, we examined the organization of axon projections in the internal capsule in horizontal brain sections. Consistent with the defects observed in netrin-1 mutant mice (7), DCC staining revealed that the internal capsule was strongly disorganized in Trio -/- mice. The axonal projections formed a parallel array of fibers in the wt internal capsule, but not in the Trio -/- mice, where they formed irregular and intermingled bundles (Fig. 7B). The defects observed in the Trio-null embryos were not due to a problem with the expression of netrin-1 since both in wt and in Trio-null embryos netrin-1 had the same expression pattern (data not shown). Thus, these results indicate that Trio plays a significant role in netrin-1/DCC-dependent projections in the developing spinal cord and brain.

Discussion

The findings presented here support a role for the RhoGEF Trio in axon outgrowth and guidance in mammals. We show that Trio and DCC interact in embryonic brain, most likely independently of netrin-1. Co-expression of DCC and Trio with Nck-1 and PAK1 suggests that Trio/DCC interaction probably occurs via the interaction of Trio with PAK1. Furthermore, the N-terminus region of Trio comprising the Sec-14 and the spectrin domains mediates the interaction with PAK1. However, it still remains to be determined whether the interaction between Trio and PAK1 is direct. Altogether, these data are consistent with the results obtained in *D. melanogaster* where D-Trio genetically interacts with DOCK, PAK, and Rac in controlling axon guidance of photoreceptors (36). Since Nck-1 binds to DCC through its first and third SH3 domains (27), and PAK1 binds to the second SH3 domain of Nck-1 (8), it is tempting to postulate that a cascade of molecular events implicating Nck-1/PAK interaction serves to bridge Trio to DCC. The mechanisms by which Trio becomes activated when netrin-1 binds to DCC remain unknown but may involve phosphorylation by FAK or the Src family kinase Fyn (32, 34).

We show here for the first time that netrin-1 treatment of embryonic brains stimulates Rac1 activity. This Rac1 activation is completely abolished in the absence of Trio, suggesting that Trio-related kalirin does not compensate for the lack of Trio in brain. Trio also activates nucleotide exchange on both RhoG and Rac1 through its first GEFD1 domain (6). Unfortunately, we could not determine whether netrin-1 is able to stimulate RhoG activity in mouse brains because of the lack of specific anti-RhoG antibodies. However, altering the specific RhoG pathway did not inhibit DCC-induced neurite outgrowth in N1E-115 cells, suggesting that it is unlikely that RhoG mediates DCC-induced Rac1 activation.

We have previously shown that human Trio plays a role in NGF-induced neurite outgrowth (11), but the role of mammalian Trio in axon outgrowth and guidance remained poorly characterized. We took advantage of the Trio-null mice to examine the axon outgrowth and

guidance of Trio-deficient neurons. Our findings argue for a specific role of the GEF Trio in axon outgrowth induced by netrin-1. The cortical neurons of the Trio-null mice are defective in extending neurites in response to netrin-1, while they are able to extend neurites in response to glutamate, which has been proposed to act through different signaling pathways, including Ca^{2+}-dependent activation of Rho GTPases (19, 23, 49). Likewise, commissural axon outgrowth from Trio -/- spinal cord explants is also reduced in response to netrin-1, while netrin-1-independent outgrowth is not affected. These data show that Trio-null neurons are not completely defective in neurite outgrowth but are specifically impaired in their axon response to netrin-1.

Netrin-1 and DCC-null mice present several defects in the developing spinal cord and brain commissures (13, 43). We have compared these defects with those observed in the Trio-null mice (Table 1). Interestingly, Trio -/- embryos show defects in the anterior commissure that are more severe than those observed in DCC- and netrin-1-deficient mice, suggesting that Trio plays a prominent role in brain morphogenesis. In addition, the observations that Trio heterozygous mutant embryos present an intermediate phenotype with defasciculation of the axons of the anterior commissure suggest that Trio is involved not only in netrin-1-induced axon outgrowth but also in guidance. The spinal commissural axons of the netrin-1- and DCC-mutant mice also show major deviations from normal trajectories only in the ventral part of the spinal cord. In the absence of Trio, the phenotype is milder, but the axon bundles are clearly defasciculated when approaching the floor plate, showing that Trio plays a role in the netrin-1-dependent pathfinding of commissural axons. Similarly, Trio contributes to the guidance of cortico-cortical projections along the corpus callosum, although corpus callosum defects appear milder in Trio -/- embryos than in netrin-1 or DCC mutant mice. In the case of the corpus callosum, one could speculate that two populations of axons can be differentiated among the cortical axons projecting along the corpus callosum, one population in which netrin-1 response is totally dependent on Trio and would thus be defective in Trio -/- brains, and another population that is not dependent on Trio and thus could project

normally. Alternatively, Trio could be partially redundant with another protein in all these neurons. In addition to cortico-cortical projections, cortical axons also project to sub-cortical targets, including the thalamus and the spinal cord. These sub-cortical projections navigate in the internal capsule, in which also extend reciprocal thalamic projections in route towards the cortex. Netrin-1 has also been implicated in both cortico-thalamic and thalamo-cortical axon guidance (7, 41). Interestingly, we have detected a disorganization of the internal capsule in Trio -/- brains, which is also observed in netrin-1-deficient mice, supporting the hypothesis that Trio is implicated in both reciprocal pathways. In conclusion, the defects presented in the Trio -/- mice are observed in the same classes of axons that are affected in netrin-1 and DCC-deficient animals, eventhough the phenotypes are generally milder, except for the anterior commissure (Table 1).

Much evidence now suggests that several intracellular pathways, including MAPK, phosphatidylinositol signaling, tyrosine phosphorylation, and activation of Rac1, act in concert to mediate the response of axons to netrin-1 (3). Therefore, it is not surprising that the *in vivo* phenotypes of the Trio-null embryos do not reproduce exactly those seen in the netrin-1 and DCC-null mice because the Trio-null embryos are defective in Rac1 activation, but not in the other netrin-1-activated signaling pathways. Future studies will help to define how these signaling pathways are interconnected in order to achieve a directed response of axons to netrin-1.

In conclusion, our study shows that Trio mediates netrin-1/DCC-induced Rac1 activation and that the role of mammalian Trio in axon guidance reflects the conserved signaling mechanisms involved in neural development throughout evolution.

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Figure Legends

Figure 1: Trio defective in Rac1 activation inhibits DCC-induced neurite outgrowth in N1E-115 neuroblastoma cells.

(A) Schematic of Trio and Trio mutant proteins. TrioAEP is a dominant negative form of Trio, and corresponds to Trio (1-2308) containing triple mutations (stars) in the GEFD1 and adjacent SH3 domain. DH: Dbl homology; PH: Pleckstrin homology; SH3: Src homology 3, Ig: Immunoglobulin g (B) N1E-115 cells were transfected with the indicated plasmids and cells exhibiting neurite outgrowth were counted 24 hours after transfection. The values correspond to the average of at least three independent experiments. Error bars represent standard deviation (SD).

Figure 2: Interaction of DCC and Trio

(A) Lysates of HEK-293 cells transfected with pEGFP-Trio and pRK5-DCC were submitted to immunoprecipitation (IP) using anti-DCC antibodies (DCC) or mouse Immunoglobulin G (IgG) coupled to protein G-sepharose beads. Immunoprecipitated proteins (IP) and 10% of the total cell lysates (TCL) were submitted to SDS-PAGE, and GFP-Trio and DCC were detected by western blotting using anti-DCC and anti-GFP antibodies. (B) Lysates of E18.5 mouse brains were submitted to immunoprecipitation (IP) using anti-Trio or normal rabbit IgGs coupled to protein G-Sepharose beads. Immunoprecipitated proteins and 10% of the total cell lysates (TCL) were submitted to SDS-PAGE, and the presence of DCC and Trio was detected by western blotting using the appropriate antibodies. (C and D) Lysates of COS-7 cells transfected with pRK5 (EV), pRK5-DCC, pEGFP-Trio, pRK5-HA-Nck-1 or pRK5myc-PAK1, alone or as indicated, were incubated with GST, GST-Nck (C) or GST-PAK (D). GST pull-down proteins (pull-down) and 10% of the total cell lysates (TCL) were submitted to SDS-PAGE, and proteins were detected by Western blotting analysis using anti-DCC, anti-GFP, anti-PAK1 and anti-Nck-1 antibodies. (E)

Lysates of COS-7 cells transfected with pEGFP-Trio (1-1813), -Trio (1813-3038), -Trio (1-1203) or -Trio (1203-1813) were incubated with GST or GST-PAK. GST pull-down proteins (pull-down) and 8% of the total cell lysates (TCL) were submitted to SDS-PAGE, and proteins were detected by Western blotting analysis using anti-GFP antibodies.

Figure 3: Netrin-1-induced Rac1 activation is impaired in Trio null-embryonic brains

(A) GTP-loaded Rac1 was pulled-down using GST-PAK-PBD from lysates of +/+ or Trio -/- embryonic brains treated or not with netrin-1 for the indicated time. Upper panel, GTP-bound Rac1 was detected by western blotting using anti-Rac1 antibodies. Lower panel, total cell lysates probed for Rac1 indicated equal amounts of GTPase. Quantification of Rac1 activity corresponds to the average of at least three independent experiments. P-value < 0.01. (B) As in A, except that DCC blocking antibodies (4 µg/ml) were added before netrin-1 stimulation (5 minutes). Mouse anti-GFP antibodies were used as a negative control and had no effect on netrin-1-induced Rac1 activation. Quantification of Rac1 activity corresponds to the average of at least three independent experiments. P-value = 0.012 (Student T-Test). For (A) and (B) panels, error bars represent SD.

Figure 4: Trio is required for netrin-1-induced axon outgrowth

(A) Neurite outgrowth of +/+ or Trio -/- cortical neurons expressing GFP at DIV 1.5 treated with control buffer, netrin-1 or glutamate for 24 hours. Scale bar, 25µm. (B) Quantification of average axon length of cortical neurons presented in panel A. Values are represented as a percentage of average axon length of wt cortical neurons at DIV 1.5 incubated with control buffer. When indicated, neurons were incubated with control Igs or DCC blocking antibodies before netrin-1 addition. All P values are < 0.001. ** represents a comparison to wt neurons expressing GFP, except for the dotted line that refers to GFP transfected Trio-null neurons. n=8 for +/+ and n=10 for -/- embryos. (C) Distribution of axon length from panel B. (D) E11.5 dorsal spinal cord explants

from +/+ or Trio -/- embryos were incubated with control buffer or netrin-1 for 35h. Scale bar, 100µm. (E) Quantification of the average length of axon bundles per explant after 35h incubation with netrin-1 (n= 10 for Trio +/+, n=4 for Trio -/-. **, $p<0.001$) or after 70h in the absence of netrin-1 (n=3 for +/+ and -/-).

Figure 5: Commissural axon projections are defective in Trio-null embryos

(A) Upper panels: Trajectories of commissural axons are visualized using anti-DCC antibodies in sections of +/+ or Trio -/- E11.5 embryos. Lower panels: Enlargement of the corresponding images. Scale bar, 80µm. (B) Left: schematic representing normal commissural axons that project from the dorsal spinal cord towards the ventral floor plate. Right: In Trio -/- embryos, commissural axons are defasciculated when they reach the ventral floor plate (see arrows in A and B). (C and D) The thickness of axon bundles in the dorsal and ventral spinal cords was quantified by measuring the width of the DCC-stained axons (red) relative to the width of the spinal cord (blue) as depicted in (D). (E and F) Axon defasciculation in Trio -/- embryos was quantified by measuring DCC-stained area (red) relative to the total area of the spinal cord (blue) as depicted in (F). $p<0.001$, Student T-Test. Error bars represent SD. (n=5 for +/+ and n=7 for -/-).

Figure 6: The anterior commissure is absent in Trio-null embryos

(A) Neuropilin2 (Nrp2) immunostaining on horizontal serial brain sections from E17 +/+ (a), +/- (b) or -/- (c) embryos (n=6 for +/+ and -/-, n=4 for +/-). In the heterozygous embryos, the anterior branch of the commissure (AC) is defasciculated, which is illustrated by several roots exiting the cortex at lateral positions (see arrows in b). In the Trio-null embryos, the commissure is absent (c). Scale bar, 80µm. (B) Eosin staining on coronal brain sections from E17 +/- (a, b, c) and -/- (d, e, f) embryos. Defasciculated fibers are present in the heterozygous embryos (b, white arrows), whereas

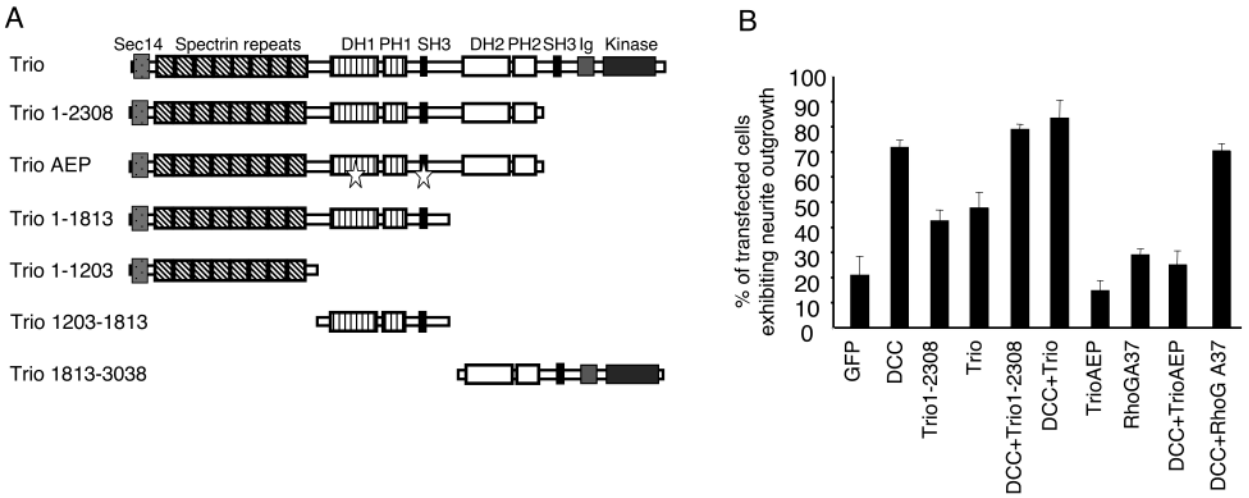
they are absent in the Trio-null embryos (e). In more posterior sections, anterior commissural fibers are detected in the heterozygous (c, black arrows) but not in Trio-null brains (f). Scale bar, 300µm.

Figure 7: Defects in the corpus callosum and internal capsule in Trio-null embryos

A) DCC immunostaining on horizontal brain sections from E18.5 +/+ and Trio -/- embryos showing the corpus callosum (CC) region. In the Trio-null embryos, the corpus callosum appears slightly abnormal with some defasciculated fibers (arrows) in the horizontal sections. The right panel represents the quantification of corpus callosum thickness along the dorso-ventral axis in +/+ and Trio -/- embryos. Quantification has been obtained by counting the number of horizontal sections in which the corpus callosum is present divided by the total number of sections. The corpus callosum thickness of the Trio-null embryos is expressed relative to the thickness of the wt corpus callosum along the dorso-ventral axis. n=5 for +/+ embryos, n=8 for Trio -/- embryos. P-value<0.05. Scale bar, 50µm. B) DCC immunostaining on horizontal brain sections from E18.5 +/+ and Trio -/- embryos showing the internal capsule (IC) region. DCC positive fibers are clearly disorganized in the internal capsule of Trio -/- embryos. Two different examples are shown. This defect was observed in 8 out of 9 Trio -/- embryos. Scale bar, 50µm.

Table 1: Comparison of the phenotypes observed in the Trio-null embryos with netrin-1- and DCC-deficient embryos

Four different netrin-1 and DCC-dependent neuronal projections were examined in the Trio-null embryos, namely the spinal commissural axon projections, the anterior commissure, the corpus callosum and the internal capsule. The figure depicts the comparison between the phenotypes observed in Trio-, netrin-1-, and DCC-deficient embryos according to (7, 13, 43). WT: wild-type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Trio AEP
Trio 1-1813
Trio 1-1203
Trio 1203-1813
Trio 1813-3038
B
% of transfected cells exhibiting neurite outgrowth
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90
80
70
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40
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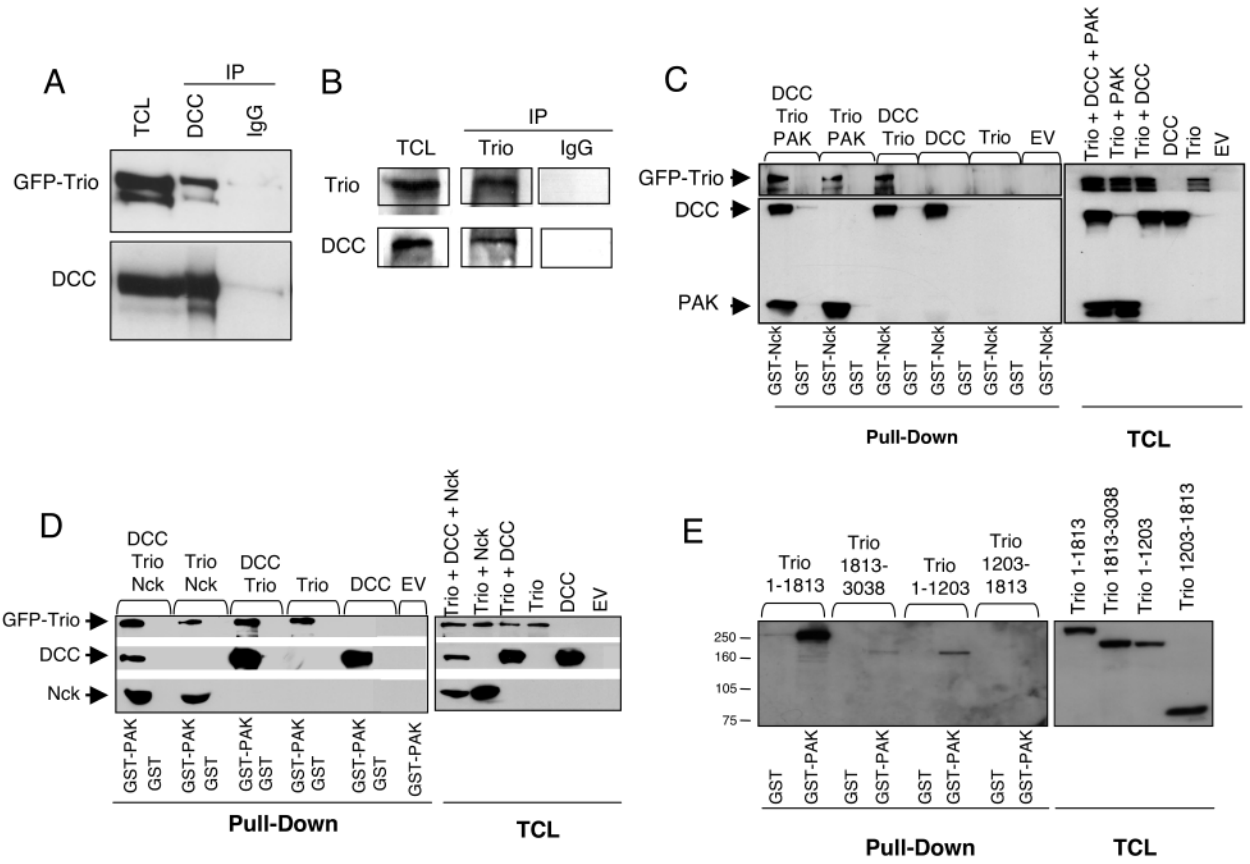
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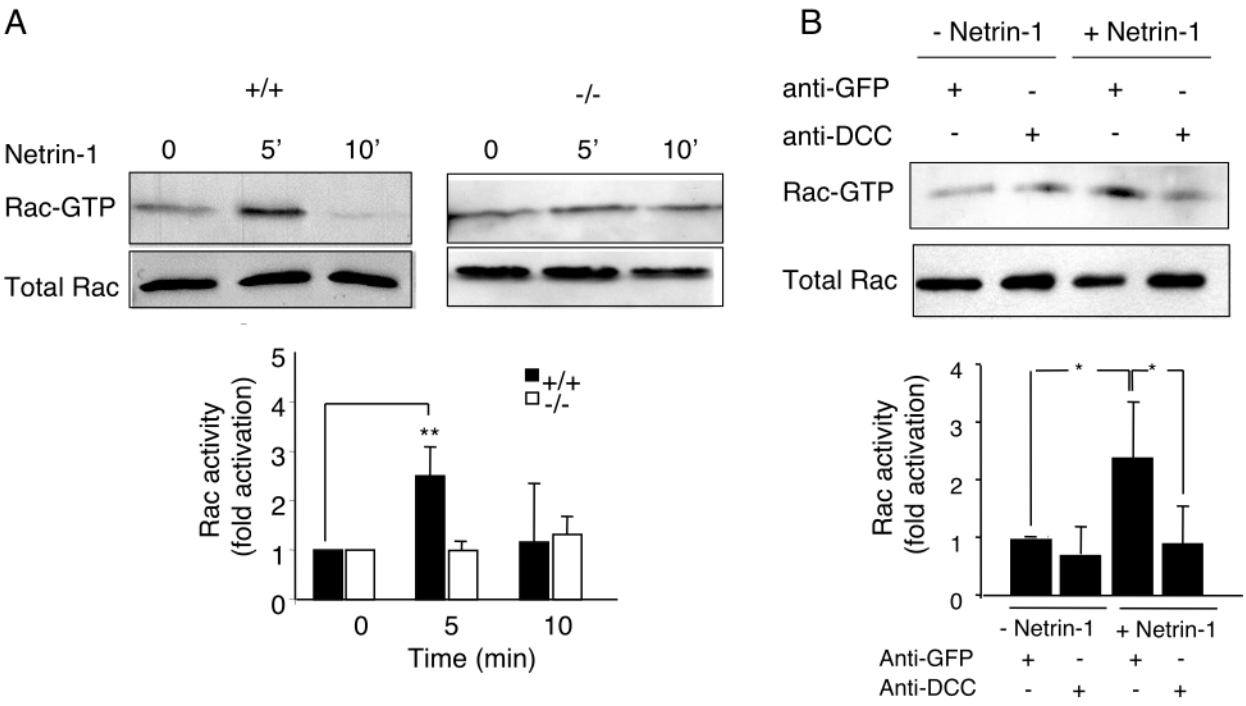
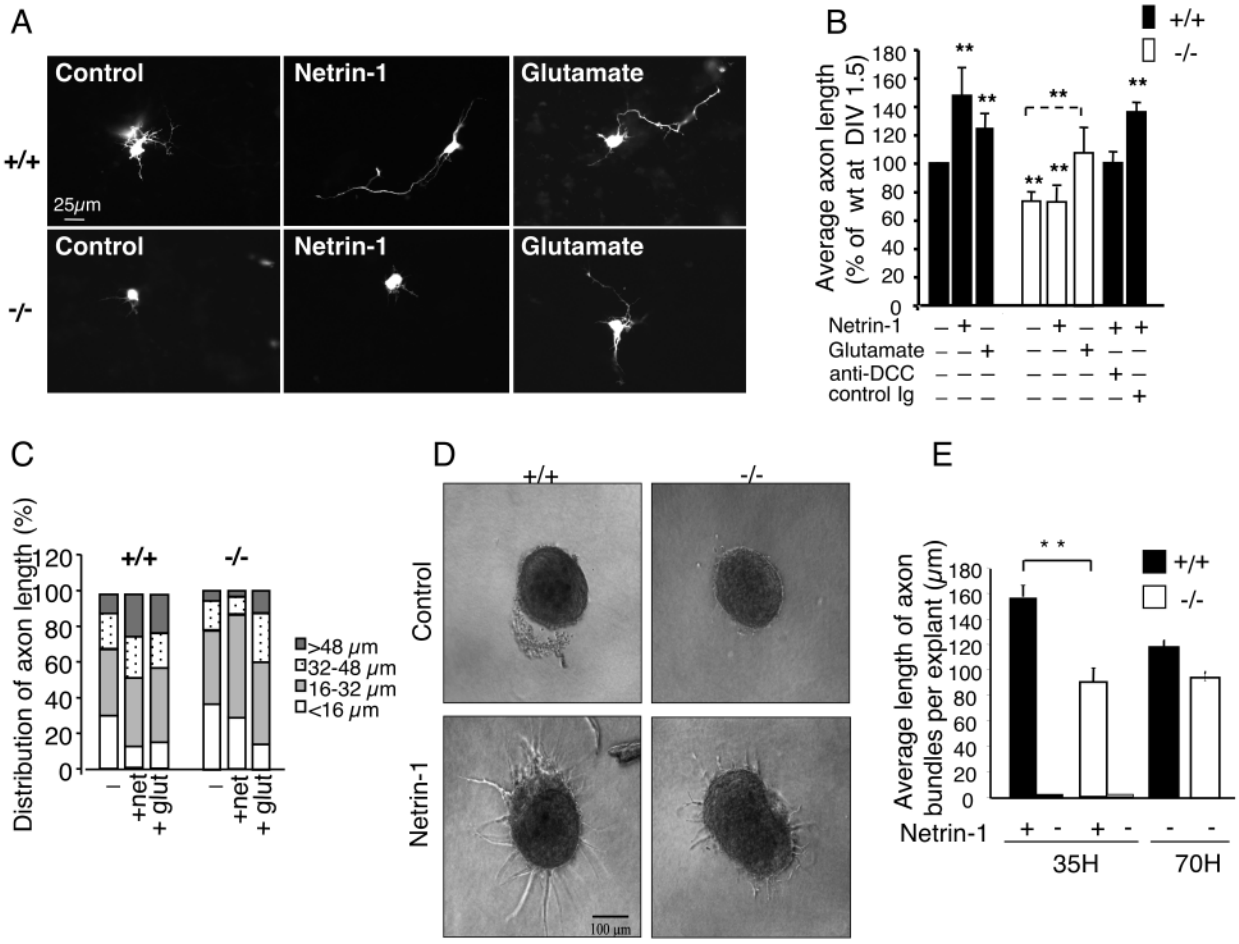
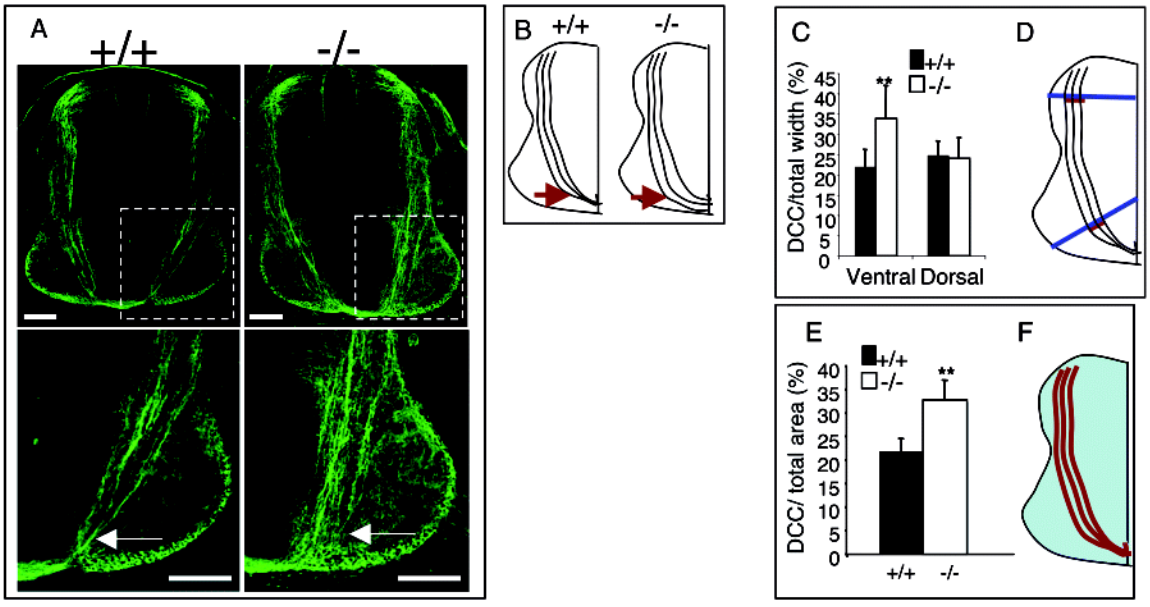
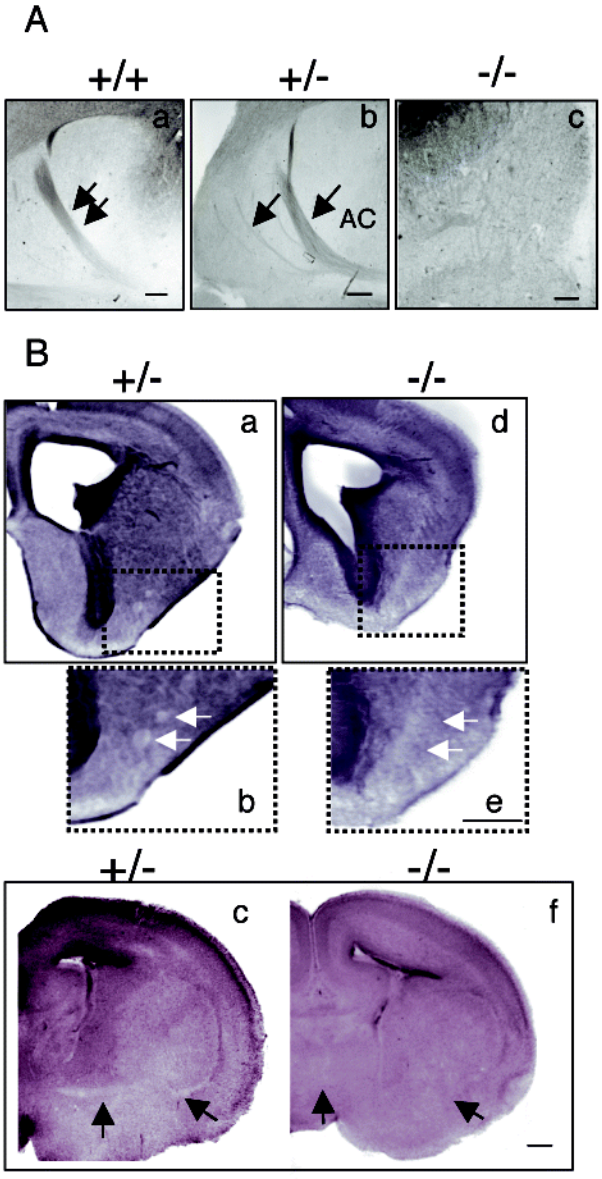
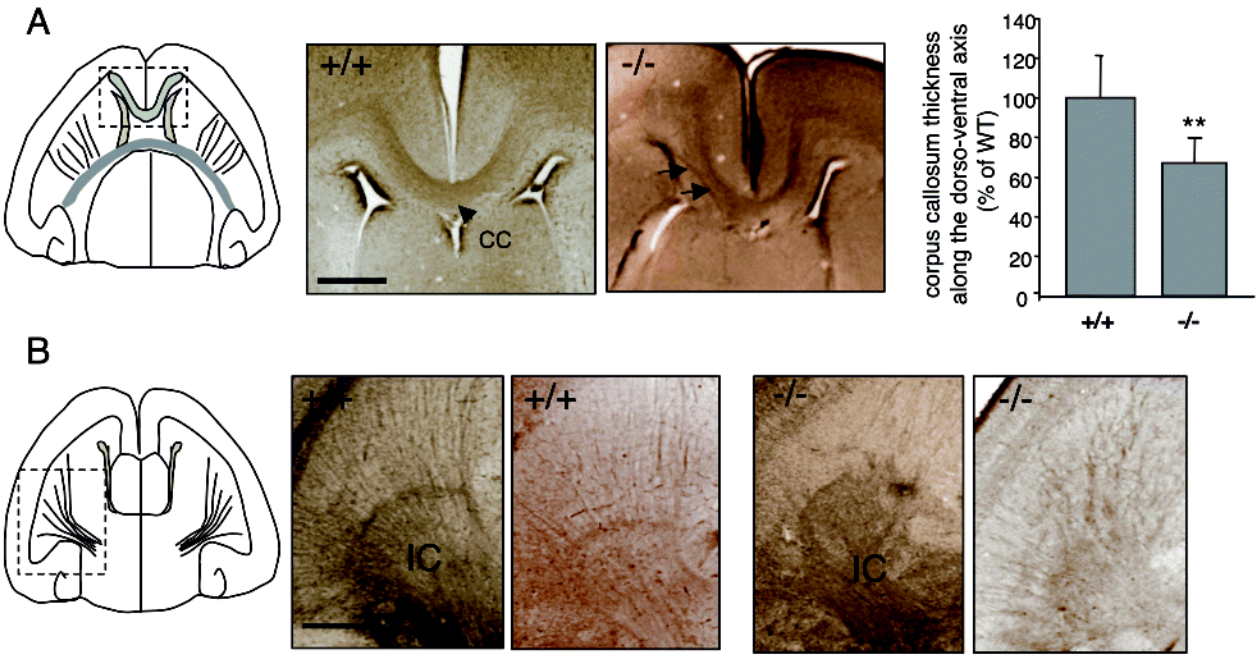
Pull-Down
TCL

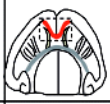
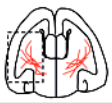

A
+/+
-/-
Netrin-1
0
5'
10'
0
5'
10'
Rac-GTP
Total Rac
Rac activity
(fold activation)
+/+
-/-
**
Time (min)
B
- Netrin-1
+ Netrin-1
anti-GFP
anti-DCC
Rac-GTP
Total Rac
Rac activity
(fold activation)
*
*
- Netrin-1
+ Netrin-1
Anti-GFP
Anti-DCC


A
Control
Netrin-1
Glutamate
+/+
-/-
25μm
B
+/+
-/-
Average axon length (% of wt at DIV 1.5)
Netrin-1
Glutamate
anti-DCC
control Ig
C
Distribution of axon length (%)
+/+
-/-
>48 μm
32-48 μm
16-32 μm
<16 μm
–
+net
+ glut
D
+/+
-/-
Control
Netrin-1
100 μm
E
Average length of axon bundles per explant (μm)
+/+
-/-
Netrin-1
35H
70H


A
+/+
-/-
B
+/+
-/-
C
+/+
-/-
DCC/total width (%)
45
40
35
30
25
20
15
10
5
0
**
Ventral Dorsal
D
E
+/+
-/-
DCC/ total area (%)
40
35
30
25
20
15
10
5
0
**
+/+
-/-
F


A
+/+
+/-
-/-
a
b
c
AC
B
+/-
-/-
a
d
b
e
+/-
-/-
c
f


A
+/+
-/-
CC
corpus callosum thickness
along the dorso-ventral axis
(% of WT)
140
120
100
80
60
40
20
0
**
+/+
-/-
B
+/+
+/+
-/-
-/-
IC
IC

	WT	Trio mutant	Netrin/DCC mutants
Spinal commissural axon projections		Defasciculated 	Absent 
Anterior commissure		Defasciculated in Trio -/+ Absent in Trio -/- 	Absent or severely reduced 
Corpus callosum		Reduced (30%) with some aberrant bundles 	Absent 
Internal capsule		Disorganized 	Disorganized and reduced