The role of the RhoGEF Trio in brain development

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ABSTRACT

Netrins are a small family of secreted proteins that guide growing axons during neural development by binding to the receptor DCC (Deleted in colorectal cancer). Our lab and others have previously shown that the activity of the Rho family GTPases Rac1 and Cdc42 are essential for DCC- mediated neurite outgrowth. Rac1 and Cdc42 act as molecular switches, mediating cytoskeleton remodelling when they are active and bound to GTP. Rac1 and Cdc42 are regulated positively by GEFs (guanine exchange factors) and negatively by GAPs (GTPase-activating proteins). Since DCC does not interact directly with Rac1, there should be an indirect link between DCC and Rac1. Trio is a GEF that activates Rac1 and RhoA. The orthologs of Trio in *C.elegans* (unc-73) and in *D. melanogaster* have been shown to play important roles in axon guidance, suggesting that mammalian Trio may link DCC to Rac1 activation. Here, we investigated how netrin-1 and its respective guidance receptor DCC are linked to Rac1 through studying the role of Trio in this signaling pathway. We found that Trio, Nck1, PAK1, and DCC are present in the same signaling complex, and that netrin-1-induced Rac1 activation 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 severely impaired in Trio -/- spinal cord explants and commissural axon projections are defective in Trio -/- embryos. In addition to defects in spinal cord development, 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 also defective in Trio -/- embryos. Thus, Trio through its ability to activate Rac1 mediates netrin-1 signaling in axon growth and guidance.

RÉSUMÉ

Le facteur de guidage chémotropique nétrine-1 favorise la croissance axonale à travers son récepteur DCC (Deleted in Colorectal Cancer) via l'activation de Rac1. Cependant, le facteur d'échange nucléotidique (GEF) qui lie nétrin-1/DCC à Rac1 n'a pas encore été identifié. Nous démontrons que Trio est la protéine GEF impliquée dans ce phénomène. Nous avons trouvé que Trio, Nck1, PAK1, et DCC sont présents dans le même complexe de signalisation et que l'activation de Rac1 induite par nétrine-1 est inhibée en absence de Trio. Les neurones corticaux Trio-/- échouent dans l'extension de neurites en réponse à la nétrine-1 alors qu'ils répondent à la stimulation par le glutamate. Par conséquent, l'induction de la croissance des axones commissuraux est sérieusement entravée dans les explants de moelle épinière des embryons Trio -/-. En plus du défaut dans le développement de la moelle épinière, les commissures antérieures sont absentes dans les embryons Trio-/-, et les projections axonales, qui forment la capsule interne et le corpus callosum, sont aussi affectées dans les embryons Trio -/-. Donc, par sa capacité d'activer Rac1, Trio favorise la signalisation par la nétrine-1 dans la croissance et le guidage axonale.

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CONTRIBUTIONS OF AUTHORS

This thesis is a manuscript-based structure. According to the faculty regulations, manuscripts co-authored by others must be accompanied with an explicit statement as to who contributed to such work and to what extent. Copyright waivers from co-authors appear in the appendix. This work has been accepted to be published in Molecular and Cellular Biology journal.

Chapter 3

Trio mediates netrin-1-induced Rac1 activation and axon guidance

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I, Atefeh Ghogha, performed all the experiments presented in:

Figure 3.1: Neurite outgrowth experiments using N1E-115 neuroblastoma cells.

Figure 3.2A: Co-immunoprecipitation using HEK-293 cells.

Figure 3.6: Dissection of the embryonic dorsal spinal cord, the explant assays and quantification of the data.

Figure 3.7: Immunohistochemistry on embryonic spinal cords.

This study submitted to MCB is the result of a 50% collaboration between the laboratories of Dr. Debant and Dr. Lamarche-Vane and although, I have not performed the experiments of Figures 3.3, 3.4, 3.5, 3.8 and 3.9, I was completely involved in the rationale and design of the experiments, as well as interpretation of the data and trouble-shooting.

Anne Briançon-Marjollet and Camille Auziol performed the experiments shown in Figure 3.3.

Anne Briançon-Marjollet performed the experiments in Figures 3.2B, 3.4, 3.5, 3.8 and 3.9.

Ibtissem Triki performed the experiments in Figure 3.2C and D. Both Ibtissem Triki and I were involved in the maintenance, breeding and genotyping of Trio mice in McGill University.

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Both Anne Briançon-Marjollet and I contributed to the writing of the manuscript with Drs. Debant and Lamarche-Vane.

The experiments included in Appendix I, were performed in collaboration with Ibtissem Triki. The affinity column experiment and preparation of the peptides were performed by Ibtissem Triki.

LIST OF ABBREVIATIONS

CNS	Central nervous system
cAMP	Cyclic adenosine monophosphate
Cdc42	Cell division cycle
DCC	Deleted in colorectal cancer
E13	Embryonic day 13
FNIII	Fibronectin Type III
GAP	GTPase activating protein
GDI	Guanine nucleotide dissociation inhibitor
GDP	Guanine diphosphate
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
GPI	Glycosylphosphatidylinositol
GST	Glutathione S-transferase
GTP	Guanine triphosphate
Ig	Immunoglobulin
MAPK	Mitogen-activated protein kinase
PAK	P21 activated kinase
PBS	Phosphate-buffered saline
PC12	Pheochromocytoma cell line
PI3-K	Phosphatidylinositol 3-kinase
PKA	Protein kinase A
PKC	Protein kinase C
PMSF	Phenylmethylsulfony
Rac	Ras-related C3 botulinum toxin substrate
LAR	Leukocyte common antigen-related
RGC	Retinal ganglion cell
RGM	Repulsive Guidance Molecule
Rho	Ras homologous
Robo	Roundabout
ROCK	Rho kinase
RT	Reverse transcriptase
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SH2	Src-homology 2
SH3	Src-homology 3
UNC or **Unc-**	Uncoordinated
NCAM	Neural Cell Adhesion Molecule
PNS	Peripheral nervous system

Shh	Sonic hedgehog homolog
NGF	Nerve growth factor
MLC	Myosin light chain
ROK	Rho-associated kinase
PAM	Peptidylglycine-alpha-amidating mono-oxygenase
CaMKII	Calcium/calmodulin-dependent protein kinase II
PTK2	Protein tyrosine kinase 2
SFKs	Src-family kinases
FAK	Focal adhesion kinase
PP1	Protein phosphatase 1
PLCg	Phospholipase C gamma
MAGE	Melanoma antigen
ADD	Addiction dependence domain
XIAP	X-linked inhibitor of apoptosis
JNK	C-jun N-term kinase
SrGAPs	Slit-robo GAPs
LOH	Loss of heterozygosity

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CHAPTER 1

INTRODUCTION

General Introduction

Processing the large amount of information in the central nervous system (CNS) is achieved by the precise network of neuronal connections. The nervous system is a complex structure. In a fly, the CNS contains more than 100,000 nerve cells that are uniquely specified and precisely connected to each other (Ghysen et al., 1986). In human brain, however, this network consists of billions of diverse cell types which are interconnected to make a functional nervous system. How this large number of cells are wired together during development to produce such a complex system remains an open question in developmental neurobiology.

One of the first attempts to find the answer to this question was initiated by Santiago Ramon y Cajal more than a century ago using isolated pieces of embryonic frog nerve where he showed that the tip of the neuron or what he called growth cone is a motile structure and plays an important role in axon outgrowth and guidance (Cajal, 1911).

In the 20th century, due to the development of modern techniques in cell and molecular biology, studying the growth and guidance of axons during development became an active area in research. Today, we know that in the embryonic environment, there are different guidance cues that attract or repel the growing axons via influencing the growth cone (Yu and Bargmann, 2001). By regulating cytoskeletal remodelling in the cell, through the dynamic regulation of filopodia and lamellipodia formation, guidance cues direct growth cone migration (Gallo et al., 2002). The small Rho GTPases are recognized as regulators of the actin cytoskeleton, which depending on being active or inactive can remodel cytoskeletal dynamics and the migration of the neuronal growth cone (Hall, 1998; Tessier-Lavigne and Goodman, 1996).

Netrin is an important family of chemotropic cues, which are known to guide the growth cone during development (Tessier-Lavigne and Goodman, 1996). The study presented in this thesis describes more detailed evidence of intracellular mechanisms mediating the response of a growing axon to netrin-1 and its receptor DCC by studying the role of Trio, a specific guanine nucleotide exchange factor (GEF) for Rac1 and the Serine/Threonine protein kinase PAK1 in this signalling pathway.

CHAPTER 2

LITERATURE REVIEW

2.1. General Mechanisms of Axon Guidance

In the embryonic environment, a growing axon meets a variety of guidance cues during the challenge to find its proper target. Firstly, the axon should correctly recognize the set of right cues in its environment and secondly, it should be able to track the signals throughout the distance it has to follow until the final goal, which is sometimes relatively long. The growth cone located at the tip of the axon functions as a sensor that quickly detects and responds to the right cues.

The growth cone structure consists of the organization of two major cytoskeletal components, microtubules and actin filaments, which are highly regulated polarized polymers (Dent and Gertler, 2003). Microtubules are found in the organelle-rich central domain of the growth cone. In the peripheral domain, actin filaments form finger-shaped structures known as filopodia, which give extra sensibility and motility to the growth cone. In addition, they form lamellipodia structures, which are sheet-like extensions at the edge of the cells containing a cross-linked actin filament meshwork (Mueller, 1999; Tessier-Lavigne and Goodman, 1996). When a growth cone turns toward attractive cues, the concentration of actin filaments increases rapidly at the site where the growth cone will turn. A lamellipodia structure then forms in that direction as well as microtubule advancement into the peripheral area of the growth cone where actin is accumulated (Mallavarapu and Mitchison, 1999). In the case of repulsive cues in the way of the navigating axon, it has been shown that growth cone collapse leads to repulsive turning (Fan and Raper, 1995). Thus, extension or retraction of filopodia and lamellipodia due to polymerization or depolymerization of actin filaments helps the growth cone to probe the extracellular environment, navigate toward the guidance cues or collapse due to a repellent signal.

The nervous system grows by navigation of axons to different parts of the developing embryo, making a complex wiring network. To overcome the long distance between the axons and the final targets, newly born axons generally extend during the early stages of development when the distances are still short. Intermediate targets can also help the axon grow within short distances. Fasciculation with preceding axons and following their path is important for the group of axons that extend later in the development.

Guidance cues have been described to have either short-range or long-range effects on a growing axon (Tessier-Lavigne and Goodman, 1996). Short-range or contact cues usually stay in the vicinity of the cells where they have been produced. On the other hand, long-range cues are known to produce a gradient from their source and attract or repel axons over long distances. They include the classical members of the Netrin, Ephrin, Semaphorin and Slit families of proteins (Flanagan and Vanderhaeghen, 1998; Nakamura et al., 2000; Serafini et al., 1996; Simpson et al., 2000). The role of morphogens for embryonic patterning has also been studied in axon guidance more recently (Charron et al., 2003).

2.2 The Netrin Family of Axon Guidance Cues

Netrin, meaning "one who guides" in Sanskrit represents a small family of secreted proteins that direct axon outgrowth and guidance during embryogenesis (Kennedy et al., 1994). Members of this family are bifunctional proteins, attracting some axons and repelling others. They have been found to function as highly conserved guidance cues in different vertebrate and invertebrate species including human (Ishii et al., 1992; Lauderdale et al., 1997; Serafini et al., 1994).

Unc-6 was the first identified netrin using a genetic screen for mutations affecting axon guidance in *C.elegans* (Ishii et al., 1992). Unc-6 is expressed by an isolated population of epidermoblasts located at the ventral midline (Wadsworth et al., 1996). Mutation of unc-6 in *C.elegans* causes severe axon guidance defects (Ishii et al., 1992). Two netrins have been described in *D. melanogaster*, netrin-A and netrin-B, which are expressed by ventral midline glial cells and their loss of function causes deficiency in commissural and longitudinal axons in the fly (Mitchell et al., 1996). In zebrafish, only a single netrin has been recognized (Lauderdale et al., 1997).

Netrin family members are 75 KDa glycoproteins sharing the same basic structure, including three domains (VI, V and C) and an amino terminal signal peptide (Figure 2.1A). The VI and V domains have homology to amino terminal domains of laminins (Ishii et al., 1992), whereas the C domain is homologous to the tissue inhibitors of

metalloproteases (TIMPs) family of proteins (Banyai and Patthy, 1999) and is known to bind to heparin (Kappler et al., 2000).

Six netrins have been identified in vertebrates: netrins 1-4 and netrins G1 and G2 (Manitt and Kennedy, 2002). Netrin-1 is the best studied member of this family and its orthologs have been found in several vertebrate species, including humans. Netrin-1 was discovered based on its ability to promote commissural axon outgrowth from embryonic spinal cord explants (Serafini et al., 1996). It was purified from postnatal chick brain, suggesting that it may be implicated in the development of other brain parts and may have other functions than axon guidance (Kennedy et al., 1994). Netrin-G1, unlike other netrins, binds to the plasma membrane via a GPI lipid anchor linked to the C domain.

The data from a study investigating the expression of netrin-1 in some parts of the rat brain shows that the amount of netrin-1 decreases as the embryo develops and there is a great reduction in the expression of netrin-1 in the brain after birth. For instance, in the corpus striatum, netrin-1 expression is increased at the different stages of embryonic development, whereas in the adult corpus striatum, netrin-1 expression is observed in a small number of neurons (Livesey, 1997). On the other hand, netrin-1 is expressed in the floor plate as well as in the ventricular zone (VZ) of the developing spinal cord in rat at embryonic day 13 (E13). The expression of netrin-1 in this region of the brain disappears by postnatal day 10 (P10). These data show the importance of netrin-1 in the development of CNS during embryogenesis. However, netrin-1 is expressed in the adult by multiple types of neurons such as motor neurons and multiple classes of interneurons as well as myelinating glia, oligodendrocytes in the CNS and schwann cells in the peripheral nervous system (PNS), which makes netrin-1 important in the normal function of adult CNS (Livesey, 1997). Netrin-1 is also expressed during development in other types of tissues such as the olfactory system, mammary gland, pancreas, the semicircular canals in the inner ear, the surface of the developing tongue and some skeletal muscle groups in the neck (Barallobre et al., 2005)

As stated above, netrin-1 is expressed by the floor plate cells, which attract commissural neurons toward the ventral midline in vertebrates (Kennedy et al., 1994). Studies using netrin-1 knock out mice showed that the corpus callosum (which links the left and the right cerebral hemispheres) is absent and that the anterior commissure is also

defective (Serafini et al., 1996). These studies also indicate that a large number of commissural axons are disorganized and shortened and do not reach the ventral spinal cord in the netrin-1 -/- embryos. Indeed, these experiments highlighted the role of netrin-1 as a long-range guidance cue.

Although the role of netrin-1 as a long-range guidance cue has been mostly studied in the embryonic axon guidance concept, some studies indicate that netrin-1 can act as a crucial factor in short-range guidance. As an example, studies using a vertebrate visual system model showed that netrin-1 plays an important role during the development of retinal ganglion cells (RGC) (Deiner et al., 1997). These cells are the only population of cells that send out their axonal projections from the eye toward the brain through the optic nerve.

2.3. Netrin Receptors in Axon Guidance

Netrin receptors were primarily identified based on the genetic mutation of *Unc-40* and *Unc-5,* which encode transmembrane Ig superfamily members in *C.elegans* (Hedgecock et al., 1990; Leung-Hagesteijn et al., 1992). *Unc-5* mutation caused a defect in the orientation of axons projecting away from the netrin source, whereas in *Unc-40* mutated nematodes axonal extensions toward netrin-1 expressing cells were defective (Ishii et al., 1992; Keino-Masu et al., 1996; Wadsworth et al., 1996). DCC (Deleted in Colorectal Cancer), the orthologue of Unc-40 in vertebrates (Chan et al., 1996), is expressed by commissural neurons and is required for their attractive response to netrin-1 (Fazeli et al., 1997). Unc-5 family members in vertebrates have been also known to be mainly important in repelling growing axons affected by netrin-1 (Leonardo et al., 1997). More recent data indicate that netrin-1 mediated short-range repulsion is through Unc-5 family members, wheras long-range repulsion needs both Unc-5 and DCC family members (Hong et al., 1999).

In addition to DCC and Unc-5 proteins, the adenosine receptor A2b family, a G-protein-coupled receptor that induces cyclic adenosine monophosphate (cAMP) accumulation upon adenosine binding, has been recognized as a netrin-1 receptor (Corset et al., 2000). Data from this study show that A2b is a receptor for netrin-1 and upon

binding to netrin-1 induces accumulation of cAMP (Corset et al., 2000). However, it has been shown that activation of A2b receptor is not required for rat commissural axon outgrowth or *Xenopus laevis* spinal axon attraction toward netrin-1 (Stein et al., 2001). Therefore, this evidence suggests that DCC only plays an essential role in netrin-1 signaling of axon growth and guidance independent of A2B receptor activation.

2.3.1. The DCC Family of Netrin-1 Receptors

The DCC family includes DCC and neogenin in vertebrates, Frazzled in *D. melanogaster*, and Unc-40 in *C. elegans* (Chan et al., 1996; Keino-Masu et al., 1996; Kolodziej et al., 1996; Vielmetter et al., 1994). DCC was first identified as a tumor suppressor gene on human chromosome 18q. Due to loss of heterozygosity (LOH) of chromosome 18q, colorectal tumors are induced in 30% of colon cancers (Fearon et al., 1990). Interestingly, reports show that the allelic losses of 18q are infrequent in early stage tumors, but are common in primary colorectal carcinomas and in most cases of hepatic metastases. Therefore, chromosome 18q LOH may be more important in progression rather than initiation of colorectal cancer (Mehlen and Fearon, 2004). Loss of heterozygosity of chromosome 18q or decreased DCC expression have also been reported in other types of cancers, including gastric, prostate, endometrial, ovarian, esophageal, breast, testicular, glial, neuroblastoma, and hematologic malignancies (Cooper et al., 1995; Reale et al., 1994).

DCC protein is expressed at low levels in almost all normal adult tissues, including colonic mucosa, with highest levels in neural tissues (Cooper et al., 1995). Orthologues of DCC have been identified in mouse and *Xenopus* and expression studies have shown that the gene is expressed in the nervous system of all these species and is also expressed in the basal cell layer of many epithelia, such as gut, skin, lung and bladder (Mehlen et al., 1998; Pierceall et al., 1994).

It has been proposed that DCC is able to induce apoptosis in the absence of its ligand, whereas in the presence of the ligand, this ability is lost. These data indicate that DCC may have a role as a tumor-suppressor protein by inducing apoptosis when its ligand is unavailable (Bernet and Mehlen, 2007). More recent data using netrin-1

overexpressing mice have shown that DCC behaves as a dependence receptor and induces cell death unless its ligand netrin-1 is present (Mazelin et al., 2004). In these mice, where netrin-1 is overexpressed in the intestinal epithelium in order to prevent receptor induced cell death, around 50 % cell death inhibition in the intestinal epithelium was observed (Bernet and Mehlen, 2007). This inhibition of cell death supports the role of dependence receptors as tumor suppressors.

In another study, investigations have shown that DCC has also a role as a receptor for netrin-1 that mediates axon guidance during development of nervous system (Keino-Masu et al., 1996; Serafini et al., 1996). *Frazzled,* the *D. melanogaster* homologue of *DCC,* was shown to encode a netrin-1 receptor, based on the similarity of the phenotypes in the Frazzled and netrin-1 mutants (Hiramoto et al., 2000; Kolodziej et al., 1996).

DCC protein is highly expressed in the early developing nervous system with the onset of neurogenesis (Gad et al., 1997). In the developing rat spinal cord, DCC is expressed on commissural neurons as they project toward the floor plate (Keino-Masu et al., 1996). With maturation of the nervous system, expression of DCC is limited to some structures such as olfactory bulb, the hippocampus and the cerebellum where there is active neurogenesis in postnatal life (Livesey, 1997; Shu et al., 2000). Data from a study using *in situ* hybridization in the embryonic, postnatal and adult rat nervous system using netrin-1 and DCC mRNA indicates that expression of DCC and netrin-1 mRNA was complementary to one another at certain stages of development in some critical parts of the nervous system (Livesey, 1997). For instance, netrin-1 labels both the floor plate and the ventricular zone of the developing spinal cord at E13 and E16, whereas netrin-1 expression disappears from this zone of the spinal cord by P10. Interestingly, DCC expression was complementary to the netrin-1 pattern of expression, being extensively expressed in the developing spinal cord, including the cell body of motor neurons, but not in the ventral ventricular zone, the floor plate or a region lateral to the floor plate. However, DCC mRNA is strongly expressed in the developing forebrain and midbrain compared to netrin-1 and is expressed in the developing corpus striatum and cerebral cortex.

About 10 years ago, DCC knock out mice were generated and carefully studied for defects in the nervous system (Fazeli et al., 1997). Their results showed that all

homozygous mice die within 24 hours after birth due to severe phenotypes, including the inability to suckle, labored respiration, abnormal body posture and abnormal limb flexion in response to pinch stimuli. DCC knock out mice show several defects in commissural axon extension toward the floor plate throughout the developing spinal cord that are similar and even more severe to those seen in netrin-1-deficient mice. Within the dorsal spinal cord of E9.5-E11.5 DCC knock out mice, there is a reduction in the number of commissural axons, although those that do extend appear to adopt a normal dorsal-to-ventral trajectory. Few axons extend into the ventral spinal cord and reach the floor plate. In addition to defects in spinal cord development, the corpus callosum commissures and the hippocampal commissure are completely absent in these mice. The axons that normally form these commissures are present but failed to cross the midline and remained ipsilateral, projecting to aberrant locations and forming tangles. The anterior commissure in DCC knock out mice is also severely reduced. Thus, the defects seen in the brain of DCC knock out mice are similar to those observed in netrin-1-deficient mice but are more severe. Some commissural axons do reach the floor plate in the DCC knock out mice, suggesting the existence of a DCC-independent mechanism for guidance of commissural axons to the floor plate. Evidence has been provided that sonic hedgehog homolog (Shh) is the cue distinct from netrin-1 and guides commissural axons toward the floor plate through a DCC-independent mechanism (Charron et al., 2003). On the other hand, experiments using a DCC blocking antibody showed that netrin-1 biological effects are largely inhibited (Keino-Masu et al., 1996). Also, DCC knock out mice showed a very similar phenotype to netrin-1 mutant animals where commissural axons have severe defects (Serafini et al., 1996) Therefore, DCC seems to be the major receptor for netrin-1 signaling in axon guidance.

DCC is a receptor with homology to the NCAM (Neural cell adhesion molecule) family of proteins (Fearon and Vogelstein, 1990). DCC family members are transmembrane proteins with four immunoglubulin (Ig) and six fibronectin type III repeats in the extracellular domain (Round and Stein, 2007). There are three conserved motifs: P1, P2, and P3 in the cytoplasmic domain of DCC (Figure 2.1B). Recent reports highlighting the role of intracellular signaling cascades that lead to directed netrin-1-mediated axon guidance have underlined the importance of tyrosine kinase activity,

phosphatidylinositol signaling and regulation mediated by Rho GTPase in triggering directed movement of the growth cone (Meriane et al., 2004; Xie et al., 2005). There was not much known about the role of intracellular domain of DCC in axon guidance until recently, when some studies started to unravel that netrin-1/DCC interaction, expressed on growth cones, leads to the intracellular activation of small GTPase Rac-1 (Li et al., 2002b; Shekarabi and Kennedy, 2002) or the MAPK signaling pathway and helps growth cone navigation (Forcet et al., 2002). It has been also shown that the SH3/SH2 adaptor protein Nck1 interacts directly and constitutively with DCC through its SH3 domains (Li et al., 2002a). In addition, Nck1 can recruit Rac1, Cdc42 and their effectors PAK1 and N-WASP to the activated receptor (Shekarabi et al., 2005), thereby providing a direct link between DCC, Rho GTPases and numerous downstream signaling components that regulate the actin cytoskeleton.

More recent data describe that DCC functions as a tyrosine kinase-associated receptor to mediate growth cone guidance in response to netrin-1 and is regulated by tyrosine phosphorylation (Meriane et al., 2004). Protein tyrosine kinase 2 (PTK2; also known as focal adhesion kinase or FAK) and Src-family kinases (SFKs) are now known to play central roles in netrin-1-mediated attraction. Therefore, netrin-1 activates both PTK2 and Src, leading to increased tyrosine phosphorylation of DCC (Li et al., 2004). Interestingly, Src has been shown to phosphorylate PTK2 and make a binding site for the adaptor protein Grb2 (Schlaepfer and Hunter, 1996). Grb2 and PTK2 interaction together might link activation of the netrin-1 receptor to the extracellular-signal-regulated kinase 2 (ERK2) and mitogen activated protein kinase (MAPK) cascade, which is necessary for netrin-1-induced outgrowth and turning (Forcet et al., 2002). PTK2 binds to Src, phospholipase C gamma (PLCg) and phosphatidylinositol 3-kinase (PI3K), which are both important in netrin-1 signaling (Xie et al., 2005; Zhang et al., 1999).

Changes in calcium concentrations have long been known to affect the turning response of growth cone, but the signaling pathways downstream of calcium have not been clear until the past few years. It has been proposed that a large increase in calcium levels operates through calcium/calmodulin-dependent protein kinase II (CaMKII) to trigger attraction, whereas a small local increase triggers calcineurin and protein phosphatase 1 (PP1) activity to elicit axon repulsion (Wang, 2004). These two families of

proteins are able to regulate the activity of tubulin in addition to the microtubule-associated proteins MAP2 and affect the stability of microtubules (Round and Stein, 2007). Some studies report that netrin-1 plays an important role in regulation of microtubule dynamics which is consistent with the findings suggesting that MAP1B is required for netrin-1 signaling (Río et al., 2004).

Calcium signaling has been reported to be important during axon guidance via the control of Rho GTPase activity. The direct increase in cytoplasmic calcium causes activation of Rac and Ccd42 and down-regulation of RhoA in cultured cells, which requires activation of PKC and CaMKII (Jin et al., 2005). These data suggest that calcium-sensitive kinases phosphorylate GEFs or GTPase-activating proteins (GAPs) and regulate activation of Rho GTPases, resulting in cytoskeletal rearrangements and growth cone turning (Dickson, 2001).

2.3.2. Neogenin Family of Netrin-1 Receptors

Neogenin, the other member of DCC family, is expressed in neurons and non-neuronal cells in the CNS during embryogenesis (Vielmetter et al., 1994). It consists of four immunoglobulin-like domains followed by six fibronectin type III domains, a transmembrane domain and an intracellular domain and is expressed by growing nerve cells in the developing vertebrate brain (Fitzgerald et al., 2006; Rajagopalan et al., 2004; Vielmetter et al., 1997). Commissural neurons express DCC but not neogenin as they extend axons toward the floor plate (Keino-Masu et al., 1996). Neogenin binds to netrin-1 directly but there is not much know about its function in the nervous system.

Investigation of the pattern of neogenin expression shows that this protein is widely present in different tissues such as brain, skeletal muscle, heart, liver, stomach, Fduodenum, ileum, colon, kidney, lung, testis, ovary, oviduct, and uterus in mice (Rodriguez et al., 2007).

Recent studies have demonstrated that, through its novel ligand Repulsive Guidance Molecule (RGM), neogenin is involved in axonal migration and neural tube closure (Keino-Masu et al., 1996; Rajagopalan et al., 2004). Using neogenin knock out

mice, analyzing the signal transduction of RGM-a-mediated growth cone collapse, it has been recently shown that the interaction of RGM-a and neogenin is specific and important in axon repulsion (Conrad et al., 2007). These data describe that PKC and RhoA are involved in neogenin-mediated growth cone collapse. Moreover, it has been shown that neogenin is implicated in myotube formation mediated by its binding to netrin-3 (Kang et al., 2004).

There is some evidence suggesting a central role for neogenin in growth and organization of mammary glands (Hinck and Silberstein, 2005). Neogenin is expressed by myoepithelial progenitor cells (cap cells) in the mammary gland (Srinivasan et al., 2003). Neogenin loss-of-function mutants showed slower growth and a significant disorganization in the mammary end buds due to loss of adhesion between the cap and luminal cell layers, with large spaces forming under the cap cell layer (Hinck and Silberstein, 2005). These data suggest that neogenin signaling maintains the integrity of the end bud, which is required for normal growth of the mammary tree.

2.3.3. The Unc-5 Homologue Family of Netrin-1 Receptors

Unc genes were first named by Sydney Brenner on the basis of their associated uncoordinated phenotypes in mutant nematodes where he described “Some of the uncoordinated mutants have distinctive abnormalities in the nervous system” in his report (Brenner, 1974). The Unc-5 family includes Unc-5 in *C. elegans* and *D. melanogaster* and Unc-5H1, Unc-5H2, Unc-5H3 and Unc-5H4 in vertebrates (Hamelin et al., 1993; Keleman and Dickson, 2001). These transmembrane proteins have two Ig domains, two thrombospondin type I domains, a transmembrane domain, and an intracellular domain containing a ZU-5 domain (homologous to zona occludens-1 and is also involved in cell death) and a death domain (Figure 2.1B). Considering the death domain, some studies have reported that Unc-5H1 is involved in programmed cell death (Mehlen and Mazelin, 2003).

Unc-5H1, Unc-5H2 and Unc-5H3/RCM (Rostral Cerebellar Malformation) were the first homologues of *C. elegans* Unc-5 proteins that were identified in vertebrates.

Studies in *C.elegans* and *D. melanogaster* indicate that Unc-5 mediates the repulsive response to netrin (Hamelin et al., 1993; Keleman and Dickson, 2001). In mammals, Unc-5H3/RCM knock out mice show aberrant cerebellar neuron migration that results in decreased cerebellum size and abnormal morphology (Ackerman et al., 1997; Salehi et al., 2000).

Unc-5H acts as a dependence receptor to netrin-1, however the signaling mechanisms regulated by the Unc-5H proteins have just started to be unravelled. In the absence of its ligand, it has been proposed that Unc-5H1 triggers apoptosis and is consequently cleaved by caspase-3 at position 412 in its intracellular domain, but it does not necessarily imply that caspase-3 is responsible for the cleavage of Unc-5H *in vivo* (Llambi et al., 2001). In Unc-5H, the ADD (Addiction dependence domain) is supposed to be located downstream of the caspase cleavage site, including a death domain (Llambi et al., 2001). Nevertheless, it is not yet known how the death domain of Unc-5H induces apoptosis.

Moreover, it has been proposed that Unc-5H1-mediated apoptosis is mediated by its interaction with NRAGE, a protein of the MAGE (melanoma antigen) family known as regulators of apoptosis. The interaction between NRAGE and Unc-5H1 could induce activation of the apoptotic pathway by promoting the degradation of the survival protein XIAP (X-linked inhibitor of apoptosis), or by activating the JNK (c-jun N-term kinase) pathway (Williams et al., 2003).

Recently, it has been described that Unc-5H1 interacts with PICK1 (Protein interacting with Protein kinase C, PKC) and that PKC activation promotes the removal of Unc-5H1 from the cell surface of neurons and causes a decrease in netrin-1 capacity of inducing growth cone collapse (Williams et al., 2003).

Unc-5H has been reported to be important in tumorigenesis similar to DCC. It has been observed that the expression of Unc-5H is strongly reduced in more than 90 % of colorectal cancers, as well as in many other tumors (Bernet and Mehlen, 2007). Moreover, Unc-5H2 has been described to be a direct transcriptional target of the p53 tumor suppressor gene whose proapoptotic activity is dependent on the p53-dependent expression of Unc-5H2 and can be antagonized by the presence of netrin-1 (Tanikawa et al., 2003).

2.4. Role of the Small Rho GTPases in Axon Guidance

Cytoskeletal rearrangements are crucial during growth cone guidance. It has been proposed that, when a growth cone turns toward a source of attractive cues, cell surface receptors are recruited at the leading edge of the growth cone and bind to the attractive cues to make functional linkages with the underlying actin cytoskeleton and to stabilize it. This stabilization of actin networks produces tension between the central and peripheral domains, allowing the microtubules to extend and the leading edge of the growth cone to grow, forming lamellipodial actin networks and filopodial finger-shaped structures (Dent et al., 2003).

Rho GTPases comprise a subfamily of the Ras superfamily of small GTPases. The number of Rho GTPases varies from about six in *C.elegans* and *D. melanogaster* to twenty-two proteins in mammals (Jaffe and Hall, 2005). Rho GTPases act as molecular switches that cycle between an active GTP-bound state and an inactive GDP-bound state. GEFs activate the Rho GTPases and facilitate the exchange of GDP to GTP (Rossman et al., 2005), whereas GAPs stimulate the intrinsic GTPase activity of Rho proteins to hydrolyze GTP to GDP and act as negative regulators (Tcherkezian and Lamarche-Vane, 2007). Guanine nucleotide dissociation inhibitors (GDI) stabilize the inactive GDP-bound form of the protein (Olofsson et al., 1999). Plasma membrane receptors and other cellular signaling pathways upstream of the Rho GTPases activate GDIs, GAPs and GEFs. Active GTPases bind to effector proteins that transduce the downstream signal to regulate different tasks in the cell such as the morphology and growth of the cell (Figure 2.2).

In the Rho family of the small GTPases, RhoA, Rac1 and Cdc42 are the best-studied members. *In vitro* studies using N1E-115 neuroblastoma cells showed that Rho GTPases are important regulators of actin dynamics that drive growth cone motility. These data using expression of constitutively active or dominant negative mutant proteins in neuronal cells show that Rac1 and Cdc42 promote neurite outgrowth by regulating the formation of lamellipodia and filopodia in the growth cone, whereas RhoA activity inhibits neurite outgrowth (Kozma et al., 1997). *In vivo* studies in *D. melanogaster* brought more insight into the role of the GTPases in axonal growth (Luo et al., 1994). Expression of either constitutively active or dominant negative Rac1 in *D. melanogaster*

embryonic sensory neurons resulted in selective defects in axonal outgrowth, whereas mutations in Cdc42 affected both axons and dendrites (Luo et al., 1994).

Other investigations have shown that Rho GTPases are also involved in guiding growing axons. For example, down regulation of Rac1 and Cdc42 or activation of RhoA is essential for midline repulsion in *D. melanogaster* (Fritz and VanBerkum. 2002). Also, dominant negative Rac1 expression causes errors in motor neuron guidance (Kaufmann et al., 1998). Studies in *C. elegans* indicate that mutations in a Rho-like GTPase mig-2 can cause both outgrowth and occasional guidance defects (Zipkin et al., 1997). Recently, knock out mice with Rac1 deleted from the brain have been generated (Chen et al., 2007). This study indicated that although the deletion of Rac1 in VZ progenitors did not prevent axonal outgrowth of telencephalic neurons, the anterior commissure was absent and the corpus callosum and hippocampal commissural axons failed to cross the midline in these knock out embryos. The thalamocortical and corticothalamic axons were also reported to show defasciculation or projection defects. These results indicate that Rac1 has a critical role in axon guidance and in achievement of migratory competency during differentiation of the progenitors for the ventral telencephalon-derived interneurons.

2.5. GAPs and GEFs in Axon Guidance

2.5.1. RhoGAPs

RhoGAPs are a large family of proteins recognized for their ability to enhance the low intrinsic GTPase activity of Rho GTPases, which consequently lead to their inactivation (Tcherkezian and Lamarche-Vane, 2007). The first RhoGAP, P50Rho GAP, was discovered 18 years ago (Garrett et al., 1989) and, to date, more than 70 members have been characterized in eukaryotes (Tcherkezian and Lamarche-Vane, 2007).

The members of this family all share the conserved ‘GAP catalytic domain’ consisting of around 180 amino acids, which mediates binding and catalytic activity with Rho proteins. Most of Rho GAP protein members contain other motifs essential in their signaling activity, such as Src homology 2 or 3 (SH2 or SH3), pleckstrin homology (PH), Dbl homology (DH) and Serine/Threonine (Ser/Thr) kinase domains, in addition to SH3-

binding sites. It has been shown that some RhoGAPs interact with only one Rho protein such as Slit-Robo GAPs (srGAPs), whereas others can interact with all three members: RhoA, Rac1 and Cdc42 such as p190-B (Sordella et al., 2002).

The members of this family of proteins are extremely conserved during evolution. In a very simple organism, such as the mold *Dictyostelium discoideum*, around 43 RhoGAP-domain encoding genes have been identified (Vlahou and Rivero, 2006). In *C. elegans,* two RhoGAPs from 20 encoding genes have been identified, whereas in yeast this number comes to 10 genes (Tcherkezian and Lamarche-Vane, 2007). In *D. melanogaster,* around 20 different genes encode RhoGAP-domain-containing proteins and six have been characterized as GAP proteins (Bernards, 2003; Billuart et al., 2001). Finally, in the human genome, around 60 RhoGAP containing proteins have been identified and more than half of them are characterized as GAPs (Bernards, 2003; Peck et al., 2002; Venter et al., 2001).

Many different molecular mechanisms including phosphorylation and protein-protein interaction regulate RhoGAPs, which make this family of proteins important in a variety of cellular functions such as cell migration, cell differentiation, cytokinesis, neuronal morphogenesis and tumor-suppression.

In the context of axon guidance, a GAP for Cdc42 has been implicated in Slit-Robo signaling in growth cone repulsion. It has been shown that srGAP binds to Robo via its SH3 domain and this interaction leading to down-regulation of Cdc42 is enhanced in the presence of the guidance cue (Wong et al., 2001). There are some reports indicating that another member of this family, srGAP3, is crucial in the development of neuronal structures. These structures are important for normal cognitive function and mutations in this gene cause severe mental retardation (Endris et al., 2002).

Other studies using mice lacking p190-A, which is a GAP specific for RhoA, have reported that there are defects in axon outgrowth, guidance and fasciculation, as well as in neuronal morphogenesis (Brouns et al., 2001). Grit is another RhoGAP, which interacts with RhoA, Rac1 and Cdc42 (Nakamura et al., 2002). Several reports indicate that Grit interacts with TrkA, which is known as a receptor for NGF, one of the most important extracellular factors in axon outgrowth and guidance (Yamashita et al., 1999).

These results suggest that Grit regulates neurite outgrowth by modulating the Rho family of small GTPases downstream of NGF (Nerve growth factor) signaling pathway.

2.5.2. RhoGEFs

It is well understood that signals arising from extracellular factors are translated into intracellular signals, which converge to Rho GTPase activation via the regulatory proteins GEFs. GEFs were initially identified as potential oncogenes with the ability to induce transformation in NIH 3T3 cells (Eva and Aaronson, 1985). Dbl was the first GEF isolated, from B lymphomas as an oncoprotein (Hart et al., 1991; Ron et al., 1991), which shares an approximately 200 conserved amino acid in a domain known as DH domain with Cdc24 (Zheng et al., 1994). The DH and PH domains shared by all members of this family, characterize the structural domain responsible for catalyzing the GDP-GTP exchange reaction of Rho proteins. Recent studies have shown that Dbl family members are implicated in many biological processes, including neuronal axon guidance. Presently, more members of the Dbl family such as Vav (Katzav et al., 1989), Tiam-1 (Habets et al., 1994), FGD1 (Olson et al., 1996) and Trio (Debant et al., 1996) have been identified and more detailed evidence of their regulation, activation and mechanism of interaction with Rho GTPase have begun to emerge. Around 60 GEFs have been characterized in humans, which contain the two conserved domains (Venter et al., 2001), a DH domain, which is responsible for activation of the GTPases and the PH domain that is considered to be important in membrane localization by interacting with specific lipids. It has been proposed that GEFs might have multifunctional roles since most of them contain protein-protein interaction motifs other than PH and DH domains. Many mammalian GEFs are highly specific for a single GTPase, like Tiam-1, which is a Rac1 specific GEF, or FGD1, a GEF specific for Cdc42, whereas several GEFs such as Vav are less specific and can activate RhoA, Rac1 and Cdc42 (Hart et al., 1994).

There is increasing evidence that GEFs are important during axon guidance in different model systems. The data from studying Unc-73, the homologue of Trio in *C. elegans* indicates that mutants of *unc-73* have a variety of defects in axon guidance that makes the nematode nearly paralyzed (Steven et al., 1998).

DOCK180 represents another superfamily of GEFs for Rac, which was originally identified as a 180 KDa protein interacting with the proto-oncogene product c-Crk (Hasegawa et al., 1996). The orthologue of DOCK180 in *D. melanogaster*, Myoblast City (MBC), has been reported to have an essential role in dorsal closure and cytoskeletal organization during embryonic development (Erickson et al., 1997). The Rac1-specific GEF Tiam-1 has been shown to cause N1E-115 cells to form neurites and prevent LPA-induced neurite retraction (Leeuwen et al., 1997). Tiam-1 is expressed in migrating cortical neurons during neural development. In mice lacking Tiam-1 expression, cerebellar granule neurons failed to migrate (Ehler et al., 1997). Another Rac1-GEF, STEF, is also expressed in the developing CNS and causes Rac1-dependent neurite outgrowth in N1E-115 cells (Matsuo et al., 2002). The mechanisms of regulation of these GEFs in axon guidance are not totally understood, but they are likely to bind directly or indirectly to guidance receptors leading to Rho GTPase regulation.

2.6. The Trio RhoGEF Family of Proteins

2.6.1. Trio RhoGEF

The Trio family members identified in vertebrates are Trio and Kalirin (Alam et al., 1997; Colomer et al., 1997; Debant et al., 1996). Trio was identified in mammals as a binding partner of the leukocyte common antigen-related (LAR) receptor tyrosine phosphatase using a yeast-two-hybrid approach (Debant et al., 1996). LAR subfamily of receptors PTPs (RPTPs) are known as crucial players in several key aspects of cellular functions by modulating control of cellular tyrosine phosphorylation levels. LAR family members have a predominant function in nervous system development that is conserved throughout evolution (Schaapvel et al., 1998; Sommer et al., 1997). Most members of the LAR subfamily are highly expressed in axons and growth cones in both vertebrates and invertebrates during development, which suggests the role of this family of proteins in the nervous system is highly conserved (Desai et al., 1994; Schaapvel et al., 1998; Sommer et al., 1997; Tian et al., 1991).

Human Trio is a large multidomain protein which contains, two GEF domains consisting of a DH domain coupled to a PH domain, and an SH3 domain located between the two GEF domains. It also has a second SH3 domain, an Ig-like domain and a Serine/Threonine kinase domain (Figure 2.3), therefore, it is predicted that Trio might function in multiple signalling pathways (Debant et al., 1996).

Genetic and biochemical data suggest that GEF1 activates RhoG resulting in activation of Rac1 and GEF2 activates RhoA (Blangy et al., 2000; Steven et al., 1998). Investigating the role of Trio in PC12 neuronal cells shows that human Trio induces neurite outgrowth in a GEF1-dependent manner, whereas the spectrin repeats and the SH3-1 domain of Trio are crucial for neurite induction (Estrach et al., 2002).

The Trio kinase domain is similar to calcium/calmodulin regulated kinases, including the myosin light chain kinase (Debant et al., 1996). There is some evidence indicating that one site in Trio kinase domain is phosphorylated by FAK (Medley et al., 2003). It has been reported that spectrin repeats are important in interaction of Trio with the actin-filament-crosslinking protein filamin (Bellanger et al., 2000). Some data show that these repeats are essential for regulating neurite extension and neuronal morphology regulated by Kalirin (Penzes et al., 2003).

As stated previously, Trio activates the Rac pathway and/or the Rho pathway through its two GEF domains. GTP-bound Rac, together with membrane-localized Nck1/Dock, can bind and activate PAK, mediating phosphorylation of substrates that affect the actin cytoskeleton, such as inhibitory phosphorylation of myosin light chain kinase (MLC kinase). It is proposed that GTP-bound Rho might bind to and activate Rho-associated kinase (ROK), causing inhibitory phosphorylation and inactivation of MLC phosphatase (MLC PTPase) and induce changes in cytoskeletal dynamics (Bateman et al., 2000).

GEF1 also activates RhoG in vertebrates, which then activates Rac and Cdc42 signaling pathways. In invertebrates, GEF1 activates the Rac-like GTPase MIG-2, which signals through effectors which are not known. Genetic interactions from *D. melanogaster* propose that Trio is involved in the mechanism of the Abl pathway (Bateman et al., 2000).

It has been indicated that Trio binds to the FAK amino terminal domain and to the FAK kinase domain via its SH3 and kinase domains, respectively and is tyrosine phosphorylated by this protein (Medley et al., 2003). This evidence suggests that Trio may be implicated in the focal adhesion dynamics regulation apart from its role in reorganization of the actin cytoskeleton through the activation of Rho family GTPases (Medley et al., 2003).

Trio is the first RhoGEF that has been extensively studied in the context of growth cone guidance. The pattern of Trio expression using *in situ* hybridization in the postnatal rat brain shows that during postnatal development, Trio mRNA levels are high in the cerebral cortex, hippocampus, thalamus, caudate/putamen, and olfactory bulb, with lower levels in the septal nucleus, nucleus accumbens, amygdala, and hypothalamus (Ma et al., 2005). These data indicated that in major brain areas except for the cerebellum, Trio mRNA levels peaked at P1, decreasing gradually during development, having a low but detectable levels at P30. Consistent with these results, analysis of Trio protein levels in the hippocampus and cortex is also high at P1, decreasing until P30 (Ma et al., 2005). The data suggest that Trio plays an important role during neuronal development.

2.6.2. Kalirin

Kalirin was first identified through a screen for proteins interacting with peptidylglycine-alpha-amidating mono-oxygenase (PAM), a membrane enzyme essential to the synthesis of many bioactive peptides (Mains et al., 1999). Kalirin shares almost the same structure with Trio except that it encodes two Ig-like repeats and a serine/threonine kinase domain (Figure 2.3) (Seipel et al., 1999).

Whereas Trio is expressed at moderate levels in all tissues, Kalirin members expression is specific to the central nervous system in the adult rat (Alam et al., 1997; Debant et al., 1996) and according to their pattern of expression, it has been suggested that Kalirin may play a role in axon outgrowth, myoblast migration and fusion, and endocrine development (Hansel et al., 2001). Although Trio and Kalirin have some structural differences, it has been proposed that these two families of proteins possibly function in a similar manner. It has been shown that the GEF1 domain of Kalirin

activates Rac1 *in vitro* and that its expression in cultured fibroblasts induces cell spreading and lamellipodia formation (Alam et al., 1997; Mains et al., 1999; Penzes et al., 2000). Moreover, it has been shown that a region of the spectrin repeats of kalirin interacts with PAM and may regulate receptor internalization.

2.6.3. Trio Isoforms

Using different approaches, Trio isoforms have been generated, giving rise to proteins that contain one or two RhoGEF domains (GEFDs). Expression of these isoforms is at a higher level in the nervous system than the full-length Trio (Portales-Casamar et al., 2006). Trio isoforms include TrioA, TrioB, TrioC and TrioD. These four isoforms are commonly expressed in the adult and in the embryonic brain through development. Interestingly, the pattern of expression of the Trio isoforms is similar in the different regions of the adult brain except for TrioC, which is highly expressed in the cerebellum. Moreover, all the Trio isoforms are present in the peripheral nervous system (Awasaki et al., 2000; Bateman et al., 2000).

Most knowledge about Trio is based on studies of *C. elegans* and *D. melanogaster*, while the function of Trio in vertebrates is still not very clear and the mechanisms by which this GEF is activated by membrane receptors are still unknown. Unc-73 and *D. melanogaster* Trio (D-Trio) are orthologs of mammalian Trio. D-Trio is expressed throughout the developing embryo, and high levels have been detected in nervous tissues (Awasaki et al., 2000; Bateman et al., 2000; Liebl et al., 2000; Newsome et al., 2000; Steven et al., 1998). The analysis of *unc-73* and *D-Trio* mutants has indicated that this gene plays an important role to regulate axon outgrowth and guidance in invertebrates (Awasaki et al., 2000). It has been shown that *D-Trio* mutations disrupt axonal projections in both the embryonic and adult CNS (Liebl et al., 2000) and that D-Trio interacts with non-receptor tyrosine kinase Abl. *D-Trio* and *Abl* mutations mutually enhance each other, increasing the defects in the CNS and rising the rate of lethality (Newsome et al., 2000). Also, mutations in *D-Trio*, *dreadlock* (Dock), and *PAK* (effector of Rac1 and Cdc42) result in dosage-sensitive errors in axonal projections (Bateman et al., 2000; Debant et al., 1996). These data propose that Trio, Dock, and PAK function in

a common pathway to control axon guidance in invertebrates. Interaction of these proteins in vertebrates remains to be elucidated.

Athough the role of Trio in vertebrates is not clear compared to what is known in invertebrates, Trio knock out mice show that loss of this protein is lethal for the embryos during late stages of development due to abnormal skeletal muscle and neural tissue development (O'Brien et al., 2000). Loss of Trio causes cell detachment within the hippocampus and the olfactory bulb to become disorganized, whereas the overall structure of the brain is not affected (O'Brien et al., 2000). Therefore, not only is Trio important for viability, it is also crucial to mediate fine-scale mapping of neuronal cell position after the global arrangement of the brain has been established. This makes further investigation interesting to discover the role of Trio in other aspects of neuronal development such as axon guidance in vertebrate.

In a study to identify the genes which are involved in tumor progression, soft tissue sarcomas including pleomorphic, dedifferentiated liposarcomas, malignant fibrous histiocytomas and malignant peripheral nerve sheath tumors were analyzed using a DNA microarray. Results from this study show that Trio was consistently overexpressed in all of these tissues and that Trio may play a crucial role during the progression of different sarcomas (Adamowicz et al., 2006). Other data show that Trio mRNA expression is associated with invasive tumor formation in bladder cancer (Zheng et al., 2004).

The axon guidance receptors acting upstream of Trio are still not known. One candidate is the receptor tyrosine phosphatase LAR, which interacts with Trio via its cytoplasmic domain in human (Debant et al., 1996). Mutations in the gene encoding the *D. melanogaster* LAR orthologue, DLAR, disrupt the projections of many axons that are also affected in Trio mutants (Krueger et al., 1996). However, the carboxyl-terminal region of human Trio, that mediates the association with human LAR, is absent in *D-Trio* (Debant et al., 1996). Thus, although the fly Trio and DLAR act in the same growth cones, they are unlikely to function in a common pathway.

On the other hand, there is strong evidence using genetic and biochemical experiments that suggest interactions between Trio and the netrin receptor Frazzled in *D. melanogaster* (Forsthoefel et al., 2005). These reports indicate that *Abl*, *Trio*, *Ena* and

Frazzled are integrated into a complex signaling network that regulates axon guidance in the commissural axons of the fly.

Although little is known about Frazzled and its signaling to the cytoskeleton during axon guidance, it has been shown in mammals that netrin-1/DCC interaction leads to activation of GTPases Cdc42 and Rac1 which regulates the actin dynamics and neurite outgrowth (Li et al., 2002a; Li et al., 2002b; Shekarabi and Kennedy, 2002)

Figure 2.1. Structures of Netrin-1, DCC and Unc-5H1

A) Structure of Netrin-1

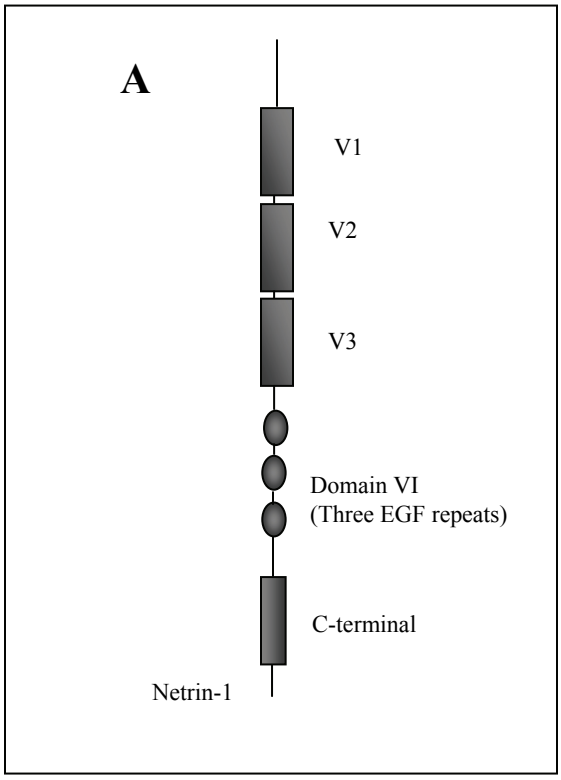
Netrin-1 is a 606 amino acid protein composed of 6 distinct domains: signal peptide, V-1, V-2, V-3, VI, and C. Domains VI and V show homology to the gamma chain of laminins. The C domain contains basic residues and is required for the binding to heparin. All domains are able to bind to Netrin receptors to various extents.

B) Structure of Unc-5 and DCC Receptors

DCC is a 1445 amino acid type I transmembrane protein. From N- to C-terminus, it contains a signal peptide sequence, 4 Ig domains, 6 FNIII repeats, a hydrophobic transmembrane domain and a short cytoplasmic tail with three conserved motifs (P1, P2, and P3). The fourth and fifth FNIII repeats have been shown to mediate binding to netrin-1.

Unc-5 is a type I transmembrane receptor which is composed of a signal peptide sequence, 2 Ig domains, two thrombospondin domains, a transmembrane domain and a cytoplasmic domain containing a ZU-5 domain (a domain of homology found in Zona Occludens-1 and Unc-5 protein) and a Death Domain. The binding of netrin-1 to Unc-5 is believed to be mediated by both Ig domains.

Adapted from: P Mehlen and F Llambi (2005). "Role of netrin-1 and netrin-1 dependence receptors in colorectal cancers". British Journal of Cancer (93, 1-6).


A
V1
V2
V3
Domain VI
(Three EGF repeats)
C-terminal
Netrin-1

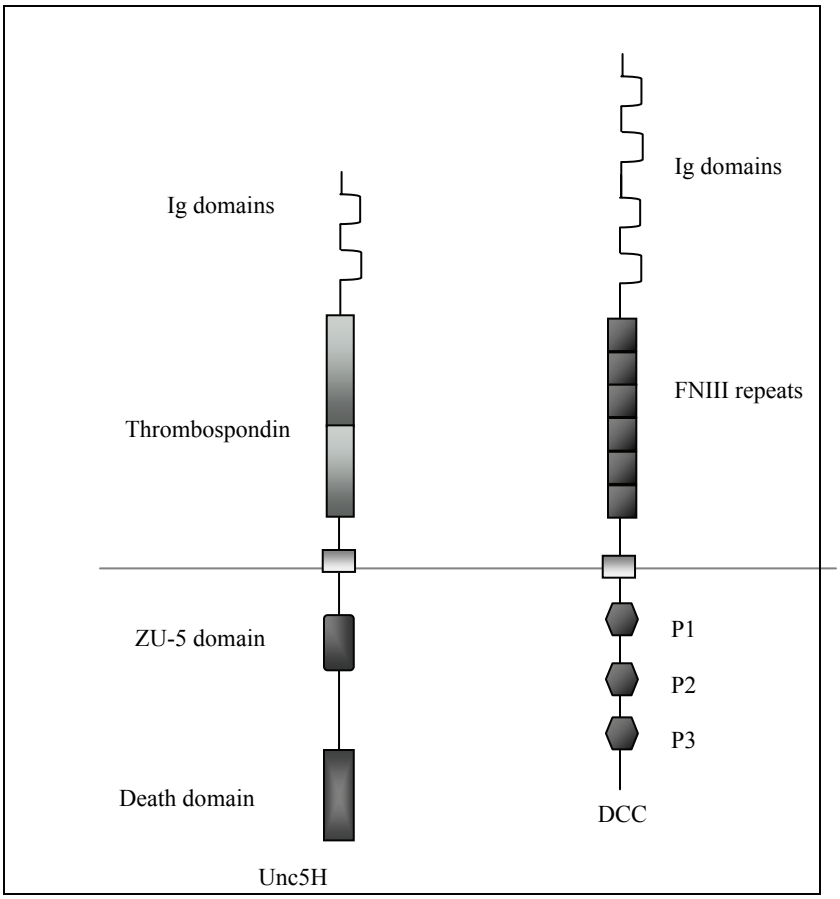

Ig domains
Thrombospondin
ZU-5 domain
Death domain
Unc5H
Ig domains
FNIII repeats
P1
P2
P3
DCC

Figure.2.2. Rho-family GTPases are key regulators of cell morphology and cell growth control.

Rho proteins act as molecular switches, "on" when bound to GTP and "off" when coupled to GDP. Rho GTPases are activated by exchange factors (GEFs) and down regulated by GAPs, in addition to guanine nucleotide dissociation inhibitors (GDIs) which are thought to block the GTPase cycle by sequestering the GDP-bound form. Activated, GTP-bound Rho proteins are believed to interact with downstream targets (effectors) which possibly mediate a signal transduction cascades towards actin cytoskeleton remodeling.

Adapted from: Tcherkezian, J., and N. Lamarche-Vane. 2007. Current knowledge of the large RhoGAP family of proteins. Biol Cell. . 99:67-86.

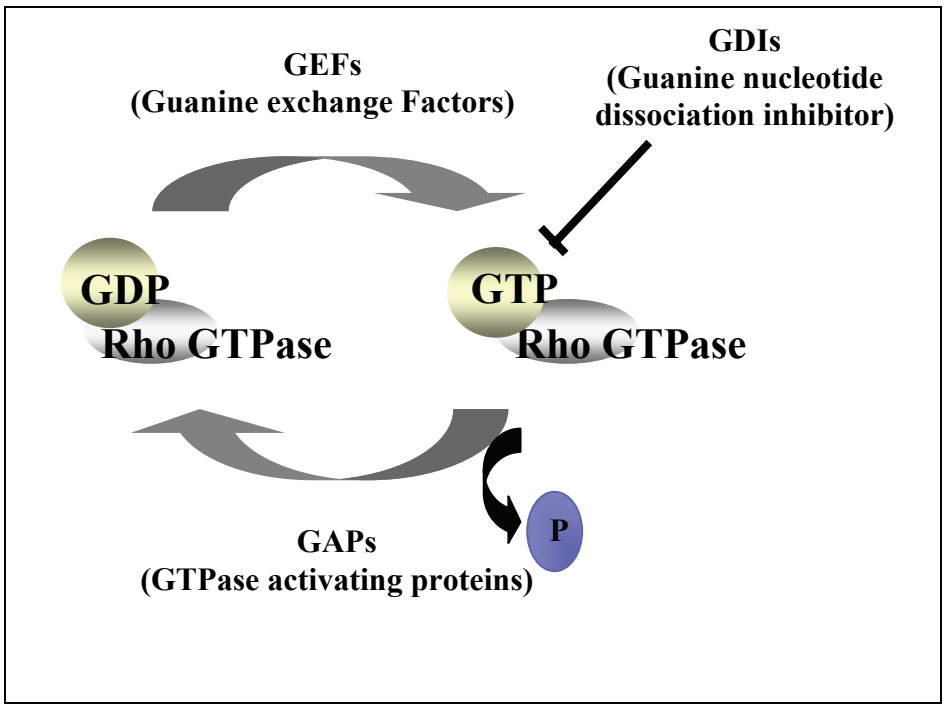
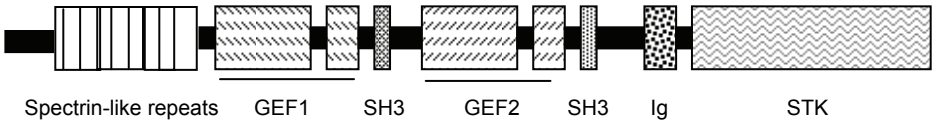
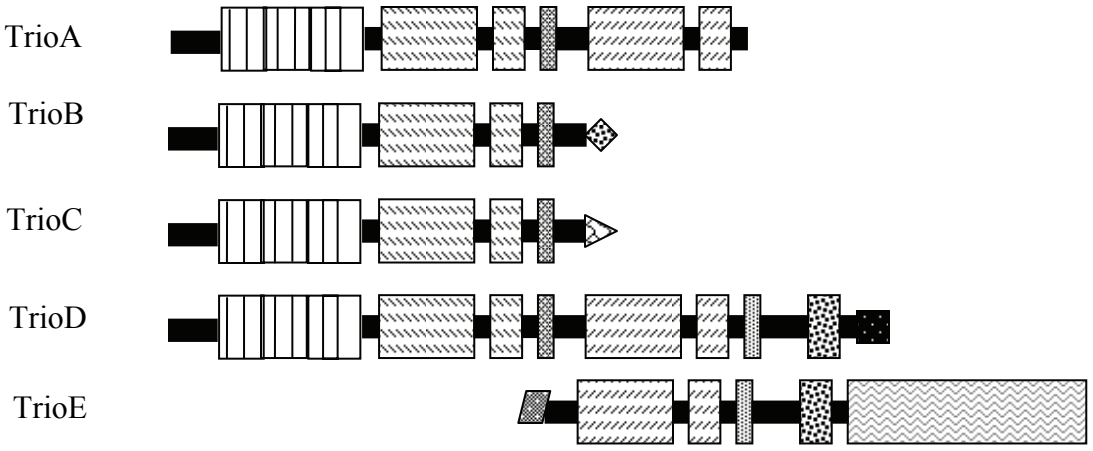

GEFs
(Guanine exchange Factors)
GDIs
(Guanine nucleotide
dissociation inhibitor)
GDP
Rho GTPase
GTP
Rho GTPase
P
GAPs
(GTPase activating proteins)

Figure 2.3. Schematic representation of the Trio family members

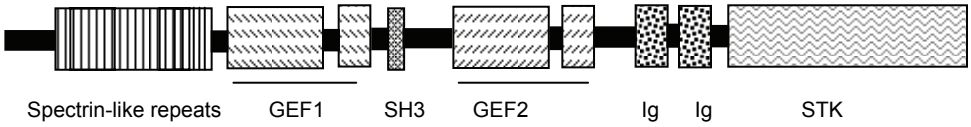
Trio family members in vertebrates (Trio and Kalirin), *C. elegans* (Unc-73) and *D. melanogaster* (D-Trio), showing spectrin repeats, two GEF domains, including DH domain, a PH domain and a Src homology 3 (SH3) domain between these GEF domains. There are some differences in the C-terminal of these proteins as human Trio encodes a second SH3 domain, an Ig like domain and a serine/threonine kinase (STK) domain, while kalirin encodes two Ig like repeats and an STK domain. Unc-73 has an Ig-like repeat and a fibronectin-like domain (FNIII), however D-Trio terminates immediately after the C-terminal PH domain.

Trio (Human)

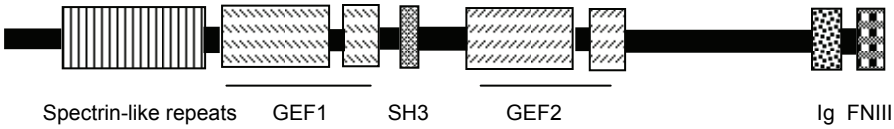




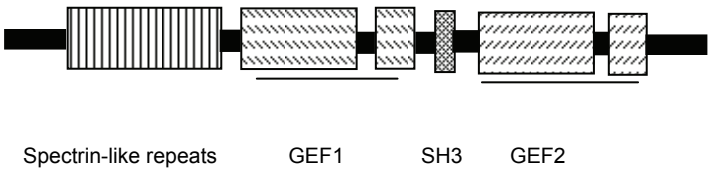
Kalirin (Human, Rat)



UNC-73 (*C.elegans*)



Trio (*D. melanogaster*)



Rationale and Objective

Rationale

Netrins are a small family of secreted proteins that guide growing axons during neural development by binding to the receptor DCC. Rho GTPases regulate multiple cellular processes affecting both cell proliferation and cytoskeleton dynamics. Their cycling between inactive GDP and active GTP bound states is regulated by GEFs and GAPs. It has been shown that the Rho family of GTPases, specifically Rac1 and Cdc42, are essential for DCC mediated neurite outgrowth. Rac1 and Cdc42 act as molecular switches, mediating cytoskeleton remodelling when they are active and bound to GTP. Since DCC does not interact directly with Rac1, there should be an indirect link between DCC and Rac1 activation. Trio is a GEF that activates Rac1 and RhoA. The orthologs of Trio in *C.elegans* (Unc-73) and in *D. melanogaster* have been shown to play important roles in axon guidance, suggesting that mammalian Trio may link DCC to Rac1 activation.

Objective

To investigate the role of guanine-nucleotide-exchange factor Trio, in netrin-1/DCC signaling pathways.

Preface to Chapter 3

The detailed process of intracellular mechanisms of netrin-1 and DCC mediating axon outgrowth and guidance is still unclear. However, many lines of evidence indicate that Rho GTPases are regulators of cytoskeletal dynamics in growth cone guidance. It has been proposed that the activation of Rac1 or Cdc42 by attractive guidance cues induces the formation of filopodia and lamellipodia, which leads to growth cone extension and attraction, whereas repulsion of the growth cone involves activation of RhoA. Rho GTPases are activated by GEFs, which provoke navigation of the growth cone in the developing nervous system. It has been shown that Rac1 and Cdc42 are activated downstream of DCC signaling, but the GEF which links DCC to activation of these Rho GTPases is not known. Trio is a GEF that has been shown to be important in neuronal extension in PC12 cells. Here, using biochemical and cellular strategies, and taking advantage of Trio knock out mice, we investigated role of Trio in activation of Rho GTPases specifically Rac1, downstream of nertin-1 and DCC signaling pathway.

CHAPTER 3

Trio Mediates Netrin Rac1 Activation and Axon Guidance

Trio Mediates Netrin-1-Induced Rac1 Activation and Axon Guidance

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3.1. Abstract

The chemotropic guidance cue netrin-1 promotes axon outgrowth through its receptor DCC (Deleted in Colorectal Cancer) via activation of Rac1. However, the guanine nucleotide exchange factor (GEF) linking netrin-1/DCC to Rac1 activation has not yet been identified. Here, we show that Trio is this GEF. We found that Trio, Nck1, PAK1 and DCC are present in the same signaling complex, and that netrin-1-induced Rac1 activation is impaired in the absence of Trio. Trio -/- cortical neurons fail to extend neurites in response to netrin-1, whereas they are able to respond to glutamate. Accordingly, netrin-1-induced commissural axon outgrowth is severely impaired in Trio -/- spinal cord explants and commissural axon projections are defective in Trio -/- embryos. In addition to defects in spinal cord development, 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 also defective in Trio -/- embryos. Thus, Trio through its ability to activate Rac1 mediates netrin-1 signaling in axon growth and guidance.

3.2. 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 responds to them by triggering signaling pathways that regulate growth cone motility (Guan and Rao, 2003; Huber et al., 2003). Netrins are a family of secreted proteins that control axon outgrowth and guidance in multiple vertebrate and invertebrate species (Barallobre et al., 2005). 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 (Kennedy et al., 1994; Serafini et al., 1994). Since then, netrin-1 has been shown to promote outgrowth of a wide variety of axons, including growing cortical axons (Liu et al., 2004; Richards et al., 1997).

Two families of netrin-1 receptors in mammals have been identified; the DCC (Deleted in Colorectal Cancer) family, comprising DCC and neogenin, and the Unc-5 family of proteins (Ackerman et al., 1997; Keino-Masu et al., 1996; Leonardo et al., 1997). DCC mediates growth cone attraction induced by netrin-1 (Ackerman et al., 1997; Keino-Masu et al., 1996; Leonardo et al., 1997; Serafini et al., 1996), whereas the repulsive effect of netrin-1 is mediated by the Unc-5 family of netrin receptors, alone or in combination with DCC (Hong et al., 1999).

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 of different DCC-binding proteins including the protein tyrosine kinases FAK, Src and Fyn, the Nck1 adaptor protein, and PITP-alpha (Li et al., 2004; Li et al., 2002a; Liu et al., 2004; Ren et al., 2004; Xie et al., 2005).

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 (Li et al., 2004; Meriane et al., 2004). 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 (Barallobre et al., 2005). 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 (Govek et al., 2005). Small GTPases are molecular switches that oscillate between an inactive GDP-bound state and an active GTP-bound state, and are activated by GEFs that accelerate the GDP/GTP exchange (Rossman et al., 2005). Cellular and genetic studies have shown that Rac, Cdc42 and RhoG promote neurite extension and growth cone motility in response to guidance cues, whereas RhoA mediates neurite retraction through growth cone collapse (Dickson, 2001). We and others have shown that the binding of netrin-1 to DCC activates the small GTPase Rac1 (Li et al., 2002b; Shekarabi and Kennedy, 2002), and that the adaptor protein Nck1 is required for this activation (Li et al., 2002a). 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 (Blangy et al., 2000; Debant et al., 1996). However, functional analysis indicates that the role of Trio in all organisms mainly depends on the catalytic activity of GEFD1. Genetic analysis of Trio orthologs in *C. elegans* (Unc-73) and in *D. melanogaster* have established Trio as a key component in the regulation of axon guidance and cell migration (Awasaki et al., 2000; Bateman et al., 2000; Bellanger et al., 1998; Liebl et al., 2000; Newsome et al., 2000; Steven et al., 1998). Moreover, D-Trio, the kinase Abl, the Abl substrate Ena, and the netrin receptor Frazzled are part of a signaling complex that regulates axon guidance at the CNS midline in *D. melanogaster* (Forsthoefel et al., 2005). 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 (Estrach et al., 2002). Moreover, targeted disruption of

Trio in mouse resulted in embryonic lethality between E15.5 and birth, suggesting that Trio is essential for late embryonic development, probably by playing critical roles in neural tissue and fetal skeletal muscle formation (O'Brien et al., 2000). However, the function of mammalian Trio in axon guidance remains to be determined. In addition, the upstream signaling pathways leading to Trio activation in axon guidance and in the development of the nervous system 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, Nck1, PAK1 and DCC are present in the same signaling complex and that netrin-1-induced Rac1 activation is abolished in Trio -/- embryonic brains. Cortical neurons are defective in extending neurites in response to netrin-1, whereas they respond to glutamate stimulation. Likewise, netrin-1-induced axon outgrowth is also severely reduced in Trio -/- spinal cord explants. Finally, netrin-1 and DCC-dependent neuronal projections are impaired in Trio deficient mouse embryos.

3.3. Experimental Procedures

Trio Constracts

The GFP Trio constructs, GFP-RhoGA37, pRK5-DCC, pGEX4T2-DCC-C, and pRK5myc-PAK have been previously described (Estrach et al., 2002; Lamarche et al., 1996; Li et al., 2002a; Li et al., 2002b). The Nck1 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 (O'Brien et al., 2000). Trio heterozygous mice have been kindly provided by Dr. Michel Streuli (Harvard Medical School). To obtain Trio-null embryos, female of Trio +/- Balb/C mice were crossed with Trio +/- males. 24 hours post-coitum is designated as embryonic day 0 (E0). 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 (O'Brien et al., 2000).

Cell culture, transfection, and immunoprecipitation

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 (Li et al., 2002b). 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) 10 µg/ml aprotinin, and 10 µg/ml leupeptin. 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.

GST-Pull Down

Total E17 brains or 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, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 1 mM PMSF). Lysates were then incubated for 2h at 4°C with 30µg of either GST, GST-DCC, GST-PAK, or GST-Nck1 fusion proteins. 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 (Portales-Casamar et al., 2006), anti-Nck1 (BD Transduction Laboratories), anti-DCC (BD Biosciences), 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 similar to the method described elsewhere (Falk et al., 2005; Llambi et al., 2005). 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 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 to nitrocellulose. Membranes were immunoblotted with anti-Rac1 antibody (BD Transduction Laboratories Inc).

Immunofluorescence Microscopy

Neurons were fixed and permeabilized as previously described (Estrach et al., 2002). 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 (Li et al., 2002b). 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 (Meijering et al., 2004) 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 E 11.5 wt, Trio +/- or -/- embryos were dissected and cultured in three-dimensional collagen type I (BD Biosciences) gels as described previously (Tessier-Lavigne et al., 1988) (See appendix II). Recombinant chick netrin-1 protein was produced and purified as described (Serafini et al., 1994). 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 (Falk et al., 2005). 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 (Okada et al., 2006). Quantification analysis of axon defasciculation in Trio -/- embryos was performed on sections positioned at the forelimb of wild type and Trio -/- embryos.

3.4. 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 (Li et al., 2002b). 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 (Figure 3.1), which drastically reduces its *in vitro* exchange activity towards RhoG/Rac1 (Estrach et al., 2002). 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, suggesting that Trio mediates netrin-1/DCC-induced neurite outgrowth in N1E-115 cells (Figure 3.1). The GEFD1 domain of Trio has been previously shown to be active on both Rac1 and RhoG small GTPases (Blangy et al., 2000).

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 (Figure 3.1), in contrast to RacN17, which blocked DCC effect on neurite extension (Li et al. 2002b). Therefore, it is likely that Trio participates via Rac1 and not RhoG in the intracellular mechanisms regulated by netrin-1/DCC leading to neurite outgrowth.

Trio, Nck1, PAK1 and DCC are present in the same signaling complex in a netrin-1-independent manner

To determine whether Trio interacts with DCC, GFP-Trio was co-expressed with DCC in HEK-293 cells that do not express endogenous DCC or netrin-1 (Li et al., 2002b; Shekarabi and Kennedy, 2002). We found that Trio was able to co-precipitate with DCC in the absence of netrin-1 stimulation, with no band corresponding to Trio in immunoprecipitates using normal mouse IgGs (Figure 3.2A). To confirm the interaction of endogenous Trio with DCC in embryonic brains, embryonic brain lysates were incubated with the recombinant intracellular domain of DCC expressed as a glutathione S-transferase (GST) fusion protein in *E.coli*. As shown in Figure 3.2B, Trio and two neuronal-specific isoforms of Trio (Portales-Casamar et al., 2006) were identified to co-precipitate with GST-DCC, with no band corresponding to Trio in the negative GST control.

To characterize the interaction between Trio and DCC, we examined whether Trio associates with DCC via the adaptor protein Nck1 and the serine/threonine protein kinase PAK1. Indeed, Rac1, PAK1 and DOCK, the *D. melanogaster* ortholog of Nck1, interact genetically with D-Trio during axon guidance of *D. melanogaster* photoreceptors (Newsome et al., 2000). Furthermore, PAK1 is known to interact with the second SH3 domain of Nck1 (Buday et al., 2002) whereas the cytoplasmic domain of DCC interacts with the first and third SH3 domains of Nck1 (Li et al., 2002a). We performed GST-pull down experiments using either recombinant GST-Nck1 (Figure 3.2C) or GST-PAK1 (Figure 3.2D) and lysates from COS-7 cells expressing GFP-Trio, DCC, Nck1, PAK1, alone or together. Interestingly, we found that Trio alone interacted with PAK1 but not with Nck1, whereas DCC was able to interact with both proteins (Figure 3.2C and D). When PAK1 or DCC were co-expressed with Trio, Trio was now able to co-precipitate with GST-Nck1 (Figure 3.2C). Finally, DCC, Trio, PAK1 and Nck-1 were found to co-precipitate together (Figure 3.2C and D) suggesting that Trio associates with DCC via a Nck1-PAK1 molecular interaction. Together, these results demonstrate that a multimolecular signaling complex comprising of Trio, Nck1, PAK1 and DCC is assembled in a netrin-1-independent manner.

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 (Li et al., 2002b; Shekarabi and Kennedy, 2002), but this has never been tested on endogenous Rac1 expressed in brain. We found that netrin-1 addition to wild-type embryonic mouse brains induced a rapid Rac1 activation with a peak at 5 minutes of stimulation (Figure 3.3A). 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 (Figure 3.3B). 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 Figure 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.

Defects in axon outgrowth of Trio -/- cortical neurons

The experiments described above show that Trio is essential for netrin-1-induced Rac1 activation and neurite outgrowth. We then examined the neuronal morphology of Trio -/- cortical neurons, which has never been characterized before. Since the Trio-null mice die between E15 and birth (O'Brien et al., 2000), we measured the length and number of neurites in dissociated cortical neurons from wt or Trio -/- E14.5 embryos at different days *in vitro* (DIV) of cell culture. The longest neurite (which we consider the axon) rapidly elongated in wt but not in Trio -/- neurons expressing Green Fluorescent Protein (GFP) (Figure 3.4A and B). Overexpression of GFP-Trio in mutant neurons was able to rescue the axon outgrowth defect in Trio-null neurons, showing that the defect was due to the lack of Trio protein (Figure 3.4A and C). When the distribution of axon length was analyzed at 3DIV in wt and Trio -/- neurons expressing GFP, the percentage of short axons (<40μm) in mutant neurons was 55% compared to 27% in wt neurons whereas the percentage of long axons (> 120μm) was significantly reduced in Trio-null neurons compared to the percentage for wt neurons (Figure 3.4D). The expression of GFP-Trio in Trio-deficient neurons was able to rescue the altered axon length

distribution. In addition, we found that the number of neurites was decreased in Trio-null neurons compared to wt neurons after 3DIV (Figure 3.4E). Taken together, these results indicate that cortical neurons from Trio -/- embryos show significant defects in axon outgrowth *in vitro*.

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 (Liu et al., 2004; Ren et al., 2004; Richards et al., 1997). 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 dissociated cortical neurons from wt or Trio-null embryos. As shown in Figure 3.5A, 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 (Figure 3.5A and B). In contrast, netrin-1 failed to stimulate axon outgrowth of Trio-null cortical neurons (Figure 3.5A and B). Consistently, analysis of the distribution of axon length in neurons showed that netrin-1 increased the percentage of long axons (> 48μm) in wt and not in mutant neurons (Figure 3.5D). 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 (Figure 3.5E). To determine whether the defect in neurite outgrowth of Trio -/- cortical neurons was specific to netrin-1 stimulation, we tested the ability of Trio -/- neurons to extend neurites in response to glutamate, which has been shown to stimulate growth cone motility by different pathways including Ca^{2+}-dependent activation of Rho GTPases (Jin et al., 2005; Kreibich et al., 2004; Zheng et al., 1996). As shown in Figure 3.5A and B, glutamate stimulated axon outgrowth in both wt and Trio-deficient neuronal cells at a similar level. 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 (Figure 3.5D). Eventhough the average axon length of Trio null neurons was lower than the one of wt neurons (Figure 3.4B), 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) (Figure 3.5C). Altogether, these data show that Trio plays a specific role in netrin-1-induced axon outgrowth in cortical neurons.

Netrin-1-induced axon outgrowth is severely impaired 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 Figure 3.6A, 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 was reduced 2-fold compared to wt explants (Figure 3.6A and B). In addition, the number of axon bundles was severely decreased in explants from Trio -/- dorsal spinal cords (Figure 3.6B). Interestingly, the loss of one Trio allele also affected the response of the explants to netrin-1 (Figure 3.6A and B). 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 Figures 3.6C and D, 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 important defects in spinal cord and brain development

To determine the role of Trio in netrin-1 function *in vivo*, we next investigated whether the different commissures controlled by netrin-1/DCC were affected in Trio -/- embryos. We first examined the axon projections of the commissural neurons in the spinal cord of wt and Trio-null littermates by immunostaining with anti-DCC antibodies

(Figure 3.7). In wt embryos, commissural axons are directed ventrally towards the floor plate of the developing spinal cord, which secretes the chemoattractant netrin-1 (Kennedy et al., 1994). In Trio -/- embryos, commissural axons could reach the floor plate, but they appeared highly defasciculated, with several axon bundles in the ventral spinal cord (Figure 3.7A and E). These results demonstrate that Trio is necessary for proper axon pathfinding of commissural neurons towards the floor plate.

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 (Fazeli et al., 1997; Serafini et al., 1996). Therefore, we examined the anterior commissure projections in sections of Trio-null brains (Figure 3.8). Several horizontal sections 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 (Figure 3.8A, compare a to f-h). A few remnant axons projecting out of the cortex were identified, yet with aberrant localization (Figure 3.8A, arrows in g, h). 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 (Figure 3.8A, b-d). At the midline crossing level, the neuropilin 2 (NRP2) staining in the heterozygous embryos showed that the fibers were able to cross the midline (Figure 3.8A, e). To confirm that the anterior commissure was absent in Trio -/- embryos, we analyzed different coronal sections of Trio +/- and -/- embryonic brains. As shown in Figure 3.8B, defasciculated fibers were present in the heterozygous embryos but were completely absent in the Trio-null embryos. 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 (Figure 3.9A). 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 (Figure 3.9A). Therefore, the corpus callosum is defective 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 (Braisted et al., 2000), 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 (Figure 3.9B). Thus, these findings strongly support a role for Trio in netrin-1/DCC-dependent projections in the developing spinal cord and embryonic brain.

3.5. Discussion

Our findings support a role for mammalian Trio in axon outgrowth and guidance induced by netrin-1. First, we show that Trio, Nck1, PAK1 and DCC are present in the same signaling complex in a netrin-1-independent manner. Second, netrin-1-induced Rac1 activation is defective in Trio -/- embryonic brains. Third, cortical neurons from Trio-null embryos do not extend neurites in response to netrin-1. Fourth, netrin-1-induced commissural axon outgrowth is impaired in Trio-null spinal cord explants. Finally, Trio-deficient mouse embryos show important defects in DCC-and netrin-1-dependent axon projections in the developing spinal cord and embryonic brain.

We demonstrate that DCC, Nck1, PAK1, and Trio are present in the same signaling complex. These data are consistent with the results obtained in *D. melanogaster* where D-Trio genetically interacts with DOCK, PAK1, and Rac1 in controlling axon guidance of photoreceptors (Newsome et al., 2000). Since Nck1 binds to DCC through its first and third SH3 domains (Li et al., 2002a), and PAK1 binds to the second SH3 domain of Nck1 (Buday et al., 2002), it is tempting to postulate that a cascade of molecular events implicating Nck1/PAK1 interaction serves to bridge Trio to DCC (Figure 3.10). The mechanisms by which Trio becomes activated when netrin-1 binds to DCC remains unknown but may involve phosphorylation by FAK or the Src family kinase Fyn (Medley et al., 2003; Meriane et al., 2004).

We show here 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 (Blangy et al., 2000). 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 participates in NGF-induced neurite outgrowth (Estrach et al., 2002), 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 (Jin et al., 2005; Kreibich et al., 2004; Zheng et al., 1996). Likewise, commissural neurons from Trio -/- spinal cord explants also show important defects in axon extension in response to netrin-1, while the 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 (Fazeli et al., 1997; Serafini et al., 1996). 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. Consistently, the few remnant anterior commissural axons that could be detected in Trio -/- brains were aberrantly localized. Likewise, in the absence of Trio, the commissural axons of the spinal cord are able to reach the floor plate but the axon bundles are highly defasciculated in the ventral spinal cord, demonstrating that Trio is necessary for the proper pathfinding of axons attracted by netrin-1. Similarly, Trio participates 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. The fact that axon outgrowth and guidance of commissural neurons or cortical projections to the corpus callosum are not completely abolished in the Trio-null embryos is not surprising since netrin-1 binding to DCC promotes, in addition to Rac1 activation, several intracellular pathways such as MAPK and phosphatidylinositol signaling important for the response of axons to netrin-1 (Barallobre et al., 2005). 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.

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 (Braisted et al., 2000; Richards et al., 1997). Interestingly, we have detected a dramatic disorganization of the internal capsule in Trio -/- brains, supporting the hypothesis that Trio is implicated in both reciprocal pathways.

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

Figures and Figure Legends

Figure 3.1: Trio defective in Rac1 activation inhibits DCC-induced neurite outgrowth in N1E-115 neuroblastoma cells.

N1E-115 cells were transfected with the indicated plasmids using the LipofectAMINE reagent (Invitrogen), according to the manufacturer's protocol. 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).

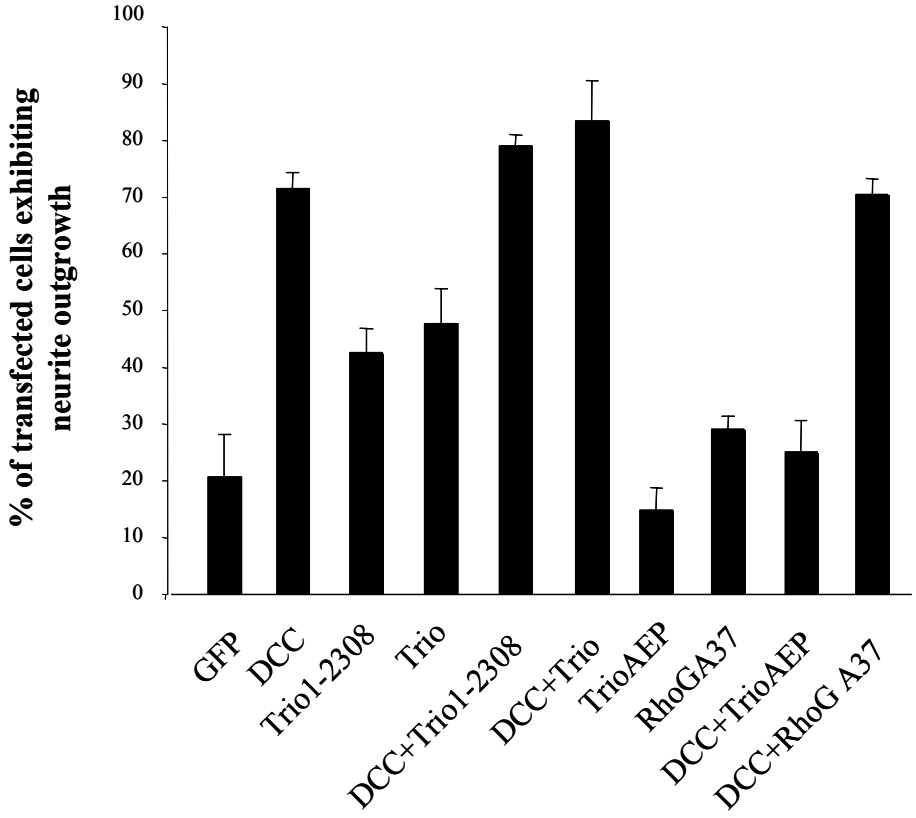
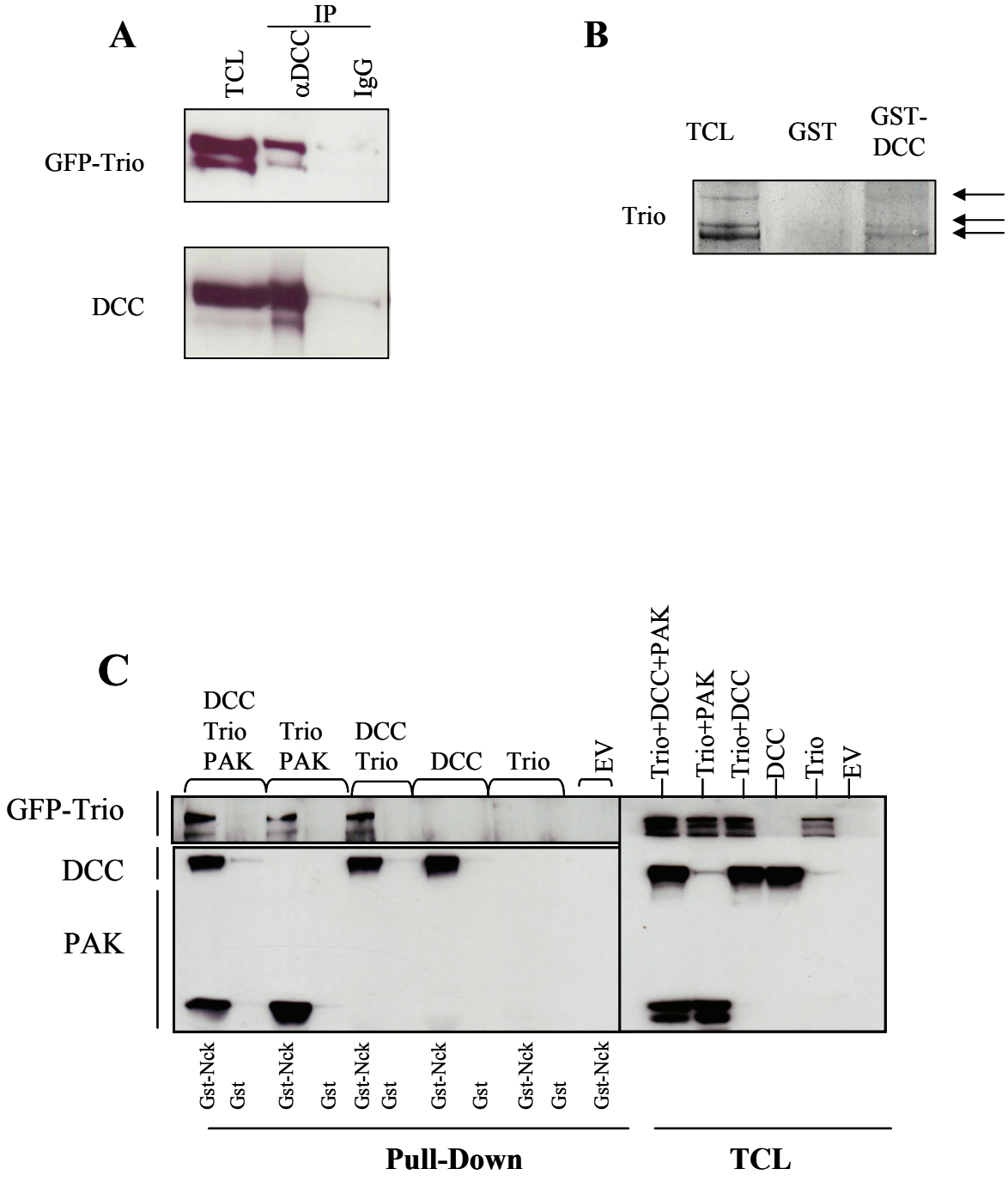

% of transfected cells exhibiting neurite outgrowth
100
90
80
70
60
50
40
30
20
10
0
GFP
DCC
Trio1-2308
Trio
DCC+Trio1-2308
DCC+Trio
TrioAEP
RhoGA37
DCC+TrioAEP
DCC+RhoG A37

Figure 3.2: Trio, Nck1, PAK1 and DCC are present in the same signaling complex

(A) Lysates of HEK-293 cells transfected with pEGFP-Trio and pRK5-DCC were submitted to co-immunoprecipitation (co-IP) using anti-DCC antibodies (αDCC) or mouse Immunoglobulin G (IgG) coupled to protein G-sepharose beads. Total cell lysates (TCL) and immunoprecipitated proteins (IP) 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 E17 brains were incubated with either GST or GST-DCC fusion proteins coupled to glutathione-Sepharose beads. Trio was detected by western blotting using polyclonal anti-Trio antibodies.

(C and D) Lysates of COS-7 cells transfected with pRK5 (empty vector), pRK5-DCC, pEGFP-Trio, pRK5-HA-Nck1 or pRK5myc-PAK1, alone or as indicated, were incubated with either GST, GST-Nck1 (C) or GST-PAK (D). Total cell lysates (TCL) and precipitated proteins (pull-down) were submitted to SDS-PAGE, and proteins were detected by Western blotting analysis using anti-DCC, anti-GFP, anti-PAK1 and anti-Nck1 antibodies.


A
IP
TCL
αDCC
IgG
GFP-Trio
DCC
B
TCL
GST
GST-DCC
Trio
C
DCC
Trio
PAK
Trio
PAK
DCC
Trio
DCC
Trio
EV
Trio+DCC+PAK
Trio+PAK
Trio+DCC
DCC
Trio
EV
GFP-Trio
DCC
PAK
Gst-Nck
Gst
Gst-Nck
Gst
Gst-Nck
Gst
Gst-Nck
Gst
Gst-Nck
Gst
Gst-Nck
Pull-Down
TCL

D

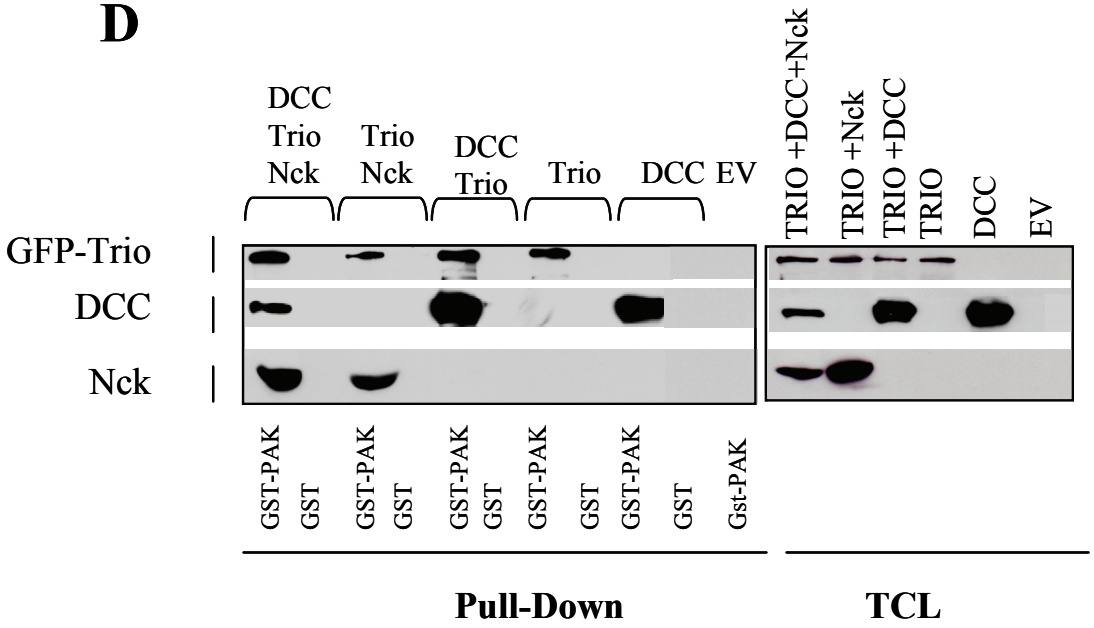
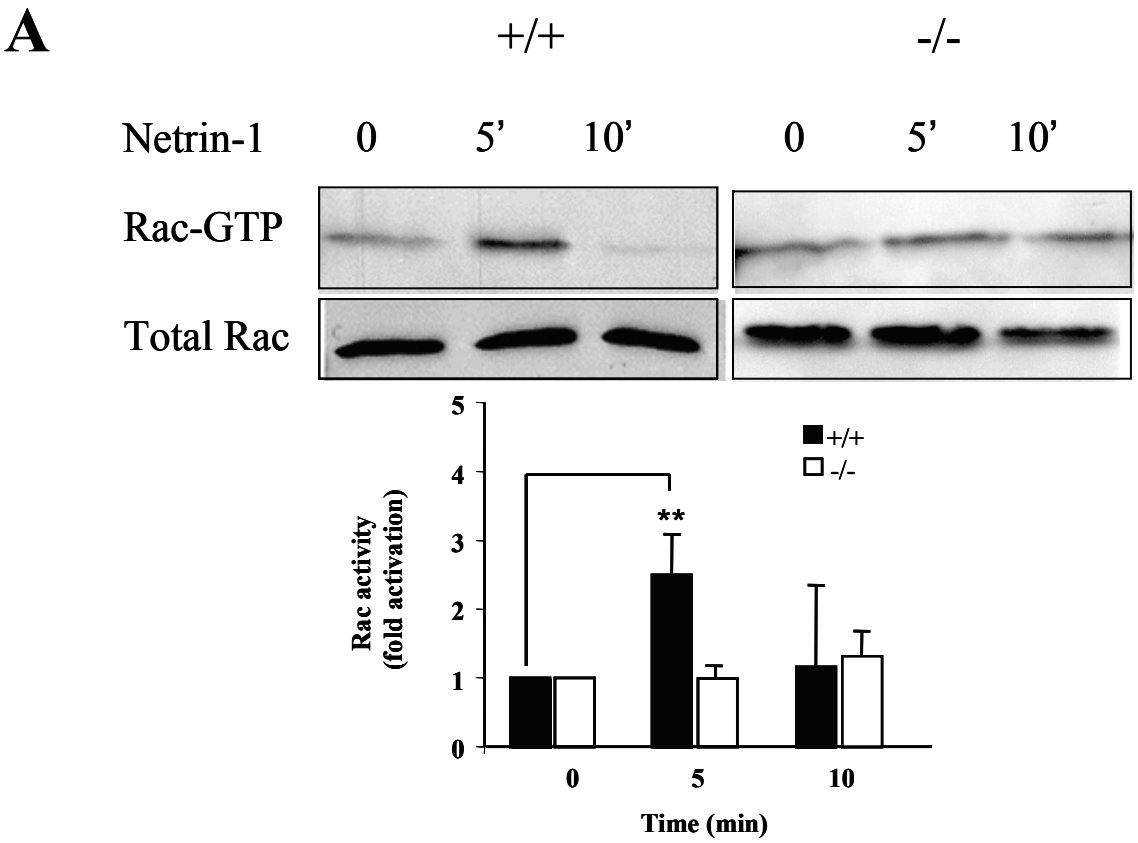

DCC
Trio
Nck
Trio
Nck
DCC
Trio
Trio
DCC
EV
TRIO +DCC+Nck
TRIO +Nck
TRIO +DCC
TRIO
DCC
EV
GFP-Trio
DCC
Nck
GST-PAK
GST
GST-PAK
GST
GST-PAK
GST
GST-PAK
GST
GST-PAK
GST
Gst-PAK
Pull-Down
TCL

Figure 3.3: Netrin-1-induced Rac1 activation is impaired in Trio null-embryonic brains

(A) GTP-loaded Rac1 was precipitated using GST-PAK-PBD from lysates of wild-type 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. Error bars represent SD. P-value < 0.01.

(B) The experiment was performed as described in (A), except that DCC blocking antibodies or anti-GFP as control antibodies (4 µg/ml) were added before netrin-1 stimulation (5 minutes). Quantification of Rac1 activity corresponds to the average of at least three independent experiments. P-value < 0.05 (Student T-Test). Error bars represent SD.


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

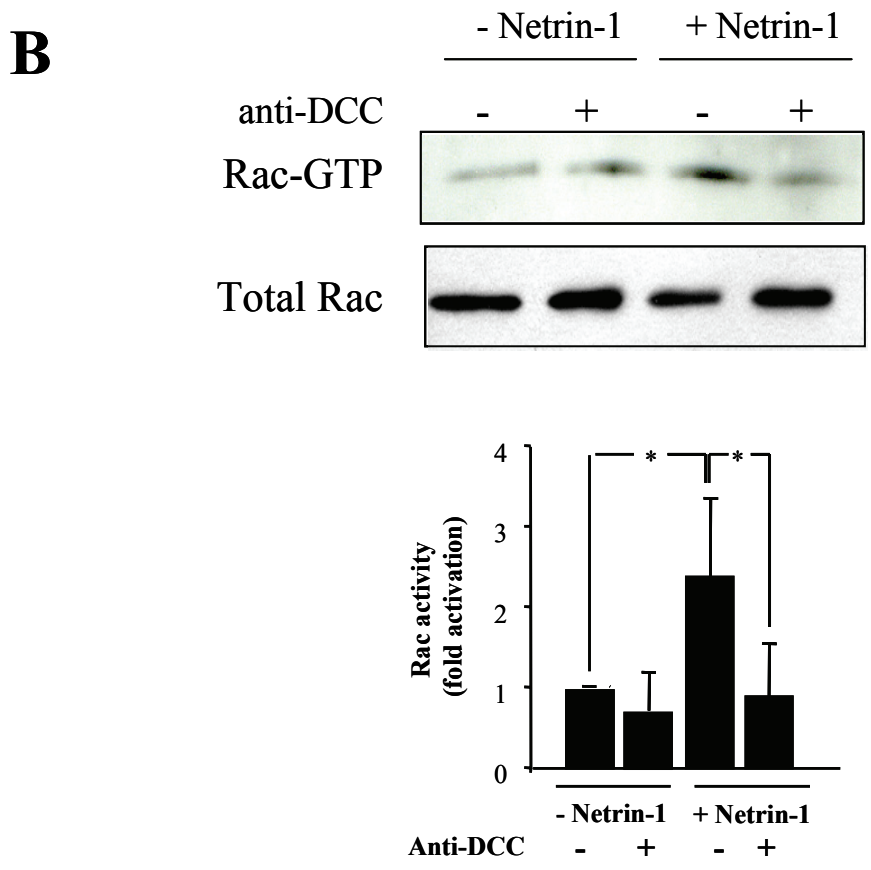

B
- Netrin-1
+ Netrin-1
anti-DCC
Rac-GTP
Total Rac
Rac activity
(fold activation)
*
*
- Netrin-1
+ Netrin-1
Anti-DCC

Figure 3.4: Defects in axon outgrowth in Trio -/- cortical neurons

(A) Neurite outgrowth of cortical neurons from +/+ or Trio -/- embryos after 3 days *in vitro* (DIV3) of cell culture. Cells were transfected with GFP or GFP-Trio as indicated. Scale bar= 25μm.

(B) Quantification of average axon length of +/+ or Trio -/- cortical neurons after 1.5, 2, or 3 DIV. Values are represented as a percentage of the average axon length of wt cortical neurons expressing GFP at DIV 1.5. $p< 0.001$ between +/+ and -/- at DIV 1.5; $p= 0.01$ between +/+ and -/- at DIV 2; $p= 0.02$ between +/+ and -/- at DIV 3. n=6 for +/+ and -/- embryos.

(C) Quantification of average axon length of cortical neurons transfected with GFP or GFP-Trio at DIV 3. Values are represented as a percentage of average axon length of wt cortical neurons expressing GFP at DIV 3. $p= 0.001$ between GFP-transfected +/+ and Trio -/- neurons; $p<=0.01$ between Trio -/- neurons transfected with GFP or GFP-Trio. n=5 for +/+ and -/- embryos.

(D) Distribution of average axon length from panel C.

(E) Quantification of the number of neurites of +/+ or Trio -/- cortical neurons. $p= 0.002$ between +/+ and -/- cortical neurons at DIV 3. Error bars represent SD.

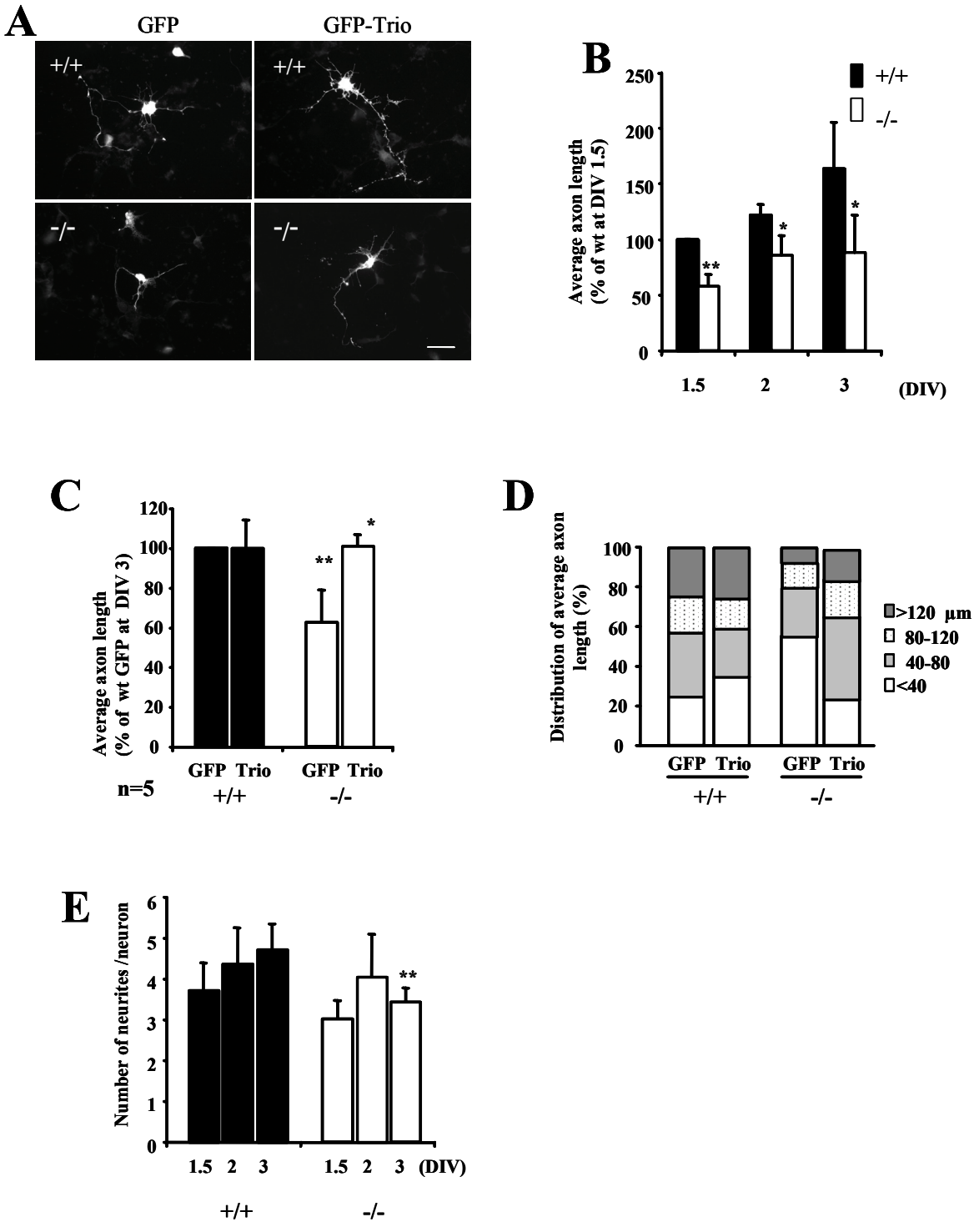

A
GFP
GFP-Trio
+/+
+/+
-/-
-/-
B
Average axon length
(% of wt at DIV 1.5)
250
200
150
100
50
0
+/+
-/-
**
*
*
1.5
2
3
(DIV)
C
Average axon length
(% of wt GFP at DIV 3)
120
100
80
60
40
20
0
**
*
GFP Trio
GFP Trio
n=5
+/+
-/-
D
Distribution of average axon
length (%)
100
80
60
40
20
0
>120 µm
80-120
40-80
<40
GFP Trio
GFP Trio
+/+
-/-
E
Number of neurites /neuron
6
5
4
3
2
1
0
**
1.5 2 3
1.5 2 3 (DIV)
+/+
-/-

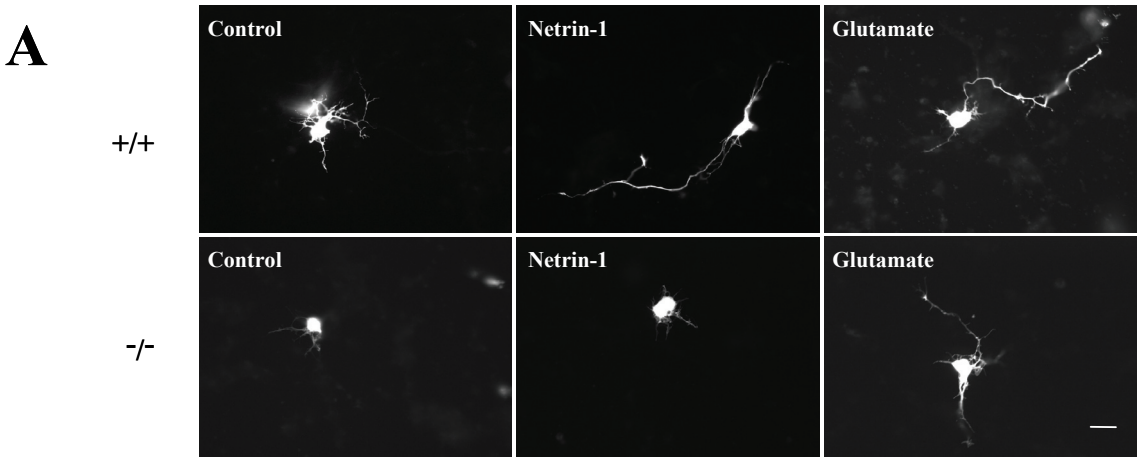
Figure 3.5: Trio-null cortical neurons do not extend neurites in response to netrin-1

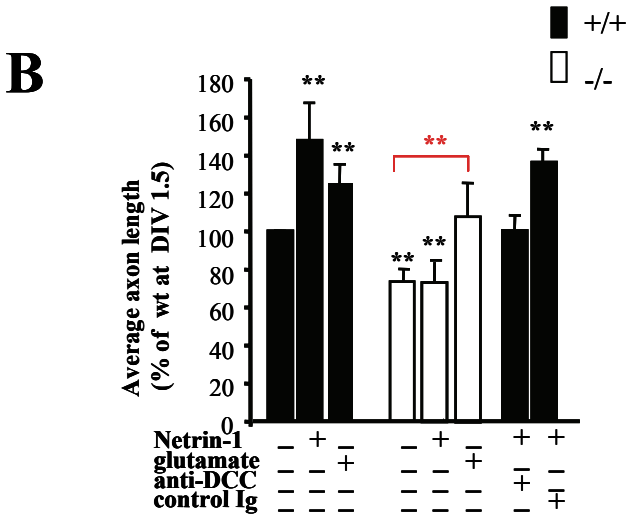
(A) Neurite outgrowth of +/+ or Trio -/- cortical neurons expressing GFP at DIV 1.5 treated with control buffer, netrin-1 (250 ng/ml) or glutamate (50μM) for 24 hours. Scale bar= 25μm.

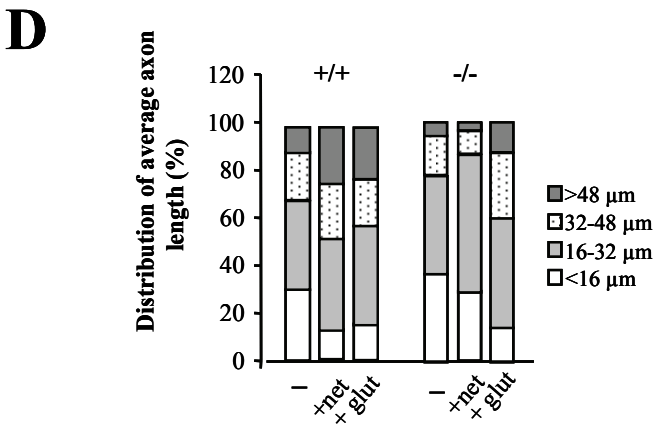
(B) Quantification of average axon length of cortical neurons presented in panel A. Values are represented as a percentage of average axon length of wt cortical neurons at DIV 1.5 incubated with control buffer. When indicated, neurons were incubated with control IgGs or DCC blocking antibodies (4 μg/ml) before netrin-1 addition. All P values are < 0.001. Black ** represent a comparison to wt neurons expressing GFP. Red ** refers to GFP transfected Trio-null neurons. n=8 for +/+ and n=10 for -/- embryos.

(C) Ratio of axon length of +/+ or Trio -/- neurons treated with netrin-1 or glutamate versus control. $p < 0.0001$.

(D) Distribution of average axon length from panel B. (E) The expression of DCC in lysates of E17 +/+ and Trio -/- brains was detected by western blotting analysis using anti-DCC antibodies.


A
Control
Netrin-1
Glutamate
+/+
Control
Netrin-1
Glutamate
-/-


B
+/+
-/-
Average axon length (% of wt at DIV 1.5)
180
160
140
120
100
80
60
40
20
0
**
**
**
**
**
Netrin-1
glutamate
anti-DCC
control Ig


D
Distribution of average axon length (%)
120
100
80
60
40
20
0
+/+
-/-
>48 µm
32-48 µm
16-32 µm
<16 µm
–
+net
+ glut
–
+net
+ glut

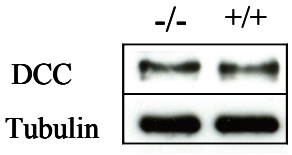

E
-/-
+/+
DCC
Tubulin

Figure 3.6: Netrin-1-induced commissural axon outgrowth is severely impaired in Trio -/- spinal cord explants

(A) E11.5 dorsal spinal cord explants from +/+, +/-, or Trio -/- embryos were incubated with control buffer or netrin-1 (500 ng/ml) for 35 hours. Scale bar= 100μm.

(B) Quantification of the number and average length of axon bundles per explant. n= 10 for Trio +/+, n= 7 for Trio +/-, n=4 for Trio -/-. Black **, $p<0.001$; red **, $p<0.0005$.

(C) E11.5 dorsal spinal cord explants from +/+ or Trio -/- embryos were cultured for 70 hours in the absence of netrin-1.

(D) Quantification of the average length of axon bundles per explant. n=3 for +/+ and -/-.

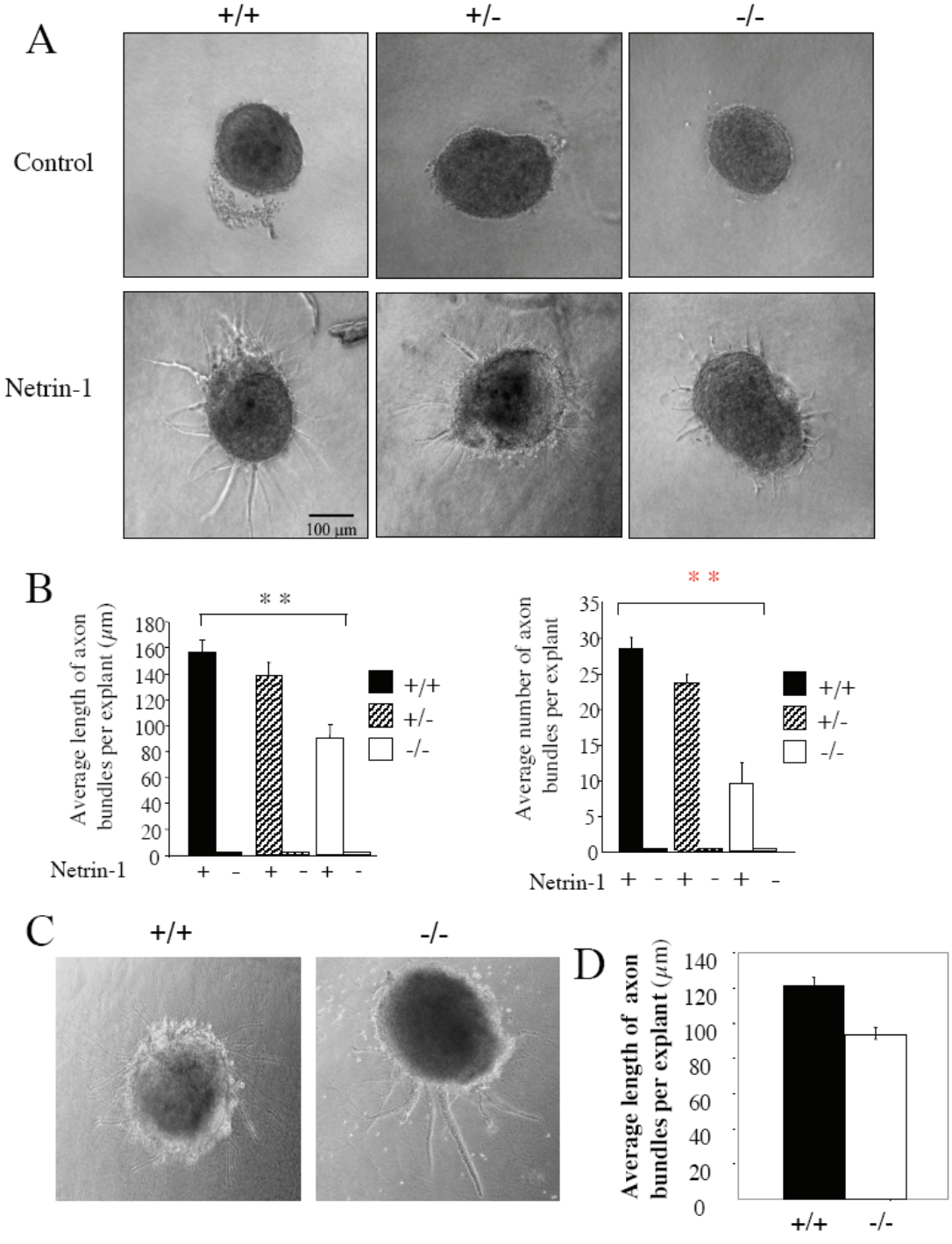

A
+/+
+/-
-/-
Control
Netrin-1
100 µm
B
Average length of axon bundles per explant (µm)
Netrin-1
+ - + - + -
+/+
+/-
-/-
Average number of axon bundles per explant
C
+/+
-/-
D
Average length of axon bundles per explant (µm)
+/+
-/-

Figure 3.7: Commissural axon projections are defective in Trio-null embryos

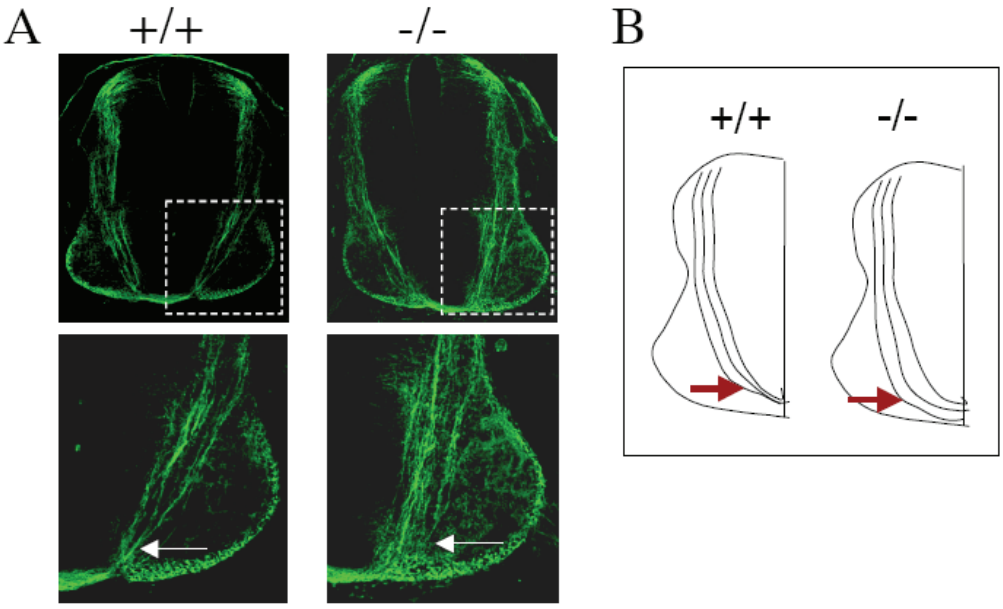
(A) Upper panels: Trajectories of commissural axons are visualized using anti-DCC antibodies in sections of +/+ or Trio -/- E11.5 embryos. Lower panels: Enlargement of the corresponding images. Scale bar, 80μm.

(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 (arrows in A and B).

(C) 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 (D).

(E) 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

(F). $p<0.001$, Student T-Test. Error bars represent SD. (n=5 for +/+ and n=7 for -/-).


A
+/+
-/-
B
+/+
-/-

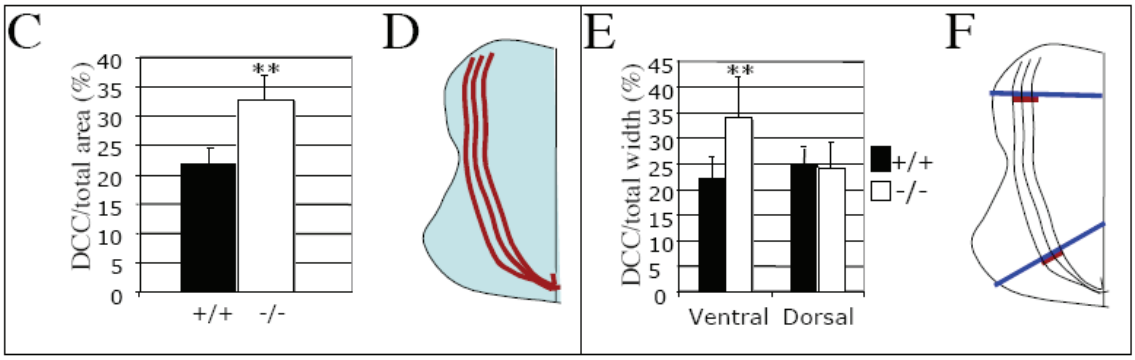
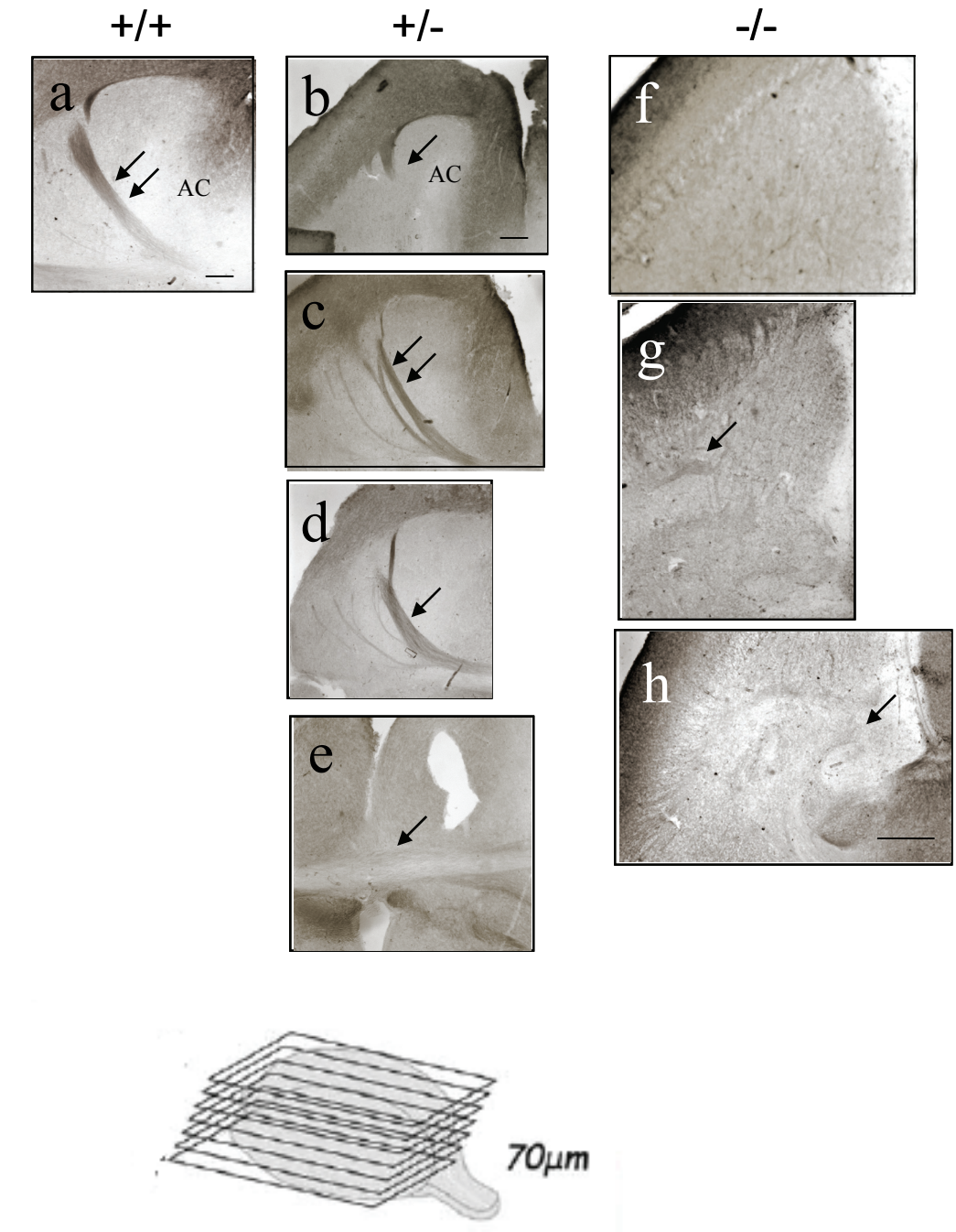

C
DCC/total area (%)
40
35
30
25
20
15
10
5
0
**
+/+
-/-
D
E
DCC/total width (%)
45
40
35
30
25
20
15
10
5
0
**
+/+
-/-
Ventral
Dorsal
F

Figure 3.8: Trio null embryos show important defects in brain commissures

(A) Neuropilin2 (Nrp2) immunostaining on horizontal serial brain sections from E17 +/+ (a) +/- (b, c, d, e) or -/- (f, g, h) 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, c, d, and e). In the Trio null embryos, the commissure is absent, even at a higher magnification than the wt (compare f with a). Some remnant anterior commissural axons can still be detected in some cases (arrows in g, h). Bar: 80µm in +/+ and +/-, 200µm in -/-.

(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). Bar 300µm.


A
+/+
+/-
-/-
a
AC
b
AC
c
d
e
f
g
h
70µm

B

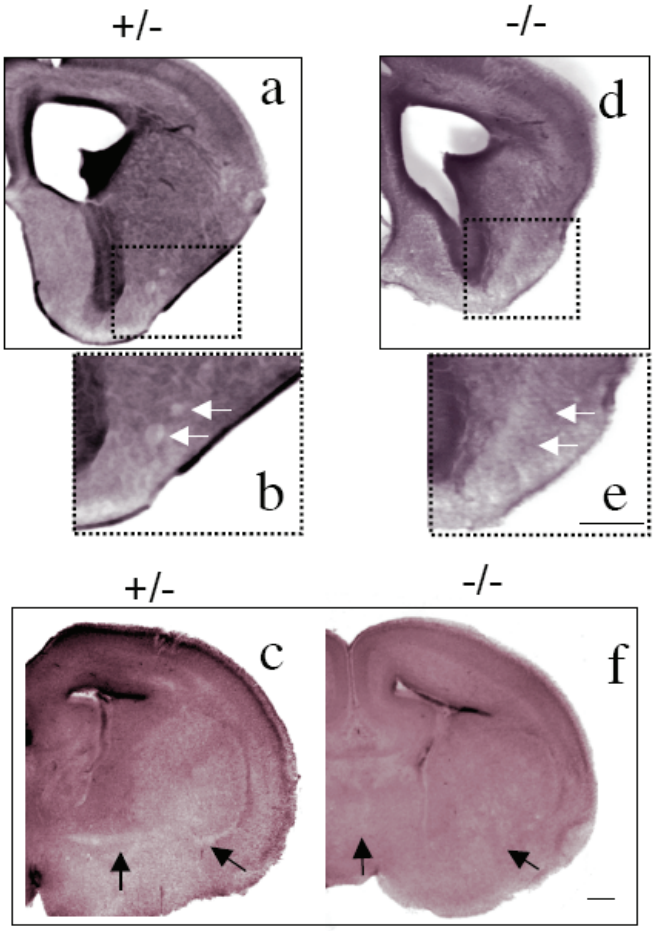


Figure 3.9: Defects in the corpus callosum and internal capsule in Trio null embryos

(A) Upper panel: 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. Lower panel: 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. 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 observed. Scale bar 50µm.

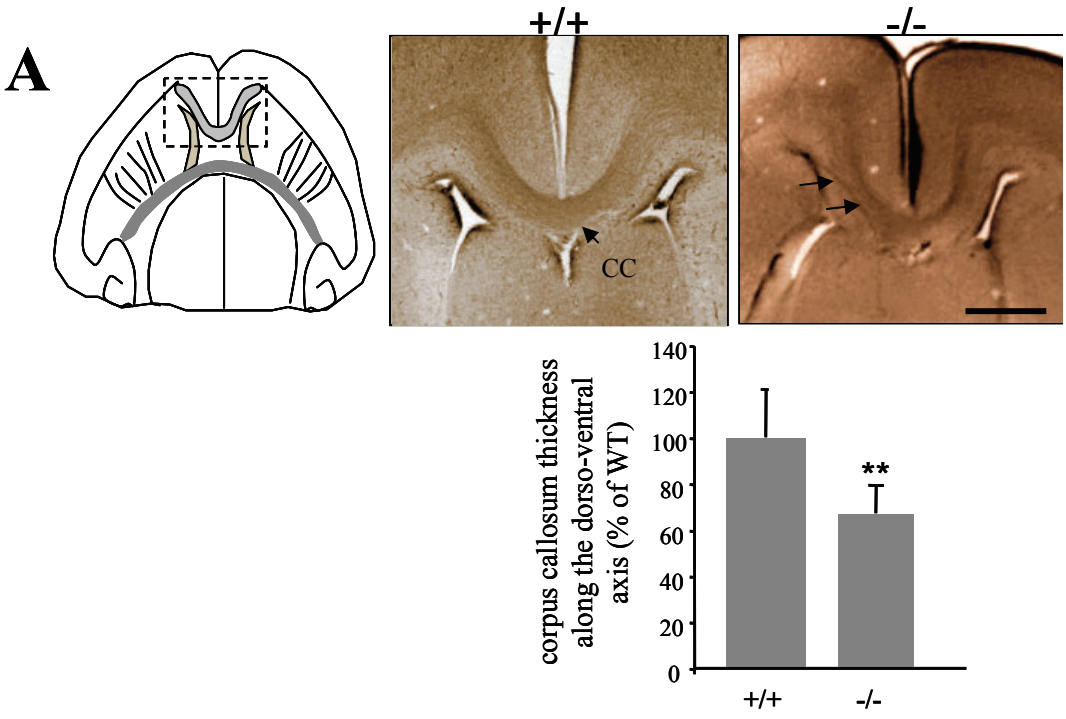
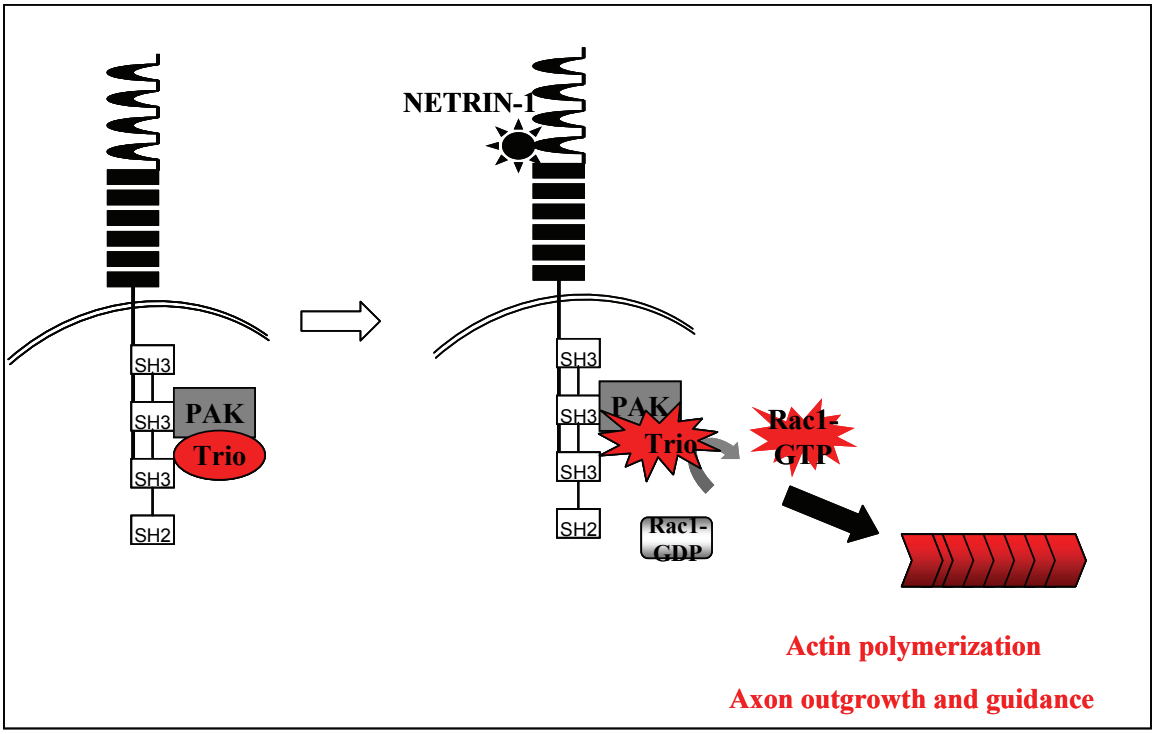

A
+/+
-/-
CC
corpus callosum thickness along the dorso-ventral axis (% of WT)
140
120
100
80
60
40
20
0
**
+/+
-/-
B
+/+
-/-
IC

Figure 3.10: Rac1 activation by Trio in netrin-1/DCC signalling

Trio is recruited to the DCC signalling complex with PAK1 and Nck1. Netrin-1 binding to DCC induces the activation of Trio that stimulates Rac1 activity and leads to actin polymerization, axon outgrowth, and guidance. The mechanism by which Trio is activated remains to be determined.


NETRIN-1
SH3
SH3
SH3
SH2
PAK
Trio
SH3
SH3
SH3
SH2
PAK
Trio
Rac1-GTP
Rac1-GDP
Actin polymerization
Axon outgrowth and guidance

CHAPTER 4

Final Conclusion and Summary

4.1. Final Conclusion

As an axon navigates in the developing nervous system, the growth cone changes its shape by remodeling of actin filaments depending on the type and concentration of external cues. It is well-known that during axon guidance, external signals are integrated into the intracellular signaling cascade through membrane receptors, which finally interact with GTPases. It is believed that the three most studied members of the Rho family of small GTPases; Cdc42, Rac1, and RhoA, are important signaling molecules within the growth cone, and are recognized as regulators of actin polymerization. Filopodia and lamellipodia structures are formed due to activation of Cdc42 and Rac1, respectively by attractive guidance cues, whereas RhoA-mediated filopodia and lamellipodia retraction happens due to repulsive cues leading to growth cone collapse (Mueller, 1999). These structures help the growth cone sense the signals in the environment, grow towards it or collapse and change direction according to the nature of the guidance cue.

There is compelling evidence supporting the role of RhoA, Rac1 and Cdc42 as important signaling elements downstream of most guidance cue receptors. Our interest was to study the role of Rho GTPase family members and specifically Rac1, Cdc42 and RhoA proteins downstream of netrin-1 as the ligand and DCC as the intracellular receptor in the developing nervous system.

The netrin-1/DCC-mediated signaling pathways have been the interest of many researchers in the past years due to its role in cell growth, differentiation and migration, which is very important in Neuroscience, Developmental Biology and Cancer therapy. After almost a decade of this ligand/receptor identification, many studies are focused on identification of new families of proteins downstream of this pathway which lead to activation of Rho GTPases and actin reorganization in the cell. In order to find DCC-binding partners which are implicated in the regulation of Rac1 activity, it has been previously identified by our group that the SH3/SH2 adaptor protein. Nck1 interacts directly and constitutively with DCC through its SH3 domains (Li et al., 2002a).

Interestingly, genetic analysis of dreadlocks (DOCK), the *D. melanogaster* ortholog of mammalian Nck1, supports a role for Nck1 in axon guidance. In a genetic screen, DOCK was identified as an essential gene for proper photoreceptor axon targeting

and fasciculation in *D. melanogaster* (Garrity et al., 1996). It has been also shown that DOCK interacts genetically with Rac, its specific GEF, Trio and the Ser/Thr protein kinase PAK1 in *D. melanogaster* (Newsome et al., 2000). This encouraged us to investigate the role of Trio as an activator of Rac1 in the signaling pathway mediated by the netrin-1 and its receptor DCC.

Trio, the DH-GEF family member is a multifunctional protein that due to containing three enzymatic domains (two GEF domains and a protein kinase serine/threonine kinase domain) and its implication in coordinating actin cytoskeletal reorganization and cell growth regulation is of particular interest in the field. Accumulated evidence suggests that Trio is crucial in neural development in invertebrates such as *C. elegans* and *D. melanogaster* as well as in the development of neural tissues and skeletal muscle in mouse.

Here, GST-pull down experiments show that DCC interacts with both PAK1 and Nck1, whereas Trio only interacts with PAK1. When PAK1 or DCC were co-expressed with Trio, Trio was able to co-precipitate with GST-Nck1. Finally, DCC, Trio, PAK1 and Nck1 were found to co-precipitate together suggesting that Trio associates with DCC via Nck1-PAK1 molecular interactions.

On the other hand, the results of our study indicate that DCC, Trio, PAK1 and Nck1 co-precipitate together indicating that Trio is present in a complex together with DCC, Nck1 and PAK1 which is in good agreement with the results obtained in *D. melanogaster*, based on genetically interaction of D-Trio with DOCK, PAK1, and Rac1 in the photoreceptor neurons of fly. This suggests that Trio could be associated with DCC via Nck1-PAK1 molecular interactions. However, the mechanisms by which Trio becomes activated after netrin-1 and DCC binding is unknown but it may involve phosphorylation by FAK or the Src family kinase Fyn since there is evidence that netrin-1 induces DCC phosphorylation on Ser and Thr residues in embryonic commissural neurons (Meriane et al., 2004). In addition, it has been indicated that Trio binds to the amino-terminal domain and to the kinase domain of FAK via its SH3 and kinase domains, respectively (Medley et al., 2003). This could be another possibility for Trio acting downstream of the netrin-1 and DCC signaling pathway, being involved in the

regulation of focal adhesion dynamics in addition to effecting changes in the actin cytoskeleton through the activation of Rho family GTPases.

Complete absence of the anterior branch of the anterior commissure in the Trio-null embryos has been reported in previous studies indicating that Trio is required for the formatting of the anterior commissure (Ma et al., 2005). It is also indicated that in the Trio mutant embryos, the corpus callosum is present but there is reduction in the dorso-ventral thickness of this structure, suggesting a role for Trio in correct targeting in this structure of the brain (Ma et al., 2005). In addition, netrin-1-null mice are also defective in the thalamo-cortical reciprocal projections in internal capsule. Altogether, these data indicating defects in the spinal cord and brain development of netrin-1 knock out and Trio-null embryos suggest that Trio plays an important role in the guidance of different Netrin-1-mediated axon projections.

In addition to the role of Trio in the embryonic neural development, some studies have attracted attention to importance of Trio members in the adult nervous system. To support this theory, one can point to the high expression of Trio members in the adult rat brain and their localized expression to postsynaptic dendrites (Alam et al., 1997; Penzes et al., 2000). On the other hand, there is data supporting the role of Trio members in receptor internalization at the synapse, which could be mediated by GTPase activation considering role of Rac and Rho in endocytosis pathways (Ellis and Mellor., 2000).

The present study has provided insights into the role of guanine nucleotide exchange factor Trio, downstream of netrin-1/DCC signalling pathway, highlighting its role in axon guidance.

4.2. Summary of Original Findings

In this study, we showed that Trio plays an important role as a GEF in axon outgrowth and guidance induced by netrin-1. Our results also provide evidence that Trio, Nck1, PAK1 and DCC are in the same signalling complex independently of netrin-1 and that in Trio -/- embryos, Rac1 activation induction by netrin-1 is abolished. Our data with cultures of cortical neurons from Trio knock out embryos show that these neurons are not able to extend neurits in presence of netrin-1. In Trio -/- spinal cord commissural explants, commissural axons are unable to grow in respond to netrin-1, which highlights the role of Trio in correct axon growth. Axonal projections in Trio-null mice also seem to be defective in the brain and spinal cord.

There is strong evidence supporting overexpression of some members of the Rho family of GTPases in human diseases, such as different cancers, cardiovascular disease and neurodegenerative diseases suggesting that these proteins are involved in giving cells the ability to change their normal growth or migration. Although the precise mechanisms by which Rho GTPases participate in carcinogenesis are still not fully understood, a large amount of work has been done in this field and it is becoming more obvious that Rho protein overexpression has an obvious role in tumor initiation, progression and metastasis in some human tumors. Indeed, Rho GTPases are potential candidates for therapeutic interventions and findings their activator proteins can potentially be used for target therapies for different diseases.

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Appendices

Appendix I

Identification of DCC interacting proteins

Rationale:

Netrins are a small family of secreted proteins that guide growing axons during neural development by binding to the receptor DCC. The intracellular mechanisms mediating the response of axons to netrin-1 stimulation via its receptor DCC have been extensively studied over the past few years (Barallobre et al., 2005). It has been showed by our group and others that Rac1 and Cdc42 are essential mediators of neurite outgrowth initiated by netrin-1 and DCC (Li et al., 2002b; Shekarabi and Kennedy, 2002), but the other molecules involved in this pathway linking DCC to Rac1 activation are not identified yet.

Seeking for a more detailed image of netrin-1/DCC intracellular mechanisms, results from our group demonstrated that netrin-1 induces DCC Tyrosine phosphorylation (Meriane et al., 2004). The data from *in vitro* experiments in this study showed that DCC is specifically phosphorylated by the Src family kinase Fyn, on its Y1418 and this phosphorylation site is required to mediate Rac1 activation and neurite outgrowth.

It is of great interest to determine the proteins interacting with the specific phosphorylation sites of DCC, and how they influence the responses of developing axons to netrin-1. To find DCC-binding partners involved in the regulation of Rac1 activity mediated by netrin-1/DCC, a proteomic approach was used since we assumed that PY1418 could recruit interacting proteins that may contain an SH2 or PTB domain.

Objective:

Identify new possible partners involved in the netrin-1/DCC signalling pathway leading to neurite outgrowth.

Experimental Procedures:

Affinity column: An affinity column was prepared using the phosphopeptide corresponding to amino acids 1409 to 1423 of DCC, phosphorylated on tyrosine 1418 (KPTEDPASVYPEQDDL) coupled to affigel (Biorad). The unphosphorylated peptide was used as a negative control. Protein lysates from total rat embryonic brains were loaded on the column and proteins bound to the phosphopeptide were eluted using a gradient of sodium chloride by Fast Protein Liquid Chromatography. The eluted proteins were separated by SDS-PAGE and proteins were detected on the SDS-PAGE gel using silver-staining method (Figure1). The protein bands present in the phosphopeptide affinity purification but not in the control peptide were cut and sent to be identified by Mass-spectrometry.

Silver staining method: Mass-spectrometry compatible SilverQuest™ silver staining kit was used (Invitrogen). The staining was performed according to the manufacture's protocol. The two bands shown in figure1 were sent for Mass-Spectrometry analysis.

Cell culture and GST pull down: COS-7 cells were cultured and transfected with DCC constructs using LipofectAMINE 2000 reagent (Invitrogen) according to manufacturer's instructions. Cells were lysed in buffer A (25 mM Hepes pH 7.5, 1% NP40, 10 mM $MgCl_2$, 100 mM NaCl, 5% Glycerol, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 1 mM PMSF). Lysates were then incubated for 2h at 4°C with 30µg of either GST or GST-ezrin fusion proteins. Total cell lysates and GST-pull down associated proteins were resolved by SDS-PAGE and transferred on nitrocellulose. Membranes were immunoblotted with anti-DCC (BD Biosciences Inc.).

Results and Discussion:

Mass-Spectrometry results from the two bands (Figure 1) are summarized in table 1. The MS/MS data yield 4 peptides matching 10% of the sequence of ezrin. Ezrin is a member of the ERM (ezrin, radixin, moesin) family of actin-membrane linkers, which play important roles in the regulation of actin filaments at the plasma membrane (Tsukita et al., 1997). ERM linkers are concentrated in cell-surface structures which are rich in actin, such as microvilli and filopodia, and their impaired expression or inactivation severely affects cell surface morphology, motility, and adhesion (Bretscher et al., 2000). Ezrin consists of an N-terminal FERM domain, an alpha-helical region, a proline-rich domain, and a C-terminal actin-binding (AB) region (Figure 2).

To confirm the data obtained from Mass-Spectrometry, a GST Pull down experiment was performed to investigate possible interaction between DCC and ezrin. We found that DCC expressed in COS-7 cells was able to interact with GST-Ezrin, suggesting that ezrin may interact with the cytoplasmic domain of DCC (Figure 3).

It has been previously reported by our group that DCC associates with the actin cytoskeleton through unidentified proteins (Li et al. 2002b). Interestingly, data from *in vitro* assays have previously reported that DCC interacts with the N-terminal of ezrin, but its role in netrin-1 signaling and axon guidance has not been investigated (Martin et al., 2006). In addition, in response to activation of RhoA and Rac1, the ERM proteins have been shown to mediate their effects on actin reorganization.

These data indicate that ezrin is a potential candidate as a linker between DCC and the actin cytoskeleton. The role of ezrin as an effector protein mediating the effects of netrin-1/DCC signaling on axon outgrowth and guidance was not further investigated by the candidate since she was involved in another project, which is comprehensively explained in this thesis.

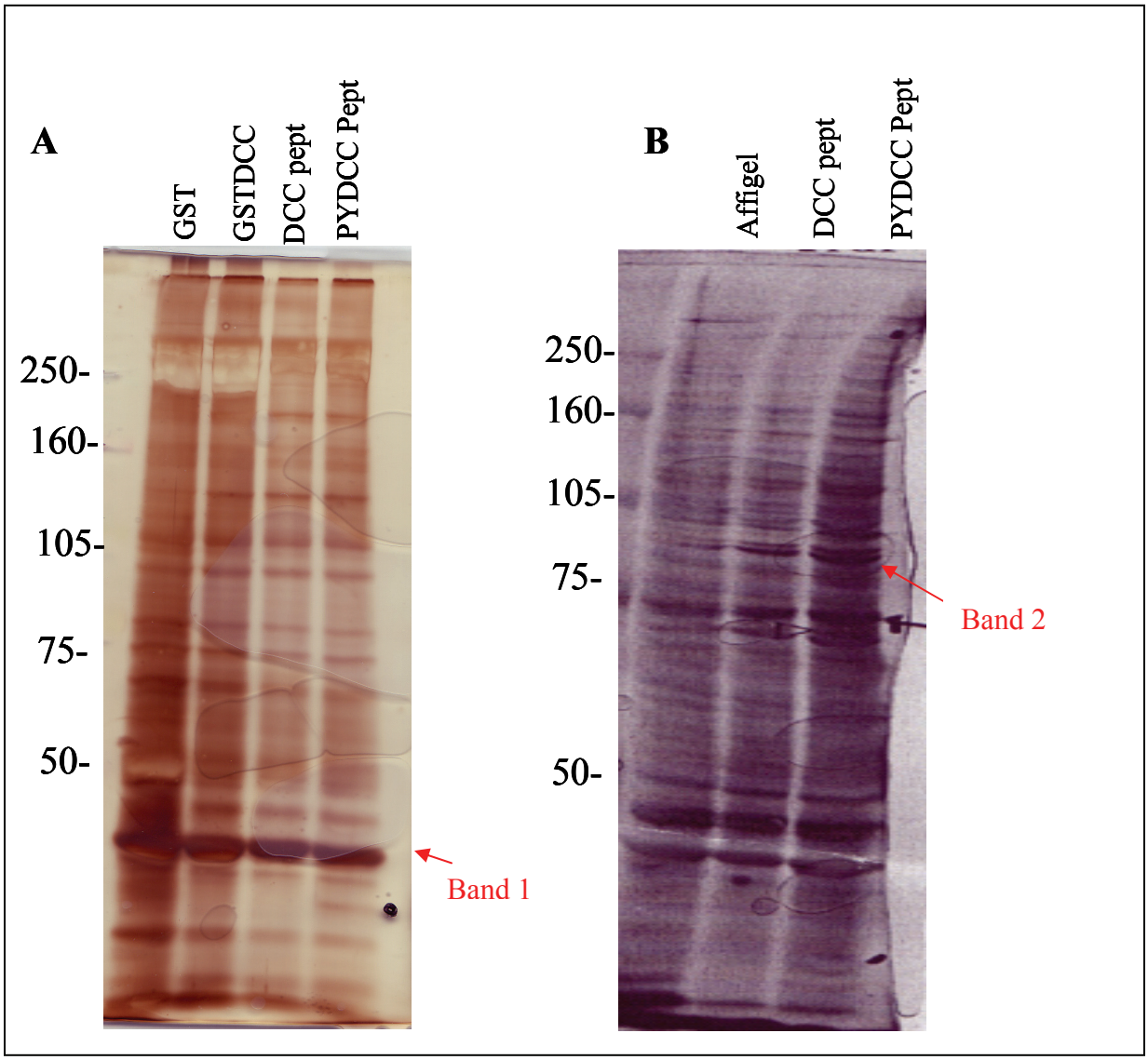

Figure 1. Silver-stained gels resulted from total rat embryonic brain lysates incubated with the affinity column, prepared with the phosphopeptide, showing the bands sent for Mass-spectrometry. Gel B was not scanned before sending to Mass-spectrometry and only a paper print of the gel was available to be used in this thesis. Red arrows show the bands sent for Mass-spectrometry.

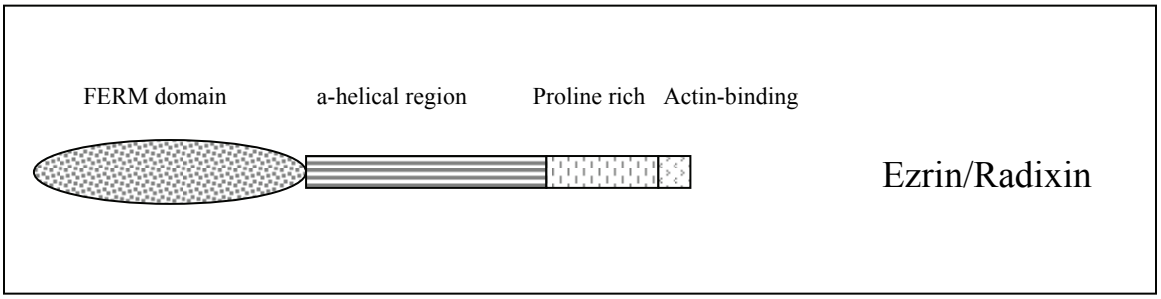

Figure 2. Schematic representations of ezrin, showing an N-terminal membrane-binding domain, followed by an α-helical domain and a C-terminal actin-binding domain.

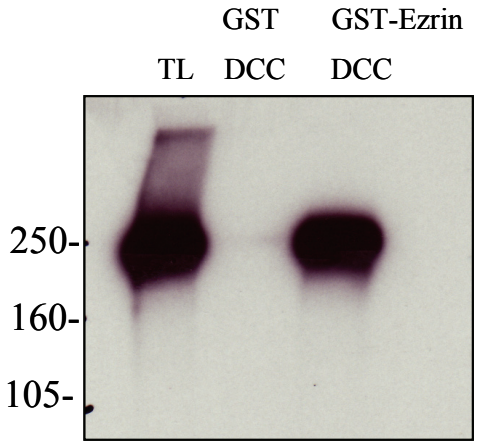

Figure 3. GST Pull down experiment using COS-7 cells lysates expressing DCC. Cell lysates were incubated with GST or GST-Ezrin .Western Blot anti-DCC.

Table1. List of some proteins detected by Mass-Spectrometry analysis from gels shown in figure 1.

Band 1 (Mass-Spectrometry analysis)	Band 2 (Mass-Spectrometry analysis)
Glutamate oxaloacetate transaminase 2 [Rattus norvegicus]	**Msn (Moesin) protein [Mus musculus]**
GTP-binding regulatory protein G	Pyruvate kinase [Mus musculus]
Guanine nucleotide binding protein, alpha 13 [Rattus norvegicus]	Poly (A) binding protien [Mus musculus]
Guanine nucleotide binding protein, alpha z subunit [Rattus norvegicus]	GTP-binding regulatory protein alpha-13 chain
Similar to 53BP1 protein [Rattus norvegicus]	TNF receptor-associated protein 1 [Mus musculus]
Golgi-associated protein GCP360 [Rattus norvegicus]	Villin 2 [Mus musculus]

Appendix II

Dissection of embryonic dorsal spinal cord and the explant assay

Staged 11.5 day pregnant mouse (Trio +/-) was sacrificed by placing in a Co_2 box. The mouse abdomen was saturated with alcohol and using sterilized instruments, the skin was cut to expose the peritoneum. Uterus was then opened and embryonic sacs were placed into disposable petri dish. Embryonic sacs were opened using sterile scissors and embryos were released in cold Hanks Buffer medium. Each embryo was then given a number and was placed in a clean petrie dish containing fresh cold Hanks Buffer medium.

Dorsal spinal cord was dissected as described previously (Tessier-Lavigne et al., 1988). Briefly, each embryo was dissected separately in cold Hanks Buffer medium. The spinal cord was dissected using fine forceps after removing the head and the limbs. The spinal cord was carefully released from the surrounding tissue using fine forceps and was placed in a small petrie dish containing cold fresh PBS. The head of each embryo was kept frozen for genotyping. The dorsal part of the spinal cord was then cut into small explants.

To make the embedding material, collagen (BD Biosciences), Dulbecco's Modified Eagle's Medium (DMEM) 10X medium (Gibco) and sodium bicarbonate 1M were mixed proportionally (8:1:1).

Explants were then embedded in three-dimensional collagen in a four-well plate and kept at 37°C for 30 minutes before adding the medium.

500µl Neurobasal Medium (Gibco) pre-warmed and supplemented with 2% Glutamine and Penicillin/Streptomycin was added to each well gently, with or without (500 ng/ml) netrin-1. Images were captured after 35 hours and 70 hours using a digital camera on a microscope (Axiovert 135) with a 40x phase-contrast objective lens. The total length of the axon bundles was calculated using Northern Eclipse software.

Appendix III

Authors copyright

September 25, 2007

Dear Ms. Atefeh Ghogha,

As a coauthor on the following manuscript:

Anne Briançon-Marjollet, Atefeh Ghogha, Homaira Nawabi, Ibtissem Triki, Camille Auziol, Sylvie Fromont, Karim Chebli, Jean-François Cloutier, Valérie Castellani, Anne Debant and Nathalie Lamarche-Vane. **Trio mediates netrin-1-induced Rac1 activation and axon guidance,** *Submitted to* Mol. Cell. Biol. 2007.

We authorized you to include the above submitted manuscript as part of your Master's thesis.

Yours sincerely,

Anne Debant, PhD.
Associate Professer
CRBM-CNRS, UMR5237
Université Montpellier I et II, IFR 122
Montpellier 34293, France

Camille Auziol, PhD Candidate
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Montpellier 34293, France

September 25, 2007

Dear Ms. Atefeh Ghogha,

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Yours sincerely,

Jean-Francois Cloutier, PhD.
Assistant Professor
Montreal Neurological Institute
3801 University Street
Montreal, Quebec
H3A 2B4

September 25, 2007

Dear Ms. Atefeh Ghogha,

As a coauthor on the following manuscript:

Anne Briançon-Marjollet, Atefeh Ghogha, Homaira Nawabi, Ibtissem Triki, Camille Auziol, Sylvie Fromont, Karim Chebli, Jean-François Cloutier, Valérie Castellani, Anne Debant and Nathalie Lamarche-Vane. **Trio mediates netrin-1-induced Rac1 activation and axon guidance,** *Submitted to* Mol. Cell. Biol. 2007.

We authorized you to include the above submitted manuscript as part of your Master's thesis.

Yours sincerely,

Valérie Castellani, PhD.
Associate Professor
CGMC UMR-CNRS 5534
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69622 Villeurbanne, France

Homaira Nawabi, PhD Candidate
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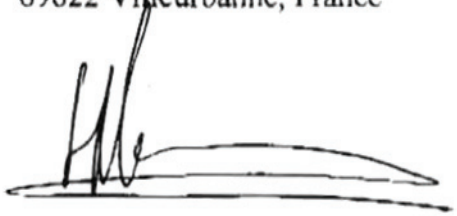

September 25, 2007

Dear Ms. Atefeh Ghogha,

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I authorized you to include the above submitted manuscript as part of your Master's thesis.

Yours sincerely,

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September 25, 2007

Dear Ms. Atefeh Ghogha,

As a coauthor on the following manuscript:

Anne Briançon-Marjollet, Atefeh Ghogha, Homaira Nawabi, Ibtissem Triki, Camille Auziol, Sylvie Fromont, Karim Chebli, Jean-François Cloutier, Valérie Castellani, Anne Debant and Nathalie Lamarche-Vane. **Trio mediates netrin-1-induced Rac1 activation and axon guidance,** *Submitted to* Mol. Cell Biol. 2007.

I authorized you to include the above submitted manuscript as part of your Master's thesis.

Yours sincerely,

Nathalie Lamarche-Vane, PhD.
Associate Professor
McGill University
3640 University Street
Montreal, Quebec
H3A 2B2

October 22nd, 2007

Dear Ms. Atefeh Ghogha,

As a coauthor on the following manuscript:

Anne Briançon-Marjollet, Atefeh Ghogha, Homaira Nawabi, Ibtissem Triki, Camille Auziol, Sylvie Fromont, Karim Chebli, Jean-François Cloutier, Valérie Castellani, Anne Debant and Nathalie Lamarche-Vane. **Trio mediates netrin-1-induced Rac1 activation and axon guidance,** *Submitted to* Mol. Cell. Biol. 2007.

I authorized you to include the above submitted manuscript as part of your Master's thesis.

Yours sincerely,

Ibtissem Triki
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Research and Development
2100 Cunard Street
Laval (Québec) H7S 2G5

October 22nd, 2007

Dear Ms. Atefeh Ghogha,

As a coauthor on the following manuscript:

Anne Briançon-Marjollet, Atefeh Ghogha, Homaira Nawabi, Ibtissem Triki, Camille Auziol, Sylvie Fromont, Karim Chebli, Jean-François Cloutier, Valérie Castellani, Anne Debant and Nathalie Lamarche-Vane. **Trio mediates netrin-1-induced Rac1 activation and axon guidance,** *Submitted to* Mol. Cell. Biol. 2007.

I authorized you to include the above submitted manuscript as part of your Master's thesis.

Yours sincerely,

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Appendix IV

Letter of acceptance from the publisher

Date: 7 janvier 2008 13:58:10 HNE
To: nathalie.lamarche@mcgill.ca
Subject: Decision on manuscript MCB00998-07 Version 3

Dr. Nathalie Lamarche-Vane
McGill University
Anatomy and Cell Biology
3640 University street
Montreal, Quebec H3A 2B2
Canada

Re: Trio mediates netrin-1-induced Rac1 activation in axon outgrowth and guidance (MCB00998-07 Version 3)

Dear Dr. Lamarche-Vane:

Your manuscript has been accepted, and I am forwarding it to the ASM Journals Department for publication. For your reference, ASM Journals' address is given below. Before it can be scheduled for publication, your manuscript must be checked by the ASM production editor to make sure that all elements meet the technical requirements for publication. Becky Zwadyk, the production editor for Molecular and Cellular Biology (MCB), will contact you if anything needs to be revised before copyediting and production can begin.

An official letter of acceptance and a copyright transfer agreement will be sent to the person listed as the corresponding author on the manuscript title page once your article has been scheduled for an issue.

Thank you for submitting your paper to MCB.

Sincerely,
Dafna Bar-Sagi
Editor, Molecular and Cellular Biology (MCB)

Journals Department
American Society for Microbiology
1752 N St., NW
Washington, DC 20036
journalsrr@asmusa.org
1-202-942-9214
1-202-942-935

Appendix V

Certificates

APPROVED

www.mcgill.ca/rgo/animal/

1

McGill University Animal Care Committee
RENEWAL of Animal Use Protocol

For: Research ☒ Teaching ☐ project

Protocol #: 4529
Approval end date: ~~Jan 31, 2007~~
Facility Committee: MED
Renewal#: 1st (2nd)
EXTENSION: MARCH 31, 2007

Principal Investigator:	Nathalie Lamarche-Vane	**Protocol #**	4529
Protocol Title:	Intracellular mechanisms regulated by the axon guidance cue netrin-1	**Phone:**	1784
Unit, Dept. & Address:	Anatomy and Cell Biology, 3640 University street, H3A 2B2	**Fax:**	5047
Email: nathalie.lamarche@mcgill.ca	**Level:** Associate ~~Professor~~ B	**Funding source:**	CIHR MOP-14701
Start of Funding: 2002	**End of Funding:** 2007		
Emergency contact #1 + phone #s			
Emergency contact #2 + phone #s	Nathalie Lamarche (514-273-6537) and Ibtissem Triki (450-972-1442) 514-398-2329		

11473 15511 18685 20537 20568

1. Personnel and Qualifications

List the names of the Principal Investigator and of all individuals who will be in contact with animals in this study and their employment classification (investigator, technician, research assistant, undergraduate/ graduate student, fellow). If an undergraduate student is involved, the role of the student and the supervision received must be described. Training is mandatory for all personnel listed here. Refer to *www.animalcare.mcgill.ca* for details. Each person listed in this section must sign. *(Space will expand as needed)*

Name	Classification	Animal Related Training Information	Occupational Health Program *	Signature *"Has read the original full protocol"*
Lamarche-Vane, Nathalie, Principal investigator , theory course, no				[signature]
Ibtissem Triki, research assistant, theory course, rat and mouse workshop, no				[signature]
Atefeh Ghogha, M.Sc. student, theory course, rat and mouse workshop, no				[signature]
VANESSA VINCENT, AHT, MCINTYRE ANIMAL FACILITIES				
FRANCINE TREMBLAY - AHT, GENOMIC ANIMAL FACILITIES				

* Indicate for each person, if participating in the local OHP Program, see http://www.mcgill.ca/rgo/animal/occupational/ for details.

Approved by:

2. Approval Signatures

Principal Investigator/ Course Director	[signature]	Date: January 30, 2006
Chair, Facility Animal Care Committee	Louise Larose	Date: April 20, 2006
UACC Veterinarian LW	[signature]	Date: March 14, 2006
Chairperson, Ethics Subcommittee *(D level or Teaching Protocols Only)*	—	Date: —
Approved Animal Use Period	Start: Feb 1, 2006	End: ~~Jan 31, 2007~~ EXTENSION: MARCH 31, 2007

3. Summary (in language that will be understood by members of the general public)

AIMS AND BENEFITS: Describe, in a short paragraph, the overall aim of the study and its potential benefit to human/animal health or to the advancement of scientific knowledge ***(was section 5a in main protocol).***

In my laboratory, we are studying the molecular mechanisms induced by the chemotropic factor netrin-1 and its receptors. This research help to understand how neuronal growth cones are guided toward their appropriate

Received Feb 3/06

TOTAL P.02

nd certifies with the applicant that the experiment will be in accordance with the principles outlined in the "Laboratory Biosafety Guidelines" prepared by Health Canada and the MRC, and in the "McGill Laboratory Biosafety Manual". Containment Level (circle 1): 1 (2) 3 4

Principal Investigator or course director: Nlamarre (SIGNATURE) date: 25 04 2002 (day month year)

Chairperson, Biohazards Committee: (SIGNATURE) date: 01-05-2002 (day month year)

Dr. CORNELIUS J. BRIEDIS
Dept. of Microbiology & Immunology
McGill University
3775 University Street
Montreal, Quebec H3A 2B4
Canada

Approved period: 01-05-2002 (day month year) ending 31-03-2007 (day month year)

* as defined in the "McGill Laboratory Biosafety Manual"

2nd REVISION, JANUARY 1996

4. RESEARCH PERSONNEL: (attach additional sheets if preferred)

Name	Department	Check appropriate classification				Fellow
		Investigator	Technician & Research Assistant	Student		
				Undergraduate	Graduate	
Nathalie Lamarche-Vane	Anatomy and Cell Biology	X				
Ibtissem triki	idem		X			
Mayya Meriane	idem					X
Xiaodong Li	idem				X	
Eric Danek	idem				X	
Joseph Tcherkezian	idem				X	

5. EMERGENCY: Person(s) designated to handle emergencies

Name: Dr. Alex bell Phone No: work: 398-6352 home: 721-5088

Name: Phone No: work: home:

6. Briefly describe:

i) the biohazardous material involved (e.g. bacteria, viruses, human tissues) & designated biosafety risk group

1. cDNAs constructs microinjected or transfected into cells (Swiss 3T3 fibroblasts, N1E115 neuroblastoma cells, Cos-7 cells or primary rat commissural neurons)

2. GST fusion proteins expressed in E.coli

3. Yeast two-hybrid system