Netrin-1 Signaling:

Cellular Consequences and Molecular Mechanisms

by

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Abstract

During the embryonic development of the nervous system, groups of multipotent cells proliferate, migrate, and differentiate to become neurons. The new born neurons need to make precise connections with their targets. These may be a great distance away from their soma. To make these connections, neurons send their axon through a biochemically complex environment and their growth cones may make many guidance decisions on the ways to their targets.

The existence of axon guidance molecules was inferred by the early classical neuro-anatomists. We now know of multiple examples of secreted or membrane-bound proteins that either attract or repel axonal growth cones by causing the growth cone to collapse or extend. The molecular mechanisms that regulate growth cone collapse or extension are closely linked to reorganization of the cytoskeleton. The small Rho GTPases, Cdc42, Rac, and Rho play key roles in neuronal growth cones by regulating the organization of the cytoskeleton.

Netrins are a small family of secreted guidance cues that are implicated in the attraction and repulsion of axons during the development of the nervous system. The netrin-1 receptor deleted in colorectal cancer (DCC) is highly expressed by commissural neurons in the developing spinal cord. The findings described here show that DCC is present at the tips of filopodia and the edges of lamellipodia of HEK293T, NG108-15 neuroblastoma-glioma cells, and the growth cones of embryonic rat spinal commissural neurons. Furthermore, netrin-1 protein causes an increase in filopodia number and surface area of embryonic rat commissural neuron growth cones and the cell lines when transfected to express DCC. Further experiments indicated that netrin-1 activates the small GTPases Cdc42 and Rac1 in the cell lines and the embryonic rat commissural

spinal cord neurons. The activation of Cdc42 and Rac1 by netrin-1 requires DCC. Furthermore, netrin-1 causes increased phosphorylation of Pak1, an effector for both Cdc42 and Rac1. Expression of a dominant negative form of N-WASP, an effector for Cdc42, blocks the netrin-1 induced morphological changes in the growth cones of commissural neurons. Evidence is presented that netrin-1 induces the formation of a complex of proteins interacting with the intra-cellular domain of DCC that includes Nck1, Cdc42, Rac1, Pak1, and N-WASP. The findings described lead to a model whereby netrin-1 binding to DCC triggers the activation of Cdc42 and Rac1, which leads to actin based membrane extension and changes in growth cone morphology.

Résumè

Au cours du développement embryonnaire du système nerveux central, plusieurs cellules souches prolifèrent, migrent et se différencient en neurones. Ces nouveaux neurones doivent établir des connexions précises avec leur tissu cible et parfois cette cible se trouve à une grande distance du soma. Afin d'établir ces connexions, les neurones étendent leur axone à travers un environnement biochimiquement fort complexe et en cours de route, leurs cônes de croissance doivent prendre plusieurs décisions afin d'attendre leurs cibles.

L'existence de molécules pouvant guider l'axone fut proposée, il y a plusieurs années, par certains neuro-anatomistes. Depuis, plusieurs protéines sécrétées ou attachées à la membrane ont été identifiées. Elles peuvent avoir un effet attractif ou répulsif sur les cônes de croissance en causant leur extension ou leur rétraction. Les mécanismes moléculaires régulant ces phénomènes sont étroitement liés à la réorganisation du cytosquelette.

Les nétrines forment une petite famille de protéines causant l'attraction ou la répulsion des axones au cours du développement de système nerveux central. Le récepteur « deleted in colorectal cancer » (DCC) à la nétrine-1 est largement exprimé par la moelle épinière durant son développement. Les résultats rapportés dans cette thèse démontrent que DCC est présent à l'extrémité des filopodes et en bordure des lamellipodes des cellules HEK293T, des glioblastomes NG108-15 et des cônes de croissance des neurones commissuraux provenant de moelle embryonnaire de rat. Également, nous avons observé que la nétrine-1 induisait l'augmentation du nombre des filopodes et de la surface cellulaire des lignées de cellules où DCC avait été transfecté.

Ces résultats se sont avérés également reproductibles au niveau des cônes de croissance des neurones commissuraux.

Des expériences complémentaires indiquent que dans ces mêmes cellules, la nétrine-1 via son récepteur DCC peut activer certaines petites GTPases (Cdc42 et Rac1). Également, elle induit la phosphorylation de PAK1, un effecteur de Cdc42 et de Rac1. L'expression d'un «dominant négatif» pour N-WASP, un autre effecteur de Cdc42, bloque les modifications morphologiques des cônes de croissance de neurones commissuraux induites par la nétrine-1. Nous démontrons également que nétrine-1 induit la formation d'un complexe de protéines interagissant avec le domaine intracellulaire de DCC. Celui-ci est composé de Nck1, Cdc42, Rac1, Pak1 et de N-WASP. Les résultats décrits dans cette thèse suggèrent un modèle selon lequel nétrine-1 se lie à DCC ce qui déclenche l'activation des Cdc42 et Rac1, qui à leur tour via l'actine entraîne des changements morphologiques au niveau du cône de croissance.

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List of Abbreviations

cAMP:	cyclic adenosine monophosphate
Cdc42:	cell division cycle 42
CNS:	central nervous system
CRIB:	Cdc42 / Rac1-interactive binding (motif)
DCC:	deleted in colorectal cancer
DOC	deoxycholate
ERK:	extra-cellular-signal-regulated kinase
GAP:	GTPase-activating protein
GDP:	guanosine diphosphate
GDI:	guanine nucleotide dissociation inhibitor
GEF:	guanine nucleotide exchange factor
GPI:	glycosylphosphatidylinositol
GTP:	guanosine triphosphate
MAPK:	mitogen-activated protein kinase
MLC:	myosin light chain
MLCK:	myosin light chain kinase
N-WASP:	neuronal-Wiskott-Aldrich syndrome protein
Pak1:	p21-activated protein kinase 1
PDL	poly-D-lysine
PI3K:	phosphoinositide 3-kinase
PNS:	peripheral nervous system
Rac1:	Ras-related C3 botulinum toxin substrate 1
Rho:	Ras homology

SOV	sodium ortovanedate
Unc-5:	uncoordinated 5
Unc-5H:	uncoordinated 5 homolog

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Shekarabi M, Kennedy TE. The netrin-1 receptor DCC promotes filopodia formation and cell spreading by activating Cdc42 and Rac1. *Mol Cell Neurosci* 2002 Jan; 19(1): 1-17.

Bourbonniere M, **Shekarabi M,** Nalbantoglu J. Determination of molecular weights of nucleic acid-binding proteins by UV photo-crosslinking and SDS-PAGE. *Biotechniques* 1997 Jul; 23(1): 60-2.

Shekarabi M, Bourbonniere M, Dagenais A, Nalbantoglu J. Transcriptional regulation of amyloid precursor protein during dibutyryl cyclic AMP-induced differentiation of NG108-15 cells. *J Neurochem* 1997 Mar; 68(3): 970-8.

Bourbonniere M, **Shekarabi M,** Nalbantoglu J. Enhanced expression of amyloid precursor protein in response to dibutyryl cyclic AMP is not mediated by the transcription factor AP-2. *J Neurochem* 1997 Mar; 68(3): 909-16.

Contributions of Authors

All work reported in this thesis was performed by Seyed Masoud Shekarabi with the following exception. In chapter 3, Figure 5 has been done by Dr. J-F Bouchard.

Chapter 1

General Introduction

Cell proliferation, migration, and differentiation profoundly influence cellular fate during embryonic development. The vertebrate nervous system develops from a region of ectoderm that lies along the dorsal midline of the embryo. This region, the neural plate, then folds and forms a hollow structure called the neural tube that is composed of a neuroepithelial cell layer that generates the neurons and glia of the central nervous system (CNS). Neuroblasts (neuronal precursor cells) born in the early neuroepithelium remain in the CNS and differentiate into the neurons of the brain and spinal cord. The neural crest cells are also generated from the neuroepithelum but then migrate out of the neural tube and form peripheral tissue and neurons (reviewed by Gilbert, 1997).

A newborn neuron must still extend processes to innervate its specific target cells. To accomplish this, the axon must navigate through a very complex environment. The molecular mechanisms that guide extending axons include chemoattraction, contact attraction, chemorepulsion and contact repulsion. These molecular cues influence the direction of growth cone movement by promoting or inhibiting the movement of the growth cone on its path to a target (reviewed by Tessier-Lavigne & Goodman, 1996). A major accomplishment over the past two decades has been the identification of multiple families of proteins that function as guidance cues for extending axons.

Axonal Guidance in the Nervous System

Three remarkable contributions to our understanding of how the nervous system develops occurred in the late 19th and 20th centuries. The first were observations by Santiago Ramón y Cajal (1890) in the developing chick spinal cord. Using Golgi staining, he described:

"... This fiber [of the commissural neuron] ends...in an enlargement which may be rounded and subtle, but that may also adopt a conical appearance. This latter we shall name the growth cone, that at times displays fine and short extensions...which appear to insinuate themselves between the surrounding elements, relentlessly forging a path through the interstitial matrix"

In addition, Ramón y Cajal not only described the growth cone for the first time, but also reported the fine extensions now known as filopodia. The second turning point was the formulation of the chemoaffinity theory by Roger Sperry in 1963. In this theory, Sperry suggested the existence of at least two cytochemical gradients in the retina and the tectum that would direct the formation of a retino-topographic projection (Sperry, 1963). Friedrich Bonhoeffer and colleagues were responsible for the third key discovery in the 1970s and 80s. Using an *in vitro* assay and making stripes from anterior and posterior membrane preparations, they took the first steps toward identifying the molecular cues proposed by Sperry by demonstrating that the growth cones from neurons in the temporal half of retina can differentiate between cell membranes derived from the anterior and posterior regions of the tectum (reviewed by Baier & Bonhoeffer, 1992).

Below, I summarize key findings related to families of axon guidance cues and their mechanisms of action that have been discovered so far (Fig. 1).

The Semaphorin Family

The semaphorins are a large family of axon guidance molecules that are characterized by a conserved ~ 500 amino acid N-terminal domain called a semaphorin motif. They are present in transmembrane and secreted forms. Class 1 (membrane-bound) and class 2 (secreted) semaphorines are expressed in invertebrates. In vertebrates, semaphorins range from class 3 to class 7. Class 3 is the only secreted vertebrate semaphorin. The remaining classes (class 4-7) are transmembrane semaphorins with small cytoplasmic domains. An exception is class seven semaphorins, which are glycosylphosphatidylinositol (GPI) anchored (reviewed by Culotti & Kolodkin, 1996; Kolodkin, 1998).

Collapsin-1 (now called Sema-3A/Sema-III) was the first member of the semaphorin family identified. It was purified from embryonic chick brain membranes on the basis of its ability to induce growth cone collapse in chick sensory axons (Luo *et al.*, 1993). The grasshopper homolog of Sema-3a was identified, named Sema-1a (fasciclin IV), and shown to function as a repellent in the CNS (Winberg *et al.*, 1998). *In vitro*, Sema-3a inhibits the growth of sensory and motor axons; Sema-3b and Sema-3c repel sympathetic ganglion axons; Sema-3e and Sema-3f cause collapse of growth cones from dorsal root ganglion axons and hippocampal axons, respectively (Adams *et al.*, 1997; Chedotal *et al.*, 1998; Miyazaki *et al.*, 1999). Mutant mice lacking Sema-3a show modest abnormalities in axonal targeting in the spinal cord as well as reduced fasciculation of the trigeminal projection (Taniguchi *et al.*, 1997). The fact that more severe abnormalities are not produced in the absence of a single semaphorin is likely due to the presence of redundant molecular mechanisms operating in the developing CNS.

Semaphorins also function as attractants for extending axons. For example, Sema-3c attracts the axons of cortical neurons, and Sema-3b attracts olfactory bulb axons *in vitro* (Bagnard *et al.*, 1998). Ectopic expression of Sema-1a in grasshopper epithelium causes Ti1 axons to turn towards the region of espression (Wong *et al.*, 1997; de Castro *et al.*, 1999; Wong *et al.*, 1999). Two families of transmembrane proteins: neuropilins (1 and 2) and plexins (A1-4, B1-3, C1 and D1) are known to bind semaphorins. These proteins can form homo- and heteromeric complexes to act as a receptor complex for different semaphorins (reviewed by Fujisawa & Kitsukawa, 1998; Roskies *et al.*, 1998; Yu & Kolodkin 1999). Neuropilin-1 binds Sema-3a, Sema-3c, and Sema-3f, and both *in vitro* and *in vivo* experiments have shown that neuropilin-1 is a Sema-3a receptor (He & Tessier-Lavigne, 1997; Kitsukawa *et al.*, 1997). Neuropilin-2 binds Sema-3b, Sema-3c, and Sema-3f but not Sema-3a, and is implicated in the repulsive effects of Sema-3f *in vitro* (Chen *et al.*, 1997). Plexin A binds Sema-1a and Sema-1b in grasshopper neurons *in vitro*. Importantly, similar phenotypes have been reported for loss-of-function of plexin A and Sema-1a (Winberg *et al.*, 1998). In *D. melanogaster* plexin A controls motor and CNS axon guidance (Winberg *et al.*, 1998). Human plexin C1 binds Sema-7a and plexin B1 binds Sema-4d while class 3 semaphorins do not bind plexins (Tamagnone *et al.*, 1999). Plexin-A3, which is ubiquitously expressed in the mammalian nervous system is implicated in Sema-3f and Sema-3a signaling and regulates hippocampal axonal projections (Cheng *et al.*, 2001).

Ephrins

Ephrins and Eph receptors are a large family of ligands and receptors that mediate growth cone collapse and axon repulsion. They are thought to act by contact repulsion mechanisms in vertebrates (reviewed by Frisen *et al.*, 1999).

Eph receptors contain two fibronectin repeats on their extracellular domain and a tyrosine kinase motif in their intracellular region. The ligands, ephrins, include type A ephrins that are plasma membrane linked by GPI anchors and type B ephrins that are transmembrane proteins. EphA receptors interact with type A ephrins with the exception of EphA4 which is a receptor for both A and B ephrins. EphB receptors bind type B ephrins. There are four predicted GPI-anchored ephrins and one Eph receptor in the *C. elegans* genome (reviewed by Cutforth & Harrison, 2002). Two Eph receptors have also been identified in *D. melanogaster* (reviewed by Kullander & Klein, 2002).

The repulsive effect of ephrins and their receptors was first described *in vitro* on hippocampal and entorhinal neurons using stripe assays from membrane preparations of NIH 3T3 cells transfected with ephrin-A2 and ephrin-A3, respectively (Stein *et al.*, 1999). Substrate-bound ephrin-A5 repels retinal and ventral spinal cord neurons, while substantia nigra neurite outgrowth is inhibited by ephrin-B2 *in vitro* (Yue *et al.*, 1999). Overexpression of ephrin-A2 or ephrin-A5 by retinal axons leads to errors in targeting within the tectum, while ectopic expression of ephrin-A2 or ephrin-A5 disrupts midline crossing of axons in the optic chiasm (Dutting *et al.*, 1999; Hornberger *et al.*, 1999).

Eph mutant mice show many axon guidance errors in different regions of the CNS. For example, superior collicular neurons in EphA8 deficient mice display misrouting of axons in the cervical spinal cord. Also, in EphB3 mutant mice axons fail to cross the midline leading to defects in the formation of the corpus collosum (reviewed by Frisen *et*

al., 1999). However, ephrins and eph receptors have also been implicated in attraction/adhesion functions (reviewed by Holmberg & Frisen, 2002).

Signal transduction in this family of axon guidance molecules involves formation of a receptor complex. Ligand binding induces signaling responses not only downstream of the receptor complex (forward signaling) but also in the ephrin expressing cells (reverse signaling). Binding of receptor and ligand causes phosphorylation of the receptors and also in some cases activates other kinases in the ligand expressing cells. Target phosphoproteins appear to include components of the cytoskeleton that in turn cause growth cone collapse or repulsion (reviewed by Schmucker & Zipursky, 2001; Kullander & Klein, 2002).

Slit and the Roundabout Family of Receptors

Using a genetic screen, Seeger and collegues identified mutations that disrupt growth cone guidance at the midline of the *D. melanogaster* CNS and called them *roundabout* (*robo*) (Seeger *et al.* 1993). In *robo* mutant flies growth cones of pCC, MP1, and vMP2 neurons that normally form ipsilateral projections, inappropriately cross the midline. For example, the growth cone of RP1 neurons that normally would cross the midline only once, cross more than once in a *robo* mutant (Kidd *et al.*, 1998; Tear *et al.*, 1996). Similar phenotypes were observed in *C. elegance sax-3* mutants, which encodes the nematode homolog of Robo (Zallen *et al.*, 1998). *Robo* encodes a receptor with a high level of expression in non-crossing axons suggesting a chemorepellent role for Robo (Kidd *et al.*, 1998). The midline glial cells express Slit, which has been shown to function as a ligand for Robo and act as a repellent for non-crossing axons. Furthermore, Slit proteins repel

both migrating mesodermal cells and neuronal cell bodies (Kidd *et al.*, 1999; Bashaw & Goodman, 1999). Slit 1 and Slit 2 knockout mice exhibit severe defects in many axon projections including corticofugal, callosal, and thalamocortical tracts. These mice revealed that Slit proteins define a ventral boundary and prevent axons from crossing or recrossing the ventral regions (Bagri *et al.*, 2002). Slit 1 has also been implicated in the formation and branching of the dendrites of cortical neurons (Whitford *et al.*, 2002). Differences between *slit* and *robo* mutants strongly suggested the presence of additional receptors for slit (reviewed by Guthrie, 2001).

Mechanisms of Robo mediated signal transduction have recently been reported (reviewed by Ghose & Van Vactor, 2002). Robo activity is regulated by an interaction with the netrin-1 receptor DCC (see below) (Stein & Tessier-Lavigne, 2001; Bashaw & Goodman, 1999). The intracellular domain of Robo also interacts with the abelson (Abl) tyrosine kinase. Furthermore, the abl substrate Ena regulates Robo function (Bashaw *et al.*, 2000). Robo also appears to signal via the small GTPases by binding to a novel GAP (see below).

Netrins and Netrin Receptors: helping to guide via attraction and repulsion

Using explants from dorsal and ventral halves of embryonic rat spinal cord *in vitro*, Marc Tessier-Levigne *et al.* (1988) showed that commissural spinal neurons are attracted to the floor plate at the ventral midline. This assay provided direct evidence that diffusible factor(s) secreted from the floor plate guide commissural axon extension. Netrins are a family of secreted proteins initially purified from embryonic chick brain (Kennedy *et al.*, 1994; Serafini *et al.*, 1994). They are a highly conserved family of proteins of ~65 KD in size. They are composed of three domains: VI, V, and C. Domains VI and V are

homologous to domain VI and V of laminin. Domain C is highly charged and has some sequence similarity to carbonic anhydrase (C domain) (reviewed by Manitt and Kennedy, 2002). Five mammalian netrins have been identified so far. Mammalian netrin-1 is an ortholog of chick netrin-1 with the ability to guide commissural axons (Serafini *et al.*, 1996). Netrin-3, first cloned from mouse, is homologous to human netrin-2-like (NTN2L), and is highly expressed in the peripheral nervous system (PNS) by sensory ganglia, mesenchymal cells, and muscles during PNS development (Wang *et al.*, 1999). Netrin-G1 has six isoforms, 5 of which are membrane anchored by a GPI linkage. They are expressed in different CNS regions during development including olfactory bulb, thalamus, and deep cerebellar nuclei. A secreted form of netrin-G1 failed to attract circumferentially growing axons from the cerebellar nuclei (Nakashiba *et al.*, 2000). Mice also express netrin-G2, which like netrin-G1, has not been demonstrated to attract axon growth as a soluble protein. However, netrin-G1 and netrin-G2 both promote neurite outgrowth from neocortical and thalamic neurons when recombinant protein is applied as an immobilized substrate (Nakashiba *et al.*, 2002). Netrin-G2 is expressed in the cerebral cortex, habenular nucleus, and superior colliculus (Nakashiba *et al.*, 2002). The sequence of netrin-4 or β-netrin is more similar to the β chain of laminin than to other netrins and its expression has been detected in kidney, heart, ovary, retina, and the olfactory bulb. Secreted recombinant netrin-4 can induce neurite outgrowth from olfactory bulb explants (Koch *et al.*, 2000; Yin *et al.*, 2000).

Netrin A and netrin B make up the netrin family in *D. melanogaster*. These netrins play an important role attracting commissural axons toward the ventral midline in the developing fly CNS. The first netrin described, UNC-6, was identified as a result of a genetic screen in *C. elegans* (Hedgecock *et al.*, 1990). *Unc-6* mutants exhibit disrupted

projections for axons growing toward or away from the ventral midline (reviewed by Tessier-Lavigne & Goodman, 1996; Wadsworth, 2002).

Recent studies have revealed two classes of receptors for netrins: the UNC-5 homolog family and the DCC family. UNC-5 in C. elegans and UNC-5 homologs in mammals mediate the repellent response to netrins. The DCC family includes UNC-40 in *C. elegans*, Frazzled in *D. melanogaster*, and deleted in colorectal cancer (DCC) and neogenin in mammals. These receptors contribute to mediating both attractant and repellent responses to netrin (reviewed by Manitt and Kennedy, 2002).

Netrin receptor function has been studied in detail in *D. melanogaster*, *C. elegans* and vertebrate species. Hedgecock and *et al.* (1990) showed that in *C. elegans* the migration of pioneer axons extending either toward or away from the ventral midline is disrupted in *unc-6* mutants. *Unc-5* mutants exhibit defects in ventral to dorsal axon trajectories, while *unc-40* mutants exhibit defects in dorsal to ventral axon migration. Mutations of *frazzled*, the DCC homolog in *D. melanogaster*, causes defects in the developing fly CNS that are functionally similar to those found in *unc-40* mutants (Kolodziej *et al.*, 1996). Consistent with the *Unc-5* phenotype in *C. elegans*, *Unc-5* mutations in fly cause disruptions in a subset of motor neuron projections that normally do not cross the ventral midline (Keleman & Dickson, 2001).

In vertebrate species, DCC and neogenin were identified as candidate netrin receptors on the basis of their homology to UNC-40 and Frazzled (Keino-Masu *et al.*, 1996). Although netrin-1 binds neogenin, its function remains unknown. Mice that are deficient in either DCC or netrin-1 show very similar phenotypes, including defects in axonal trajectories and targeting to the spinal commissure, forebrain commissure, hippocampal commissure, corpus callosum, optic disc, and hypothalamus. These findings demonstrate

that netrin-1 and DCC make essential contributions to the development of axonal projections in a variety of CNS regions (Fazeli *et al.*, 1997; Serafini *et al.*, 1996). UNC-5 homologs in vertebrates have been identified. In mouse, the UNC-5 homolog 3 (UNC-5H3) or RCM (rostral cerebellar malformation) is required for appropriate cerebellar development (Ackerman *et al.*, 1997; Przyborski *et al.*, 1998). Two other vertebrate members, UNC-5H1 and UNC-5H2, were cloned from rat (Ackerman *et al.*, 1997; Leonardo *et al.*, 1997), and UNC-5H4 has recently been identified in mouse and human (Engelkamp, 2002).

Deleted in Colorectal Cancer (DCC): what is a tumor suppressor doing in the developing spinal cord?

DCC was first identified in a study of alleles lost from chromosome 18q associated with colorectal carcinomas (Fearon *et al.*, 1990). In addition, DCC expression is lost or reduced in other cancers, including stomach, breast, prostate, brain, and some leukemias (reviewed in Cho & Fearon, 1995). Loss of DCC expression is correlated with cancer progression and metastasis (Reyes-Mugica *et al.*, 1997; Reyes-Mugica *et al.*, 1998). The DCC gene is large, located at chromosome band 18q21.1 composed of over 1.35 million base pairs and including at least 29 exons. The gene encodes a transmembrane protein of approximately 1447 amino acid in size that belongs to the immunoglobulin superfamily (Ig). Its extracellular domain contains four immunoglobulin-like domains and six fibronectin type III-like domains. In its intracellular domain, ~325 amino acids, there is very little sequence similarity to other proteins other than several proline rich domains. Well-conserved DCC homologs have been found in rat, mouse, chicken, and *Xenopus*.

Human DCC shares ~85% amino acid identity with the *Xenopus* DCC homolog and ~94% with chick DCC (Hedrick *et al.*, 1994).

In vertebrate species, DCC is expressed in epithelia at a low level and in differentiated colon goblet cells (secretory cells) (Hedrick *et al.*, 1994). In the nervous system, DCC is expressed in the spinal cord, retina, hippocampus, and cerebellum. DCC protein is distributed on axons of commissural neurons, motor neurons, granule cells, and retina ganglion cells (RGCs) during the development of the spinal cord, hippocampus and retina (Deiner *et. al.*, 1997; Shu *et. al.*, 2000; Keino-Masu *et. al.*, 1996).

The one additional member of the DCC family in vertebrates is neogenin. Its expression has been described in neurons during terminal differentiation and in the gastrointestinal tract of the chick embryo (Vielmetter *et al.*, 1994). Although netrin-1 binds neogenin *in vitro,* neogenin is not expressed in commissural neurons and does not appear to contribute to their guidance. A function for neogenin remains to be identified (Keeling *et al.*, 1997; Reale *et al.*, 1994).

DCC Plays a Central Role in Growth Cone Attraction and Repulsion

Assays examining the outgrowth of axons from explants from embryonic rat spinal cord and axon guidance assays using cultured embryonic *Xenopus* neurons have demonstrated that DCC plays an essential highly conserved role directing commissural axons to the ventral midline of the developing vertebrate CNS (de Hopker *et al.*, 1997; Keino-Masu *et al.*, 1996).

In addition, netrin-1 repels trochlear motor neurons, a subset of spinal motor neurons that reside in the ventral half of spinal cord (Colamarino & Tessier-Lavigne, 1995). Interestingly, after spinal commissural axons reach the ventral midline they are repelled

from the netrin-1 producing cells (Zou *et al.*, 2000). This repulsion suggests the existence of a molecular mechanism that suppresses the netrin-1 induced attraction of commissural neurons.

Two important findings have shed light on the molecular basis of netrin-1 induced repulsion. First, growth cones of cultured spinal cord neurons from *Xenopus* turn toward a netrin-1 source *in vitro*, however, in the presence of an inhibitor of protein kinase A, the growth cones turn away from netrin-1 (Ming *et al.*, 1997). These experiments have shown that the concentration of cAMP within the growth cone dramatically influences the type of turning response made to netrin-1 (Song *et al.*, 1998; Song *et al.*, 1997; reviewed in Song & Poo, 2001). A second series of experiments indicated that a receptor complex determines the response made by a growth cone to netrin-1. A chimeric receptor composed of the extracellular domain of Robo and the intracellular domain of Frazzled (a DCC homolog) expressed in *D. melanogaster* mediated a repellent response to cells expressing netrin-1 at the midline and muscle cells that express netrin-1. A chimera made of the intracellular domain of Frazzled and extracellular domain of Robo directed an attractant response toward the Slit-producing midline cells (Bashaw & Goodman, 1999). Microinjecting mRNAs encoding *unc-5h1*, *unc-5h2* or *unc-5* into *Xenopus* spinal cord neurons cultured *in vitro* caused their growth cones to be repelled from a source of netrin-1. Protein chimeras composed of the intracellular domain of UNC-5H2 and extra-cellular domain of DCC could convert attractive responses to repellent responses toward netrin-1 in these neurons. These experiments demonstrated that UNC-5 proteins play an essential role mediating the repellent action of netrin-1 in the presence of DCC (Hong *et al.*, 1999). On the other hand, Robo and DCC directly interact in the presence of Slit protein. Slit silences netrin-1 mediated attraction in stage 22 *Xenopus* spinal neurons, but does not

affect the response of growth cones to netrin-1 in outgrowth assays (Stein & Tessier-Lavigne, 2001). This action of Slit and Robo, silencing the DCC mediated response to netrin-1, has been proposed to cause commissural axons to cross the midline only once, blocking them from re-crossing back.

Small Rho GTPases and Axon Guidance

The Rho family of small GTPases belongs to the larger Ras super-family. Rho (Ras homology) was identified first in *Aplysia* and then in human (Madaule & Axel, 1985). The Rho family is divided into 5 groups. (1) RhoA, RhoB, and RhoC; (2) Rac1, Rac2, and RhoG; (3) Cdc42 and TC10; (4) RhoD; and (5) RhoE and TTF (Foster *et al.*, 1996; reviewed by Ridley, 1996). They are low molecular weight proteins with highly conserved amino acid sequences. In the late 1980s and early 1990s these proteins were discovered to play an important role regulating cell morphology. Importantly, they were shown to be required for extracellular stimuli to induce stress fiber formation in fibroblast cells (Paterson *et al.*, 1990; Ridley & Hall, 1992). Among them RhoA, Cdc42 (cell division cycle 42), and Rac1 (Ras-related C3 botulinum toxin substrate 1) have been particularly well studied *in vitro* and *in vivo* (reviewed by Hall, 1998).

Small GTPases act as molecular switches that are transiently activated by binding GTP and inactivated by replacing GTP by GDP. This cycle is tightly regulated by other proteins: GEFs (GDP/GTP exchange factors), GAPs (GTPase-activating proteins), and GDIs (guanine nucleotide dissociation inhibitors) (reviewed by Schmidt & Hall, 1998, Cherfils & Chardin, 1999; Hoffman & Cerione, 2002).

GEFs are defined as proteins having a conserved domain of about 150 amino acids called the Dbl homology (DH) domain. The DH domain is required to control the GEF

activity toward the small GTPases. The cellular response of DH domain is coupled to another conserved domain called a pleckstrin homology (PH) domain. The exact role of the PH domain is not clear but it has been suggested to regulate the sub-cellular localization of GEFs (Zheng *et al.*, 1996). Cdc24, which was first identified in studies of budding in yeast has a close homology to Dbl oncogene proteins and regulates yeast Cdc42 (Hart *et al.*, 1991). This led to the demonstration that Dbl proteins have GEF activity in eukaryotic cells.

Recent reports have suggested the presence of other families of proteins with GEF activity but no DH domain (reviewed by Braga, 2002). In DOCK180, which interacts with the adaptor protein Crk, a domain called Docker was identified with a GEF activity toward Rac (Brugnera *et al.*, 2002). A novel GEF was also identified for Cdc42 with a new non-conventional GEF domain and no DH domain (Meller *et al.*, 2002).

It is believed that *in vivo* a small GTPase in its inactive form (GDP-bound) binds with relatively low affinity to its specific GEF(s). Binding the GEF accelerates the release of bound GDP, causing the formation of a high affinity transient binary complex between nucleotide-free GTPase and the GEF(s). This complex is very stable inside cells. Since GTP has even higher affinity for the GTPase than the GEF(s), with increasing concentrations of GTP, GTP quickly binds to the small GTPase and frees the GEF(s) (Lenzen *et al.*, 1998). Dominant negative mutants of the small GTPases were made by amino acid replacements (Ridley *et al.*, 1992; Coso *et al.*, 1995). These mutants compete with endogenous wild type GTPases for binding to the GEF(s), which subsequently produce non-active complexes. GAPs bind to the active form of the small GTPases and induce their intrinsic GTPase activity leading to GTP dissociation, and inactivation of the

GTPases. Therefore, both the activity of GEFs and GAPs are critical regulators of the activity of small Rho GTPases.

GDIs also regulate small Rho GTPases. They are believed to inhibit GEFs and even GAPs. They interact with both GDP and GTP bound forms of Rho GTPases, but act to remove them from membranes, thus playing an important role in translocating the small GTPases to the membrane upon extracellular stimulation of resting cells. Three RhoGDIs have been identified: RhoGDI-1 (Rho GDI α), RhoGDI-2 (D4/Ly-GDI or RhoGDI β), and RhoGDI-3 (RhoGDI γ)(reviewed by Olofsson, 1999).

Rho GTPases Are Required for Appropriate Axon Guidance

Multiple studies indicate that Rho GTPases play a role in axon extension and growth cone motility (Fig. 2). A role for Cdc42 and Rac1 in axon growth and guidance was initially demonstrated in *D. melanogaster* where expression of constitutively active or dominant negative forms of Cdc42 and Rac1 caused defects in axonal pathfinding. Interestingly, defects in dendritic connectivity and structure were also detected, suggesting roles in both dendritic and axonal development (Jalink *et al.*, 1994; Luo *et al.*, 1994). Rac1 has been directly implicated in growth cone function in PC12 cells, contributing to the generation of mechanical tension in elongating neurites (Lamoureux *et al.*, 1997). In *C. elegans*, a mutant of the Rho-like GTPase *mig-2* (*migration-2*) causes neuronal outgrowth defects (Zipkin *et al.*, 1997). Dominant negative expression of Rac1 in *D. melanogaster* caused errors in motor axon guidance (Kaufmann *et al.*, 1998). In *Xenopus* retinal ganglion cells (RGCs), expression of a constitutively active form of Rac1 reduced axonogenesis. Furthermore growth cone morphology was affected by expression

of dominant negative RhoA and wild type Cdc42 in these neurons (Ruchhoeft *et al.*, 1999). Growth cone collapse induced by ephrin-A2 has been shown to inactivate Rac1. Interesting, this also promoted endocytosis of the plasma membrane during growth cone collapse (Jurney *et al.*, 2002).

In many cases, the expression of both active and dominant negative forms of these GTPases produced similar phenotypes in neurons. This finding is an indication of the importance of the precise regulation of the activity of this family of proteins during neuronal outgrowth and guidance. Small Rho GTPases also mediate cell migration by regulating focal adhesions. Rho GTPase activity can instruct a growth cone to either move forward, retract, or promote adhesive interactions with ECM (extracellular matrix) (reviewed by Schmitz *et al.*, 2000). Other small Rho GTPases directly implicated in neurite outgrowth and axonal guidance include: RhoG, Rnd1 and TC10. RhoG induces neurite outgrowth when overexpressed in PC12 cells, dominant negative Cdc42 and Rac1 inhibited this action of RhoG, and active forms of RhoG increased the level of Cdc42 and Rac1 activation. These findings suggested that Rho G is an upstream regulator of Cdc42 and Rac1 (Katoh *et al.*, 2000). Rnd has also been implicated in neurite outgrowth, and specifically in Sema-3a signaling (Rohm *et al.*, 2000). TC10, which has sequence similarity to Cdc42, has been studied in nerve regeneration and caused neurite outgrowth when overexpressed in rat DRGs (Tanabe *et al.*, 2000).

Rho GTPase Activity Is Regulated by Axon Guidance Receptors

Extracellular signals are believed to regulate activation of the small Rho GTPases by regulating the activity of GEFs and GAPs. In the nervous system, a GEF called Trio (the triple functional domain protein) contains two modules of DH-PH domains, one specific

for Rac1 and the other specific for Rho. Trio was first identified as a protein interacting with the intracellular domain of the receptor tyrosine phosphatase LAR (Debant *et al.*, 1996). Trio homologs in *D. melanogaster* (Dtrio) play an important role regulating axonal guidance during development of the fly nervous system (Awasaki *et al.*, 2000; Bateman *et al.* 2000; Liebl *et al.*, 2000; Newsome *et al.*, 2000). The *C. elegans* homolog of *trio*, *unc-73*, is also required for axon guidance and cell migration (Steven, *et al.*, 1998). GEF64C is a Rho specific GEF in *D. melanogaster*, that can suppress the repellent action of Robo at the fly ventral midline when ectopically expressed (Bashaw *et al.*, 2001). Tiam1, the invasion-inducing T-lymphoma and metastasis 1 protein, is also a Rac1 GEF implicated in neurite outgrowth and axon formation (Kunda *et al.*, 2001; Leeuwen *et al.*, 1997). Ephexin is a GEF that interacts with the intracellular domain of EphA receptors in the presence of ephrin A, leading to RhoA activation and inhibition of Cdc42 and Rac1 (Shamah *et al.*, 2001). Kalirin-7 and –9, an exchange factor for Rac1, and for RhoA and Rac1 respectively, are both expressed in the brain (Hansel *et al.*, 2001). Kalirins have close homology to Trio and are present in cell fractions enriched for the postsynaptic density (Ma *et al.*, 2001; Penzes *et al.*, 2000; Penzes *et al.*, 2001; Penzes *et al.*, 2001). Collybistin is a recently described brain specific GEF for Cdc42 that is implicated in synapse formation and plasticity (Kins *et al.*, 2000). It has also been recently shown that plexin-B family members are associated with GEF proteins. PDZ-RhoGEF and LARG (leukemia-associated Rho guanine-nucleotide exchange factor) proteins interact with the intracellular domain of plexin-B family members regulated by Sema-4d (Swiercz *et al.*, 2002). This interaction has been shown to cause Rho A activation. A dominant negative form of PDZ-RhoGEF abolishes growth cone collapse in neurons in the presence of Sema-4d (Swiercz *et al.*, 2002).

Recently, Wong *et al.* (2001) have identified a new family of GAP proteins that are involved in Slit-Robo signaling (srGAPs). They have shown that srGAP1 interacts with CC3 domain of Robo and causes inhibition of Cdc42 activity but increases Rho A activity (reviewed by Ghose & Van Vactor, 2002). These investigators have suggested that Slit-Robo signaling causes growth cone collapse and repulsion by inhibiting Cdc42, since a constitutively active form of Cdc42 could block the repellent activity of Slit. Furthermore, a novel protein called PDZ-RGS3 that exhibits a GAP activity for G proteins has been shown to bind the intracellular domain of ephrin B transmembrane proteins through its PDZ domain (Lu *et al.*, 2001). Over-expression of p190 RhoGAP in neuroblastoma cells induces neurite outgrowth and mice deficient in p190 RhoGAP have axon guidance errors (Brouns *et al.*, 2001).

A role for GDIs in axon guidance has not yet been reported, but their involvement in some neural systems has been suggested. Among them, the Rab-GDP dissociation inhibitor is the most studied member in the brain where its mRNA increases in chick ciliary ganglion (CG) neurons during synaptogenesis (Ikonomov *et al.*, 1998). Interestingly, mutations in this GDI are implicated in X-linked mental retardation (D'Adamo *et al.*, 1998).

Downstream Effectors of Rho GTPases Regulate the Growth Cone Cytoskeleton

The small Rho GTPases are at the center of multiple pathways responsible for regulating cell motility, adhesion, and the cell cycle (reviewed by Marshall, 1999; Schmitz *et al.*, 2000; Tsai & Miller, 2002). There is not only cross talk between RhoGTPase family members, but also with Ras family members by two distinct mechanisms; signal divergence and signal convergence. In signal divergence, GEF(s)

activated by ligand/receptor binding can activate the small Rho and Ras GTPases in parallel, leading to a cellular response. In signal convergence, Rho GTPase effector proteins like Pak1 and PI3 Kinase can modulate the Ras pathway, leading to a cellular response (reviewed by Bar-Sagi & Hall, 2000).

Recently, the MAPK (mitogen activated protein kinase) pathway has been implicated in netrin-1/DCC signal transduction. Using HEK293T cells, Forcet *et al.* (2002) have shown that netrin-1 activates MAPK and induces phosphorylation of ERK1/2 (extracellular signal-regulated kinase 1/2). They have also shown that inhibitors of MEK1/2 abolished ERK1/2 phosphorylation induced by netrin-1, and blocked netrin-1-induced axon outgrowth and turning. It has also been suggested that MAPK activation regulates local protein synthesis and adaptation of the growth cone to netrin-1 (Ming *et al.*, 2002).

p21 Activated Kinase; an important effector for Cdc42 and Rac1

PAKs (p21 activated kinases) are serine/threonine protein kinases that are activated by the small GTPases Cdc42 and Rac1. Six different PAKs (*pak1, pak2, pak3, pak4, pak5,* and *pak6*) have been identified with different tissue expression patterns (reviewed by Bagrodia & Cerione, 1999; Jaffer & Chernoff, 2002). *Pak1*, *pak3*, and *pak6* are highly expressed in the brain. PAKs consist of a catalytic C-terminal domain that is regulated by binding activated Cdc42 and Rac1 to a conserved sequence called the CRIB (Cdc42 and Rac1 interacting binding) domain. This binding induces conformational changes in the catalytic domain, activating the kinase and leading to autophosphorylation of PAKs. The catalytic domain also contains proline rich sequences that are potential binding sites for SH3 domain containing proteins such as the adaptor protein Nck1 (Lu *et al.*, 1997).

Pak1 is an extensively studied member of the PAK family with a molecular weight of 68 kDa. It has been suggested that in its inactive form, Pak1 is present as a stable dimer, while upon stimulation by activated Cdc42 or Rac1, the activated Pak1 monomer translocates to the membrane. In fibroblasts Pak1 is detected in submembranous vesicles and PDGF treatment translocates Pak1 to membrane ruffles and into the edges of lamellipodia (Dharmawardhane *et al.*, 1997). Activated Pak1 has also been detected at sites of focal adhesions, throughout filopodia and at the edges of lamellipodia in PDGF-stimulated fibroblasts (Sells *et al.*, 2000).

Activated Pak1 phosphorylates MLCK (myosin light chain kinase) and reduces its activity toward MLC (myosin light chain) thus opposing the effects of Rho and p160 Rho kinase (Sanders *et al.*, 1999). Pak1 also influences actin polymerization and depolymerization by phosphorylating LIM kinase 1 which phosphorylates cofilin (an actin depolymerizing protein) (Edwards *et al.*, 1999). Recently, it has been demonstrated that Pak1 phosphorylates MEK1 and Raf1, thereby potentially regulating the MAP kinase and ERK pathways (Coles & Shaw, 2002; Eblen *et al.*, 2002).

Although Pak1 was initially isolated from rat brain, its role in the development of the mammalian nervous system has not been extensively studied (reviewed by Bagrodia & Cerione, 1999). In *D. melanogaster* Pak1 interacts with Dock (NCK homolog of *D. melanogaster*) and plays an important role in the guidance of photoreceptor axons (*Hing et al.*, 1999). A membrane-targeted form of Pak1 induces neurite formation in PC12 cells in the absence of NGF (Daniels *et al.*, 1998). Recently Pak1 has been implicated in Sema-4d/plexin-B1 signaling in cell lines. Pak1 is inactivated by Sema-4d, promoting its dissociation from active Rac1, thereby inhibiting actin assembly (Vikis *et al.*, 2002). Sema-3a induces sequential phosphorylation and dephosphorylation of cofilin during

growth cone collapse by regulating LIM kinase 1 activity (Aizawa *et al.*, 2001). Recently, Nck1, a Pak1 binding adaptor protein, has been implicated in netrin-1/DCC signaling by constitutively binding to DCC (Li *et al.*, 2002).

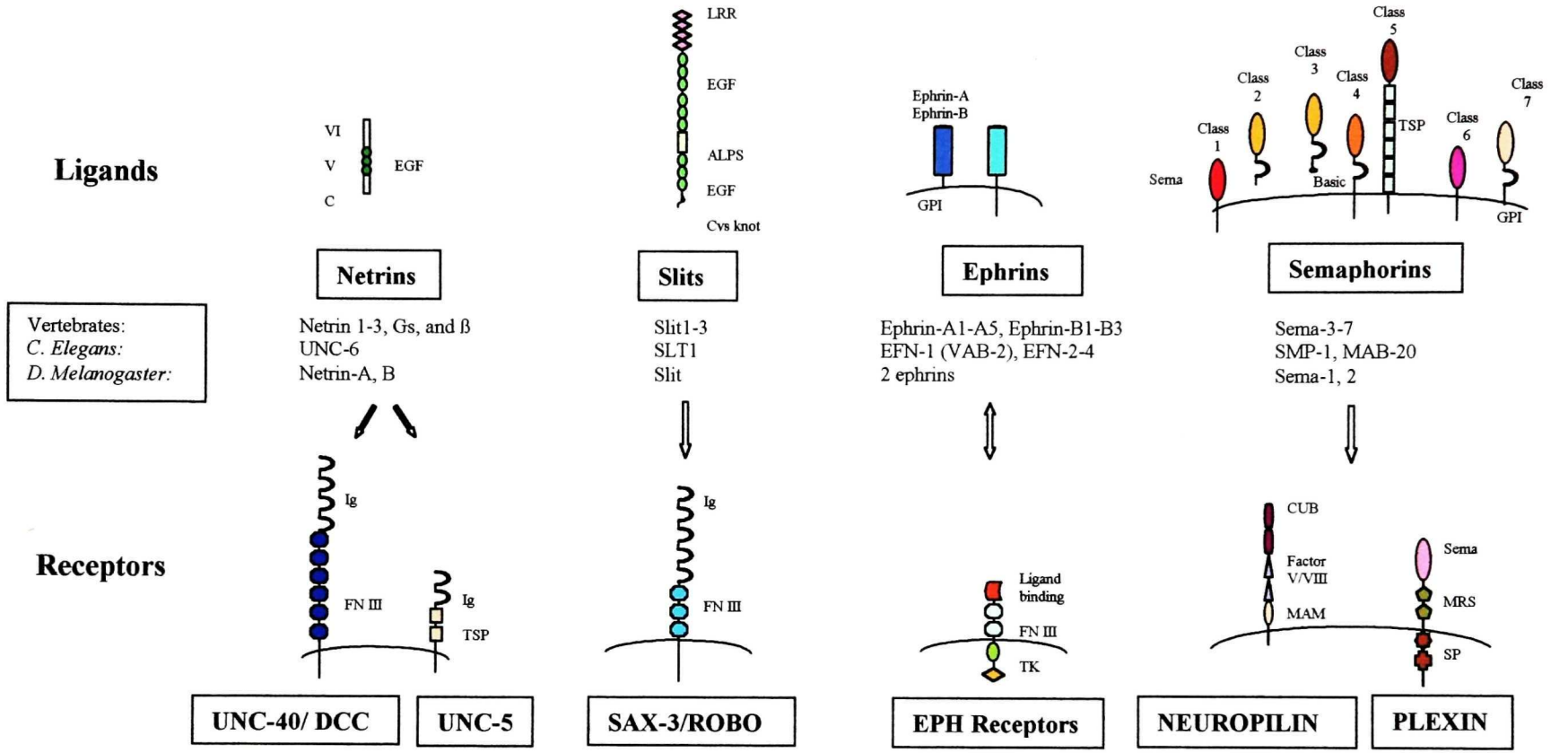
N-WASP Is an Important Effector of Activated Cdc42

N-WASP (neuronal Wiskott-Aldrich Syndrome Protein) is a neuronal homolog of WASP, an effector protein for Cdc42 and Rac1. N-WASP is ubiquitously expressed in the nervous system and is believed to play an important role in actin nucleation. Activated Cdc42 is able to activate N-WASP and to induce actin polymerization (Miki *et al.*, 1998). In its inactive form, the NH_2 terminus of N-WASP inhibits an active site in its COOH terminus. Active Cdc42 binding eliminates the inhibition and allows the COOH terminus to stimulate the Arp2/3 complex to nucleate actin (reviewed by Zigmond, 2000).

N-WASP has been shown to play an important role in filopodia formation, cytoskeletal rearrangement, actin-based motility, and neurite extension in PC12 cells and hippocampal neurons (Miki *et al.*, 1998; Miki *et al.*, 1996; Suzuki *et al.*, 1998; Banzai *et al.*, 2000). Additional molecules also regulate N-WASP activation. For example, NCK, PI3 kinase, and tyrosine kinases all appear to influence N-WASP activation (Rohatgi *et al.*, 2001).

Figure 1. Instructive cues and their receptors play an important role in axon guidance.

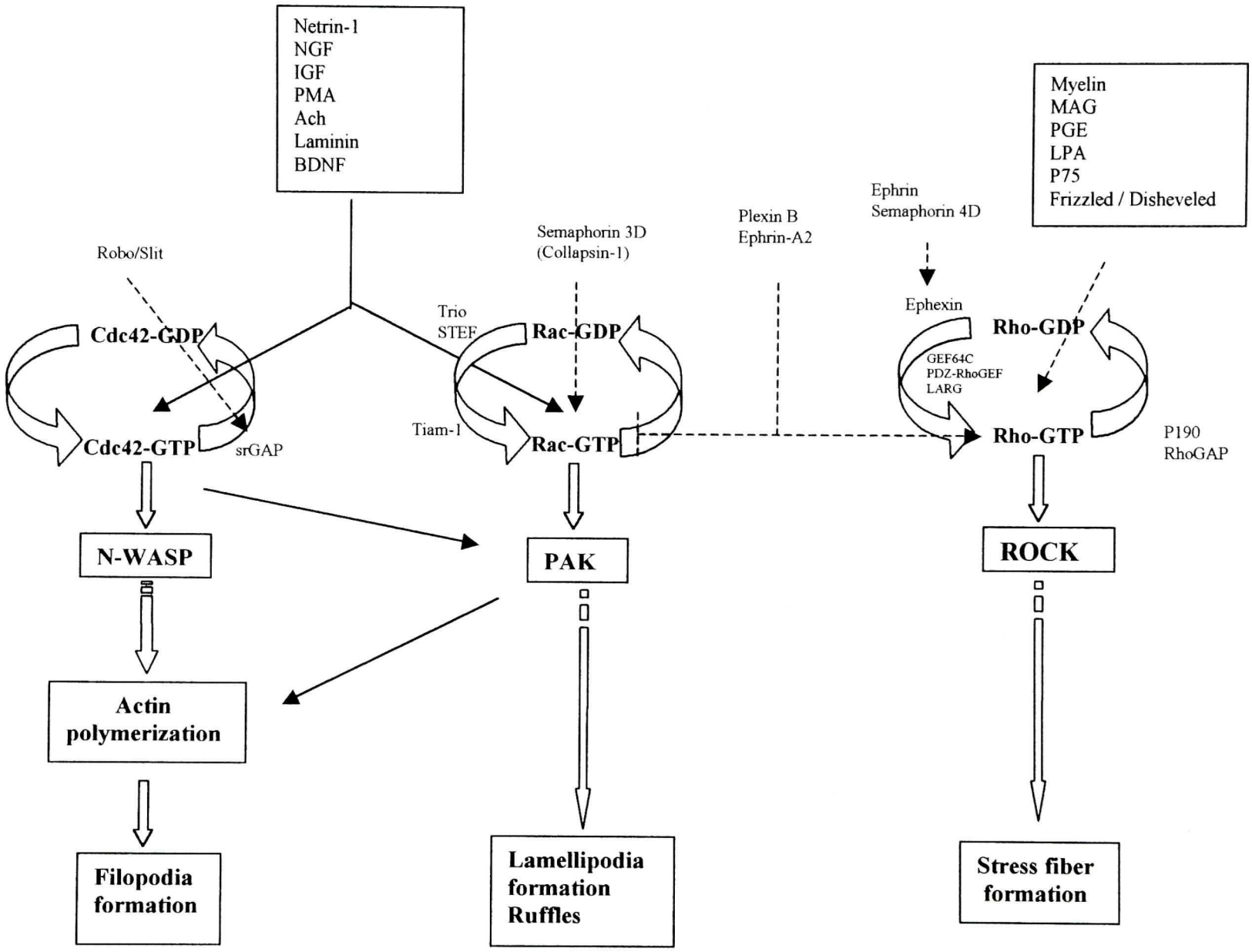
A summary of instructive cues and their receptors is shown with their response and their modifiers, which control some of their functions. ALPS, agrin-laminin-perlecan-Slit domain; C, netrin C terminus; CUB, CI/Uegf/BMP-I domain; DCC, deleted in colorectal cancer; EGF, epidermal growth factor; FNIII, fibronectin type III domain; GPI, glycosylphosphatidyl-inositol anchor; Ig, immunoglobulin domain; LRR, leucine-rich repeat; MAM, meprin / A5 antigen motif; MRS, Met tyrosine kinase-related sequence; RK, arginine / lysine-rich basic domain; SAM, sterile alpha motif; SP, 'sex and plexins' domain; TK, tyrosine kinase domain; TSP, thrombospondin domain; VI and V, homology to laminin domains VI and V, respectively.



Adapted and Modified from: Yu and Bargmann, 2001.

Figure 2. Netrin-1 like other guidance cues and extracellular signals influences growth cone dynamics by regulating the small Rho GTPases.

When GTP-bound, the small Rho GTPases are believed to be active. They are able to bind to their downstream targets and cause their activation. They then recycle back to their inactive form. This cycle is regulated by GEF, GAP, and GDI proteins, which control their activation and inactivation. It is known that N-WASP is an important effector for active Cdc42 to increase actin polymerization at the tips of filopodia. PAKs are also effectors for both Cdc42 and Rac1. PAKs regulate actin polymerization via Lim kinases. Rho interacts with ROCK to initiate stress fiber formation.



Adapted and modified from: Meyer and Feldman, 2002

Rationale and Objectives

Significant insight into the intracellular signaling mechanisms that regulate the cytoskeleton has been obtained over the last few decades. In addition, several families of extracellular cues that direct axon extension and the receptors for these cues have also been identified. Exactly how these cues and their receptors functionally modify the cytoskeleton to direct axon growth remains to be determined.

The demonstration that DCC is a receptor for netrin-1, and that is essential for the normal development of the nervous system was a surprise to many investigators. This finding provided a first step towards identifying the molecular mechanisms underlying the action of netrin-1. Netrin-1 directs the extension of commissural neurons in the embryonic spinal cord. DCC is highly expressed by these neurons and is essential for them to reach the ventral midline.

At the start of this study, signaling mechanisms downstream of DCC had not been identified. I set out to identify components of the molecular mechanism that mediate DCC/netrin-1 signal transduction. Characterizing cell lines that do not normally express DCC provided me with the opportunity to identify the consequences of expressing DCC in these cells, and investigate the signal transduction mechanisms involved. Extending this analysis to primary cultures of embryonic rat spinal commissural neurons then allowed the role of these mechanisms to be addressed in neuronal growth cones.

Chapter 2

The Netrin-1 Receptor DCC Promotes Filopodia Formation and Cell Spreading by Activating Cdc42 and Rac1

Summary

Netrins are a family of secreted proteins that function as tropic cues directing cell and axon migration during neural development. Filopodia are dynamic actin based membrane protrusions that explore the environment, interacting with extracellular cues that influence motility. We show that the netrin-1 receptor, deleted in colorectal cancer (DCC), is present at the distal tips of filopodia in the growth cones of embryonic rat spinal commissural neurons. To further investigate DCC function, we characterized the expression of netrins and netrin receptors in HEK293T cells and NG108-15 cells and found that they express netrin-1 but do not express DCC. Ectopic expression of DCC produced a striking netrin-1 dependent increase in the number of filopodia and in cell surface area. We then investigated the possible role of members of the Rho family of GTPases in generating these responses. Co-expression of DCC and dominant negative Cdc42 or dominant negative Rac1 blocked the increase in filopodia number and cell surface area, respectively. Furthermore, we found that addition of netrin-1 to cells expressing DCC produced a persistent activation of Cdc42 and Rac1. These findings

suggest that netrin-1, via DCC, influences cellular motility by regulating actin based membrane extension through the activation of Cdc42 and Rac1.

Introduction

During neurite extension, the growth cone probes the environment for cues instructing it to advance, turn, or retract. Netrins are a family of secreted proteins that function as tropic cues that direct the migration of both growth cones and cells during development (reviewed by Culotti & Merz, 1998; Kennedy, 2000). Netrin-1 is bifunctional, acting as an attractant for some cell types and as a repellent for others. A combination of genetic and biochemical analyses have identified two classes of receptors for netrin-1, the DCC family and the UNC-5 homolog family. DCC is a transmembrane member of the Ig superfamily that is required for axons to be attracted to a source of netrin-1 (Keino-Masu *et al.*, 1996). DCC also appears to collaborate with UNC-5 homologs to mediate the repellent response to netrin-1 (Ackerman *et al.*, 1997; Leonardo *et al.*, 1997; Hong *et al.*, 1999).

Filopodia and lamellipodia are dynamic actin based membrane protrusions found at the advancing edge of many types of motile cells, including the neuronal growth cone (reviewed by Bentley & O'Connor, 1994; Tanaka & Sabry, 1995). In neurons, disruption of filopodia causes errors in axon guidance (Keshishian & Bentley, 1983; Bentley & Toroian-Raymond, 1986; Chien *et al.*, 1993; Zheng *et al.*, 1996). Conversely, contact of the tip of a single filopodium with an appropriate extracellular target is sufficient to alter the trajectory of an extending axon (O'Connor *et al.*, 1990; Chien *et al.*, 1993), indicating that the receptors that interact with extracellular guidance cues are present, and perhaps enriched, at filopodial tips. Substantial evidence indicates that such guidance cues influence motility by remodeling the actin cytoskeleton (reviewed by Suter & Forscher, 1998). The Rho family of small GTPases are key components of the intracellular signal transduction cascade that regulate the organization of actin. In neuronal and non-neuronal

cells, the Rho family members Cdc42 and Rac1 regulate the reorganization of actin responsible for the formation of filopodia and lamellipodia respectively (reviewed by Hall, 1998; Dickson, 2001).

Here we demonstrate that expression of DCC causes a netrin-1 dependent increase in the number of filopodia and in the cell surface area of rodent neuroblastoma-glioma cells (NG108-15) and human embryonic kidney cells (HEK293T). We observed a striking localization of DCC protein at the distal tips of filopodia in these cells and in the growth cones of rat embryonic spinal commissural neurons. Co-expression of DCC with dominant negative Cdc42 or dominant negative Rac1 blocked the netrin-1 induced increase in filopodia number and in cell size respectively, demonstrating that these Rho GTPase family members are required for these responses. Furthermore, we show that DCC causes a netrin-1 dependent activation of Cdc42 and Rac1, consistent with these proteins being components of the signal transduction cascade activated by netrin-1. These findings suggest that netrin-1 and DCC influence cell motility by activating the Rho GTPases Cdc42 and Rac1 and causing reorganization of actin.

Materials and Methods

Cell Culture

Neuroblastoma × glioma 108-15 cells were cultured and differentiated for three days as described (Shekarabi *et al.*, 1997) except that the differentiation was performed on glass coverslips (Carolina Biological Supply, NC) in the presence of 1 μM cytosine arabinoside (ara C, Sigma) to reduce proliferation. HEK293T were grown in DMEM (Biomedia, QC) containing 10 % heat inactivated fetal bovine serum (iFBS, Biomedia) plus 2 mM glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. Both cell lines were transfected using LipofectAMINE reagent (Invitrogen, ON). Cells were plated at a density of 2.5×10^5 cells per 35 mm well. The following day, two hours prior to transfection, the media was replaced with fresh plating media. One microgram of each DNA construct was mixed with LipofectAMINE according to the manufacturer's protocol. Following transfection, the cells were trypsinized, washed, and plated on glass coverslips coated with poly-D-lysine (PDL, Sigma, MO) and laminin-1 (Collaborative, MA). Coverslips were coated by incubation with 20 μg/ml PDL at 4°C overnight, then washed with Hanks buffered salt solution (Invitrogen) and allowed to dry. They were then coated with 10 μg/ml of laminin-1 in Hank's BSS at 37°C overnight. Coverslips were washed with water and allowed to dry just before plating the cells. NG108-15 or HEK293T cells were plated at a density of 10×10^4 on 22 × 22 mm glass coverslips. HEK293T cells were serum-starved prior to stimulation with netrin-1 as described below under GTPase activation assays. The following reagents were used: purified recombinant chick netrin-1 protein (160 ng/ml; Serafini *et al.*, 1994), affinity purified rabbit polyclonal netrin antibody PN3 (25 μg/ml, Métin *et al*, 1997), purified non-immune rabbit IgG (25

μg/ml, Invitrogen); function blocking DCC monoclonal antibody (clone AF5, 10 μg/ml, Calbiochem).

For primary neuronal culture, the spinal cords of E13 rat embryos were dissected as described (Tessier-Lavigne *et al.*, 1988). The dorsal halves of embryonic spinal cords were incubated with 0.25% trypsin (Invitrogen) in S-MEM (Invitrogen) for 30 min at 37°C. The tissue was transferred to 5 ml of Neurobasal medium (Invitrogen) containing 10% iFBS and 2mM of glutamine, penicillin/streptomycin (as described above) and spun into a pellet. Tissue was then dissociated to a single cell suspension by trituration with a constricted Pasteur pipette in 1.5 ml of complete Neurobasal medium at 37°C. Five thousand cells were plated per 13 mm coverslip coated with PDL and laminin-1. Twelve hours after plating, the media was changed to fresh serum-free Neurobasal medium plus B27 supplement (1:200, Invitrogen) and 160 ng/ml of recombinant netrin-1 protein. Recombinant netrin-1 protein was purified from a HEK293T cell line secreting netrin-1 as described (Serafini *et al.*, 1994; Shirasaki *et al.*, 1996).

DNA Constructs

A cDNA containing the full-length coding sequence of rat DCC was provided by M. Tessier-Lavigne (pBS-DCC, clone D4-1, genebank U68725, Keino-Masu *et al.*, 1996). We generated the construct, pDCC-FL, in order to express full-length DCC protein without an epitope tag or GFP by partially digesting pBS-DCC with HindIII to generate an ~6Kb fragment containing the complete DCC coding sequence. This was then subcloned into the HindIII site of pCEP4 (Invitrogen). The vector pEGFP-N1 (Clontech, CA) was used to generate a DCC-GFP chimeric protein where DCC is tagged with green fluorescent protein (GFP) at its C-terminus. To produce this construct, pBS-DCC was

partially digested by HindIII and the same ~6Kb fragment was subcloned into the HindIII site of pEGFP-N1. The construct pDCC-GFP was then made by partially digesting the 6Kb fragment-pEGFP-N1 construct with BamHI to remove a fragment containing 3' UTR sequence and sequence encoding the 31 carboxyl terminal amino acids of full length DCC. The BamHI site corresponds to position 4568 in the nucleic acid sequence of DCC (genebank U68725, Keino-Masu *et al.*, 1996). The BamHI sites of the plasmid and insert were then ligated back together bringing the coding sequence of DCC in frame with that of GFP to generate pDCC-GFP. Constructs encoding Rac1 and Cdc42 contain a myc epitope tag at their N-terminus as described (Lamarche *et al.*, 1996). The GST-PAK1-CRIB construct (Sander *et al.*, 1998) was provided by J. Collard.

Reverse Transcriptase-PCR (RT-PCR)

Total RNA was purified using Trizol reagent (Invitrogen), and poly A(+) RNA isolated using the Oligotex mRNA purification kit (Qiagen, ON). Positive control mRNA was isolated from newborn rat brain, newborn mouse brain, or cultured human fetal astrocytes (provided by Dr. J. Antel, Montreal). First strand cDNA was synthesized using superscript reverse transcriptase (Invitrogen). The polymerase chain reaction (PCR) was used to amplify cDNA using the primers listed. Primers were annealed at 60°C and 35 cycles of amplification carried out. The size of the predicted amplification product is indicated.

rat netrin-1: CGG GAT CCG CGT GCG CGA CCG AGA CGA CAG T;

GGA ATT CTG GGG GAG CGG CTC TGC TGG TAG C, 462 bp

rat DCC: CCG CTC GAG TGG TCA CCG TGG GCG TTC TCA;

GGC TGG ATC CTC TGT TGG CTT GTG, 938 bp.

rat neogenin: AAC TGC AGA AAG CGG ACT CCT CTG ATA AAA TG;

TCC CCG CGG CCG GCT CCT TCC TAA A, 914 bp.

rat unc-5h1: GGA ATT CCC TCC CTC GAT CCC AAT GTG T

TCC CCG CGG GGC AGG GAA CGA AAG TAG T, 909 bp.

rat unc-5h2: GCT CTA GAG TCG CGG CAG CAG GTG GAG GAA

GGA ATT CAG GGG GCG GCT TTT AGG GTC GTT, 771 bp.

human netrin-1: GCC GCC ACT GCC ATT ACT GC

GAG GGG CTT GAT TTT GGG ACA CTT, 527 bp

human DCC: CAA GTG CCC CGC CTC AGA ACG

GCT CCC AAC GCC ATA ACC GAT AAT, 434 bp

human neogenin: TGG CCC AGC ACC TAA CCT

TTG CCG GGC CTG TAC CAT TGA TTG, 545 bp

human unc-5h3: CCC CCG GCA CAC CTC TG

TGC TGG CGC GAA ATC TCA AT, 412 bp.

Immunocytochemistry, Actin Staining, and Quantification

Cells were rapidly rinsed with PBS pH 7.3, fixed in 4% paraformaldehyde plus 4% sucrose in PBS at room temperature (RT) for 15 min, and then permeabilized using 0.25% Triton X-100 in PBS for 5 min. Blocking was performed in 5% heat inactivated normal goat serum (HINGS) and 0.125% Triton X-100 in PBS at RT for one hour. Coverslips were incubated with monoclonal antibodies against an intracellular epitope of DCC (2 μg/ml, G97-449, Pharmingen, ON) or a myc epitope (9E10, Evan *et al.*, 1985) at 4° C overnight. Cells were then washed three times with PBS and incubated with a fluorescein-conjucated (FITC) anti-mouse IgG or a rhodamine-conjugated anti-mouse

IgG (Jackson ImmunoResearch, PA) at RT for one hour, followed by 0.5 μg/ml rhodamine phalloidin (Molecular Probes, OR) in PBS at 37° C for 15 min. The coverslips were washed with PBS and mounted using Mowiol (Aldrich, WI) containing 2.5% 1,4-Diazabicyclo [2.2.2] octane (Dabco, Sigma) to prevent photo-bleaching. Epifluorescent images were captured using a Zeiss axiovert microscope, a 100 × objective lens, and a MagnaFire CCD camera (Optronics, CA). Filopodia number and cell surface area were quantified using Northern Eclipse software (Empix Imaging, ON). A filopodium was defined as a fine cellular process, at least 3 μm long and less than 0.5 μm in diameter. The area occupied by the two-dimensional fluorescence image of each cell was defined as the cell surface area. Only isolated cells from randomly selected fields were counted. Confocal imaging was performed with a Leica LP2 laser scanning confocal microscope using a 100 × objective. Statistical analysis used ANOVA with Bonferroni adjusted probabilities (Systat, SPSS Inc, Il).

Rac1 and Cdc42 Activation Assays

To assay activation of Cdc42 and Rac1 in HEK293T cells, 3×10^6 cells were co-transfected with expression constructs encoding DCC-GFP or GFP and wild type myc tagged Cdc42 or Rac1 using lipofectAMINE (Invitrogen) in 100 mm tissue culture plates (Sarstedt, QC) and grown overnight. The following day the medium was changed to fresh DMEM plus 0.15 % BSA for 8 hours. For stimulation, 160 ng/ml of recombinant chick netrin-1 protein was added to the medium, incubated for the indicated time periods, washed with ice-cold PBS plus 1mM $MgCl_2$ and 0.1 mM $CaCl_2$ and the cells collected in lysis buffer as described (Sander *et al.*, 1998). Samples were spun at 14,000 rpm at 4°C

for 10 min to pellet cell debris and aliquots of the supernatants stored at –20°C in order to assay the total amount of Cdc42 and Rac1 protein present. The supernatants were then rocked at 4°C for one hour with 10 µl of glutathione-coupled sepharose 4B beads (Pharmacia, NJ) previously loaded with ~10 µg of bacterially produced GST-Pak1-CRIB domain fusion protein (amino acids 56-272 of Pak1, Sander *et al.*, 1998). Non-hydrolysable GTPγS was added to lysates as a positive control as described (Knaus *et al.*, 1992). Beads were washed three times with lysis buffer and eluted in SDS-PAGE sample buffer. Samples were separated by SDS-PAGE and analyzed by western blotting using either the 9E10 monoclonal antibody against a myc epitope tag, a monoclonal antibody against Rac1 (Transduction Laboratories, CA) or polyclonal antibodies against Cdc42 (Santa Cruz Biotechnology, CA). Signals were detected using the ECL chemiluminescence kit (NEN Lifescience products, MA). Densitometry and quantification of the relative levels of Cdc42 and Rac1 were carried out on scanned images of western blots using NIH Image software (United States National Institutes of Health).

Results

Analysis of Netrin-1, DCC, Neogenin, and Unc-5 Homolog Expression in NG108-15 Cells and HEK293T Cells

In order to investigate netrin-1 signal transduction, we first characterized the expression of netrin-1 and netrin-1 receptors in two cell lines: NG108-15 and HEK293T. NG108-15 cells can be proliferated in culture or differentiated using agents that elevate cAMP. Differentiated cells are post-mitotic and have some of the characteristics of cholinergic motor neurons (Nelson *et al.*, 1976). RT-PCR analysis detected expression of mRNAs encoding netrin-1 and the DCC family member neogenin in both cell lines (Fig. 1A and 1B). NG108-15 cells express UNC-5H1 (Fig 1A, lane 3 and 4) and HEK293T cells express UNC-5H3 (Fig. 1B, lane 2); however, DCC expression was not detected in either cell line. As negative controls, no product was amplified in the absence of cDNA (Fig. 1A, lane 5; Fig 1B, lane 3) or if the RT reaction step was excluded (not shown). To confirm that netrin-1 protein was produced, cell lysates were prepared and analyzed by western blotting using PN3, a polyclonal antibody against netrin-1 (Manitt *et al.*, 2001). Netrin-1 protein was detected in both cell lines, although at a substantially lower level in HEK293T cells than NG108-15 cells (Fig. 1C). Netrin-1 protein isolated from NG108-15 cells caused the outgrowth of commissural neurons from explants of embryonic day 13 rat dorsal spinal cord cultured in collagen gels indicating that the protein is functional (not shown).

Expression of DCC, in the Presence of Netrin-1, Increases the Number of Filopodia and Cell Surface Area of NG108-15 and HEK293T Cells

The absence of DCC expression by NG108-15 cells and HEK293T cells allowed us to identify the consequences of expressing DCC in these cell lines. Constructs were generated that encode full-length DCC, and a C-terminal tagged DCC-GFP fusion protein (Fig. 1D). Cells were transfected with constructs encoding DCC full-length, DCC-GFP, or GFP alone. Approximately 70% and 25% transfection efficiencies were obtained for HEK293T and NG108-15 cells respectively. Following transfection, NG108-15 cells were plated on PDL/laminin-1 coated coverslips and differentiated in the presence of 10 μM of forskolin for three days. NG108-15 cells express a relatively large amount of netrin-1 protein and no additional netrin-1 was added to the culture medium. However, because HEK293T cells produce only small amounts of netrin-1 protein (Fig. 1C), this provided the opportunity to assess the effect of growing them overnight on a substrate of PDL/laminin-1 with or without the addition of 160 ng/ml recombinant netrin-1 protein. Filamentous actin was visualized using rhodamine-conjugated phalloidin.

Expression of either full-length DCC or DCC-GFP produced a dramatic increase in cell surface area and filopodia number in both NG108-15 cells and HEK293T cells (Fig. 2 and Fig. 3; Table 1). In the NG108-15 cells, DCC expression produced an approximately four-fold increase in the number of filopodia and three fold increase in cell surface area. In HEK293T cells, an approximately 5 fold increase in filopodia number and 2.5-fold increase in cell surface area were found in the presence of netrin-1 relative to cells transfected with the GFP expression construct as a control. In both cell lines, the presence of DCC or netrin-1 function blocking antibodies significantly reduced the effects of DCC expression (Fig. 2D, 2E, 3G, and 3H).

Presence of DCC at the Tips of Filopodia in NG108-15 Cells, HEK293T Cells and Commissural Neuron Growth Cones

We observed a striking localization of DCC protein at the tips of filopodia in NG108-15 cells and HEK293T cells expressing DCC full-length or DCC-GFP. To characterize this in more detail, DCC protein and filamentous actin were imaged using confocal laser scanning microscopy. In HEK293T cells actin filaments marked the core of each filopodium and a mesh of actin was detected under the plasma membrane. DCC protein, full-length or DCC-GFP, was highly enriched at filopodia tips (Fig. 4A-4C) and a similar distribution was detected in NG108-15 cells (Fig. 4D-4F). Expression of GFP alone produced no such localization (Fig. 2A, 3A, and 3D).

To determine if endogenous DCC is similarly localized in neurons that respond to netrin-1, we examined the distribution of DCC protein and actin in the growth cones of dissociated commissural neurons obtained from the dorsal half of micro-dissected embryonic day 13 rat spinal cords. After two days *in vitro*, the cultures contained large numbers of neurons that extend long axons with well-developed growth cones. These neurons expressed both DCC and TAG-1, markers of spinal commissural neurons *in vivo* (data not shown; Dodd *et al.*, 1988; Keino-Masu *et al.*, 1996). Punctate DCC immunoreactivity was observed along the axon and within the growth cone. Consistent with the distribution found in both cell lines, DCC protein was detected at the tips of filopodia in the growth cones of commissural neurons (Fig. 4G-4I).

Cdc42 and Rac1 Are Required for the DCC-Induced Increases in Filopodia Number and Cell Surface Area

Previous studies have shown that members of the Rho family of small GTPases play an essential role in regulating the organization of the actin cytoskeleton in many cell types (reviewed by Hall, 1998). These studies indicate that activation of Cdc42 leads to the formation of microspikes and filopodia and activation of Rac1 mediates the generation of membrane ruffles and lamellipodia extension (Ridley & Hall, 1992; Ridley *et al.*, 1992; Kozma *et al.*, 1995; Nobes *et al* 1995). To determine if Cdc42 is required for the DCC induced increase in filopodia number or cell surface area, we co-transfected vectors encoding DCC-GFP or GFP alone with constructs encoding a dominant negative form of Cdc42 (N17Cdc42) into NG108-15 cells and HEK293T cells. Expression of the N17Cdc42 protein was confirmed using the 9E10 monoclonal antibody against a myc epitope tag present in the expressed protein. N17Cdc42 significantly reduced the DCC induced increase in filopodia number in both cell lines; however, the DCC induced increase in cell surface area was not affected (Fig. 5A-5D, 6A, and 6B; Table 1).

To determine if Rac1 was required for DCC function, we co-transfected vectors encoding DCC-GFP or GFP with constructs encoding a dominant negative form of Rac1 (N17Rac1) into NG108-15 cells and HEK293T cells. N17Rac1 significantly reduced the netrin-1 induced increase in cell surface area in DCC-GFP expressing cells. Filopodia number increased in the presence of DCC-GFP and N17Rac1, but to a smaller extent than in the absence of the dominant negative construct (Fig. 5E-5H, 6C, and 6D; Table 1). The reduced increase in filopodia number is likely due to the small size of the cells caused by N17Rac1. Together, these findings indicate that inhibition of Cdc42 is sufficient to block the DCC induced increase in filopodia number and that inhibition of Rac1 is sufficient to block the induced increase in cell surface area. Because these effects are separable, this

suggests that independent signals from DCC can be transmitted in parallel through Cdc42 and Rac1.

DCC Activates Cdc42 and Rac1 in the Presence of Netrin-1

The results described above indicate that active Cdc42 and Rac1 are required for DCC function. We then determined if netrin-1, via DCC, activates Cdc42 and Rac1. A downstream target of activated Cdc42 and Rac1 is $p65^{PAK}$ (p21 activated kinase, Manser *et al.*, 1994). $p65^{PAK}$ interacts with GTP-bound Cdc42 and Rac1 via a CRIB (cdc42/rac interactive binding) domain (Burbelo *et al.*, 1995). The activation state of Cdc42 and Rac1 can be measured using a GST-Pak1-CRIB domain fusion protein to isolate GTP-bound Cdc42 and Rac1 (Sander *et al.*, 1998). HEK293T cells were co-transfected with constructs encoding wild type Cdc42 or Rac1 (G12Cdc42 and G12Rac1) and vectors encoding either DCC-GFP or GFP alone. After addition of netrin-1, cells were lysed, the lysates incubated with GST-Pak1-CRIB fusion protein coupled to glutathione-sepharose beads, the complex isolated, and the amount of GTP-bound Cdc42 or Rac1 assayed using western blot analysis. We found that addition of netrin-1 to HEK293T cells expressing DCC activated both Cdc42 and Rac1. Increased GTP-bound Cdc42 and Rac1 were detected within one minute after addition of netrin-1, the earliest time point assayed (Fig. 7A and 7B). Furthermore, although both Cdc42 and Rac1 activation in DCC expressing cells peaked shortly after application of netrin-1, activation persisted at a level significantly above baseline for at least one hour, the longest time point assayed (Fig. 7 A-7D). Similar increases in GTP-bound Cdc42 and Rac1 were detected using either the DCC-GFP or the full-length DCC expression construct (Fig.7C).

Netrin-1 Independently Activates Cdc42 and Rac1 via DCC

In some cells, activation of Cdc42 has been shown to be upstream of Rac1 activation (reviewed by Hall, 1998). As described above, our findings suggest that DCC can signal independently through Cdc42 and Rac1. To address this, G12Cdc42 activation was assayed in the presence of N17Rac1. Conversely, G12Rac1 activation was assayed in the presence of N17Cdc42. HEK293T cells were simultaneously transfected with the following expression constructs: DCC-GFP, N17Rac1, and G12Cdc42, or DCC-GFP, N17Cdc42, and G12Rac1, and the level of Cdc42 or Rac1 activation assayed. Control cells were transfected with GFP, N17Rac1 and G12Cdc42, or GFP, N17Cdc42 and G12Rac1. In the presence of N17Rac1, the level of GTP-bound Cdc42 was significantly greater than control, and in the presence of N17Cdc42, netrin-1 continued to activate Rac1 (Fig. 8A and 8B). These findings are consistent with independent activation of Cdc42 and Rac1 by DCC.

Discussion

Netrin-1 and its receptor DCC guide cell migration and axon extension in the developing nervous system. Although well characterized in the embryonic CNS, these gene products are expressed in many tissues (Fearon *et al.*, 1990; Kennedy *et al.*, 1994; Manitt *et al.*, 2001; Meyerhardt *et al.*, 1999) and are likely to contribute to regulating the motility of many cell types. To investigate the mechanisms underlying netrin-1 signal transduction, we have reconstituted a netrin-1 response in cells that do not normally express DCC. Our findings indicate that in the presence of netrin-1, DCC produces a dramatic change in the organization of actin, increasing cell size and filopodia number. We show that DCC protein is present at the distal tips of filopodia, an appropriate position to interact with extracellular cues and influence filopodial extension. Our results indicate that the induced increase in filopodia number and cell size requires active Cdc42 and Rac1 and that netrin-1, through DCC, activates both Cdc42 and Rac1. These findings provide new insight into the possible signaling pathways used by netrin-1 and DCC to guide cell and axonal growth cone migration in the embryonic CNS.

Netrin-1 Is a Bifunctional Axon Guidance Cue

Netrins may act as attractants or repellents for extending axons. The filopodia formation and cell spreading effects of netrin-1 and DCC described here are similar to events occurring at the edge of a neuronal growth cone as it advances forward, suggesting that these processes share mechanisms in common with chemoattraction to netrin-1. In HEK293T and NG108-15 cells we detected mRNAs encoding other netrin receptors, neogenin and UNC-5 homologs, raising the possibility that they may contribute to the effect of netrin-1 described here. The function of neogenin is not known, but a

DCC/UNC-5 homolog complex has been implicated in mediating the chemorepellent response to netrin-1 (Hong *et al.*, 1999).

It has been shown that inhibition of the A kinase can cause the turning response of a neuronal growth cone to netrin-1 to switch from attraction to repulsion (Ming *et al.*, 1997). We found that the response of HEK293T cells to netrin-1 was similar to that of NG108-15 cells. Furthermore, addition of forskolin to the HEK293T cells did not alter the activation of Cdc42 or Rac1 by netrin-1 measured using the Pak1-CRIB pull down assay (not shown). These findings suggest at least two possibilities: that A-kinase activity is high in both of these cell lines or that the ectopic over-expression of DCC overwhelms the endogenously expressed UNC-5 homolog. Our findings indicate that activation of Cdc42 and Rac1 by netrin-1 requires DCC, and the cellular response, filopodia formation and cell spreading, is consistent with the response of neuronal growth cones extending toward a source of netrin-1.

Proposed Functions of DCC: tumor suppressor and pro-apoptotic effector

DCC was first identified as a putative tumor suppressor deleted in some forms of colorectal cancer (Fearon *et al.*, 1990; Hedrick *et al.*, 1994). When DCC was knocked out in mice, no increase in tumor frequency was found; however the significance of this finding is limited because the mice die shortly after birth and tumors may not have had time to develop (Fazeli *et al.*, 1997). Re-expression of DCC in tumor cells can suppress tumorigenicity (Tanaka *et al.*, 1991; Klingelhutz *et al.*, 1995) and further analyses of chromosomal deletions continue to support a role for DCC as a tumor suppressor in multiple types of cancer (Hilgers *et al.*, 2000; Thiagalingam *et al.*, 1996). Correlations made between DCC expression and the phenotypes of primary tumor cells suggest that

DCC does not influence tumor formation but inhibits a later step in tumor development, such as tumor cell dissemination and metastasis (Reyes-Mugica *et al.*, 1997, 1998; Kong *et al.*, 1997; Reale *et al.*, 1996). These findings are consistent with the possibility that DCC may affect cell motility

In contrast, recent studies of DCC over-expression in HEK293T cells have suggested that DCC functions as a tumor suppressor by acting as a pro-apoptotic dependence receptor (Mehlen *et al.*, 1998). These authors suggest that in the absence of netrin-1, DCC promotes apoptosis through activation of a novel pro-apoptotic caspase-dependent pathway (Forcet *et al.*, 2001). Chen *et al.*, (1999) confirmed that over-expression of DCC induces apoptosis in several carcinoma cell lines; however, in this study netrin-1 did not rescue DCC induced cell death. Furthermore, these authors reported that in some cell lines, over-expression of DCC produced cell cycle arrest but not apoptosis. We found no evidence of cell death in differentiated, post-mitotic, netrin-1 expressing NG108-15 cells expressing high levels of DCC. However, in agreement with the earlier studies, we found that mitotic HEK293T cells began to die after approximately forty-eight hours of DCC over-expression, in spite of the expression of a small amount of netrin-1 by these cells. To minimize cell death in our studies, all assays examining the effect of DCC expression in HEK293T cells were carried out within twenty-four hours of transfection, when little or no cell death has occurred. Interestingly, when examining HEK293T cells over-expressing DCC we sometimes observed cells apparently attempting to undergo cytokinesis without rounding their cell bodies, but instead containing large amounts of F-actin and extended filopodia. This suggests that over-expression of DCC may contribute to promoting apoptosis in mitotic cells by inappropriately driving the actin cytoskeleton into a condensed state that is incompatible with cell division. Such an effect on the

cytoskeleton may contribute to DCCs proposed role as a tumor suppressor, making high levels of DCC expression compatible with post-mitotic differentiation but not with mitosis. We are currently investigating this hypothesis.

Role of Cdc42 and Rac1 in Netrin-1 Signal Transduction.

Our findings lead us to conclude that a major function of DCC is to direct the organization of actin. Cellular motility requires extensive remodeling of the cytoskeleton (reviewed by Mitchison and Cramer, 1996). Members of the Rho family of GTPases, including Cdc42, Rac1, and RhoA, play a key role in regulating the organization of actin in neuronal and non-neuronal cells (reviewed by Hall, 1998). This has been particularly well characterized in Swiss 3T3 fibroblasts where Cdc42, Rac1, and RhoA activation promotes filopodia, lamellipodia, and stress fiber formation respectively (Ridley & Hall, 1992; Ridley *et al.*, 1992; Nobes *et al.*, 1995; Kozma *et al.*, 1995). There is now strong evidence that Cdc42, Rac1, and RhoA also play key roles in regulating the motility of neuronal growth cones (reviewed by Dickson, 2001). A straightforward model has been proposed where activation of Cdc42 and Rac1 promote axon growth, while activation of RhoA inhibits growth or induces growth cone collapse (Hall, 1998; Mueller, 1999). The demonstration by Ming *et al.* (1997) that inhibiting the A kinase can switch netrin-1 mediated attraction to repulsion is consistent with this model. It has been shown that the activity of RhoA is inhibited following phosphorylation by the A kinase (Lang *et al.*, 1996). As such, A kinase activity may inhibit RhoA function in neurons and promote the attractant response to netrin-1.

Rho family members regulate motility in general; however, these proteins are good candidates to mediate the response to tropic cues that instruct a cell or growth cone to

move in a particular direction. Asymmetric activation of intracellular effectors that regulate the organization of the cytoskeleton likely underlies directional motility. Supporting this, an intracellular gradient of Rac1 activation proportional to the direction of cellular movement has been visualized in fibroblasts (Kraynov *et al.*, 2000). Furthermore, the kinase $p65^{PAK}$, a downstream effector of Cdc42 and Rac1, redistributes within the cell to regions of membrane undergoing active cytoskeletal rearrangement in fibroblasts stimulated with PDGF. Notably, the translocation of Pak1 appears to precede the recruitment of F-actin suggesting that it is an early event in the cascade directing cytoskeletal reorganization (Dharmawardhane *et al.*, 1997). These findings suggest that spatial differences in the intracellular distribution of activated Rho family members contribute to moving a cell in a particular direction.

Individual filopodia can behave independently within one neuronal growth cone (Bray & Chapman, 1986; Myers & Bastiani, 1993) and contact of a single filopodium with an appropriate target is sufficient to induce a growth cone to turn (O'Connor et al., 1990). Actin polymerization occurs at the tip of a filopodium and regulating the rate of F-actin assembly has been proposed to be the dominant factor controlling the rate of filopodial extension in neuronal growth cones (Mallavarapu & Mitchison, 1999). The presence of DCC at filopodial tips suggests that upon encountering netrin-1, activation of Cdc42 could promote filopodia formation and axon extension. Our findings are consistent with the proposal that if netrin-1 bound DCC asymmetrically across the surface of a growth cone, this could produce localized activation of Cdc42 and Rac1, change the organization of actin, and cause a growth cone to turn.

Tremendous progress has been made in identifying axon guidance cues and their receptors; however the signal transduction mechanisms underlying their mode of action

are just beginning to be elucidated (reviewed by Dickson, 2001; Schmucker & Zipursky, 2001). Here we provide evidence that netrin-1 binding to DCC reorganizes the actin cytoskeleton by activating Cdc42 and Rac1. Our findings suggest that DCC can independently activate either Cdc42 or Rac1; however the intermediate effectors of this are not known. Phosphatidylinositol-3-kinase (PI3-K) activation has been implicated as a downstream effector of netrin-1 function in neurons (Ming *et al.*, 1999) and although PI3-kinase activates Rac1 in some cell types (Hall 1998), it remains to be determined if PI3-kinase is a functional intermediate between DCC and Rac1. Further studies will be required to identify the proteins linking DCC to activation of Cdc42 and Rac1, and to identify the specific downstream effectors that reorganize actin in neuronal and non-neuronal cells that respond to netrin-1.

Figure 1. Analysis of netrin and netrin receptor expression in NG108-15 cells and HEK293T cells.

Panel (A) illustrates RT-PCR amplification of cDNA from polyA(+) RNA isolated from proliferating (lane 3) or differentiated (lane 4) NG108-15 cells. PolyA(+) RNA from newborn rat brain (lane 1) or mouse brain (lane 2) were used as positive controls. Lane 5 is a negative control containing no cDNA. Panel (B) illustrates RT-PCR carried out for HEK293T cells (lane 2). RNA from cultured human fetal astrocytes (lane 1) was used as a positive control. Reactions without cDNA were negative controls (lane 3). DNA markers (M) in panels A and B are the 100bp DNA Ladder (New England BioLabs, MA). Bright bands correspond to 0.5 and 1 kb. Panel (C) illustrates the results of western blot analysis of lysates of NG108-15 and HEK293T cells using netrin antibody PN3 (panel 1). Thirty μg of total protein was loaded per lane. Panel (2) shows Ponceau S staining of total protein on the same blot. Mol wt in kDa is indicated. Panel (D) is a schematic of the structure of the proteins encoded by the constructs used: full-length DCC, DCC-GFP, and GFP alone. (Ig: Immunoglobulin like domains; FIII: fibronectin type 3 domains).

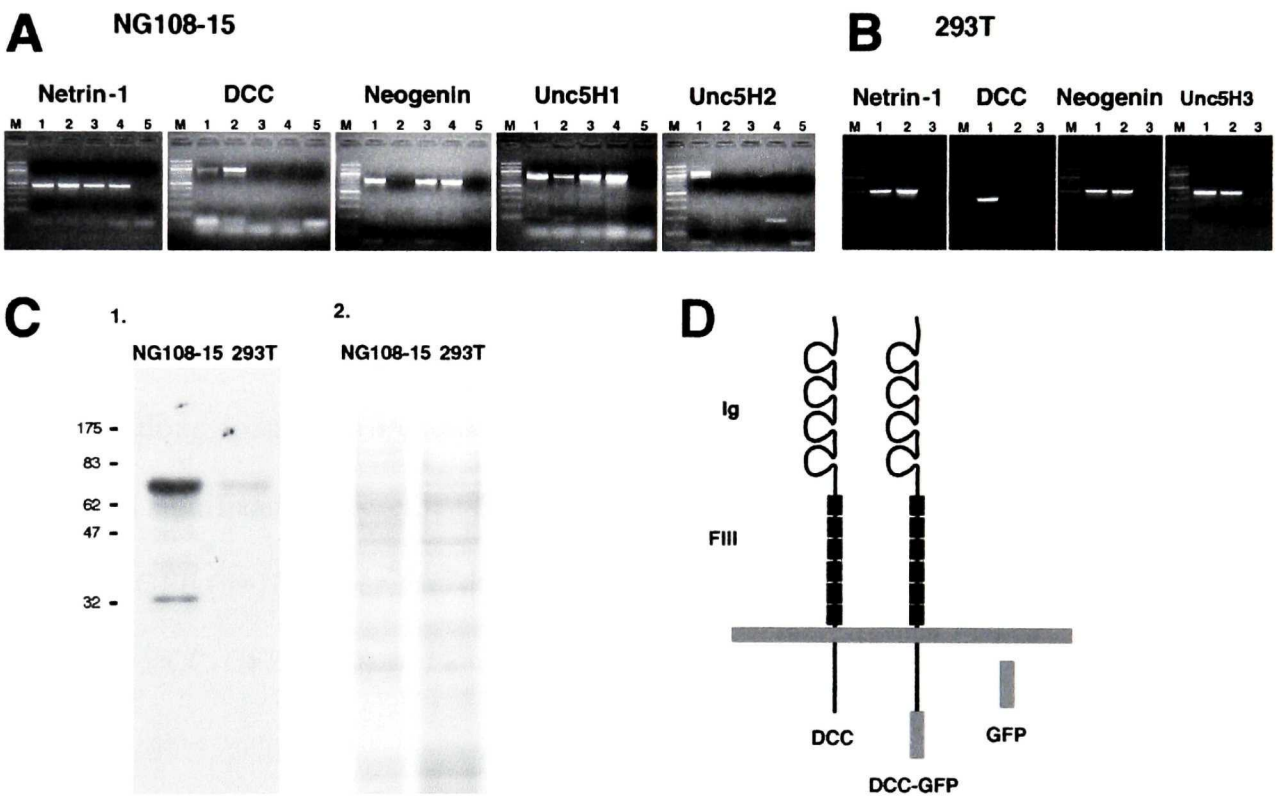
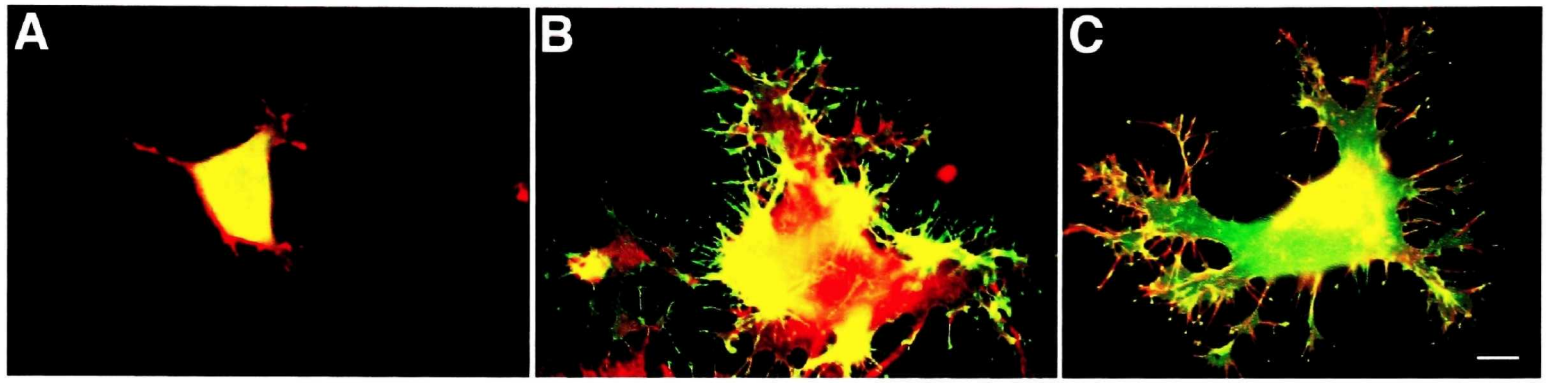

A
NG108-15
Netrin-1
DCC
Neogenin
Unc5H1
Unc5H2
M 1 2 3 4 5
B
293T
Netrin-1
DCC
Neogenin
Unc5H3
M 1 2 3
C
1.
NG108-15 293T
175
83
62
47
32
2.
NG108-15 293T
D
Ig
FIII
DCC
GFP
DCC-GFP

Figure 2. DCC increases the number of filopodia and cell surface area of NG108-15 cells.

NG108-15 cells were transfected with constructs encoding either full-length DCC, DCC-GFP, or GFP alone. Panel A illustrates the typical morphology of cells transfected with an expression construct encoding GFP alone. Panels B and C show the morphology of cells transfected with constructs encoding full-length DCC or DCC-GFP respectively. In panels A and C, the GFP-protein chimeras were visualized using the intrinsic fluorescence of GFP (green). In panel B, the distribution of full-length DCC protein was visualized using a monoclonal antibody against the C-terminal domain of DCC (G97-449) and a FITC coupled anti-mouse secondary antibody (green). Filamentous actin was visualized using rhodamine-conjugated phalloidin (red). (Scale bar = 10 μm, 100× objective lens). Expression of full length DCC or the DCC-GFP chimera significantly increased the number of filopodia per cell (panel D) and the cell surface area compared to GFP alone (panel E). Function blocking antibodies against netrin-1 (PN3) and DCC (AF5) significantly decreased the number of filopodia and cell surface area compared to DCC-GFP (n=10); error bars are SEM; * indicates $P<0.05$; GFP, n=19; DCC full length, n=20; DCC-GFP n=20).


A
B
C

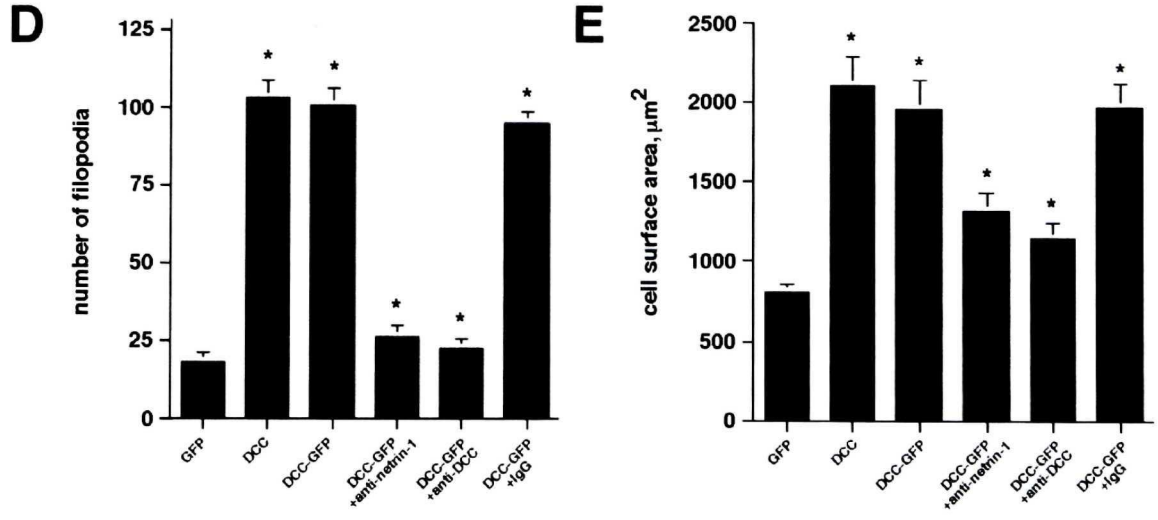
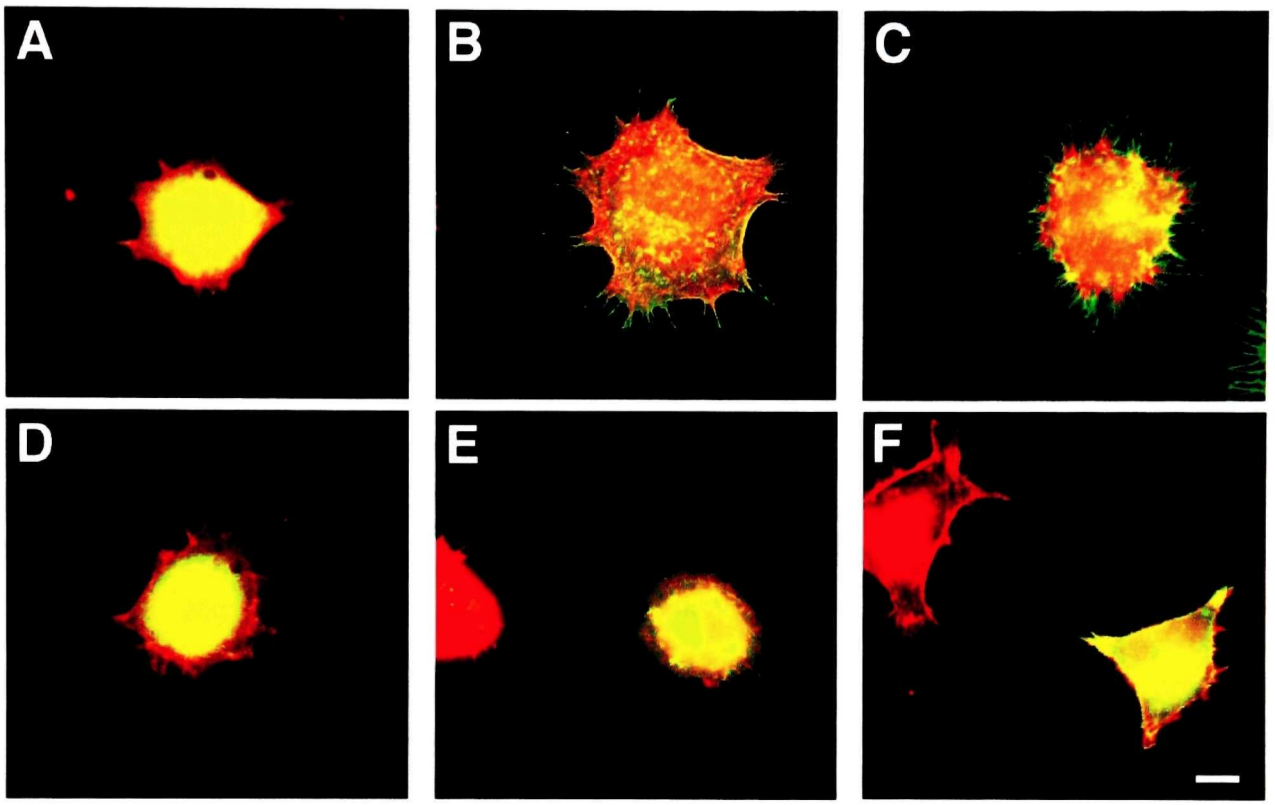
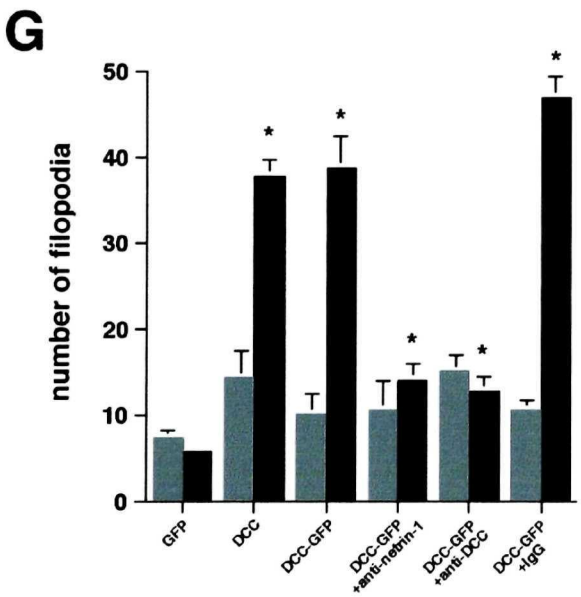

D
number of filopodia
125
100
75
50
25
0
GFP
DCC
DCC-GFP
DCC-GFP +anti-netrin-1
DCC-GFP +anti-DCC
DCC-GFP +IgG
E
cell surface area, μm2
2500
2000
1500
1000
500
0
GFP
DCC
DCC-GFP
DCC-GFP +anti-netrin-1
DCC-GFP +anti-DCC
DCC-GFP +IgG

Figure 3. DCC increases the number of filopodia and cell surface area of HEK293T cells in the presence of netrin-1.

HEK293T cells were transfected with constructs encoding either full-length DCC (B, E), DCC-GFP (C, F), or GFP (A, D) and cultured in the presence (A, B, C) or absence (D, E, F) of 160 ng/ml of purified recombinant chick netrin-1. GFP-protein chimeras were visualized using intrinsic GFP fluorescence in panels A, C, D, and F (green). Panels B and E show the distribution of full-length DCC using monoclonal anti-DCC (G97-449) and a FITC coupled anti-mouse secondary antibody (green). Filamentous actin was visualized using rhodamine-conjugated phalloidin (red). Addition of netrin-1 (G, H: black bars) in the presence of either full length DCC or DCC-GFP significantly increased the number of filopodia per cell (G) and the cell surface area (H) in the presence of netrin-1, compared to cells expressing GFP. In the absence of added netrin-1 (grey bars) expression of full length DCC or DCC-GFP did not significantly affect cell surface area (H) or the number of filopodia per cell (G) compared to cells expressing GFP. Antibodies that block the function of netrin-1 (PN3) or DCC (AF5) significantly reduced the number of filopodia and the increase in cell surface area compared to DCC-GFP. Control antibodies did not block either increase compared to the control (Scale bar = 10 μm, 100× objective lens, * indicates $P<0.05$; n=10; error bars are SEM; G and H: without netrin-1, GFP, n=19; DCC full length, n=21; DCC-GFP n=19; with netrin, GFP, n=21; DCC full length, n=20; DCC-GFP n=21).


A
B
C
D
E
F


G
number of filopodia
50
40
30
20
10
0
GFP
DCC
DCC-GFP
DCC-GFP +anti-netrin-1
DCC-GFP +anti-DCC
DCC-GFP +IgG

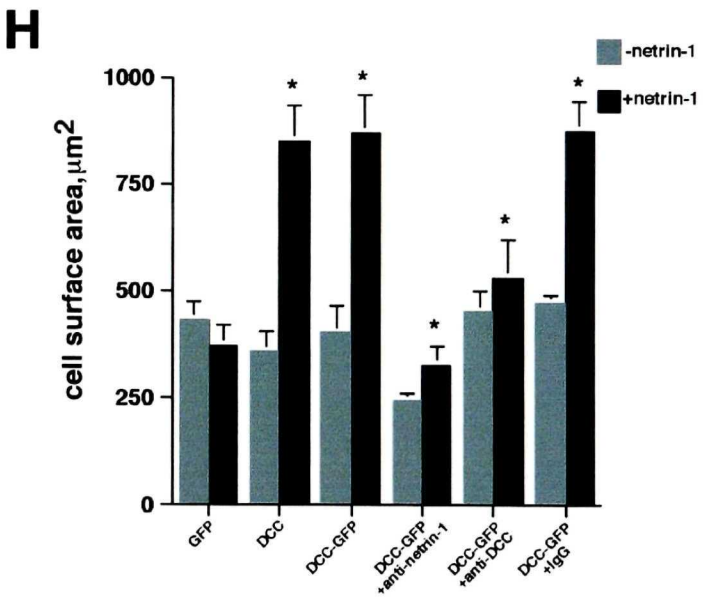

H
-netrin-1
+netrin-1
cell surface area, μm2
1000
750
500
250
0
GFP
DCC
DCC-GFP
DCC-GFP +anti-netrin-1
DCC-GFP +anti-DCC
DCC-GFP +IgG

Figure 4. DCC protein is present at the tips of filopodia in NG108-15 cells, HEK 293T cells, and rat embryonic commissural neurons.

Confocal section through the edge of a HEK293T cell (A, B, C) and the edge of an NG108-15 cell (D, E, F) transfected with DCC-GFP. GFP fluorescence (green) shows the presence of DCC-GFP protein at the distal tips of filopodia. Filamentous actin was visualized using rhodamine-coupled phalloidin (red). (A-F: confocal microscopy, 100× objective). Panels G, H, and I show the growth cone of a commissural neuron isolated from the dorsal spinal cord of an embryonic day 13 rat grown in dissociated cell culture in the presence of 160 ng/ml netrin-1. DCC protein was visualized using a monoclonal antibody against DCC (G97-449) and a secondary antibody coupled to Cy3 (H, red). F-actin was visualized using FITC coupled phalloidin (I, green) (epifluorescence microscopy, 100× objective). The arrowhead in panel G points to DCC immunoreactivity (red) at the tip of a long filopodium extending from the growth cone. The inset in panel G is an enlargement of the edge of the lamellipodium of the growth cone at the base of the filopodium identified by the arrowhead illustrating the close association between punctate DCC immunoreactivity and F-actin. (all scale bars = 10 μm)

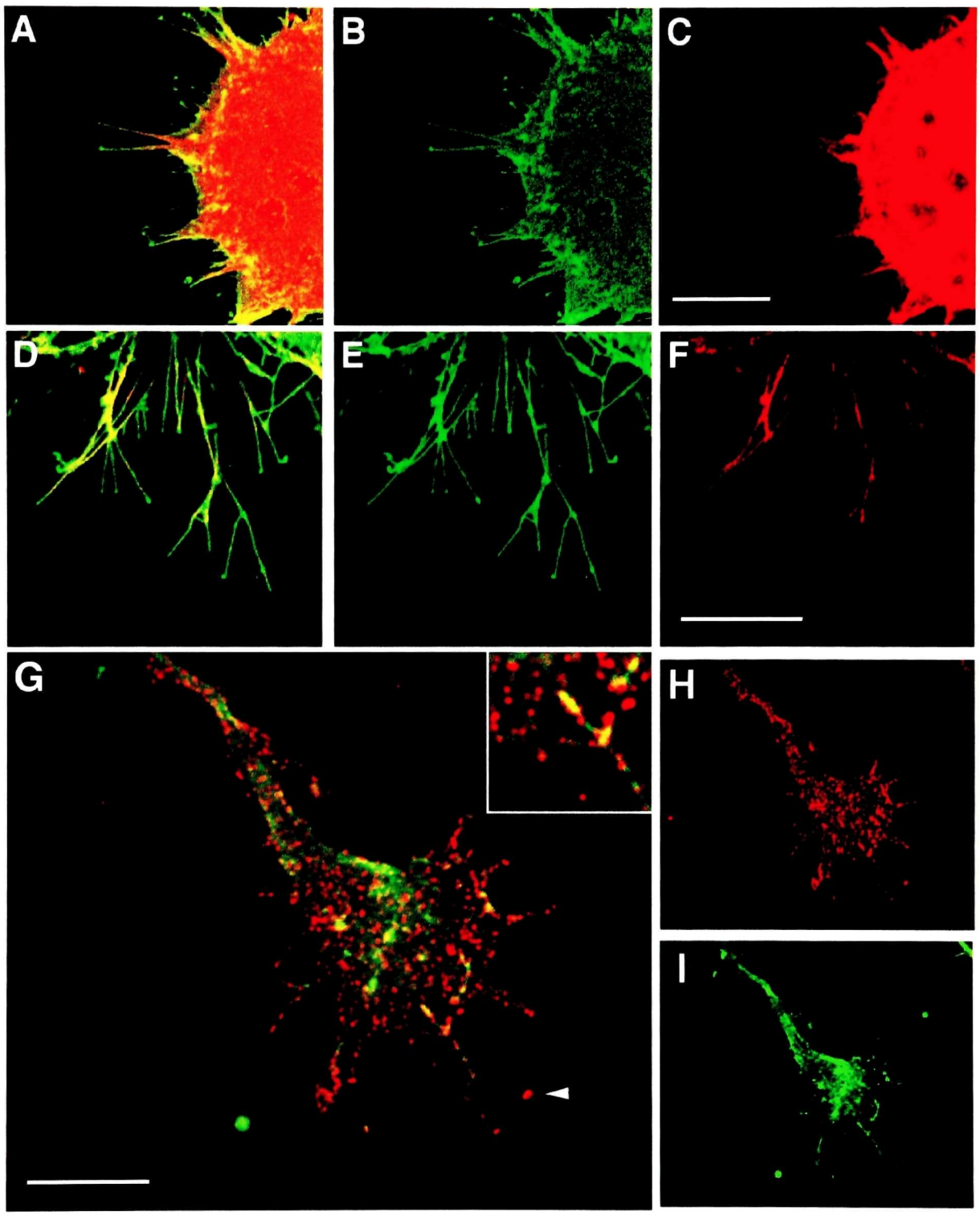

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Figure 5. Cdc42 and Rac1 are required for the DCC-induced increase in filopodia number and cell surface area, respectively.

Panels A, C, E, and G illustrate representative morphologies of NG108-15 cells co-transfected with the following expression constructs: (A) DCC-GFP and N17Cdc42, (C) GFP and N17Cdc42, (E) DCC-GFP and N17Rac1, (G) GFP and N17Rac1. Panels B, D, F, and H illustrate HEK293T cells co-transfected with the following expression constructs: (B) DCC-GFP and N17Cdc42, (D) GFP and N17Cdc42, (F) DCC-GFP and N17Rac1, and (H) GFP and N17Rac1. HEK 293T cells were cultured for 12 hours in the presence of 160 ng/ml of netrin-1 protein. DCC-GFP was visualized using the intrinsic fluorescence of GFP (green). The N17Cdc42 and N17Rac1 proteins contain a myc epitope tag that was visualized using the 9E10 antibody and an anti-mouse secondary antibody coupled to Cy3 (red). Scale bars in A and B = 10 μm.

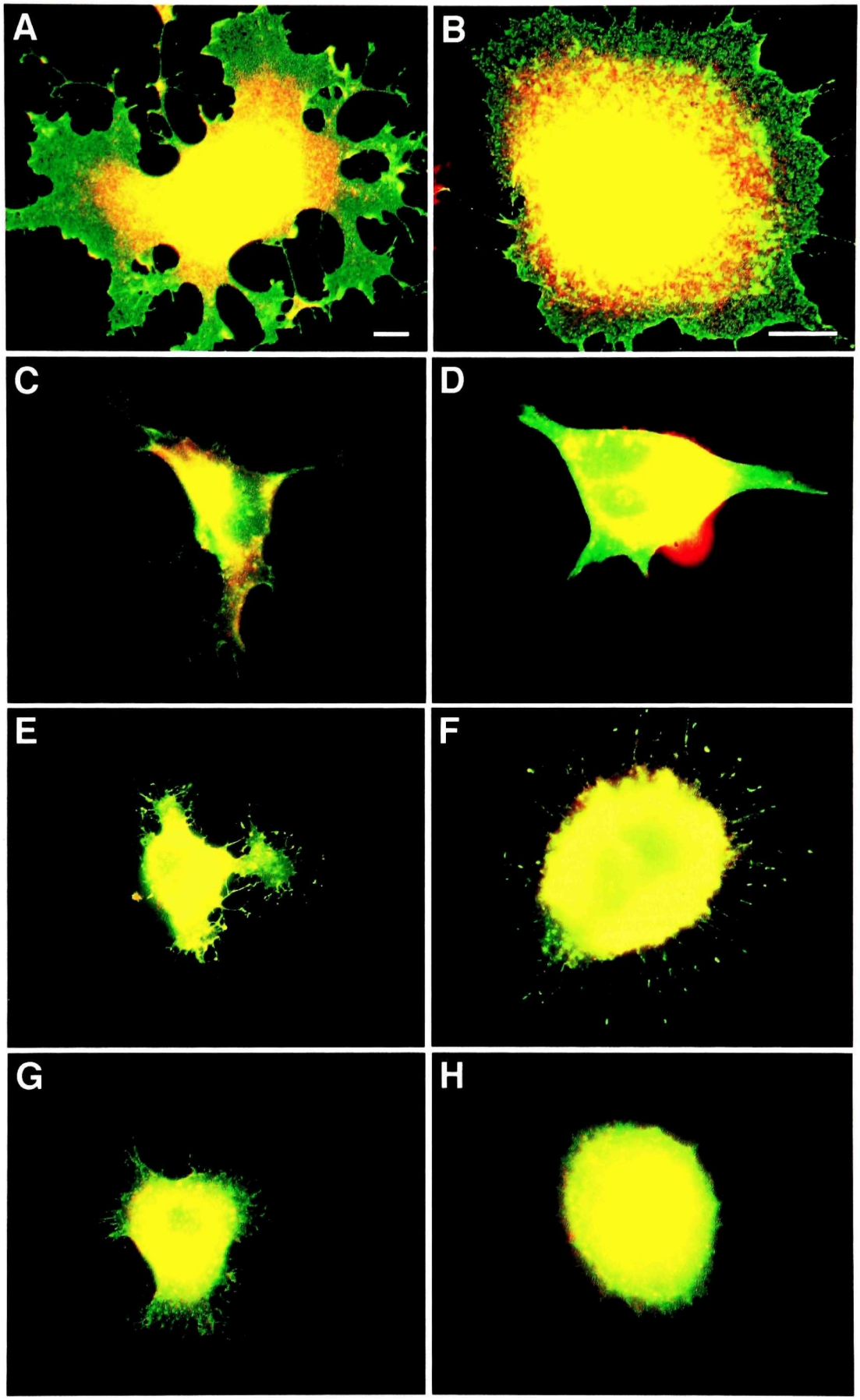
A
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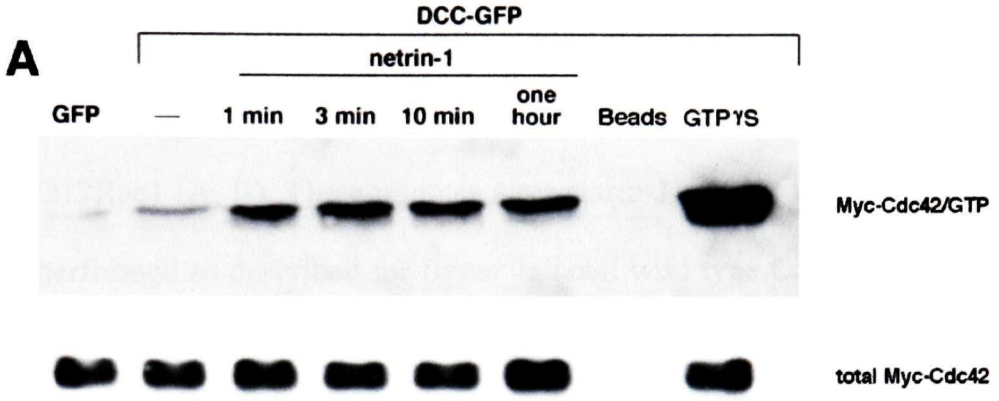
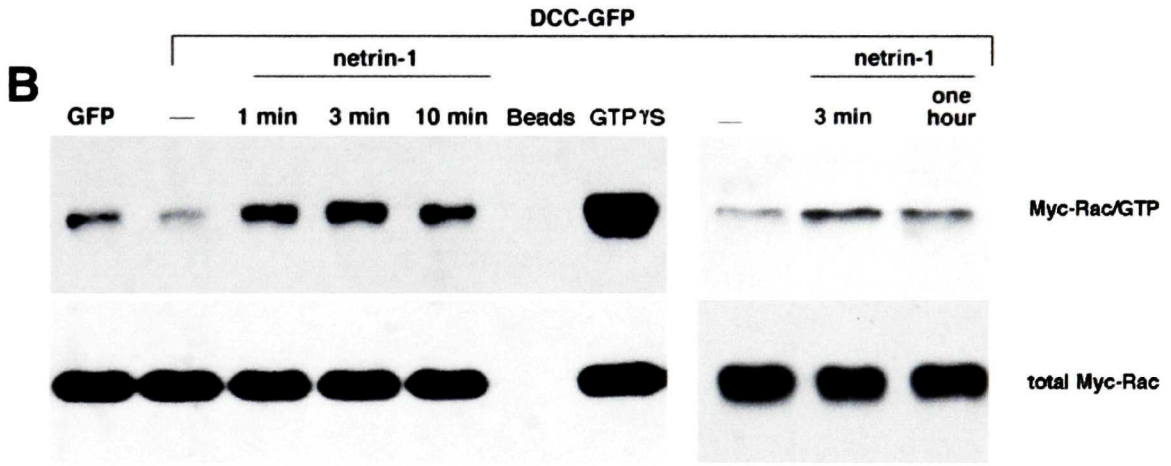
Figure 6. Cdc42 and Rac1 are required for the DCC induced increase in filopodia and cell size in NG108-15 and HEK 293 cells.

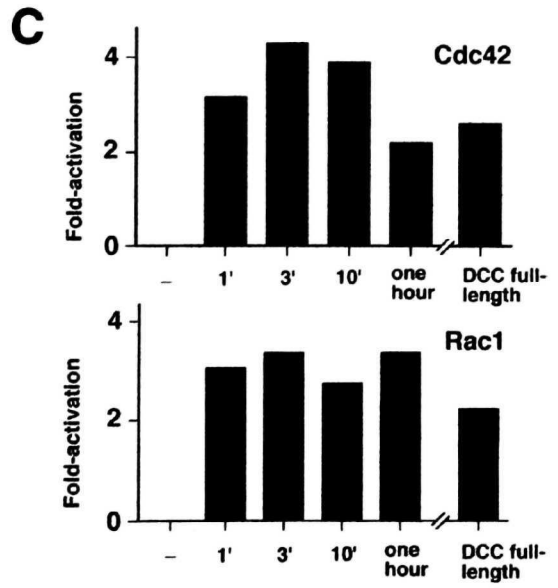
A and B show the effect of co-expression of N17Cdc42 and DCC-GFP on the number of filopodia per cell (A) and cell surface area (B). In both NG108-15 cells (black) and HEK293T cells (grey), the induced increase in filopodia number was significantly reduced by N17Cdc42 (A) but did not block the induced increase in the cell surface area (B). C and D show the effect of co-expression of N17Rac1 and DCC-GFP. The increase in cell surface area in both NG108-15 and HEK293T cells was significantly reduced by N17Rac1 (D). The significant increase in filopodia number in HEK293T cells was not affected by N17Rac1 (C); however, the increase in the number of filopodia in NG108-15 cells was significantly reduced while still remaining significantly greater than the number of filopodia in control cells. This is likely due to the effect of N17Rac on cell size. (n=10 in all cases, * indicates $P<0.05$)

Figure 7. Netrin-1 activates Cdc42 and Rac1.

HEK293T cells were co-transfected with constructs encoding DCC-GFP or GFP and G12Cdc42 (A) or G12Rac1 (B). Netrin-1 protein (160 ng/ml) was added to the cells at the indicated time points. Cell lysates were incubated with GST-Pak1-CRIB fusion protein and GTP bound Cdc42 (A) or GTP bound Rac1 (B) was isolated and analyzed by western blotting using the 9E10 monoclonal against a myc epitope tag in the recombinant proteins (upper panels of A and B). The total amount of recombinant Cdc42 or Rac1 present in cellular homogenates was also visualized using 9E10 (lower panels A and B). GFP transfection was used as a control and assayed 3 minutes after the addition of netrin-1. Non-hydrolysable GTPγS was added to lysates as a positive control. Panel C illustrates a time course of the fold change of Cdc42 and Rac1 activation following the addition of netrin-1 to cells expressing DCC-GFP. The fold change for activation induced by the DCC full-length construct at the 3 minute time point is also shown, as indicated. The table in panel D lists the ratio of GTP-bound protein compared to total protein calculated from optical densities obtained from western blot analysis. The value of n for each time point is indicated in parentheses.







D

	Cdc42	Rac1
GFP	0.056 ± 0.018 (2)	0.404± 0.158 (3)
DCC-GFP	0.334 ± 0.133 (3)	0.289± 0.08 (3)
1 min	1.063 ± 0.012 (2)	0.897 ± 0.103 (3)
3 min	1.436 ± 0.21 (3)	0.973 ± 0.084 (3)
10 min	1.305 (1)	0.783 ± 0.086 (3)
1 hour	1.153 ± 0.283 (2)	0.954 ± 0.054 (2)

± SEM is standard error of the mean

Figure 8. Independent activation of Cdc42 and Rac1 by DCC.

HEK293T cells were co-transfected with constructs encoding (A) DCC-GFP, G12Cdc42, N17Rac1, or (B) DCC-GFP, G12Rac1, N17Cdc42. The effect described in figure 7 was replicated by co-transfecting GFP or DCC-GFP, with G12Cdc42 or G12Rac1 (A, B). Three minutes after netrin-1 protein was added, activation assays were performed as described for figure 7. Total wild type Cdc42 or wild type Rac1 present in whole cell homogenates was visualized using antibodies against Cdc42 (lower panel A) or Rac1 (lower panel B). GTP-bound Cdc42 (upper panel A) or Rac1 (upper panel B) were visualized with the 9E10 antibody following pull down.

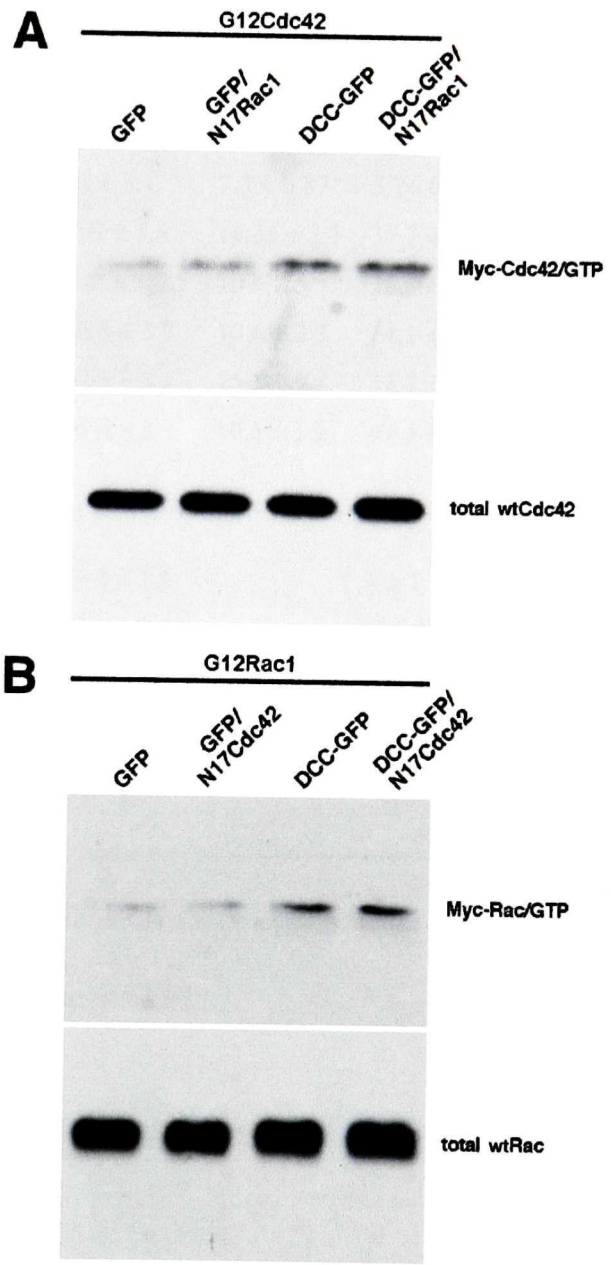

A
G12Cdc42
GFP
GFP/
N17Rac1
DCC-GFP
DCC-GFP/
N17Rac1
Myc-Cdc42/GTP
total wtCdc42
B
G12Rac1
GFP
GFP/
N17Cdc42
DCC-GFP
DCC-GFP/
N17Cdc42
Myc-Rac/GTP
total wtRac

Table I. Quantification of filopodia number and cell surface area.

Transfectants	Filopodia number			Cell surface area (μm^2)		
	NG108-15	HEK293T		NG108-15	HEK293T	
		-netrin-1	+netrin-1		-netrin-1	+netrin-1
a.						
GFP	18 ± 3.1	7.3 ± 0.87	5.7± 0.52	797 ± 56.8	428 ± 48	371 ± 47
DCC	103 ± 5.6	14.35 ± 3.1	37.7 ± 2	2104 ± 185	356 ± 48	849 ± 85
DCC-GFP	100.6 ± 5.5	10 ± 2.5	38.8 ± 3.7	1949 ± 191	399 ± 64	868 ± 90
DCC-GFP + anti-netrin-1	26.3 ± 3.7	10.6 ± 3.3	14.1 ± 1.9	1309 ± 112	239 ± 20	324 ± 46
DCC-GFP + anti-DCC	22.5 ± 3.1	15 ± 1.9	12.7 ±1.9	1134 ± 109	448 ± 51	529 ± 89
DCC-GFP + control IgG	95 ± 3.8	10.5 ± 1.2	47 ± 2.6	1957 ± 152	470 ± 22.5	873 ± 70
b.						
GFP, N17Cdc42	14.6 ± 1.8		4 ± 1	661 ± 102		463.5 ± 48
DCC-GFP, N17Cdc42	21.1 ± 1.56		14 ± 1.88	2748 ± 191		1021 ± 73
GFP, N17Rac1	20.2 ± 3.1		5.7 ± 1.3	657 ± 208		362 ± 24
DCC-GFP, N17Rac1	42 ± 5.6		33 ± 2.6	807 ± 75		434 ± 38

Note: NG108-15 and HEK293T cells were transfected with different constructs and analysed as described. Mean ± SEM.

Chapter 3

Netrin-1 Causes Growth Cone Expansion in Rat Embryonic Spinal Commissural Neurons via Activation of Cdc42, Rac1, and Pak1

Summary

Filopodia and lamellipodia are actin based membrane protrusions at the leading edge of motile cells and neuronal growth cones. Netrin-1 is a secreted protein that guides cell and axon migration during neural development. In the embryonic spinal cord, netrin-1 protein is produced by the floor plate and guides the growth cones of commissural neurons toward the ventral midline. Deleted in colorectal cancer (DCC) is a netrin-1 receptor that is required for commissural neurons to extend toward the floor plate. Extracellular cues influence growth cone shape and motility by influencing the organization of filamentous actin, the major cytoskeletal component of filopodia and lamellipodia. To investigate the mechanisms underlying the action of netrin-1 on neuronal growth cones, we have developed an assay utilizing cultures of dissociated embryonic rat spinal commissural neurons isolated by dissociating micro-dissected embryonic rat dorsal spinal cord. Netrin-1 induces an increase in the surface area and filopodia number of the growth cones of embryonic rat spinal commissural neurons. Expression of dominant negative forms of Cdc42 and Rac1 indicated that both Cdc42 and Rac1 are required for the effect of netrin-1 on commissural neuron growth cones. Furthermore, we show that

Cdc42, Rac1, and Pak1 are activated by netrin-1 in these neurons, and that activation requires DCC and PI3K.

Introduction

In the developing embryonic spinal cord, commissural neurons extend axons across the ventral midline (Tessier-Lavigne & Goodman, 1996). Netrins are a family of secreted proteins that guide migrating axons and cells during neural development (reviewed by Culotti & Merz, 1998; Kennedy, 2000). Netrin-1 is a bi-functional guidance protein (attractant and repellent) that is required for commissural neurons to extend to the ventral midline (Serafini *et al.*, 1994; Kennedy *et al.*, 1994). The receptors for netrin-1 in vertebrates include deleted in colorectal cancer (DCC), neogenin, and UNC-5 homologs. DCC is a single-pass trans-membrane receptor of the immunoglobulin (Ig) superfamily that is highly expressed in commissural neurons and required for the axons to be attracted to a source of netrin-1 (Keino-Masu *et al.*, 1996). The repellent response to netrin-1 requires either an UNC-5 homolog or a complex of an UNC-5 homolog with DCC (Ackerman *et al.*, 1997; Hong *et al.*, 1999; Leonardo *et al.*, 1997; reviewed by Song & Poo, 2001).

The leading edge of an advancing growth cone extends filopodia and lamellipodia protrusions to probe and sense the complex extracellular environment. This in turn affects the growth cone dynamics, shape, and motility (reviewed by Bentley & O'Connor, 1994; Tanaka & Sabry, 1995). A dense network of actin filaments and bundles form the main structure of lamellipodia and filopodia, and are in continuous assembly and disassembly in response to extracellular cues (Mueller, 1999). The regulation of F-actin assembly at the tips of filopodia controls filopodia extensions and retractions in growth cones (Mallavarapu & Mitchison, 1999). Asymmetric retraction of filopodia across growth cones has also been implicated in repulsive turning responses made by growth cones toward LPA (lysophosphatidic acid) (Yuan *et al.*, 2003). The presence of DCC at the tips

of filopodia and the edge of lamellipodia of commissural neurons (Shekarabi & Kennedy, 2002) supports the conclusion that DCC plays an important role in transducing the response to extracellular netrin-1.

The organization of the actin cytoskeleton is regulated by Rho GTPases that act as molecular switches by cycling between active and inactive forms (reviewed by Hall, 1998; Schmidt & Hall, 1998). Based on studies in fibroblasts, RhoA has been implicated in the formation of stress fibers, Rac1 in formation of lamellipodia, and Cdc42 in formation of filopodia (reviewed by Ridley, 2001; Luo, 2000; Mueller, 1999). The role of GTPases in growth cone dynamics and axon outgrowth has also been studied (reviewed by Dichson, 2001). Rho GTPase activity is tightly regulated and they regulate a variety of downstream effectors (reviewed by Bishop & Hall, 2000; Van Aelst & D'Souza-Schorey, 1997).

We have previously shown that the activation of two small GTPases, Cdc42 and Rac1, by netrin-1 in DCC transfected cell lines causes filopodia formation and cell spreading (Shekarabi & Kennedy, 2002). Here, we show that in embryonic rat spinal commissural neurons netrin-1 activates Cdc42 and Rac1 and that this requires DCC and PI3 kinase. The activation of Cdc42 and Rac1 by netrin-1 in these neurons triggers growth cone expansion. Furthermore, we demonstrate that Pak1, a downstream effector of Cdc42 and Rac1, is activated by netrin-1 in these neurons. These results show that netrin-1 acts through DCC to direct the reorganization of F-actin, leading to extension of the edge of the growth cone of embryonic spinal commissural neurons.

Material and Methods

Cell Culture and Explant Assays

Dorsal halves of the spinal cord of E13 (E0 = vaginal plug) rat embryos were microdissected (Fig. 1A), dissociated, and cultured as described (Shekarabi & Kennedy, 2002). For protein biochemistry, dissociated neurons were cultured in 35 mm tissue culture plates coated with poly-D-lysine (Sigma, St. Louis, MO, 20 μg/ml) at 37 °C for 2 hours in HANK's-buffered salt solution (HBSS, Invitrogen, Canada) at a density of 4×10^6 cells per plate. For immunostaining, the neurons were plated on 13 mm diameter glass coverslips (Carolina Biological Supply, Burlington, NC) at a density of 7×10^3 cells per coverslip. Coverslips had been coated with poly-D-lysine (20 μg/ml) at 4°C overnight or first poly-D-lysine followed by 10 μg/ml netrin-1 protein at 37°C overnight. Thirty-six hours after plating the cells, the cultures were washed, changed to B27-free Neurobasal (Invitrogen, CA), and incubated for another six hours prior to stimulation with 80 ng/ml purified netrin-1 protein. Cells were then either lysed and the homogenate assayed, or fixed for immunostaining as described (Shekarabi & Kennedy, 2002). For explant axon outgrowth assays, dorsal spinal cord explants were dissected from E13 rat embryos (Serafini *et al.*, 1994) and cultured in three-dimensional collagen gels as described (Tessier-Lavigne *et al.*, 1988) with 3-4 explants in each gel. Explants were cultured at 37°C for 16 hrs in Neurobasal containing, 10% IFBS, 2mM Glutamine, 1 unit/ml penicillin, and 1 μg/ml streptomycin. When added, netrin-1, LY 294002 (50 μM, Sigma) or Wortmanin (1 μM, Calbiochem) were present for the duration of the experiment.

Other Reagents

The following reagents were also used: affinity purified rabbit polyclonal netrin antibody PN3 (25 μg/ml, Metin *et al.*, 1997); purified non-immune rabbit IgG (25 μg/ml, Invitrogen); function blocking DCC monoclonal antibody, anti-DCC_{FB} (AF5, Oncogene Sci., MA); a polyclonal anti-Pak1 antibody (NEB); 1: 25 of anti-NeuN (provided by R. Mullen, U. Utah); anti-phospho Ser-198 and 203-specific Pak1 (provided by M. Greenberg, Harvard university, MA); anti-Akt and phospho Ser-473–specific Akt antibodies (provided by D. R. Kaplan, University of Toronto, ON). Expression constructs encoding GST fusion proteins of wild type Cdc42 and Rac1 were obtained from Dr. G. Bokoch (The Scripps Institute, CA) (Bagrodia *et al.*, 1995). Dominant negative (N17) and constitutive active (V12) forms of Cdc42 and Rac1 expressing adenoviruses were provided by Dr. J. Bamburg (Colorado State University, CO). Adenovirus espressing Δcof N-WASP or GFP was made as described in He *et al.* (1998).

Immunocytochemistry, Actin Staining, and Quantification

Filamentous actin was visualized using fluorescein-conjucated (FITC) phalloidin (Sigma). Number of filopodia and growth cone surface area were quantified as described (Shekarabi & Kennedy, 2002). For quantification of axon outgrowth from explants, photomicrographs were taken using a Carl Zeiss Axiovert microsope, phase-contrast optics, a 20× objective lens, Magnafire CCD camera (Optronics, CA), and analyzed using Northern Eclipse image analysis software (Empix Imaging Inc, Canada). The total length of all axon bundles emerging from each explant was measured as described (Serafini *et al.*, 1994) and expressed as mean ± SEM. The statistical significance of differences

between means was evaluated by a one-way analysis of variance with Sheffe post-hoc test (Systat, SPSS, Il).

Co-Immunoprecipitation

For co-immunoprecipitation studies, cultured commissural neurons were treated with 80 ng/ml netrin-1 protein for the time indicated and then lysed with NP40 lysis buffer (25 mM HEPES pH=7.5, 300 mM NaCl, 1% NP40, 0.25 % sodium deoxycholate, 1mM sodium orthovanadate, 1mM EDTA). The lysate was cleared by centrifugation at 15.000× g for 12 min at 4°C. Supernatants were collected and used for immunoprecipitation analyses. One µg of monoclonal anti-DCC_{IN}, raised against the DCC-ICD, was added to 600 µl of lysate (G97-449, PharMingen, Canada). For Pak1 immunoprecipitation, 2 µg polyclonal anti-Pak1 (NEB) was used. Immunocomplexes were isolated using protein G Sepharose (Sigma), washed with the lysis buffer, and resolved by SDS-PAGE followed by western blot analysis.

GTPγS Loading Assay

GTPγS loading assays were performed as described (Knaus *et al.*, 1992). Neurons cultured in 35 mm plates were treated with purified 80 ng/ml netrin-1 protein for 3 minutes and lysed in 25 mM HEPES pH=7.5, 150 mM NaCl, 1% NP40, 0.25% DOC, 5% glycerol, 25 mM NaF, 10mM $MgCl_2$, 1mM EDTA, 1mM SOV, 2 µg/ml leupeptin, 2 µg/ml aprotinin, 1 µg/ml pepstatin, and 2 mM PMSF). Inhibitors of PI3K were added to the cultures 45 min before netrin-1. Lysates were then incubated with 100 µM GTPγS in the presence of 10 mM EDTA for 12 minutes at 31°C. Activated Cdc42 and Rac1 were

pulled down with 20 µl of glutathione-coupled Sepharose 4B beads (Pharmacia, Piscataway, NJ) that had been loaded with 10 µg of bacterially expressed GST-Pak1-CRIB domain fusion protein (amino acids 56-272, Sander *et al.*, 1998). The components of the protein complex were then resolved by SDS-PAGE and western blot analysis carried out using monoclonal anti-Rac1 (Transduction Laboratories, Lexington, KY) or polyclonal anti-Cdc42 (Santa Cruz Biotechnology, Santa Cruz, CA). Signals were detected using ECL (MEN Life Science Products, Boston, MA). Densitometry and quantification were carried out using NIH Image software (United States National Institutes of Health).

GST-Cdc42 and -Rac1 Pull-Down Assay

GST-Cdc42 and GST-Rac1 fusion proteins were expressed in bacteria and isolated as described (Sander *et al.*, 1998). Cultured commissural neurons were treated with 80 ng/ml netrin-1 protein for the times indicated. Cell lysates were then incubated with GTPγS, 10 mM EDTA, and either GST-Cdc42 or GST-Rac1 fusion proteins at 31°C for 12 minutes. The complex of bound proteins was then isolated using glutathione-coupled Sepharose 4B beads as described above. The pellet was washed with lysis buffer, the bound proteins resolved by SDS-PAGE, and then western blot analysis carried out using anti-DCC_{IN} and anti-phospho-Pak1. Immunoreactivity was detected using ECL.

Results

Netrin-1 Causes DCC Dependent Expansion of Commissural Neuron Growth Cones.

In the embryonic spinal cord, commissural neurons express DCC as they extend an axon towards the floor plate at the ventral midline (Keino-Masu *et al.*, 1996). To investigate the morphological and biochemical response of these neurons to netrin-1, we developed a method of culturing these neurons *in vitro*. This involved microdissecting the dorsal half of E13 embryonic rat spinal cords that contain the commissural neuron cell bodies, dissociating this tissue, and culturing the cells (Fig. 1A and 1C). Immunostaining for NeuN, a neuronal marker, indicated that greater than 90% of the cells in these cultures are neurons (data not shown). Furthermore, these neurons express TAG-1 and DCC, both markers of embryonic spinal commissural neurons *in vivo* (Dodd *et al.*, 1988; Keino-Masu *et al.*, 1996) (Fig. 1B). These results support the conclusion that these cultures are highly enriched with cells having the properties of embryonic spinal commissural neurons.

Growth cone turning involves actin dependent membrane extension on one side of the growth cone, coordinated with partial withdrawal on the other side (reviewed by Mueller, 1999). Growth cone collapse has been used as an assay to study mechanisms underlying the action of repellent guidance cues (reviewed by Castellani & Rougon, 2002). Here we report that addition of recombinant netrin-1 protein to dissociated commissural neurons *in vitro* causes growth cone expansion (Fig. 2A–2C). Morphometric analysis of the growth cones of commissural neurons indicates that netrin-1 induces an increase in the number of filopodia and the growth cone surface area (Fig. 3A and 3C).

Evidence That Netrin-1 Acts as an Adhesive Substrate Bound Cue

We have previously reported that the majority of netrin-1 protein is not freely soluble *in vivo*, but is bound to cell surfaces or extracellular matrix (Manitt *et al.*, 2001, Manitt and Kennedy, 2002). This suggests that netrin-1 may act as a component of the extracellular matrix and mediate an adhesive function. Consistent with this, we found that growth cone expansion was induced both when soluble recombinant netrin-1 was added to the culture media (80 ng/ml) or when the coverslips were pre-coated with a netrin-1 containing solution (5 µg/ml), which was then removed before plating the neurons (Fig. 2D; Fig. 3B and 3D). A DCC function blocking monoclonal antibody (DCC_{FB}) blocked the effect of both soluble or substrate bound netrin-1, indicating that DCC is required for the expansion of commissural neuron growth cones in response to netrin-1 (Fig. 3A – 3D). Interestingly, addition of netrin-1, either soluble or substrate-bound, did not significantly affect axon outgrowth length from these cells (poly K= 40.15± 3.99; poly K + netrin= 44.48± 2.9; poly K - netrin= 42.37± 3.85; poly K - netrin+ anti-netrin = 39.98± 3.5; poly K – netrin + anti-DCC_{FB} = 33.14± 2.84 (+ soluble netrin-1, - netrin-1 substrate, and poly K indicates poly-D-lysine). This suggests that netrin-1 may influence directional choices, affecting the morphology of the growth cone, without having a dramatic effect on the rate of axon outgrowth.

Netrin-1 and DCC Dependent Growth Cone Expansion Requires Rac1 and Cdc42

Multiple signal transduction pathways lead from the cell surface to the actin cytoskeleton in different cell types. Previously, we reported that netrin-1 causes cell spreading and filopodia formation in cell lines transfected to express DCC (Shekarabi & Kennedy, 2002). These findings included the demonstration that netrin-1 independently activated Cdc42 and Rac1 in HEK293T cells and NG108-15 neuroblastoma-glioma cells.

Here, we directly assess the role of Cdc42 and Rac1 in the morphological changes induced by netrin-1 in the growth cones of embryonic rat commissural neurons. Dissociated neurons were infected with adenoviruses encoding either dominant negative or constitutively active forms of Cdc42 and Rac1. Infection with adenoviruses expressing GFP served as control. The virally encoded Cdc42, Rac1, and GFP were all myc epitope tagged. Forty-eight hours post-infection the neurons were treated with netrin-1, fixed, and then immunostained with the 9E10 monoclonal antibody against the myc epitope, and stained with FITC coupled phalloidin to visualize F-actin. Quantification of growth cone surface area and filopodia number revealed that expression of dominant negative Cdc42 (N17Cdc42), but not GFP alone, produced a significant decrease in the ability of netrin-1 to induce growth cone filopodia formation (Fig. 3E). Growth cone surface area was also decreased by N17Cdc42 in neurons exposed to netrin-1, suggesting that Cdc42 activation contributes to growth cone spreading. This provided a morphological indication suggesting that Cdc42 may be upstream of netrin-1-induced Rac1 activation in embryonic rat commissural neurons (Fig. 3F). Expression of dominant negative Rac1 (N17Rac1) completely blocked the netrin-1-induced increase in growth cone surface area (Fig. 3F). This also caused a decrease in the number of filopodia, although this was still higher than control neurons (Fig. 3E). The reduced number of filopodia in the presence of dominant negative Rac1 may be secondary to the decrease in the overall size of the growth cone, and does not necessarily indicate that Rac1 plays a role in activating Cdc42 in these cells. However, expression of either constitutively active Cdc42 (CACdc42) or constitutively active Rac1 (CARac1) is sufficient to increase both growth cone surface area and filopodia number in these neurons (Fig. 3E and 3F). These findings suggest that while

Cdc42 may be functionally upstream of Rac1 in commissural neurons, over-expressed CARac1 may be able to activate Cdc42 in these cells.

Activation of Cdc42 and Rac1 by Netrin-1 in Rat Embryonic Spinal Commissural Neurons

A technical challenge we encountered when examining the activation of Cdc42 and Rac1 was the relatively small number of commissural neurons obtained following micro-dissection and the low level of endogenous Cdc42 and Rac1 in these cells. This limitation was overcome by adapting a GTPγS loading assay. Rho-GTPases are activated by proteins called GEFs (guanosine exchange factors) that catalyze the exchange of GDP for GTP. GTPγS binds irreversibly to Rho-GTPases and is not hydrolyzed, trapping them in an activated state. By incubating GPTγS in lysates of commissural neurons and isolating GTPγS bound endogenous Cdc42 and Rac1 using the GST-Pak1-CRIB fusion protein, it was possible to visualize and quantify RhoGTPase activation following treatment with netrin-1. The results of this assay indicate that application of netrin-1 rapidly activates both Cdc42 and Rac1 in embryonic commissural neurons (Fig. 4A, 4F, and 4G). Notably, because the assay measures the accumulation of GTPγS bound Cdc42 or Rac1, this finding suggests that application of netrin-1 leads to the activation of a GEF for these GTPases. Application of anti-DCC_{FB} in combination with netrin-1 demonstrated that activation of Cdc42 and Rac1 requires DCC (Fig. 4B, 4F, and 4G). Interestingly, adenoviral mediated expression of N17Cdc42 or N17Rac1 provided evidence that netrin-1 mediated activation of Cdc42 is independent of Rac1, while activation of Rac1 appears to be dependent on Cdc42 (Fig. 4C). This is consistent with the morphological findings

described above (Fig. 3). Together, these findings suggest that Cdc42 activation is functionally upstream of Rac1 activation in embryonic rat spinal commissural neurons (Fig. 4C).

Activation of Cdc42 and Rac1 by Netrin-1 in Rat Embryonic Spinal Commissural Neurons Requires PI3 Kinase

PI3 kinase (PI3K) plays multiple important roles during neural development (reviewed by Rodgers & Theibert, 2002). Of particular relevance here, PI3K regulates Rho GTPase activation (reviewed by Fruman *et al.*, 1998), and directly regulates certain GEFs (Innocenti *et al.*, 2003; Fukuda *et al.*, 2002). Application of membrane permeable inhibitors of PI3K, 50 μM LY294002 and 1 μM Wortmannin, to cultured commissural neurons indicated that PI3K is required for netrin-1 to activate Rac1 and Cdc42 (Fig. 4D). The effectiveness of PI3K inhibition was monitored by western blot analysis of Akt phosphorylation, a downstream target of PI3K (Fig. 4D). Anti-phospho-Akt and western blot analysis of commissural neuron homogenates indicated that netrin-1 did not affect phosphorylation of Akt at early time points, less than 30 min, but that at later time points a small increase in phospho-Akt was detected (Fig. 4E). These findings suggest that activation of PI3K is not required for the activation of Cdc42 and Rac1, but that PI3K activation may contribute to the response to netrin-1 at later time points.

To investigate the functional consequence of inhibiting PI3K, axon outgrowth assays were performed using dissected explants of dorsal E13 embryonic spinal cords cultured in a collagen gel. These experiments have shown that the PI3 kinase inhibitors caused a decrease in the total axon bundle length of the explants treated with both inhibitors and netrin-1 (Fig. 5A and 5B). The decrease in the total axon bundle length was

dependent on the netrin-1 concentration. The reduction was significant in concentrations over 100 ng/ml of netrin-1 protein (Fig. 5A). Cells in the explants continue to appear healthy and phase bright following treatment, suggesting that the PI3K inhibitors are not triggering apoptosis at the time points examined. Furthermore, no increase in TUNEL staining of similarly treated dissociated commissural neurons was observed following application of the PI3K inhibitors (data not shown).

Netrin-1 Activates Pak1, a Downstream Effector of Rac1 and Cdc42

Having found that netrin-1 activates Cdc42 and Rac1, we then examined if downstream effectors of these proteins might also be activated in commissural neurons. Activation of the serine/threonine kinase Pak1, an effector for both Cdc42 and Rac1, can be assessed by monitoring serine-198 and -203 phosphorylations of Pak1. Using an antibody against phospho Ser-198 and 203-specific Pak1 (Shamah *et. al.*, 2001; Sells *et. al.*, 2000), we investigated the possibility that netrin-1 might activate Pak1 in commissural neurons. By staining dissociated commissural neurons, an increase in phospho-Pak1 (pPak1) was detected immunocytochemically in growth cones within 5 min of adding netrin-1 (Fig. 6A–6F). The relative levels of pPak1 in lysates of these cells were then detected on western blots. Application of netrin-1 induced activation of Pak1 within 5 min of addition of netrin-1 protein to the cultures. The level of pPak1 remained elevated for at least one hour, but had decreased to the level of control within 2 hours. Interestingly, coincident application of netrin-1 and either the DCC function blocking antibody, LY294002, or Wortmanin, demonstrated that netrin-1 induced activation of Pak1 requires DCC and PI3K (Fig. 7A). The phosphorylation of Pak1 observed here, implicates the involvement of a well-established pathway through which activation of

Cdc42 and Rac1 by netrin-1 could promote the polymerization of actin and influence growth cone motility.

Pak1 binds Nck1, an adaptor protein containing two SH3 domains that bind constitutively to proline rich sequences in the DCC-ICD (Li *et al.*, 2002). Expression of dominant negative Nck1 inhibited DCC induced extension of neurite-like processes from N1E-115 neuroblastoma cells and blocked DCC dependent activation of Rac1 by netrin-1 in fibroblasts, suggesting that Nck1 is required for DCC signal transduction (Li *et al.*, 2002). To determine if an interaction between Pak1 and Nck1 might contribute to the netrin-1-induced increase in commissural neuron growth cone surface area and filopodia number, we used a cell permeable peptide that acts as a dominant negative inhibitor of Pak1 binding to Nck1. The Pak-peptide consists of 13 amino acids that correspond to the first proline-rich domain of Pak1 fused to the polybasic sequence of the HIV Tat protein, which facilitates peptide entry into cells. Application of this peptide inhibits endothelial cell migration and angiogenesis (Kiosses *et al.*, 2002). A corresponding control peptide, mutated at two prolines required for binding to the Nck1 SH3 domain, had no effect on angiogenesis and cell migration.

Dissociated embryonic rat commissural neurons were cultured as described above. The peptides, Pak-peptide or control (20 μg/ml), were added to the cultures 45 min before the addition of netrin-1 (80 ng/ml). Addition of Pak1 peptide to primary cultures of dissociated commissural neurons blocked the netrin-1-induced increase in the growth cone surface area and filopodia number while application of the control peptide did not (Fig. 7B and 7C). These findings support the conclusion that an interaction between Pak1 and Nck1 is required for netrin-1-induced expansion of the growth cones of embryonic rat spinal commissural neurons.

Netrin-1 Induces Formation of a Complex of DCC, Pak1, Cdc42, and Rac1.

Activated Cdc42 and Rac1 bind directly to Pak1 to regulate its activation (Bagrodia & Cerione, 1999). To determine if netrin-1 promotes an interaction between activated Cdc42 and Rac1 with Pak1, we again used the GTPγS loading assay described above. Purified GST-Cdc42 or GST-Rac1 fusion proteins were added to lysates of dissociated commissural neurons in the presence of GTPγS and interacting proteins isolated and identified by western blotting. The results obtained show that addition of netrin-1 protein to commissural neurons promotes the binding of activated Cdc42 and Rac1 to pPak1 and to DCC (Fig. 8A). Increased binding to DCC and pPak1 were observed in homogenates derived from cells that had been treated with netrin-1 for 30 min. These findings suggest that application of netrin-1 causes a DCC dependent activation of Cdc42, Rac1 and Pak1 in spinal commissural neurons and promotes the formation of a complex that includes DCC, Nck1, Cdc42, Rac1, and Pak1.

We then tested this hypothesis by attempting to co-immunoprecipitate (co-IP) members of this complex with endogenous DCC. It was possible to IP a complex containing DCC and Pak1 from lysates of the dissociated commissural neurons cultured with netrin-1 protein. Pak1 was detected following IP using an antibody against DCC (Fig. 8B). Conversely, DCC and Nck1 were detected using an antibody to IP Pak1 (Fig. 8C) Increased amounts of DCC and Pak1 were co-immunoprecipiated following application of netrin-1, indicating that netrin-1 promotes the formation of a complex that includes Pak1 and DCC. Nck1 has been previously shown to bind constitutively to the DCC-ICD via its first and third SH3 domains (Li *et al.*, 2001). Pak1 binds to the second SH3 domain of Nck1 (Hing *et al.*, 1999). Together, these findings support a model in

which Nck1 is bound to proline rich regions in DCC via its 1st and 3rd SH3 domains, leaving the 2nd SH3 domain of Nck1 able to bind to Pak1. Interestingly, following co-IP using anti-Pak1, netrin-1 substantially reduces the amount of Nck1 in the immunoprecipitate (Fig. 8C). This is consistent with the majority of Nck1 and Pak1 being present as a constitutive complex in the cytoplasm (Lu *et al.*, 1997). Addition of netrin-1 to the cells disrupts the cytoplasmic complex, while increasing the relative amount of Nck1 interacting with DCC on the plasma membrane.

A Possible Role for βPIX as a GEF Downstream of Netrin-1

PIX are a family of Dbl homology domain containing proteins that have GEF activity for both Cdc42 and Rac1 (Manser *et al.*, 1998). β1PIX and β2PIX are encoded by two genes and multiple variants are produced by alternative mRNA splicing (Manser *et al.*, 1998). Both β1PIX and β2PIX are expressed in brain; however β2PIX is more abundant in brain tissue (Koh *et al.*, 2001). βPIX immunoreactivity, ~78 kDa, was isolated bound to GST-Rac1 from a lysate of dissociated commissural neurons (Fig. 8D). This finding indicates that βPIX is expressed by these neurons and raises the possibility that βPIX may function as a GEF, activating Cdc42 and possibly Rac1 downstream of DCC. Further studies will be required to characterize the role of βPIX in commissural neurons in response to netrin-1.

N-WASP Is Required for Filopodia Formation via Netrin-1 in Commissural Neurons

A major downstream effector of active Cdc42 that regulates actin polymerization is the neuronal Wiskott-Aldrich syndrome protein (N-WASP) (Mullins, 2000).

Immunocytochemical analysis detected N-WASP protein throughout commissural neuron growth cones, including immunoreactivity within filopodia (Fig. 9A). To investigate a role for N-WASP in the response of spinal commissural neuron growth cones to netrin-1, an adenovirus expressing a Flag tagged dominant negative mutant form of N-WASP was utilized. The mutant N-WASP (Δcof N-WASP) contains a four-amino acid deletion in its C- terminal domain that cannot direct actin reorganization and acts as a dominant-negative blocking Cdc42-induced filopodium formation and neurite extension (Banzai *et al.*, 2000). Cultured commissural neurons were infected with 20 MOI of Δcof N-WASP or GFP adenoviruses in the presence of netrin-1 protein. Forty-eight hours later the cells were fixed and stained with an antibody against the Flag tag to identify the cells expressing Δcof N-WASP (Fig. 9B and 9C). Quantification of the morphology of the growth cones of these cells indicates that interfering with N-WASP function blocks both the netrin-1 dependent increase in filopodia number and growth cone surface area. Using GST-Cdc42 fusion protein we were then able to detect increasing association of netrin-1-induced activated Cdc42 with N-WASP and Pak1 in the commissural neurons (Fig. 9D). These results suggest that netrin-1 causes activation of Pak1 and N-WASP via Cdc42.

Discussion

Membrane ruffling, lamellipodia formation, and the initiation of actin polymerization at the tips of filopodia, drive the expansion, extension, and motility of growth cones (reviewed by Mueller, 1999). Growth cone turning is thought to be the result of directed extension of the growth cone caused by the restricted polymerization of actin within the growth cone (reviewed by Bentley, 1994). Actin polymerization at the tips of filopodia and the edges of lamellipodia is regulated by the Rho family of small GTPases (Mallavarapu, 1999).

Well studied members of the Rho family of small GTPases include Cdc42, Rac1, and Rho A. These GTPases have been implicated in the expansion, extension, and collapse of growth cones (reviewed by Meyer & Feldman, 2002). They are binary molecular switches that cycle between active GTP bound and inactive GDP bound forms. GEFs, GAPs, and GDIs regulate GTPase activity by facilitating activation or inactivation (reviewed by Hall, 1998; Hoffman, 2002). Active forms of these GTPases bind and activate their downstream targets leading to actin polymerization, regulating adhesion, gene expression, and intracellular trafficking (reviewed by Schmitz, 2000). A role for RhoGTPases in the response to netrin-1 in neuronal growth cones has not previously been reported.

Chapter 2 describes results indicating that netrin-1 protein increases cell spreading and filopodia formation by activating Cdc42 and Rac1 in cell lines transfected to express DCC (Shekarabi & Kennedy, 2002). The findings presented here show that netrin-1 causes an increase in the surface area and filopodia number of the growth cones of rat embryonic spinal commissural neurons. This netrin-1 induced change in growth cone morphology requires the Rho GTPases Cdc42 and Rac1. In addition, netrin-1 activates

Cdc42 and Rac1, and their effector the serine-threonine kinase Pak1, in these neurons. Evidence is provided that the activation of Cdc42, Rac1, and Pak1, require DCC and PI3K. Furthermore, evidence is provided that the addition of netrin-1 to rat embryonic commissural neurons promotes the formation of a complex of effector proteins with the DCC-ICD that includes Cdc42, Rac1, NcK1, Pak1, and N-WASP.

The results described here used a GTPγS loading assay to visualize activated Cdc42 or Rac1 in the presence and absence of netrin-1 protein in the commissural neurons. The results obtained provide evidence that an as yet unidentified GEF(s) is activated by netrin-1, and that this in turn leads to the activation of Cdc42 and Rac1. The possibility that netrin-1 may also regulate GAP(s) or GDI(s) remains to be investigated. Activation of Cdc42 in the presence of dominant negative Rac1 implies that netrin-1 activates a GEF with the capacity to activate Cdc42. Conversely, the absence of Rac1 activation in the presence of a dominant negative form of Cdc42 suggests that the major pathway of activation is first through Cdc42, which then leads to activation of Rac1. A requirement for Rac1 activation on activation of Cdc42 has been reported previously (Nobes & Hall, 1995; Kozma *et al.*, 1995). Importantly, this differs from our earlier report that in HEK293T and NG108-15 cells that Cdc42 and Rac1 could each be activated independently by netrin-1, implying the presence of a GEF(s) with the capacity to activate both Cdc42 or Rac1 (Shekarabi and Kennedy, 2002). The difference observed likely reflects cell specific differences in the GEF proteins expressed. Here we provide evidence that the GEF protein βPIX is expressed by embryonic rat commissural neurons, however further studies will be required to determine if βPIX is involved in the response of growth cones to netrin-1. However, activation of βPIX requires PI3K, and if βPIX is the GEF for

DCC, this may explain the finding that inhibitors of PI3K block the response to netrin-1. In addition, Forcet *et al.* (2002) have provided evidence that netrin-1 activates MEK1/2 leading to activation of ERK1/2. MEK and ERK can function as upstream activators of βPIX, suggesting a model where netrin-1 binds to DCC, this activates MEK and ERK, and the activation of βPIX then leads to activation of Cdc42 (further discussed in chapter 4). This is an attractive hypothesis, however, GEFs acting downstream of netrin-1 and DCC remain to be identified.

A Complex of DCC, Nck1, and Pak1 Contributes to the Regulation of the Orgranization of Actin in Rat Embryonic Commissural Neurons

Pak1 is an important mediator between the small Rho GTPases and downstream effectors that regulate actin polymerization (reviewed by Bagrodia & Cerione, 1999). The DCC-ICD interacts constitutively with the SH2 and SH3 domain containing adaptor protein Nck1 in commissural neurons (Li *et al.*, 2002). Nck acts as a scaffold, binding via an SH3 domain to Pak1 (Bokoch *et al.*, 1996; reviewed by Li *et al.*, 2001). The findings presented here indicate that netrin-1 activates Pak1 and promotes the formation of a macromolecular complex containing activated Pak1 and DCC.

Although Pak1 has not previously been shown to be activated by netrin-1, inhibition of Pak1 has been reported to play a role in growth cone collapse. Semapohorins are a family of secreted and transmembrane proteins that trigger growth cone collapse. Sema-4d signaling via plexin-B1 has been shown to cause inactivation of Pak1 leading to inhibition of actin assembly (Vikis *et al.*, 2002). LIM kinase 1 is an important effector regulating actin polymerization that acts downstream of Pak1 (Edwards *et al.*, 1999). By

regulating LIM kinase 1, Sema-3a induces sequential phosphorylation and dephosphorylation of cofilin during growth cone collapse (Aizawa *et al.*, 2001). Together with the results presented here, these findings suggest that Pak1 is an important player regulating the response of neuronal growth cones to extracellular guidance cues.

Forcet *et al.* (2002) have shown that netrin-1 activates the MAPK (mitogen activated protein kinase) pathway by inducing ERK1 and ERK2 phosphorylation in transfected cell lines. Inhibitors of MEK1 and MEK2 blocked netrin-1 dependent axon outgrowth and turning. Pak1 can also phosphorylate and activate MEK1 (Cole & Show, 2002; Eblen *et al.*, 2002). This suggests a second model in which netrin-1 regulates activation of the MAPK pathway downstream of Cdc42, Rac1, and Pak1 activation. The findings presented here indicate that netrin-1 activates Pak1 in embryonic commissural neurons, however, determining if Pak1 is upstream or downstream of MAPK activation will require additional analysis.

N-WASP also binds to Nck1 and activated Cdc42 (Rohatgi *et al.*, 2001). By recruiting and activating the Arp2/3 complex, N-WASP plays a key role in promoting the nucleation and polymerization of F-actin (reviewed by Mullins, 2000). The findings presented here provide evidence that N-WASP is expressed in embryonic rat spinal commissural neurons and is required for the netrin-1 induced changes in growth cone morphology. Furthermore, addition of netrin-1 promoted the formation of a complex that includes Cdc42, Pak1, and N-WASP.

On the basis of the findings described, the following chapter presents a model of the molecular mechanism that trigger growth cone expansion in response to netrin-1, and growth cone turning in response to a gradient of netrin-1.

Figure 1. Analysis of dissociated embryonic rat spinal cord commissural neurons *in vitro.*

(A) Schematic illustrating the micro-dissection of the dorsal half of the rat embryonic day 13 (E13) spinal cord which was used as a source of commissural neurons for dissociated cell culture. DCC and Tag-1 both markers of commissural neurons, are expressed by the cells *in vitro*. A monoclonal IgG against the C-terminal domain of DCC, anti-DCC_{IN}, was used to detect endogenous DCC (green), and the 4D7 monoclonal IgM was used to detect Tag-1 (red) (panel B, 20× objective). Panel C shows a higher magnification image of the growth cones of dissociated commissural neurons, labeled for DCC (red) and filamentous actin using FITC-coupled phalloidin (green). 100× objective, Scale bars correspond to 10 μm. D; dorsal, V; ventral, CN; commissural neurons, MN; motor neurons.

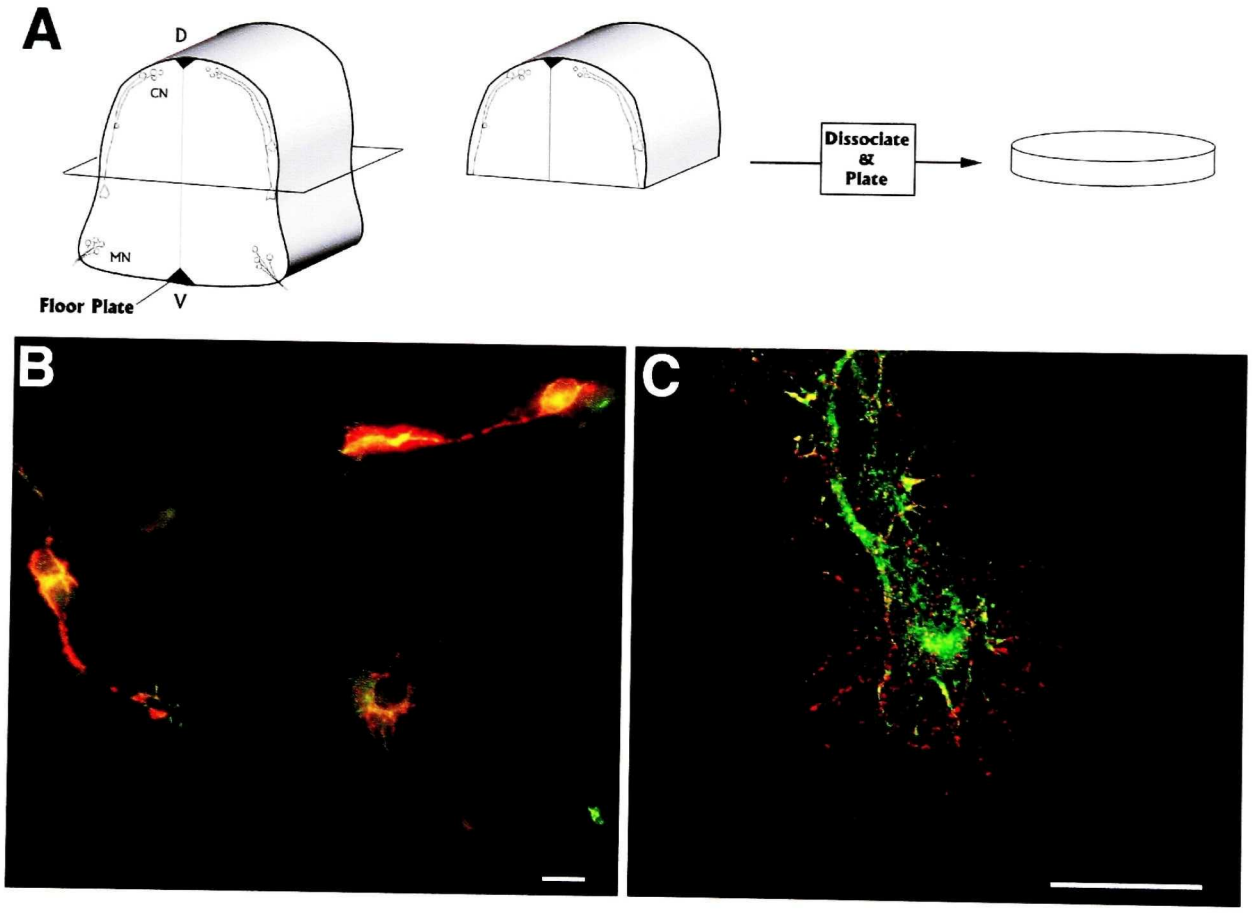

A
D
CN
MN
Floor Plate
V
Dissociate
&
Plate
B
C

Figure 2. Netrin-1 causes DCC dependent embryonic rat spinal commissural neuron growth cone expansion.

Dissociated spinal commissural neurons were grown on a substrate of poly-D-lysine (A - C). In panel A, no netrin-1 was added to the culture. Panel B illustrates the effect of application of 80 ng/ml netrin-1 protein to the culture media for 5 minutes. Panel C shows the effect of 30 min application of soluble recombinant netrin-1 (80 ng/ml). Unlike the growth cones shown in panels A-C, that received soluble recombinant netrin-1 protein, neurons in panel D were grown on a coverslip coated with a substrate of poly-D-lysine and netrin-1 protein. DCC immunoreactivity is shown in red. F-actin in the growth cones was visualized using FITC-coupled phalloidin (green). Scale bar corresponds to 10 μm, 100× objective.

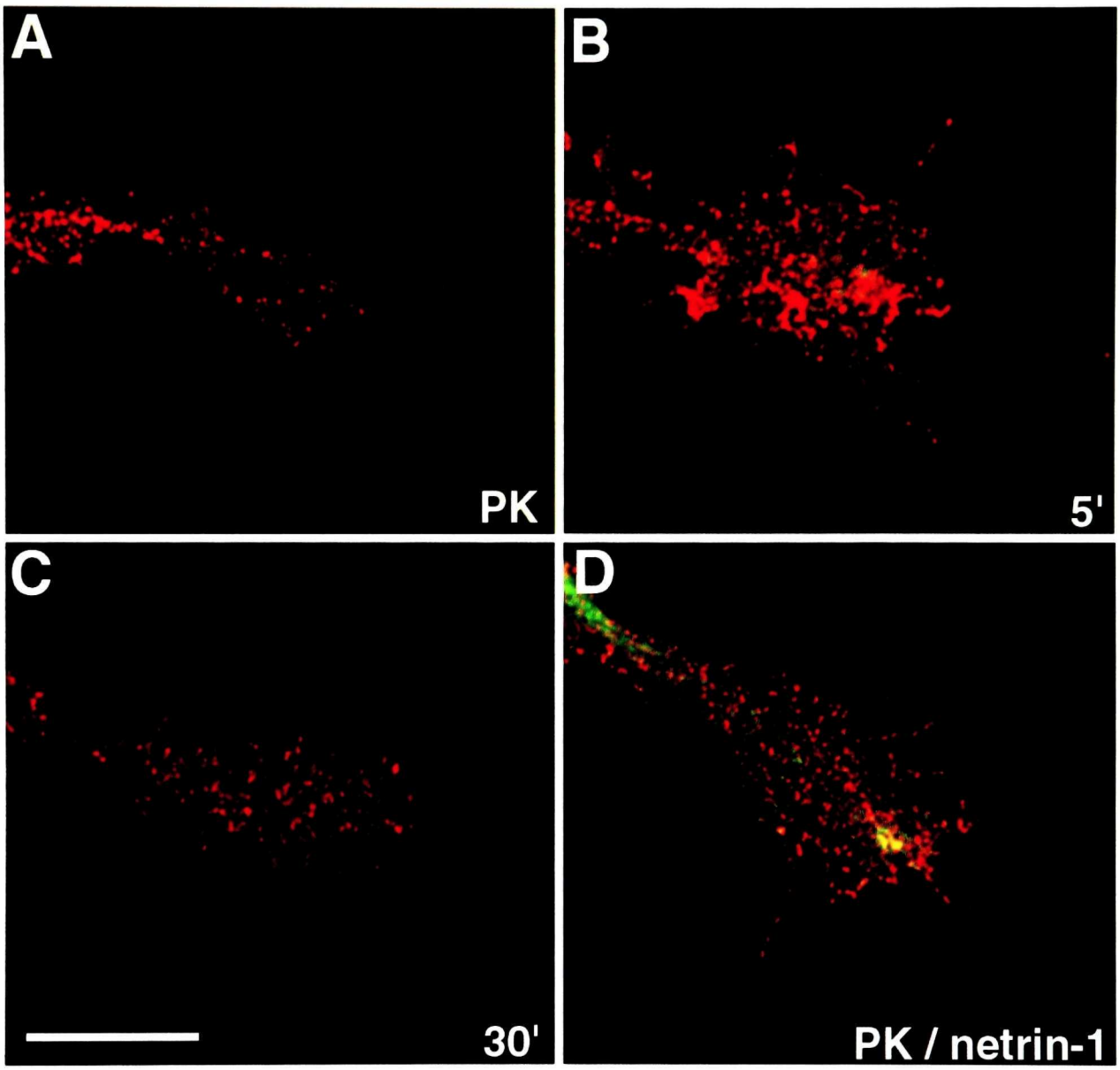

A
PK
B
5'
C
30'
D
PK / netrin-1

Figure 3. Netrin-1 causes an increase in commissural neuron growth cone surface area and filopodia number via a mechanism that requires Cdc42 and Rac1.

Presentation of netrin-1 as either a soluble or substrate-bound cue increased the surface area and number of filopodia per growth cone compared to the untreated neurons (A-D). Application of antibodies that block either netrin-1 (PN3) or DCC (AF5) function decreased the surface area and number of filopodia per growth cone compared to the netrin-1 treated neurons (n=25, error bars are SEM). To manipulate Cdc42 and Rac1 function, commissural neurons were infected with adenovirus' expressing either of N17Cdc42, N17Rac1, CACdc42, CARac1 or GFP alone (E and F). Forty-eight hours after infection, netrin-1 protein was added to the cultures. Thirty min later the cultures were fixed and immunostained using the 9E10 monoclonal antibody to detect the myc epitope tag of the recombinant proteins. Growth cone filopodia number and surface area were measured in myc-tagged, immunopositive neurons. Dominant negative Cdc42 (N17Cdc42) significantly decreased the number of filopodia per growth cone compared to the adeniovirus expressing GFP in the presence of netrin-1 protein. Dominant Rac1 (N17Rac1) produced a significant decrease in the number of filopodia per growth cone and in growth cone surface area (n=25) compared to the adeniovirus expressing GFP in the presence of netrin-1 protein. In the absence of exogenous netrin-1, constitutively active Cdc42 (CACdc42) or constitutively active Rac1 (CARac1) expression significantly increased both filopodia number and growth cone surface area compared to the adeniovirus expressing GFP (n= 15) (error bars, SEM). * indicates a p value of < 0.05, and ** indicates a p value of < 0.005.

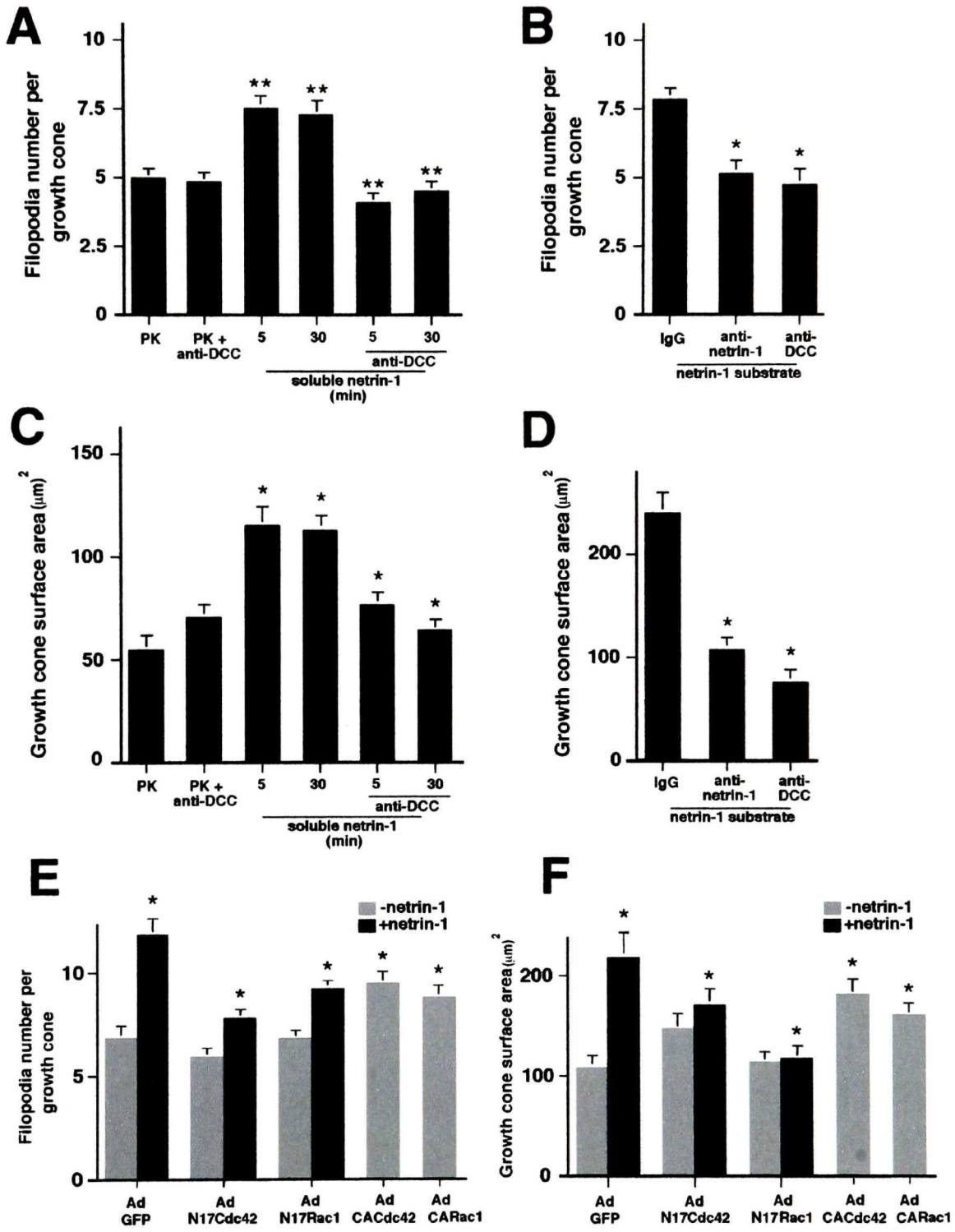

A
Filopodia number per growth cone
10
7.5
5
2.5
0
**
**
**
**
PK
PK + anti-DCC
5
30
5
30
anti-DCC
soluble netrin-1 (min)
B
Filopodia number per growth cone
10
7.5
5
2.5
0
*
*
IgG
anti-netrin-1
anti-DCC
netrin-1 substrate
C
Growth cone surface area (μm)2
150
100
50
0
*
*
*
*
PK
PK + anti-DCC
5
30
5
30
anti-DCC
soluble netrin-1 (min)
D
Growth cone surface area (μm)2
200
100
0
*
*
IgG
anti-netrin-1
anti-DCC
netrin-1 substrate
E
-netrin-1
+netrin-1
Filopodia number per growth cone
10
5
0
*
*
*
*
*
Ad GFP
Ad N17Cdc42
Ad N17Rac1
Ad CACdc42
Ad CARac1
F
-netrin-1
+netrin-1
Growth cone surface area (μm)2
200
100
0
*
*
*
*
*
Ad GFP
Ad N17Cdc42
Ad N17Rac1
Ad CACdc42
Ad CARac1

Figure 4. Activation of Cdc42 and Rac1 by netrin-1 in rat embryonic spinal commissural neurons requires DCC and PI3 Kinase.

Dissociated embryonic spinal commissural neurons were treated with 80 ng/ml netrin-1 or carrier for 3 minutes. The neurons were then lysed and incubated with GTPγS at 31°C. GTPγS bound Cdc42 and Rac1 were isolated using GST-Pak1-CRIB fusion protein and assayed by western blotting with antibodies against Rac1 or Cdc42 (A, F, and G). Cell lysates were probed using anti-DCC to confirm that equal amounts of total protein were loaded per lane. Panels B, F, and G show that addition of anti-DCC_{FB} significantly reduced the activation of Cdc42 and Rac1 by netrin-1. To determine if Cdc42 or Rac1 influence the activation of each other, cultured embryonic spinal commissural neurons were infected with adenoviral vectors (MOI=20) encoding N17Cdc42, N17Rac1, or GFP as a control, grown for forty-eight hrs and assayed as described above (C). To investigate a possible role for PI3 kinase in netrin-1 activation of Rho GTPases, the PI3 kinase inhibitors LY294002 (50 μM) or Wortmanin (1 μM) were applied for 45 minutes prior to the addition of netrin-1 protein. Panels D, F, and G illustrate the effect of 3 min stimulation with netrin-1. Panel E shows a time course following application of netrin-1. Interestingly, although PI3 kinase inhibitors blocked the activation of Cdc42 and Rac1, by monitoring phospho-Akt, panel E indicates that addition of netrin-1 does not activate PI3 kinase at early time points. However, an increase in phospho-Akt was detected after 30 min, suggesting that PI3 kinase may be activated at later time points.

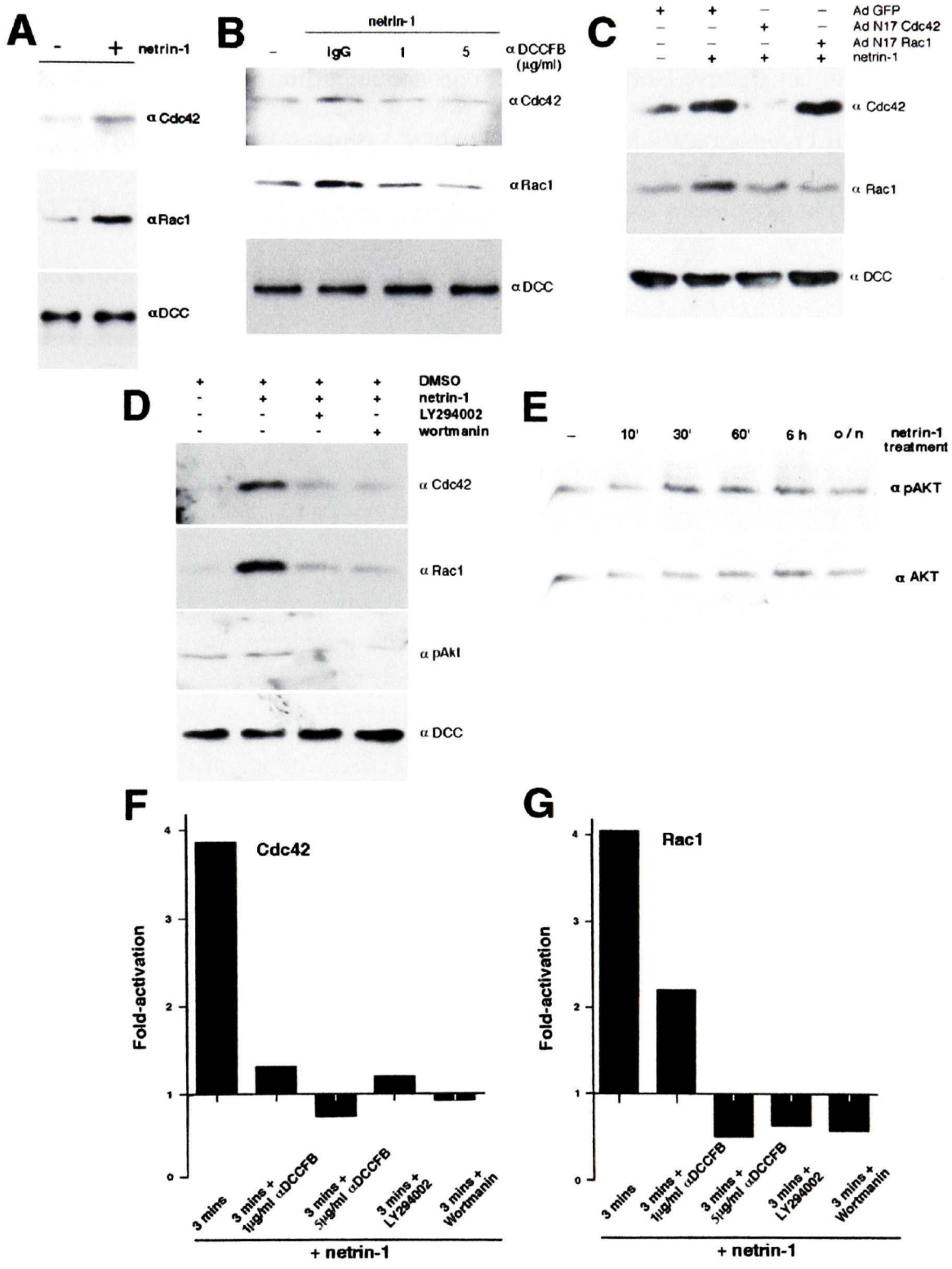
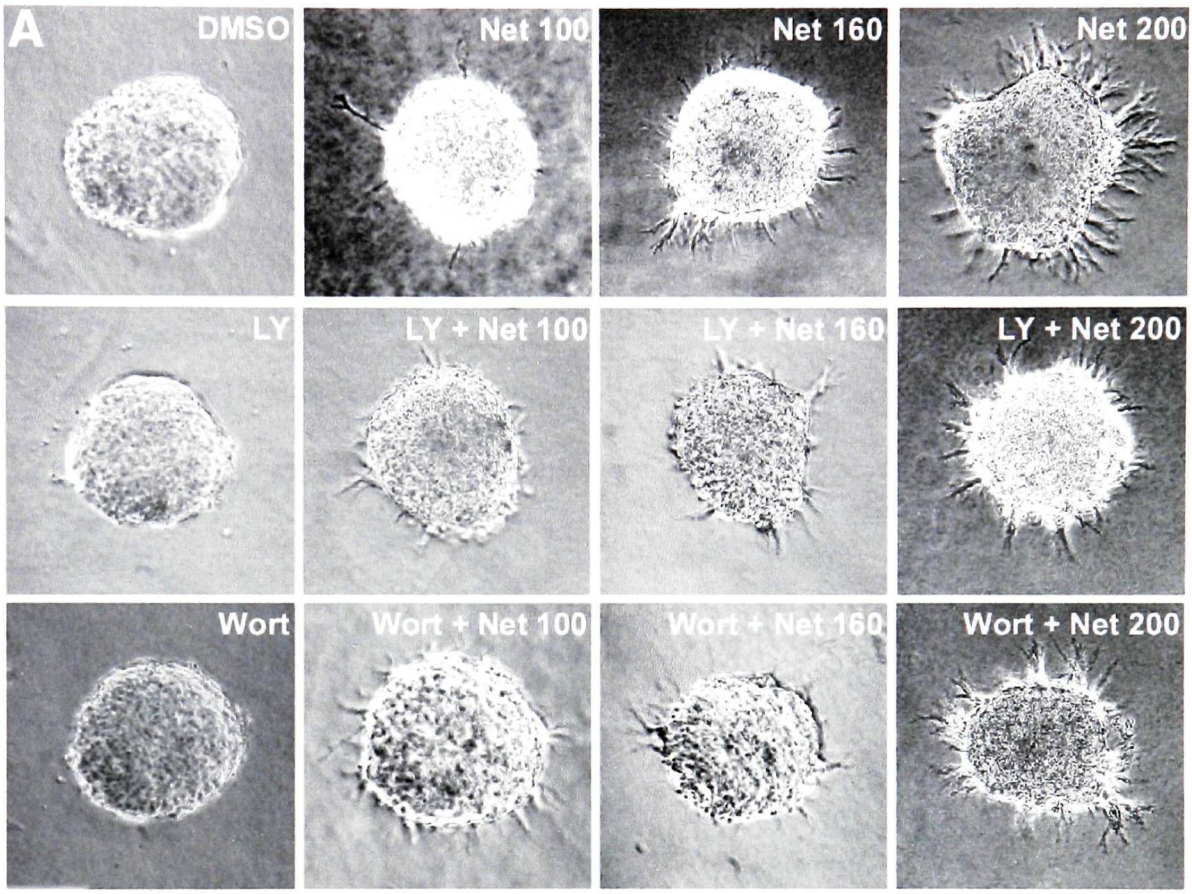

A
- + netrin-1
α Cdc42
αRac1
αDCC
B
netrin-1
- IgG 1 5
α DCCFB (μg/ml)
α Cdc42
αRac1
αDCC
C
+ + - - Ad GFP
- - + - Ad N17 Cdc42
- - - + Ad N17 Rac1
- + + + netrin-1
α Cdc42
α Rac1
α DCC
D
+ + + + DMSO
- + + + netrin-1
- - + - LY294002
- - - + wortmanin
α Cdc42
α Rac1
α pAkt
α DCC
E
- 10' 30' 60' 6 h o/n netrin-1 treatment
α pAKT
α AKT
F
Cdc42
Fold-activation
0 1 2 3 4
3 mins
3 mins + 1μg/ml αDCCFB
3 mins + 5μg/ml αDCCFB
3 mins + LY294002
3 mins + Wortmanin
+ netrin-1
G
Rac1
Fold-activation
0 1 2 3 4
3 mins
3 mins + 1μg/ml αDCCFB
3 mins + 5μg/ml αDCCFB
3 mins + LY294002
3 mins + Wortmanin
+ netrin-1

Figure 5. PI3 kinase inhibitors decrease axon outgrowth from netrin-1 treated embryonic dorsal spinal cord explants.

Dorsal halves of E13 rat spinal cords were cultured in a 3D collagen matrix in the absence or presence of different concentrations of netrin-1 protein and in the presence or absence of PI3 kinase inhibitors LY294002 (50 μM) and Wortmanin (1 μM) for 16 hours (A). The total length of axon outgrowth per explant was measured and plotted (B). Both inhibitors decreased the total length of the outgrowth per explant in a netrin-1 concentration dependent way.


A
DMSO
Net 100
Net 160
Net 200
LY
LY + Net 100
LY + Net 160
LY + Net 200
Wort
Wort + Net 100
Wort + Net 160
Wort + Net 200

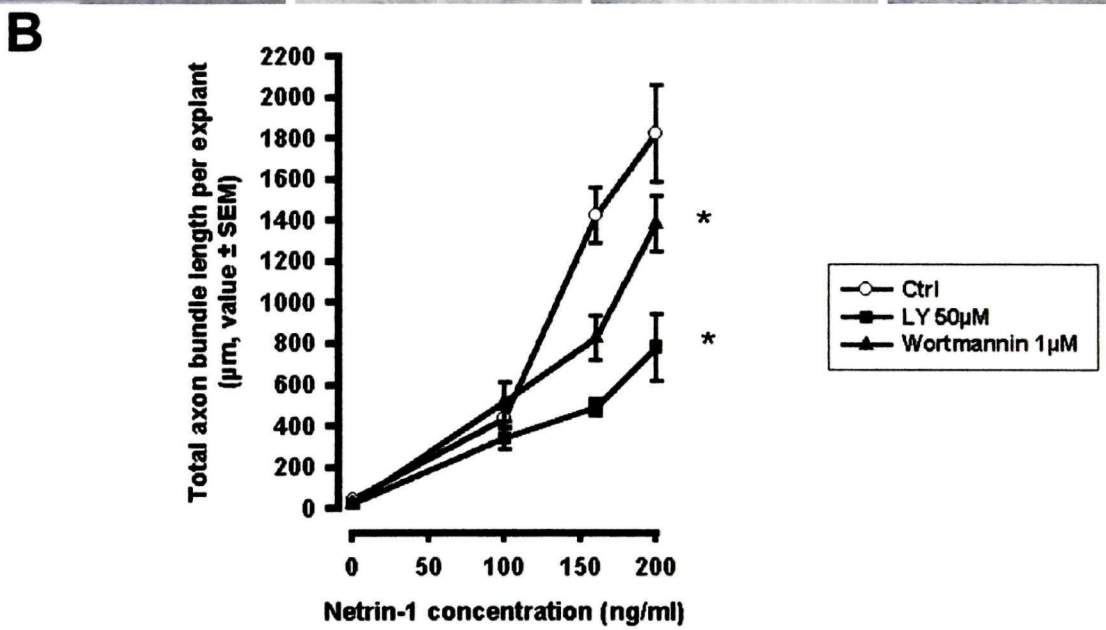
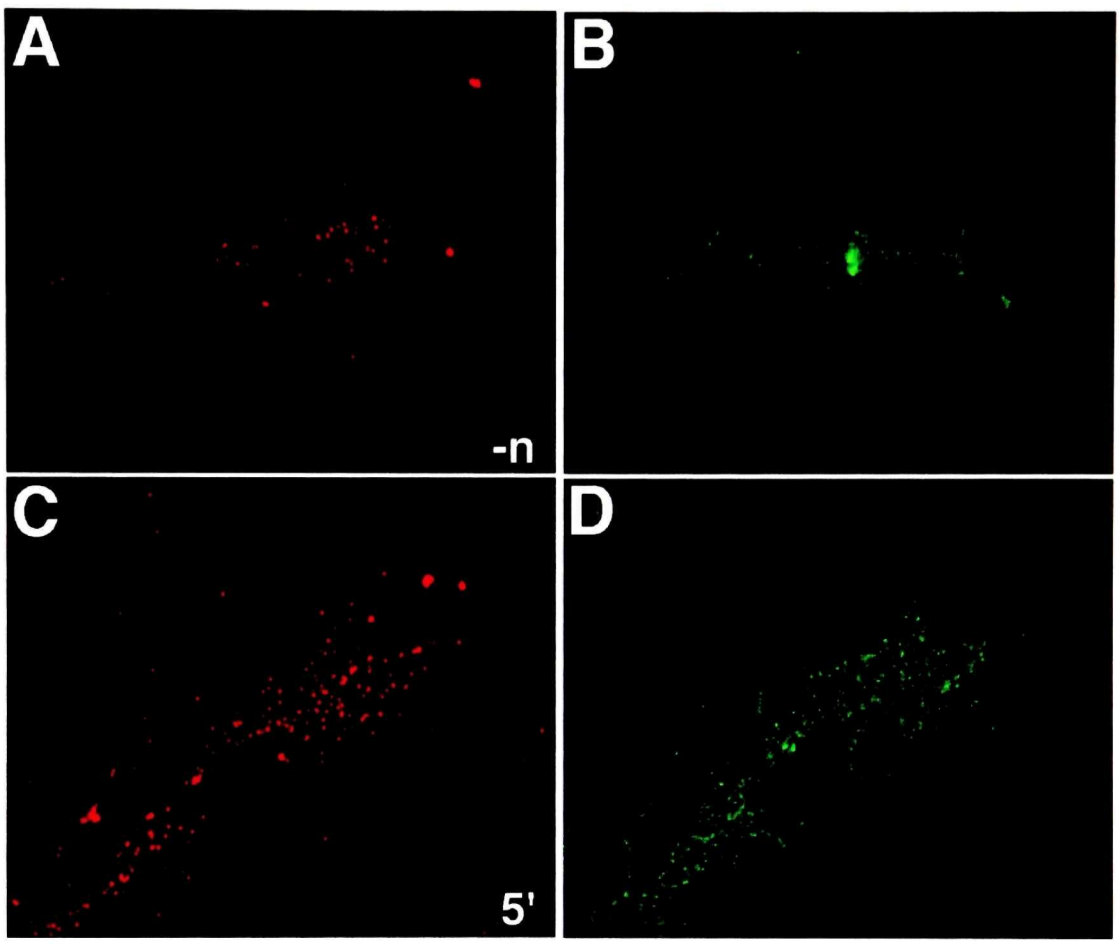

B
Total axon bundle length per explant (µm, value ± SEM)
2200
2000
1800
1600
1400
1200
1000
800
600
400
200
0
0
50
100
150
200
Netrin-1 concentration (ng/ml)
*
*
Ctrl
LY 50µM
Wortmannin 1µM

Figure 6. Netrin-1 activates Pak1, a downstream effector of Rac1 and Cdc42.

Cultures of dissociated spinal commissural neurons were prepared as described above (A-D) and treated with netrin-1 protein for 5 min (C and D). The neurons were then fixed and double stained with antibodies against phosphorylated Pak1 (red) and DCC (green). Higher phospho-Pak1 immunoreactivity was detected in growth cones exposed to netrin-1. Images were captured using a cooled CCD camera and identical exposure times in each of the different conditions, allowing the intensity of fluorescence to be compared. Panel F shows quantification of phospho-Pak1 immunoreactivity as the percentage of the growth cone surface area above threshold. (n= 28) (error bars, SEM). * indicates a p value of < 0.05.


A
-n
B
C
5'
D

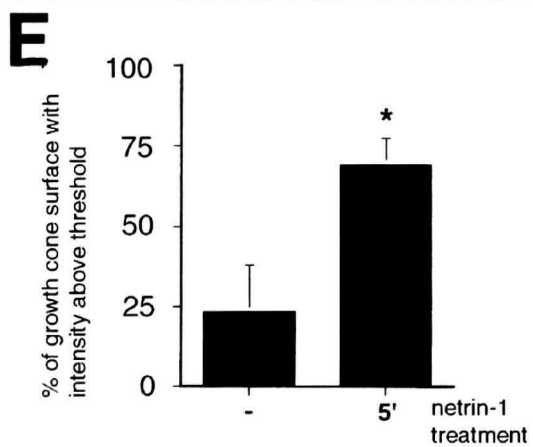

E
100
75
50
25
0
*
% of growth cone surface with intensity above threshold
-
5'
netrin-1 treatment

Figure 7. Activation of Pak1 by netrin-1 requires DCC and PI3 kinase.

Netrin-1 protein (80 ng/ml) was applied to dissociated commissural neurons for the times indicated. In some cases, PI3K inhibitors or the DCC function-blocking antibody were added to the cultures 1 hour before netrin-1. Panel A shows the results of western blot analysis using an antibody against phospho-Pak1. The same blot was reprobed with an antibody against Pak1 to confirm that equal amounts of total protein had been loaded per lane. Netrin-1-induced an increase in phospho-Pak1, which was dependent on PI3 kinase and DCC. Panel B and C show the results of adding the Pak peptide, a cell permeable competitive inhibitor of Pak1 binding to the adaptor protein Nck1. The control peptide contains amino acid substitutions for two prolines that are required for SH3 domain binding. Addition of the Pak peptide to cultures of dissociated commissural neurons for 40 min blocked the netrin-1-induced increase in filopodia number and growth cone surface area compared to the control peptide in the presence of netrin-1 protein. The control peptide produced no effect (B and C). These experiments suggest that Pak1 binding to Nck1 is required for netrin-1-induced commissural neuron growth cone expansion (n= 25) (error bars, SEM). * indicates a p value of < 0.05, and ** indicates a p value of < 0.005.

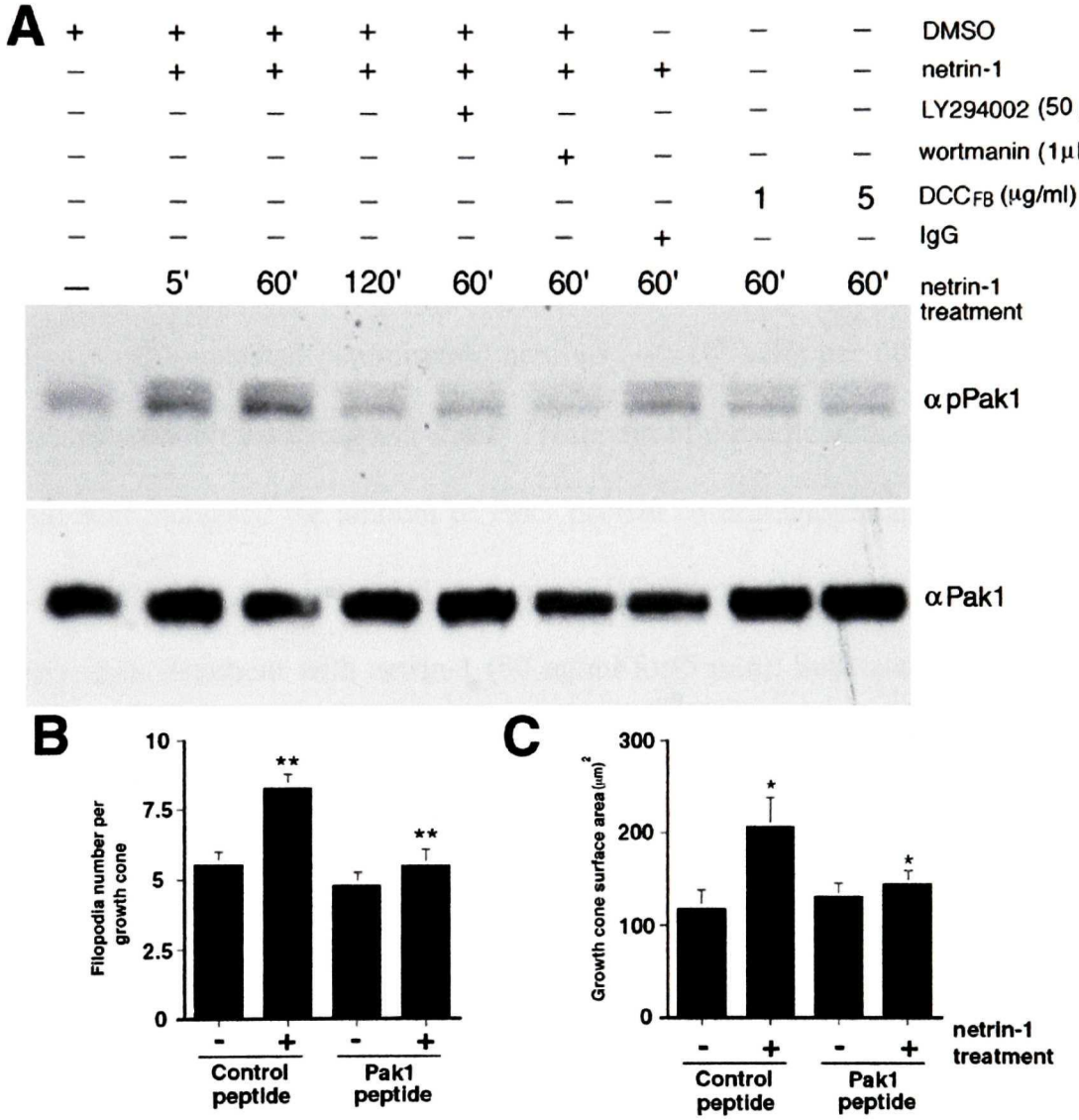

A
DMSO
netrin-1
LY294002 (50 μM)
wortmanin (1μM)
DCC FB (μg/ml)
IgG
netrin-1 treatment
— 5' 60' 120' 60' 60' 60' 60' 60'
αpPak1
αPak1
B
Filopodia number per growth cone
10
7.5
5
2.5
0
**
**
- + - +
Control peptide
Pak1 peptide
C
Growth cone surface area (μm)²
300
200
100
0
*
*
- + - +
Control peptide
Pak1 peptide
netrin-1 treatment

Figure 8. Evidence that netrin-1 induces the formation of a complex that includes Cdc42, Rac1, Pak1, and DCC.

(A) Following treatment with netrin-1, cell lysates of dissociated commissural neurons were prepared as described in Figure 4. Purified recombinant protein encoding GST-Cdc42 or GST-Rac1 was added to lysates along with GTPγS. The GST fusion protein complex was then isolated and separated using SDS-PAGE. Treatment with netrin-1 promoted the formation of a complex of proteins that included phospho-Pak1, DCC, Cdc42, and Rac1. Increased binding activity was observed following a minimum of 5 min treatment with netrin-1. (B) DCC IP (1.5 μg anti-DCC_{IN}) was carried out using lysates of dissociated commissural neurons (~4×10^6 cells per 60 mm culture dish) and then immunobloted using anti-Pak1. Treatment of the cells with netrin-1 (80 ng/ml for 5 minutes) increased the amount of Pak1 protein co-immunoprecipitating with DCC. (C) Consistent with this, increased amounts of DCC were detected in a co-IP with anti-Pak1 following treatment with netrin-1 (80 ng/ml for 5 min). Substantially less Nck1 protein was isolated by anti-Pak1 IP following treatment with netrin-1. (D) βPix immunoreactivity, ~78 kDa, was detected following GST-Rac1 pull down from homogenates of cultured dissociated commissural neurons. As a positive control, an immunorective band of the same molecular weight was detected in PC12 cells.

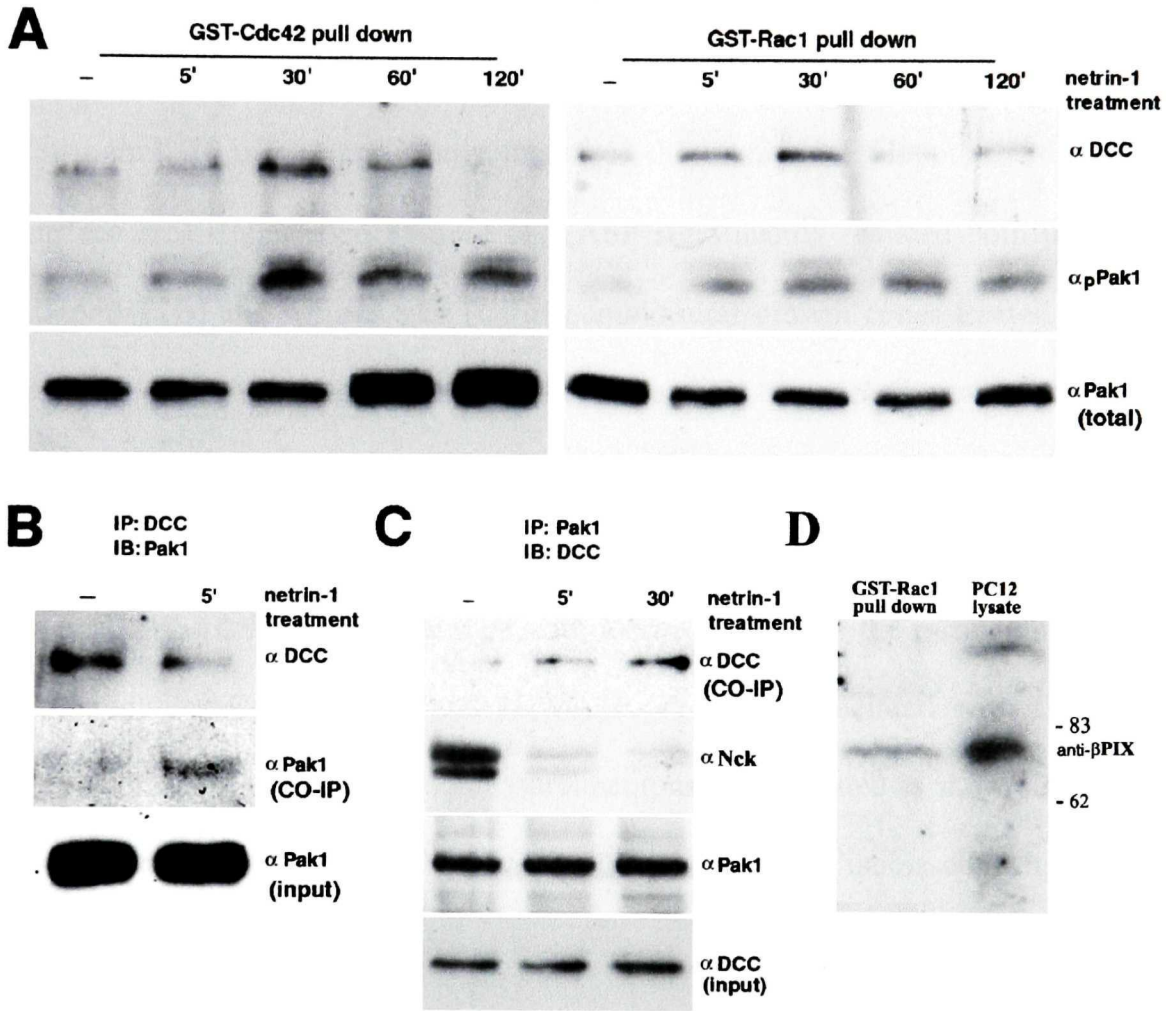
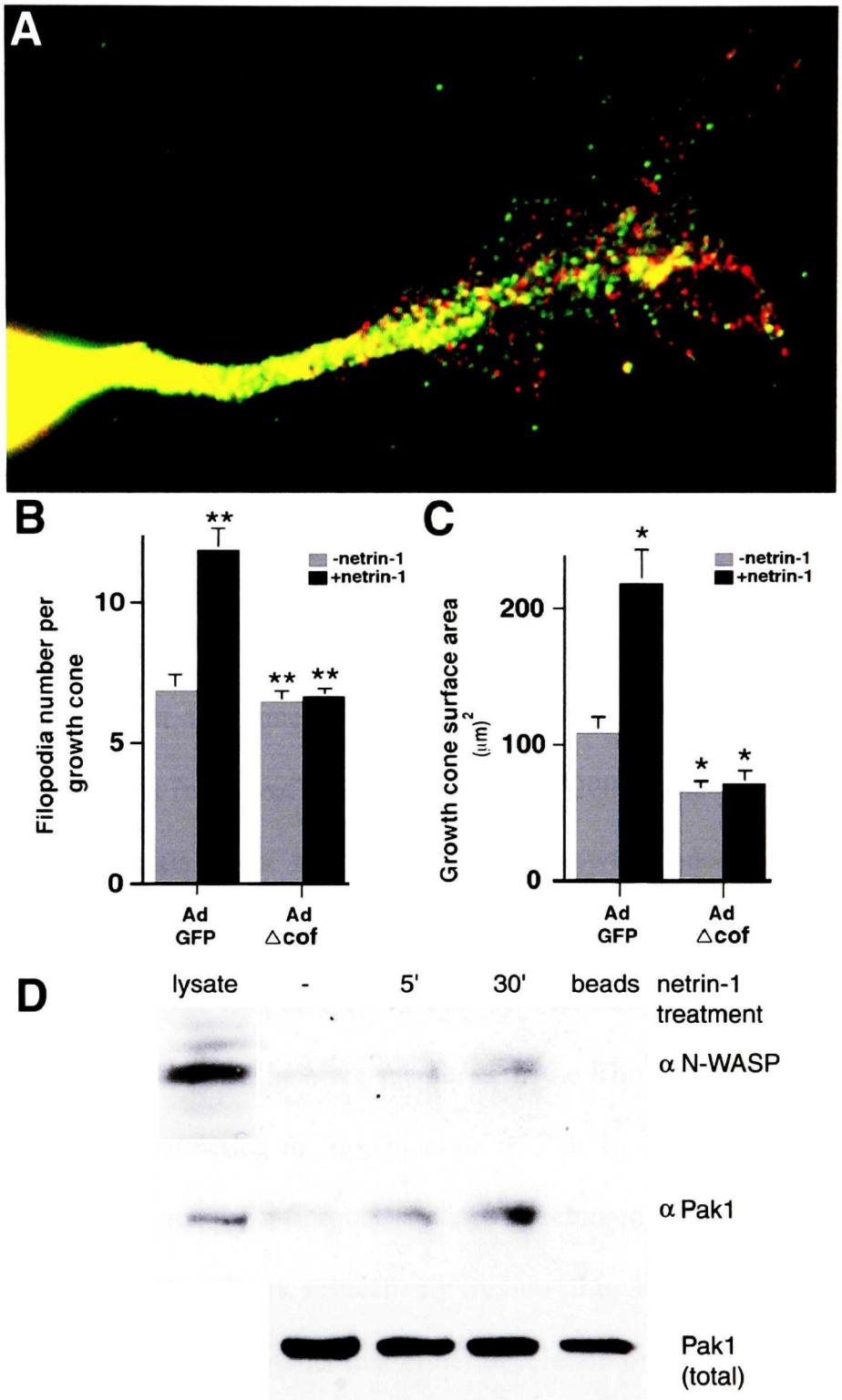

A
GST-Cdc42 pull down
GST-Rac1 pull down
–
5'
30'
60'
120'
netrin-1 treatment
α DCC
αpPak1
αPak1 (total)
B
IP: DCC
IB: Pak1
–
5'
netrin-1 treatment
α DCC
α Pak1 (CO-IP)
α Pak1 (input)
C
IP: Pak1
IB: DCC
–
5'
30'
netrin-1 treatment
α DCC (CO-IP)
α Nck
α Pak1
α DCC (input)
D
GST-Rac1 pull down
PC12 lysate
- 83
anti-βPIX
- 62

Figure 9. N-WASP is required for netrin-1 induced filopodia formation in commissural neurons.

(A) Dissociated commissural neurons were immunostained with antibodies against DCC (green) and N-WASP (red). Immunoreactivity of both N-WASP and DCC was distributed along the axon, growth cone, and filopodia of commissural neurons. (B and C) Following infection with adenoviral vectors encoding either GFP or Δcof N-WASP, growth cone morphology was quantified. Consistent with figure 3, addition of netrin-1 (80 ng/ml, 30 min) significantly increased the number of filopodia and growth cone surface area. Expression of Δcof N-WASP significantly reduced both the number of filopodia (B) and surface area (C) of commissural growth cones treated with netrin-1 compared to adenovirus espressing GFP. Notably, in panel C expression of Δcof N-WASP significantly reduced the growth cone surface area below that either in the presence of netrin-1 or control (n=15) (error bars, SEM). * indicates $p < 0.05$, ** indicates $p < 0.005$. (D) Treatment with netrin-1 promotes the formation of a complex that includes Cdc42, Pak1 and N-WASP. Proteins associated with GST-Cdc42 in homogenates of dissociated commissural neurons were isolated as shown in figure 8. The association of N-WASP and Pak1 with activated Cdc42 was increased by netrin-1.


A
B
Filopodia number per growth cone
10
5
0
**
**
**
-netrin-1
+netrin-1
Ad GFP
Ad △cof
C
Growth cone surface area (μm)²
200
100
0
*
*
*
-netrin-1
+netrin-1
Ad GFP
Ad △cof
D
lysate
-
5'
30'
beads
netrin-1 treatment
α N-WASP
α Pak1
Pak1 (total)

Chapter 4

General Discussion

The Rho family of Small GTPases and Cellular Motility

Understanding how the Rho family of small GTPases regulates cell motility has made great progress in recent years. Many of these insights are now being applied to the complex processes regulating axon outgrowth and turning during neural development. Filopodia and lamellipodial protrusions are required for a cell or a growth cone to move and turn. Filopodia and lamellipodia probe and develop adhesive contacts with the extracellular matrix (reviewed by Wood & Martin, 2002), and also function to transmit extracellular signals from the ECM to the growth cone and the soma. Actin polymerization and depolymerization occurs at the edge of the growth cone, within the lamellipodium and the filopodia (reviewed by Takenawa & Miki, 2001; Wear *et al.*, 2000). Cdc42, Rac1, and RhoA are members of the Rho family of small GTPases that play key roles in regulating the organization of F-actin, and have been well studied in multiple cellular contexts. In fibroblasts, they are required for the formation of filopodia, lamellipodia, and stress fibers, respectively (reviewed by Hall, 1998). The action of these proteins is regulated by proteins that either accelerate or inhibit Rho GTPase activity: GEFs, GAPs, and GDIs (reviewed by Mueller, 1999). In neuronal growth cones, several

reports have now documented the involvement of Rho GTPases downstream of repellent cues and their receptors during growth cone collapse (reviewed by Dickson, 2001).

This thesis reports that netrin-1 induces the formation of filopodia and increases cell surface of HEK293T cells, NG108-15 cells, and cultured embryonic rat spinal commissural neuron growth cones. Absence of DCC expression was first documented in the cell lines, and then these cells were transfected to express DCC, and the phenotype produced by this expression was identified. This led to the demonstration of netrin-1-induced, DCC dependent filopodia formation and spreading of both the cell lines. Subsequent analysis demonstrated that the small Rho GTPases Cdc42 and Rac1 are required for these effects of netrin-1, and that application of netrin-1 activates Cdc42 and Rac1 in both DCC expressing cell lines and embryonic rat spinal commissural neurons. Investigation of the downstream targets of activated Cdc42 and Rac1 in the commissural neurons led to the findings that Pak1 is activated by netrin-1. The activation of Pak1 requires active Cdc42 and Rac1. Furthermore, PI3 kinase and DCC were required for netrin-1 to activate Pak1.

DCC Inactivation, Cancer, Apoptosis, and Motility

Loss of DCC expression is thought to promote tumor progression. As its name indicates, DCC was first identified due to gene deletion in some forms of colon cancer (Fearon *et al.*, 1990; Hedrick *et al.*, 1994). DCC is expressed by goblet cells in the adult colon (Hedrick *et al.*, 1994), but DCC function in this context is not known. In the embryonic nervous system, DCC plays an essential role in regulating the motility of some types of axonal growth cones (Keino-Masu *et al.*, 1996). DCC is required for these axons

to navigate to their appropriate targets (Keino-Masu *et al.*, 1996). The findings described here indicate that DCC is a powerful regulator of the organization of F-actin.

Deregulation of a mechanism that controls the structure of the cytoskeleton could contribute to tumor progression. However, this interpretation generates a paradox. In the nervous system, DCC is required for cells and growth cones to migrate correctly. In the adult colon, loss of DCC expression is associated with increased metastasis and tumor progression. In one case, DCC regulates growth cone movement, while in colon cancer DCC seems to prevent cell movement. An obvious possibility is that DCC plays an important role regulating the structure of the cytoskeleton in both cases, but that the different outcomes for the cell result from the two different cellular contexts. With this in mind, it will likely be important to identify what other receptors and ligands work with DCC to regulate cellular movement in different contexts.

Members of the UNC-5 homolog family of proteins form a netrin receptor complex with DCC, and evidence suggests that this interaction can switch the response to netrin-1 from attraction to repulsion (Hong *et al.*, 1999). Furthermore, in the embryonic nervous system, a *cis* interaction of DCC with the transmembrane protein Roundabout (robo) blocks the response of a growth cone to netrin-1 (Bagri *et al.*, 2002; reviewed by Guthrie, 2001). Such findings reinforce the conclusion that identifying the proteins that make up the netrin receptor complex is important for understanding DCC function.

Overexpression of DCC in certain cell lines causes cell death (Mehlan *et al.*, 1998; Chen *et al.*, 1999). Although the precise pathway through which DCC induces apoptosis is not clear, evidence has been presented that in the absence of netrin-1, DCC activates caspase-9 through caspase-3 and induces apoptosis by a cytochrome c and Apaf-1 independent pathway (Forcet *et al.*, 2001). Alternatively, the reorganization of the actin

cytoskeleton by DCC may also contribute to the induction of cell death in cells overexpressing DCC. Massive induction of actin polymerization is not compatible with cell division and proliferation. Recently several groups have described transgenic mice expressing constitutively active Rac1 and Cdc42 (reviewed by Bustelo, 2002; Gomez *et al.*, 2000; Na *et al.*, 1999). In mice expressing constitutively active Cdc42 (QL61), a large increase in apoptosis has been reported in thymus, peripheral lymph organs, spleen, and lymph nodes (Na *et al.*, 1999). Because of the functional link found between DCC and Cdc42, it will be of interest to determine if these mice have an altered probability of developing colon or other cancers.

It has been shown that lack of appropriate cell-matrix interactions triggers a process called anoikis, in which the cells go through apoptosis (reviewed by Frisch & Screaton, 2001). Anoikis is a major cellular process in the colonic epithelium, where there is a balance between cellular proliferation and apoptosis (reviewed by Shanmugathasan & Jothy, 2000). Anoikis has been implicated in integrin-dependent cell adhesion, in which apoptosis is induced due to failed integrin-matrix interactions (reviewed by Frisch & Ruoslahti, 1997). It has also been shown that Cdc42 and Rac1 regulate anoikis and protect MDCK cells and primary mouse fibroblasts from apoptosis (Coniglio *et al.*, 2001; Zugasti *et al.*, 2001). DCC may contribute to the activation of Cdc42 and Rac1 in differentiated goblet cells in the colon preventing them from apoptosis by anoikis. On the other hand, DCC expression may help the goblet cells maintain contact with extracellular matrices, thus restricting their migration.

Signal Transduction Mechanisms Downstream of DCC and Netrin-1

Netrin-1 is a bi-functional cue with the potential to either attract or repel the same growth cone. This property of netrin-1 has been shown to be dependent on the concentration of cAMP or Ca^{2+} in the growth cone, and the expression of UNC-5 homologs by the neuron (Ming *et al.*, 1997; Song *et al.*, 1997; Hong *et al.*, 2000; reviewed by Song & Poo, 2001). In the context of high intracellular concentration of cAMP or Ca^{2+}, netrin-1 acts as an attractant, but in low cAMP or Ca^{2+}, netrin-1 induces a repellent response (reviewed by Song & Poo, 2001). Recently, we have found that cAMP and protein kinase A activation is implicated in rapid translocation of cytoplasmic vesicles pre-packed with DCC to the plasma membrane (Bouchard *et al.*, 2002; manuscript in preparation). Importantly, Cdc42 and Rac1 have been implicated in regulating the fusion of cytoplasmic vesicles with the plasma membrane (reviewed by Ridley, 2001). As such, activation of Cdc42 and Rac1 may contribute to the translocation of DCC to the plasma membrane triggered by PKA.

Downstream effectors of Cdc42 and Rac1 in netrin-1/DCC signaling were identified in the spinal commissural neurons. These studies revealed an important role for the Cdc42 and Rac1 effector, the serine/threonine kinase Pak1, which is activated by netrin-1 in these neurons. Pak1 is a key regulator of cell motility (reviewed by Bagrotia, 1999; Daniels & Bokoch, 1999). Activated Pak1 has been shown to accumulate at sites of focal adhesion, throughout filopodia, and within the body and edges of lamellipodia in platelet-derived growth factor stimulated NIH-3T3 cells. Activation of Pak1 is dependent on PI3 kinase, and Src family kinases (Sells *et al.*, 2000). Furthermore, it has been suggested that adhesion to extracellular matrix influences the effectiveness of coupling Rac1 to Pak1 (reviewed by Symons, 2000).

Activated Pak1 binds Nck1 (Bokoch *et al.*, 1996; reviewed by Li *et al.*, 2001). We have previously shown that Nck1 is expressed by embryonic spinal commissural neurons and that Nck1 binds intracellular DCC. These results suggest that the interaction of Pak1 with Nck1 may trigger signaling pathways downstream of DCC. Nck1 is known to bind activated N-WASP (Rohatgi *et al.*, 2001). N-WASP, and the associated Arp 2/3 complex, is a main effector of F-actin polymerization in filopodia (reviewed by Mullins, 2000). A likely scenario is that Cdc42, activated by netrin-1, binds N-WASP and facilitates the Nck1/N-WASP interaction. Chapter 3 provided evidence that N-WASP is present throughout the axons and growth cones of spinal commissural neurons, and localized at the tips of filopodia. Furthermore, expression of a dominant negative form of N-WASP blocked the netrin-1 induced morphological changes in the growth cone, indicating that this protein plays a functional role in commissural neuron growth cones. Further studies will be required to determine if the N-WASP associated Arp 2/3 complex is activated by netrin-1 and plays a role in commissural axon turning.

A major event underlying Pak1 function is that Pak1 translocates to the plasma membrane when activated. Importantly, this translocation to the plasma membrane involves binding to Nck1. Importantly, targeting Pak1 to the plasma membrane induces Pak1 activation and neurite outgrowth (Daniels *et al.*, 1999; Lu & Mayer, 1999). The findings reported here describe an increase in activated Pak1 immunoreactivity in the growth cone of spinal commissural neurons when exposed to netrin-1. Pak1 directly regulates components of the cellular machinery that nucleate F-actin. These include LIM kinase, a kinase that regulates cofilin, and myosin light chain kinase (MLCK). MLCK subsequently phosphorylates the myosin light chain (MLC). The possibility that these proteins may be regulated by Pak1, downstream of netrin-1, remains to be demonstrated.

Evidence has been provided that netrin-1 activates the MAP kinase pathway by activating MEK1/2, followed by ERK1/2 (Forcet *et al.*, 2001). These authors suggest that MAP kinase activation plays a key role mediating the directional response of growth cones to netrin-1. Interestingly, the effects of PKA activation on axon extension, described above, could act through PKA regulating ERK activity. However, the exact contribution of PKA in neurons will require additional analysis as PKA has been argued to activate or inhibit ERK, depending on the cell types (reviewed by Stork & Schmitt, 2002). The serine/threonine kinase Pak1 also activates ERK in some contexts (Coles & Shaw, 2002; Eblen *et al.*, 2002), suggesting that Pak1 may be upstream of the netrin-1 dependent MAP kinase activation described by Forcet *et al.* (2001).

The findings presented provide evidence that PI3K is required for netrin-1 to activate Cdc42, Rac1, and Pak1. Roles for PI3 kinase in cell migration, chemoattraction, and chemotaxis have been characterized (reviewed by Wu *et al.*, 2002; Stephens *et al.*, 2002). PI3K plays a key role in pathways involving the small Rho GTPases (reviewed by Fruman *et al.*, 1998; Heldin *et al.*, 1998; Jimenez *et al.*, 2000). Importantly, PI3K is part of the signal transduction machinery downstream of receptor tyrosine kinases such as Trks (reviewed by Kaplan, 2002). In this pathway, PI3K is downstream of Ras and upstream of Rac1 activation. As discussed in chapter 3, PI3K has also been shown to act downstream of Cdc42 and Rac1 (reviewed by Stephens *et al.*, 2002). Furthermore, association of Pak1 with PI3K induces actin phosphorylation and reorganization (Papakonstanti & Stournaras, 2002). This thesis reports that Akt, an important effector protein phosphorylated by PI3K, is phosphorylated following application of netrin-1. However increased phosphorylation of Akt was not observed at the earliest time points after adding netrin-1, but only first detected after 30 minutes. This suggests that although

active PI3K is required for activation of Cdc42, Rac1, and Pak1, activation of PI3K appears to be a later event in the cascade of events triggered by application of netrin-1. The precise contribution of PI3K to netrin-1 signal transduction, upstream or downstream of Cdc42 and Rac1, remains to be determined.

Evidence that PI3K regulates the activity of certain GEF proteins (Innocenti *et al.*, 2003; Fukuda *et al.*, 2002), identifies a possible contribution of PI3K to netrin-1 signaling upstream of Cdc42. Based on its expression in commissural neurons, we have suggested that βPIX may function as a GEF downstream of netrin-1, activating Cdc42. PI3K binds βPIX and the complex activates Cdc42 and Rac1 (Yoshii et al., 1999). The observation that inhibitors of PI3K block the activation of Cdc42, Rac1, and Pak1, by netrin-1 could be due to inhibition of the activation of a GEF, such as βPIX, downstream of DCC. Furthermore, βPIX is regulated by phosphorylation and the cascade of MEK>ERK>Pak2 has been shown to be upstream of βPIX activation (Shin *et al.*, 2002). This suggests that the activation of MEK by netrin-1 (Forcet *et al.*, 2001) described above, could begin a cascade that leads from MEK>ERK>Pak2>βPIX>Cdc42>Rac1>Pak1, followed by activation of the downstream effectors that lead to the reorganization of F-actin and changes in the morphology of the growth cone. However, the precise ordering of this proposed cascade of activation will require additional study.

Model for Netrin-1-Induced DCC Dependent Growth Cone Spreading

On the basis of the findings presented here, the following model of the signal transduction events underlying netrin-1 induced growth cone spreading is presented. Prior to the exposure of a growth cone to netrin-1 protein, a constitutive complex of DCC and

Nck1 provides the backbone for a series of molecular events (Fig. 1A). Netrin-1/DCC binding causes activation of a GEF, possibly βPIX. The activated GEF is able to catalyze GDP-bound Cdc42 to its active GTP-bound form, which in turn promotes activation of Rac1, and the binding of both Cdc42 and Rac1 to Pak1. This triggers Pak1 activation. Pak1 bound to the second SH3 domain of Nck1 exposes its binding domain to Cdc42-induced activated N-WASP (Fig. 1B). Activated N-WASP activates the Arp 2/3 complex, leading to actin nucleation and polymerization.

Incorporating the evidence that netrin-1 activates MEK1/2 and causes ERK1/2 phosphorylation (Forcet *et al.*, 2001) suggests that activation of Pak2 by ERK1/2, could be the upstream event that activates βPIX. Interestingly, Pak1 has been shown to activate MEK1/2 (Coles & Show, 2002; Eblen *et al.*, 2002), suggesting that activation in this pathway may loop back on itself, perhaps promoting the maintenance of a complex of proteins that signals the response to netrin-1 (Fig. 1C).

Model for the Signal Transduction Mechanisms Underlying Netrin-1-Induced, DCC Dependent Growth Cone Turning

Polymerization of F-actin occurs at the tips of filopodia (Mallavarapu, & Mitchison, 1999). The findings presented here indicated that DCC is localized at the tips of filopodia of commissural neuron axonal growth cones, an appropriate location to influence filopodial extension. The experiments described investigated the response of neuronal growth cones to application of a uniform concentration of netrin-1, but they do not directly address netrin-1 mediated growth cone turning. Contact of a single filopodium with an appropriate target is sufficient to cause a growth cone to turn (O'Connor, *et al.*,

1990). Treatment with a uniform concentration of netrin-1 increases the number of commissural growth cone filopodia. If continuing the studies reported here, it would be of interest to determine if application of a gradient of netrin-1 to a growth cone causes an asymmetric increase in the number of filopodia. Similarly, determining if a gradient of netrin-1 produced an asymmetric distribution of activated Cdc42, Rac1, and Pak1 within the growth cone would be a valuable extension of the results. This might be accomplished using either a substrate bound graded distribution of netrin-1, or a gradient of soluble netrin-1 puffed from a pipette across a neuronal growth cone.

On the basis of the results presented, the following speculative model for the signal transduction events that induce a growth cone to turn in response to netrin-1 is proposed. A gradient of netrin-1 protein may result in the formation of an asymmetric distribution of DCC across the growth cone (Fig. 2) enriched at the tips of filopodia and the edges of lamellipodia (Fig. 2-a and Fig. 2-b). Stabilization of DCC on the growth cone surface, via an adhesive interaction with extracellular netrin-1 may contribute to generating localized enrichments of DCC. Additionally, insertion of DCC protein from secretory vesicles into the plasma membrane may also contribute to increasing the local concentration of DCC. The graded distribution of DCC bound to netrin-1 across the plasma membrane of the growth cone would then be predicted to produce an asymmetric activation of signal transduction mechanisms that lead to the reorganization of F-actin (Fig. 1B). As such, the presence of a netrin-1 gradient may result in greater polymerization of F-actin on one side of the growth cone (Fig 2-c). The outcome of this would be increased extension of filopodia, lamellipodia and membrane ruffles on one side of the growth cone, generating a leading edge that moves up the netrin-1 gradient.

While the results presented here now allow such a model to be proposed, additional analysis will be required to test the model, and identify the complete mechanism underlying the ability of netrin-1 to turn a growth cone.

Figure 1. Model for netrin-1 signal transduction: intracellular domain of DCC recruits a complex of signaling proteins.

Illustration of our working model of the molecular mechanisms that act downstream of netrin-1/DCC signaling. (A) Nck1 is believed to bind to DCC constitutively via its 1 st and 3 rd SH3 domain. (B) Activation of a GEF leads to the activation of Cdc42 and Rac1, and this leads to activation of Pak1. Here we hypothesize that the GEF may be βPIX. Activated Cdc42 is also able to activate N-WASP, which together with the Arp2/3 complex promotes actin nucleation. Panel C incorporates findings indicating that netrin-1 activates MEK, which then activates ERK. Here we propose that a cascade leading from MEK>ERK>Pak2 may regulate the activtion of βPIX.

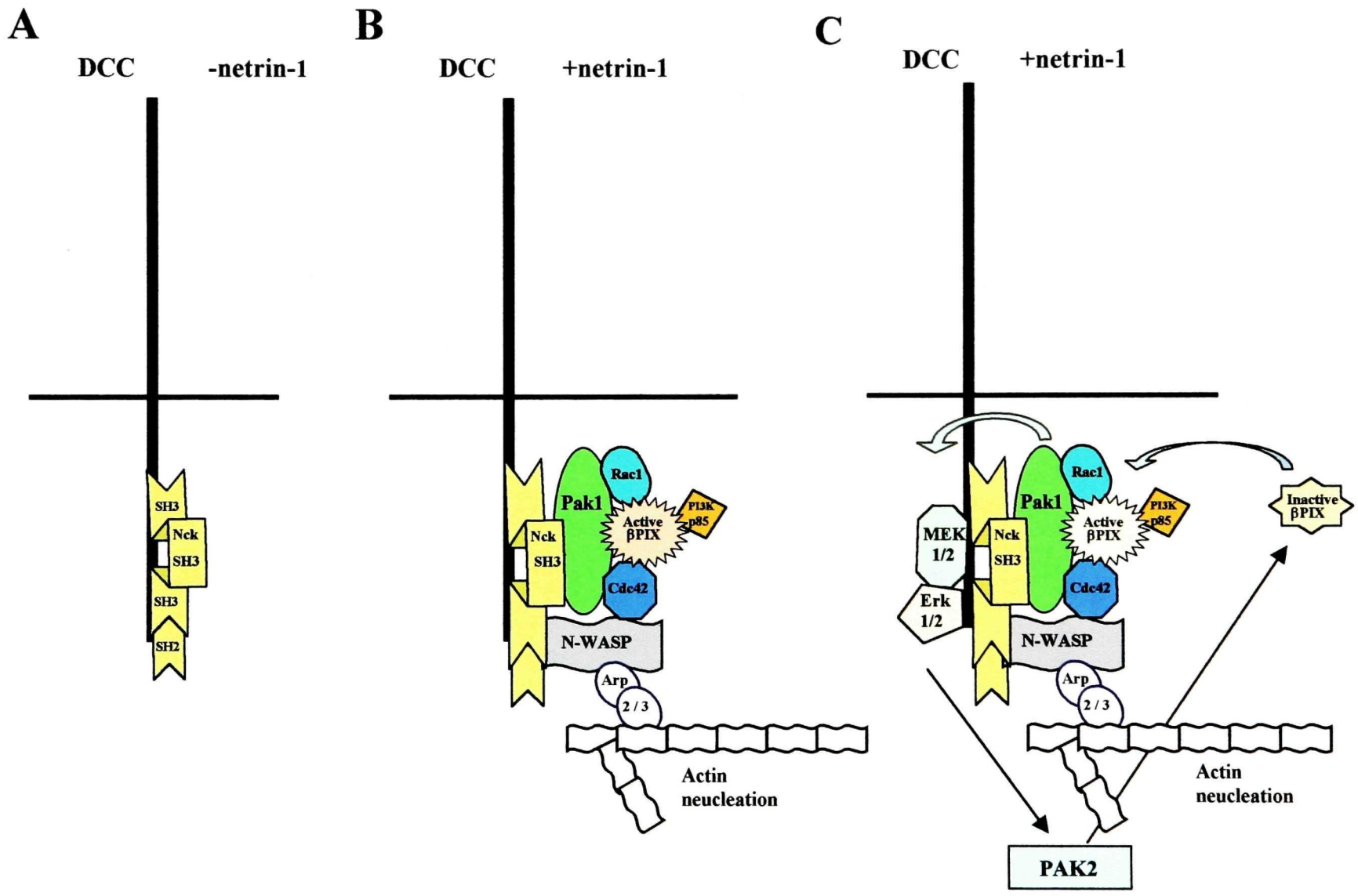
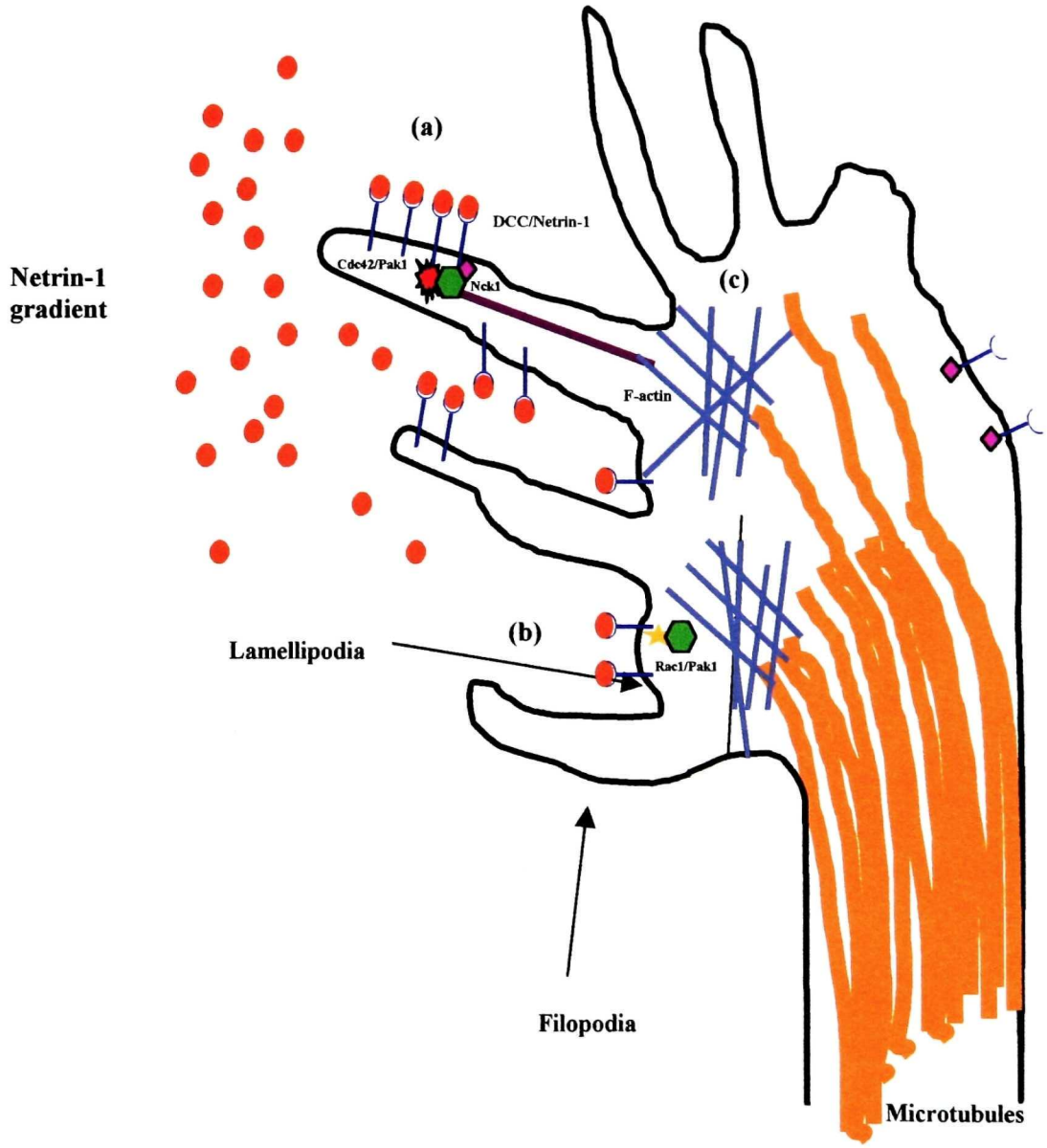

A
DCC
-netrin-1
SH3
Nck
SH3
SH3
SH2
B
DCC
+netrin-1
Rac1
Pak1
Active
βPIX
PI3K
p85
Nck
SH3
Cdc42
N-WASP
Arp
2 / 3
Actin
neucleation
C
DCC
+netrin-1
Rac1
Pak1
Active
βPIX
PI3K
p85
Inactive
βPIX
MEK
1/2
Nck
SH3
Erk
1/2
Cdc42
N-WASP
Arp
2 / 3
Actin
neucleation
PAK2

Figure 2. A gradient of netrin-1 protein may direct the organization of an asymmetric distribution of DCC and the cellular machinery that regulates actin.

Initial contact of a gradient of netrin-1 protein with the tips of filopodia projecting from a neuronal growth cone (a) recruits and stabilizes DCC protein on one side of the growth cone. Netrin-1 signaling through DCC then activates actin polymerization leading to the asymmetrical extension of the actin cytoskeleton, including at the tips of filopodia and lamellipodial membrane extension (b and c). The directed production of actin based membrane extensions leads to directional movement of a growth cone.


Netrin-1
gradient
(a)
DCC/Netrin-1
Cdc42/Pak1
Nck1
(c)
F-actin
(b)
Lamellipodia
Rac1/Pak1
Filopodia
Microtubules

Bibliography

Adams,R.H., Lohrum,M., Klostermann,A., Betz,H., and Puschel,A.W. (1997). The chemorepulsive activity of secreted semaphorins is regulated by furin-dependent proteolytic processing. EMBO J. *16*, 6077-6086.

Awasaki,T., Saito,M., Sone,M., Suzuki,E., Sakai,R., Ito,K., and Hama,C. (2000). The Drosophila trio plays an essential role in patterning of axons by regulating their directional extension. Neuron *26*, 119-131.

Bagnard,D., Lohrum,M., Uziel,D., Puschel,A.W., and Bolz,J. (1998). Semaphorins act as attractive and repulsive guidance signals during the development of cortical projections. Development *125*, 5043-5053.

Bagri,A., Marin,O., Plump,A.S., Mak,J., Pleasure,S.J., Rubenstein,J.L., and Tessier-Lavigne,M. (2002). Slit proteins prevent midline crossing and determine the dorsoventral position of major axonal pathways in the mammalian forebrain. Neuron *33*, 233-248.

Bagrodia,S., Taylor,S.J., Creasy,C.L., Chernoff,J., and Cerione,R.A. (1995). Identification of a mouse p21Cdc42/Rac activated kinase. J. Biol. Chem. *270*, 22731-22737.

Bagrodia,S. and Cerione,R.A. (1999). Pak to the future. Trends Cell Biol. *9*, 350-355.

Baier,H. and Bonhoeffer,F. (1992). Axon guidance by gradients of a target-derived component. Science *255*, 472-475.

Banzai,Y., Miki,H., Yamaguchi,H., and Takenawa,T. (2000). Essential role of neural Wiskott-Aldrich syndrome protein in neurite extension in PC12 cells and rat hippocampal primary culture cells. J. Biol. Chem. *275*, 11987-11992.

Bar-Sagi,D. and Hall,A. (2000). Ras and Rho GTPases: a family reunion. Cell *103*, 227-238.

Bashaw,G.J. and Goodman,C.S. (1999). Chimeric axon guidance receptors: the cytoplasmic domains of slit and netrin receptors specify attraction versus repulsion. Cell *97*, 917-926.

Bashaw,G.J., Kidd,T., Murray,D., Pawson,T., and Goodman,C.S. (2000). Repulsive axon guidance: Abelson and Enabled play opposing roles downstream of the roundabout receptor. Cell *101*, 703-715.

Bashaw,G.J., Hu,H., Nobes,C.D., and Goodman,C.S. (2001). A novel Dbl family RhoGEF promotes Rho-dependent axon attraction to the central nervous system midline in Drosophila and overcomes Robo repulsion. J. Cell Biol. *155*, 1117-1122.

Bateman,J., Shu,H., and Van Vactor,D. (2000). The guanine nucleotide exchange factor trio mediates axonal development in the Drosophila embryo. Neuron *26*, 93-106.

Batlle,E., Henderson,J.T., Beghtel,H., van den Born,M.M., Sancho,E., Huls,G., Meeldijk,J., Robertson,J., van de,W.M., Pawson,T., and Clevers,H. (2002). Beta-catenin and TCF mediate cell positioning in the intestinal epithelium by controlling the expression of EphB/ephrinB. Cell *111*, 251-263.

Bentley,D. and Toroian-Raymond,A. (1986). Disoriented pathfinding by pioneer neurone growth cones deprived of filopodia by cytochalasin treatment. Nature *323*, 712-715.

Bentley,D. and O'Connor,T.P. (1994). Cytoskeletal events in growth cone steering. Curr. Opin. Neurobiol. *4*, 43-48.

Bishop,A.L. and Hall,A. (2000). Rho GTPases and their effector proteins. Biochem. J. *348 Pt 2*, 241-255.

Bokoch,G.M., Wang,Y., Bohl,B.P., Sells,M.A., Quilliam,L.A., and Knaus,U.G. (1996). Interaction of the Nck adapter protein with p21-activated kinase (PAK1). J. Biol. Chem. *271*, 25746-25749.

Bouchard, J-F., Roux, P.P., Shekarabi, M., Barker, P.A., Kennedy, T.E. cAMP elevation rapidly translocates DCC to the plasma membrane and promotes netrin-1 dependent axon extension. Program No. 729.14. *2002 Abstract Viewer/Itinerary Planner*. Washington, DC: Society for Neuroscience, 2002. Online.

Braga,V.M. (2002). GEF without a Dbl domain? Nat. Cell Biol. *4*, E188-E190.

Bray,D. and Chapman,K. (1985). Analysis of microspike movements on the neuronal growth cone. J. Neurosci. *5*, 3204-3213.

Brouns,M.R., Matheson,S.F., and Settleman,J. (2001). p190 RhoGAP is the principal Src substrate in brain and regulates axon outgrowth, guidance and fasciculation. Nat. Cell Biol. *3*, 361-367.

Brugnera,E., Haney,L., Grimsley,C., Lu,M., Walk,S.F., Tosello-Trampont,A.C., Macara,I.G., Madhani,H., Fink,G.R., and Ravichandran,K.S. (2002). Unconventional

Rac-GEF activity is mediated through the Dock180 ELMO complex. Nat. Cell Biol. *4*, 574-582.

Buday,L., Wunderlich,L., and Tamas,P. (2002). The Nck family of adapter proteins. Regulators of actin cytoskeleton. Cell Signal. *14*, 723-731.

Burbelo,P.D., Drechsel,D., and Hall,A. (1995). A conserved binding motif defines numerous candidate target proteins for both Cdc42 and Rac GTPases. J. Biol. Chem. *270*, 29071-29074.

Bustelo,X.R. (2002). Knocked out by Rho/Rac T-cell biology. Histol. Histopathol. *17*, 871-875.

Carter,N., Nakamoto,T., Hirai,H., and Hunter,T. (2002). EphrinA1-induced cytoskeletal re-organization requires FAK and p130(cas). Nat. Cell Biol. *4*, 565-573.

Castellani,V. and Rougon,G. (2002). Control of semaphorin signaling. Curr. Opin. Neurobiol. *12*, 532-541.

Chedotal,A., Del Rio,J.A., Ruiz,M., He,Z., Borrell,V., de Castro,F., Ezan,F., Goodman,C.S., Tessier-Lavigne,M., Sotelo,C., and Soriano,E. (1998). Semaphorins III and IV repel hippocampal axons via two distinct receptors. Development *125*, 4313-4323.

Chen,H., Chedotal,A., He,Z., Goodman,C.S., and Tessier-Lavigne,M. (1997). Neuropilin-2, a novel member of the neuropilin family, is a high affinity receptor for the semaphorins Sema E and Sema IV but not Sema III. Neuron *19*, 547-559.

Chen,Y.Q., Hsieh,J.T., Yao,F., Fang,B., Pong,R.C., Cipriano,S.C., and Krepulat,F. (1999). Induction of apoptosis and G2/M cell cycle arrest by DCC. Oncogene *18*, 2747-2754.

Cheng,H.J., Bagri,A., Yaron,A., Stein,E., Pleasure,S.J., and Tessier-Lavigne,M. (2001). Plexin-A3 mediates semaphorin signaling and regulates the development of hippocampal axonal projections. Neuron *32*, 249-263.

Cherfils,J. and Chardin,P. (1999). GEFs: structural basis for their activation of small GTP-binding proteins. Trends Biochem. Sci. *24*, 306-311.

Chien,C.B., Rosenthal,D.E., Harris,W.A., and Holt,C.E. (1993). Navigational errors made by growth cones without filopodia in the embryonic Xenopus brain. Neuron *11*, 237-251.

Cho,K.R. and Fearon,E.R. (1995). DCC: linking tumor suppressor genes and altered cell surface interactions in cancer? Curr. Opin. Genet. Dev. *5*, 72-78.

Colamarino,S.A. and Tessier-Lavigne,M. (1995). The axonal chemoattractant netrin-1 is also a chemorepellent for trochlear motor axons. Cell *81*, 621-629.

Colavita,A. and Culotti,J.G. (1998). Suppressors of ectopic UNC-5 growth cone steering identify eight genes involved in axon guidance in Caenorhabditis elegans. Dev. Biol. *194*, 72-85.

Coles,L.C. and Shaw,P.E. (2002). PAK1 primes MEK1 for phosphorylation by Raf-1 kinase during cross- cascade activation of the ERK pathway. Oncogene *21*, 2236-2244.

Coniglio,S.J., Jou,T.S., and Symons,M. (2001). Rac1 protects epithelial cells against anoikis. J. Biol. Chem. *276*, 28113-28120.

Contractor,A., Rogers,C., Maron,C., Henkemeyer,M., Swanson,G.T., and Heinemann,S.F. (2002). Trans-synaptic Eph receptor-ephrin signaling in hippocampal mossy fiber LTP. Science *296*, 1864-1869.

Cooke,J.E. and Moens,C.B. (2002). Boundary formation in the hindbrain: Eph only it were simple.. Trends Neurosci. *25*, 260-267.

Corset,V., Nguyen-Ba-Charvet,K.T., Forcet,C., Moyse,E., Chedotal,A., and Mehlen,P. (2000). Netrin-1-mediated axon outgrowth and cAMP production requires interaction with adenosine A2b receptor. Nature *407* , 747-750.

Coso,O.A., Chiariello,M., Yu,J.C., Teramoto,H., Crespo,P., Xu,N., Miki,T., and Gutkind,J.S. (1995). The small GTP-binding proteins Rac1 and Cdc42 regulate the activity of the JNK/SAPK signaling pathway. Cell *81*, 1137-1146.

Culotti,J.G. and Kolodkin,A.L. (1996). Functions of netrins and semaphorins in axon guidance. Curr. Opin. Neurobiol. *6*, 81-88.

Culotti,J.G. and Merz,D.C. (1998). DCC and netrins. Curr. Opin. Cell Biol. *10*, 609-613.

Cutforth,T. and Harrison,C.J. (2002). Ephs and ephrins close ranks. Trends Neurosci. *25*, 332-334.

D'Adamo,P., Menegon,A., Lo,N.C., Grasso,M., Gulisano,M., Tamanini,F., Bienvenu,T., Gedeon,A.K., Oostra,B., Wu,S.K., Tandon,A., Valtorta,F., Balch,W.E., Chelly,J., and

Toniolo,D. (1998). Mutations in GDI1 are responsible for X-linked non-specific mental retardation. Nat. Genet. *19*, 134-139.

Daniels,R.H., Hall,P.S., and Bokoch,G.M. (1998). Membrane targeting of p21-activated kinase 1 (PAK1) induces neurite outgrowth from PC12 cells. EMBO J. *17*, 754-764.

Daniels,R.H. and Bokoch,G.M. (1999). p21-activated protein kinase: a crucial component of morphological signaling? Trends Biochem. Sci. *24*, 350-355.

de Castro,F., Hu,L., Drabkin,H., Sotelo,C., and Chedotal,A. (1999). Chemoattraction and chemorepulsion of olfactory bulb axons by different secreted semaphorins. J. Neurosci. *19*, 4428-4436.

de,l.T., Jr., Hopker,V.H., Ming,G.L., Poo,M.M., Tessier-Lavigne,M., Hemmati-Brivanlou,A., and Holt,C.E. (1997). Turning of retinal growth cones in a netrin-1 gradient mediated by the netrin receptor DCC. Neuron *19*, 1211-1224.

Debant,A., Serra-Pages,C., Seipel,K., O'Brien,S., Tang,M., Park,S.H., and Streuli,M. (1996). The multidomain protein Trio binds the LAR transmembrane tyrosine phosphatase, contains a protein kinase domain, and has separate rac- specific and rho-

specific guanine nucleotide exchange factor domains. Proc. Natl. Acad. Sci. U. S. A *93*, 5466-5471.

Deiner,M.S., Kennedy,T.E., Fazeli,A., Serafini,T., Tessier-Lavigne,M., and Sretavan,D.W. (1997). Netrin-1 and DCC mediate axon guidance locally at the optic disc: loss of function leads to optic nerve hypoplasia. Neuron *19*, 575-589.

Dharmawardhane,S., Sanders,L.C., Martin,S.S., Daniels,R.H., and Bokoch,G.M. (1997). Localization of p21-activated kinase 1 (PAK1) to pinocytic vesicles and cortical actin structures in stimulated cells. J. Cell Biol. *138*, 1265-1278.

Dickson,B.J. (2001). Rho GTPases in growth cone guidance. Curr. Opin. Neurobiol. *11*, 103-110.

Dodd,J., Morton,S.B., Karagogeos,D., Yamamoto,M., and Jessell,T.M. (1988). Spatial regulation of axonal glycoprotein expression on subsets of embryonic spinal neurons. Neuron *1*, 105-116.

Dutting,D., Handwerker,C., and Drescher,U. (1999). Topographic targeting and pathfinding errors of retinal axons following overexpression of ephrinA ligands on retinal ganglion cell axons. Dev. Biol. *216*, 297-311.

Eblen,S.T., Slack,J.K., Weber,M.J., and Catling,A.D. (2002). Rac-PAK signaling stimulates extracellular signal-regulated kinase (ERK) activation by regulating formation of MEK1-ERK complexes. Mol. Cell Biol. *22*, 6023-6033.

Edwards,D.C., Sanders,L.C., Bokoch,G.M., and Gill,G.N. (1999). Activation of LIM-kinase by Pak1 couples Rac/Cdc42 GTPase signalling to actin cytoskeletal dynamics. Nat. Cell Biol. *1*, 253-259.

Engelkamp,D. (2002). Cloning of three mouse Unc5 genes and their expression patterns at mid- gestation. Mech. Dev. *118*, 191-197.

Evan,G.I., Lewis,G.K., Ramsay,G., and Bishop,J.M. (1985). Isolation of monoclonal antibodies specific for human c-myc proto- oncogene product. Mol. Cell Biol. *5*, 3610-3616.

Fazeli,A., Dickinson,S.L., Hermiston,M.L., Tighe,R.V., Steen,R.G., Small,C.G., Stoeckli,E.T., Keino-Masu,K., Masu,M., Rayburn,H., Simons,J., Bronson,R.T., Gordon,J.I., Tessier-Lavigne,M., and Weinberg,R.A. (1997). Phenotype of mice lacking functional Deleted in colorectal cancer (Dcc) gene. Nature *386*, 796-804.

Fearon,E.R., Cho,K.R., Nigro,J.M., Kern,S.E., Simons,J.W., Ruppert,J.M., Hamilton,S.R., Preisinger,A.C., Thomas,G., Kinzler,K.W., and . (1990). Identification of a chromosome 18q gene that is altered in colorectal cancers. Science *247*, 49-56.

Fearon,E.R. (1996). DCC: is there a connection between tumorigenesis and cell guidance molecules? Biochim. Biophys. Acta *1288*, M17-M23.

Forcet,C., Ye,X., Granger,L., Corset,V., Shin,H., Bredesen,D.E., and Mehlen,P. (2001). The dependence receptor DCC (deleted in colorectal cancer) defines an alternative mechanism for caspase activation. Proc. Natl. Acad. Sci. U. S. A *98*, 3416-3421.

Forcet,C., Stein,E., Pays,L., Corset,V., Llambi,F., Tessier-Lavigne,M., and Mehlen,P. (2002). Netrin-1-mediated axon outgrowth requires deleted in colorectal cancer-dependent MAPK activation. Nature *417*, 443-447.

Foster,R., Hu,K.Q., Lu,Y., Nolan,K.M., Thissen,J., and Settleman,J. (1996). Identification of a novel human Rho protein with unusual properties: GTPase deficiency and in vivo farnesylation. Mol. Cell Biol. *16*, 2689-2699.

Frisen,J., Holmberg,J., and Barbacid,M. (1999). Ephrins and their Eph receptors: multitalented directors of embryonic development. EMBO J. *18*, 5159-5165.

Fruman,D.A., Meyers,R.E., and Cantley,L.C. (1998). Phosphoinositide kinases. Annu. Rev. Biochem. *67*, 481-507.

Fujisawa,H. and Kitsukawa,T. (1998). Receptors for collapsin/semaphorins. Curr. Opin. Neurobiol. *8*, 587-592.

Fukata,M. and Kaibuchi,K. (2001). Rho-family GTPases in cadherin-mediated cell-cell adhesion. Nat. Rev. Mol. Cell Biol. *2*, 887-897.

Fukuda,T., Kiuchi,K., and Takahashi,M. (2002). Novel mechanism of regulation of Rac activity and lamellipodia formation by RET tyrosine kinase. J. Biol. Chem. *277*, 19114-19121.

Gardiner,E.M., Pestonjamasp,K.N., Bohl,B.P., Chamberlain,C., Hahn,K.M., and Bokoch,G.M. (2002). Spatial and temporal analysis of Rac activation during live neutrophil chemotaxis. Curr. Biol. *12*, 2029-2034.

Ghose,A. and Van Vactor,D. (2002). GAPs in Slit-Robo signaling. Bioessays *24*, 401-404.

Gilbert, S. F., In Developmental Biology (1997). 5th edition, Sinauer Publications.

Gitai,Z., Yu,T.W., Lundquist,E.A., Tessier-Lavigne,M., and Bargmann,C.I. (2003). The Netrin Receptor UNC-40/DCC Stimulates Axon Attraction and Outgrowth through Enabled and, in Parallel, Rac and UNC-115/AbLIM. Neuron *37*, 53-65.

Gomez,M., Tybulewicz,V., and Cantrell,D.A. (2000). Control of pre-T cell proliferation and differentiation by the GTPase Rac-I. Nat. Immunol. *1*, 348-352.

Guthrie,S. (2001). Axon guidance: Robos make the rules. Curr. Biol. *11*, R300-R303.

Hall,A. (1998). Rho GTPases and the actin cytoskeleton. Science *279*, 509-514.

Hansel,D.E., Quinones,M.E., Ronnett,G.V., and Eipper,B.A. (2001). Kalirin, a GDP/GTP exchange factor of the Dbl family, is localized to nerve, muscle, and endocrine tissue during embryonic rat development. J. Histochem. Cytochem. *49*, 833-844.

Hart, M.J., Eva,A., Evans,T., Aaronson,S.A., and Cerione,R.A. (1991). Catalysis of guanine nucleotide exchange on the CDC42Hs protein by the dbl oncogene product. Nature *354*, 311-314.

He TC, Zhou S, da Costa LT, Yu J, Kinzler KW, Vogelstein B. (1998). A simplified system for generating recombinant adenoviruses. Proc Natl Acad Sci U S A. Mar 3; 95(5): 2509-14.

He, Z. and Tessier-Lavigne,M. (1997). Neuropilin is a receptor for the axonal chemorepellent Semaphorin III. Cell *90*, 739-751.

Hedgecock,E.M., Culotti,J.G., and Hall,D.H. (1990). The unc-5, unc-6, and unc-40 genes guide circumferential migrations of pioneer axons and mesodermal cells on the epidermis in C. elegans. Neuron *4*, 61-85.

Hedrick,L., Cho,K.R., Fearon,E.R., Wu,T.C., Kinzler,K.W., and Vogelstein,B. (1994). The DCC gene product in cellular differentiation and colorectal tumorigenesis. Genes Dev. *8*, 1174-1183.

Heldin,C.H., Ostman,A., and Ronnstrand,L. (1998). Signal transduction via platelet-derived growth factor receptors. Biochim. Biophys. Acta *1378*, F79-113.

Hilgers,W., Song,J.J., Haye,M., Hruban,R.R., Kern,S.E., and Fearon,E.R. (2000). Homozygous deletions inactivate DCC, but not MADH4/DPC4/SMAD4, in a subset of pancreatic and biliary cancers. Genes Chromosomes. Cancer *27*, 353-357.

Hing,H., Xiao,J., Harden,N., Lim,L., and Zipursky,S.L. (1999). Pak functions downstream of Dock to regulate photoreceptor axon guidance in Drosophila. Cell *97*, 853-863.

Hoffman,G.R. and Cerione,R.A. (2002). Signaling to the Rho GTPases: networking with the DH domain. FEBS Lett. *513*, 85-91.

Holmberg, J. and Frisen, J. (2002). Ephrins are not only unattractive. Trends Neurosci. May; 25(5):239-43.

Holmes,G.P., Negus,K., Burridge,L., Raman,S., Algar,E., Yamada,T., and Little,M.H. (1998). Distinct but overlapping expression patterns of two vertebrate slit homologs implies functional roles in CNS development and organogenesis. Mech. Dev. *79*, 57-72.

Hong,K., Hinck,L., Nishiyama,M., Poo,M.M., Tessier-Lavigne,M., and Stein,E. (1999). A ligand-gated association between cytoplasmic domains of UNC5 and DCC family receptors converts netrin-induced growth cone attraction to repulsion. Cell *97*, 927-941.

Hong,K., Nishiyama,M., Henley,J., Tessier-Lavigne,M., and Poo,M. (2000). Calcium signalling in the guidance of nerve growth by netrin-1. Nature *403*, 93-98.

Hornberger,M.R., Dutting,D., Ciossek,T., Yamada,T., Handwerker,C., Lang,S., Weth,F., Huf,J., Wessel,R., Logan,C., Tanaka,H., and Drescher,U. (1999). Modulation of EphA receptor function by coexpressed ephrinA ligands on retinal ganglion cell axons. Neuron *22*, 731-742.

Ikonomov,O.C., Kulesa,M.C., Shisheva,A.C., and Jacob,M.H. (1998). Innervation and target tissue interactions induce Rab-GDP dissociation inhibitor (GDI) expression during peripheral synapse formation in developing chick ciliary ganglion neurons in situ. J. Neurosci. *18*, 6331-6339.

Innocenti,M., Frittoli,E., Ponzanelli,I., Falck,J.R., Brachmann,S.M., Di Fiore,P.P., and Scita,G. (2003). Phosphoinositide 3-kinase activates Rac by entering in a complex with Eps8, Abi1, and Sos-1. J. Cell Biol. *160*, 17-23.

Jaffer,Z.M. and Chernoff,J. (2002). p21-Activated kinases: three more join the Pak. Int. J. Biochem. Cell Biol. *34*, 713-717.

Jalink,K., van Corven,E.J., Hengeveld,T., Morii,N., Narumiya,S., and Moolenaar,W.H. (1994). Inhibition of lysophosphatidate- and thrombin-induced neurite retraction and neuronal cell rounding by ADP ribosylation of the small GTP-binding protein Rho. J. Cell Biol. *126*, 801-810.

Jimenez,C., Portela,R.A., Mellado,M., Rodriguez-Frade,J.M., Collard,J., Serrano,A., Martinez,A., Avila,J., and Carrera,A.C. (2000). Role of the PI3K regulatory subunit in the control of actin organization and cell migration. J. Cell Biol. *151*, 249-262.

Jurney,W.M., Gallo,G., Letourneau,P.C., and McLoon,S.C. (2002). Rac1-Mediated Endocytosis during Ephrin-A2- and Semaphorin 3A-Induced Growth Cone Collapse. J. Neurosci. *22*, 6019-6028.

Kaplan,D., Zirrgiebel,U., and Atwal,J. (2000). Center stage for NGF in peripheral (but not central) sensory neuron outgrowth. Neuron *25*, 253-254.

Katoh,H., Yasui,H., Yamaguchi,Y., Aoki,J., Fujita,H., Mori,K., and Negishi,M. (2000). Small GTPase RhoG is a key regulator for neurite outgrowth in PC12 cells. Mol. Cell Biol. *20*, 7378-7387.

Kaufmann,N., Wills,Z.P., and Van Vactor,D. (1998). Drosophila Rac1 controls motor axon guidance. Development *125*, 453-461.

Keeling,S.L., Gad,J.M., and Cooper,H.M. (1997). Mouse Neogenin, a DCC-like molecule, has four splice variants and is expressed widely in the adult mouse and during embryogenesis. Oncogene *15*, 691-700.

Keino-Masu,K., Masu,M., Hinck,L., Leonardo,E.D., Chan,S.S., Culotti,J.G., and Tessier-Lavigne,M. (1996). Deleted in Colorectal Cancer (DCC) encodes a netrin receptor. Cell *87*, 175-185.

Keleman,K. and Dickson,B.J. (2001). Short- and long-range repulsion by the *Drosophila unc-5* netrin receptor. Neuron *32*, 605-617.

Kennedy,T.E., Serafini,T., de,l.T., Jr., and Tessier-Lavigne,M. (1994). Netrins are diffusible chemotropic factors for commissural axons in the embryonic spinal cord. Cell *78*, 425-435.

Kennedy,T.E. (2000). Cellular mechanisms of netrin function: long-range and short-range actions. Biochem. Cell Biol. *78*, 569-575.

Keshishian,H. and Bentley,D. (1983). Embryogenesis of peripheral nerve pathways in grasshopper legs. I. The initial nerve pathway to the CNS. Dev. Biol. *96*, 89-102.

Kidd,T., Brose,K., Mitchell,K.J., Fetter,R.D., Tessier-Lavigne,M., Goodman,C.S., and Tear,G. (1998). Roundabout controls axon crossing of the CNS midline and defines a novel subfamily of evolutionarily conserved guidance receptors. Cell *92*, 205-215.

Kidd,T., Bland,K.S., and Goodman,C.S. (1999). Slit is the midline repellent for the robo receptor in Drosophila. Cell *96*, 785-794.

Kins,S., Betz,H., and Kirsch,J. (2000). Collybistin, a newly identified brain-specific GEF, induces submembrane clustering of gephyrin. Nat. Neurosci. *3*, 22-29.

Kiosses,W.B., Hood,J., Yang,S., Gerritsen,M.E., Cheresh,D.A., Alderson,N., and Schwartz,M.A. (2002). A dominant-negative p65 PAK peptide inhibits angiogenesis. Circ. Res. *90*, 697-702.

Kitsukawa,T., Shimizu,M., Sanbo,M., Hirata,T., Taniguchi,M., Bekku,Y., Yagi,T., and Fujisawa,H. (1997). Neuropilin-semaphorin III/D-mediated chemorepulsive signals play a crucial role in peripheral nerve projection in mice. Neuron *19*, 995-1005.

Klingelhutz,A.J., Hedrick,L., Cho,K.R., and McDougall,J.K. (1995). The DCC gene suppresses the malignant phenotype of transformed human epithelial cells. Oncogene *10*, 1581-1586.

Knaus,U.G., Heyworth,P.G., Kinsella,B.T., Curnutte,J.T., and Bokoch,G.M. (1992). Purification and characterization of Rac 2. A cytosolic GTP-binding protein that regulates human neutrophil NADPH oxidase. J. Biol. Chem. *267*, 23575-23582.

Koch,M., Murrell,J.R., Hunter,D.D., Olson,P.F., Jin,W., Keene,D.R., Brunken,W.J., and Burgeson,R.E. (2000). A novel member of the netrin family, beta-netrin, shares homology with the beta chain of laminin: identification, expression, and functional characterization. J. Cell Biol. *151* , 221-234.

Kolodkin,A.L. (1998). Semaphorin-mediated neuronal growth cone guidance. Prog. Brain Res. *117*, 115-132.

Kolodziej,P.A., Timpe,L.C., Mitchell,K.J., Fried,S.R., Goodman,C.S., Jan,L.Y., and Jan,Y.N. (1996). frazzled encodes a Drosophila member of the DCC immunoglobulin subfamily and is required for CNS and motor axon guidance. Cell *87*, 197-204.

Kong,X.T., Choi,S.H., Inoue,A., Xu,F., Chen,T., Takita,J., Yokota,J., Bessho,F., Yanagisawa,M., Hanada,R., Yamamoto,K., and Hayashi,Y. (1997). Expression and mutational analysis of the DCC, DPC4, and MADR2/JV18-1 genes in neuroblastoma. Cancer Res. *57*, 3772-3778.

Kozma,R., Ahmed,S., Best,A., and Lim,L. (1995). The Ras-related protein Cdc42Hs and bradykinin promote formation of peripheral actin microspikes and filopodia in Swiss 3T3 fibroblasts. Mol. Cell Biol. *15*, 1942-1952.

Kramer,S.G., Kidd,T., Simpson,J.H., and Goodman,C.S. (2001). Switching repulsion to attraction: changing responses to slit during transition in mesoderm migration. Science *292*, 737-740.

Kraynov,V.S., Chamberlain,C., Bokoch,G.M., Schwartz,M.A., Slabaugh,S., and Hahn,K.M. (2000). Localized Rac activation dynamics visualized in living cells. Science *290*, 333-337.

Kullander,K. and Klein,R. (2002). Mechanisms and functions of eph and ephrin signalling. Nat. Rev. Mol. Cell Biol. *3*, 475-486.

Kunda,P., Paglini,G., Quiroga,S., Kosik,K., and Caceres,A. (2001). Evidence for the involvement of Tiam1 in axon formation. J. Neurosci. *21*, 2361-2372.

Lamarche,N., Tapon,N., Stowers,L., Burbelo,P.D., Aspenstrom,P., Bridges,T., Chant,J., and Hall,A. (1996). Rac and Cdc42 induce actin polymerization and G1 cell cycle progression independently of p65PAK and the JNK/SAPK MAP kinase cascade. Cell *87*, 519-529.

Lamoureux,P., Altun-Gultekin,Z.F., Lin,C., Wagner,J.A., and Heidemann,S.R. (1997). Rac is required for growth cone function but not neurite assembly. J. Cell Sci. *110 (Pt 5)*, 635-641.

Lang,P., Gesbert,F., Delespine-Carmagnat,M., Stancou,R., Pouchelet,M., and Bertoglio,J. (1996). Protein kinase A phosphorylation of RhoA mediates the morphological and functional effects of cyclic AMP in cytotoxic lymphocytes. EMBO J. *15*, 510-519.

Leeuwen,F.N., Kain,H.E., Kammen,R.A., Michiels,F., Kranenburg,O.W., and Collard,J.G. (1997). The guanine nucleotide exchange factor Tiam1 affects neuronal morphology; opposing roles for the small GTPases Rac and Rho. J. Cell Biol. *139*, 797-807.

Lenzen,C., Cool,R.H., Prinz,H., Kuhlmann,J., and Wittinghofer,A. (1998). Kinetic analysis by fluorescence of the interaction between Ras and the catalytic domain of the guanine nucleotide exchange factor Cdc25Mm. Biochemistry *37*, 7420-7430.

Leonardo,E.D., Hinck,L., Masu,M., Keino-Masu,K., Ackerman,S.L., and Tessier-Lavigne,M. (1997). Vertebrate homologues of C. elegans UNC-5 are candidate netrin receptors. Nature *386*, 833-838.

Li,W. and She,H. (2000). The SH2 and SH3 adapter Nck: a two-gene family and a linker between tyrosine kinases and multiple signaling networks. Histol. Histopathol. *15*, 947-955.

Li,W., Fan,J., and Woodley,D.T. (2001). Nck/Dock: an adapter between cell surface receptors and the actin cytoskeleton. Oncogene *20*, 6403-6417.

Liebl,E.C., Forsthoefel,D.J., Franco,L.S., Sample,S.H., Hess,J.E., Cowger,J.A., Chandler,M.P., Shupert,A.M., and Seeger,M.A. (2000). Dosage-sensitive, reciprocal genetic interactions between the Abl tyrosine kinase and the putative GEF trio reveal trio's role in axon pathfinding. Neuron *26*, 107-118.

Llambi,F., Causeret,F., Bloch-Gallego,E., and Mehlen,P. (2001). Netrin-1 acts as a survival factor via its receptors UNC5H and DCC. EMBO J. *20*, 2715-2722.

Lu,Q., Sun,E.E., Klein,R.S., and Flanagan,J.G. (2001). Ephrin-B reverse signaling is mediated by a novel PDZ-RGS protein and selectively inhibits G protein-coupled chemoattraction. Cell *105*, 69-79.

Lu,W., Katz,S., Gupta,R., and Mayer,B.J. (1997). Activation of Pak by membrane localization mediated by an SH3 domain from the adaptor protein Nck. Curr. Biol. *7*, 85-94.

Lu,W. and Mayer,B.J. (1999). Mechanism of activation of Pak1 kinase by membrane localization. Oncogene *18*, 797-806.

Luo,L., Liao,Y.J., Jan,L.Y., and Jan,Y.N. (1994). Distinct morphogenetic functions of similar small GTPases: Drosophila Drac1 is involved in axonal outgrowth and myoblast fusion. Genes Dev. *8*, 1787-1802.

Luo,L., Hensch,T.K., Ackerman,L., Barbel,S., Jan,L.Y., and Jan,Y.N. (1996). Differential effects of the Rac GTPase on Purkinje cell axons and dendritic trunks and spines. Nature *379*, 837-840.

Luo,L. (2000). Rho GTPases in neuronal morphogenesis. Nat. Rev. Neurosci. *1*, 173-180.

Luo,Y., Raible,D., and Raper,J.A. (1993). Collapsin: a protein in brain that induces the collapse and paralysis of neuronal growth cones. Cell *75*, 217-227.

Ma,X.M., Johnson,R.C., Mains,R.E., and Eipper,B.A. (2001). Expression of kalirin, a neuronal GDP/GTP exchange factor of the trio family, in the central nervous system of the adult rat. J. Comp Neurol. *429* , 388-402.

Madaule,P. and Axel,R. (1985). A novel ras-related gene family. Cell *41*, 31-40.

Mallavarapu,A. and Mitchison,T. (1999). Regulated actin cytoskeleton assembly at filopodium tips controls their extension and retraction. J. Cell Biol. *146*, 1097-1106.

Manitt,C., Colicos,M.A., Thompson,K.M., Rousselle,E., Peterson,A.C., and Kennedy,T.E. (2001). Widespread expression of netrin-1 by neurons and oligodendrocytes in the adult mammalian spinal cord. J. Neurosci. *21*, 3911-3922.

Manitt,C. and Kennedy,T.E. (2002). Where the rubber meets the road: netrin expression and function in developing and adult nervous systems. Prog. Brain Res. *137*, 425-442.

Manser,E., Leung,T., Salihuddin,H., Zhao,Z.S., and Lim,L. (1994). A brain serine/threonine protein kinase activated by Cdc42 and Rac1. Nature *367*, 40-46.

Manser,E., Loo,T.H., Koh,C.G., Zhao,Z.S., Chen,X.Q., Tan,L., Tan,I., Leung,T., and Lim,L. (1998). PAK kinases are directly coupled to the PIX family of nucleotide exchange factors. Mol. Cell *1*, 183-192.

Marshall,C. (1999). How do small GTPase signal transduction pathways regulate cell cycle entry? Curr. Opin. Cell Biol. *11*, 732-736.

McIntire,S.L., Garriga,G., White,J., Jacobson,D., and Horvitz,H.R. (1992). Genes necessary for directed axonal elongation or fasciculation in C. elegans. Neuron *8*, 307-322.

Mehlen,P., Rabizadeh,S., Snipas,S.J., Assa-Munt,N., Salvesen,G.S., and Bredesen,D.E. (1998). The DCC gene product induces apoptosis by a mechanism requiring receptor proteolysis. Nature *395*, 801-804.

Meller,N., Irani-Tehrani,M., Kiosses,W.B., Del Pozo,M.A., and Schwartz,M.A. (2002). Zizimin1, a novel Cdc42 activator, reveals a new GEF domain for Rho proteins. Nat. Cell Biol. *4*, 639-647.

Metin,C., Deleglise,D., Serafini,T., Kennedy,T.E., and Tessier-Lavigne,M. (1997). A role for netrin-1 in the guidance of cortical efferents. Development *124*, 5063-5074.

Meyer,G. and Feldman,E.L. (2002). Signaling mechanisms that regulate actin-based motility processes in the nervous system. J. Neurochem. *83*, 490-503.

Meyerhardt,J.A., Caca,K., Eckstrand,B.C., Hu,G., Lengauer,C., Banavali,S., Look,A.T., and Fearon,E.R. (1999). Netrin-1: interaction with deleted in colorectal cancer (DCC) and alterations in brain tumors and neuroblastomas. Cell Growth Differ. *10*, 35-42.

Miki,H., Miura,K., and Takenawa,T. (1996). N-WASP, a novel actin-depolymerizing protein, regulates the cortical cytoskeletal rearrangement in a PIP2-dependent manner downstream of tyrosine kinases. EMBO J. *15*, 5326-5335.

Miki,H., Sasaki,T., Takai,Y., and Takenawa,T. (1998). Induction of filopodium formation by a WASP-related actin- depolymerizing protein N-WASP. Nature *391*, 93-96.

Ming,G., Song,H., Berninger,B., Inagaki,N., Tessier-Lavigne,M., and Poo,M. (1999). Phospholipase C-gamma and phosphoinositide 3-kinase mediate cytoplasmic signaling in nerve growth cone guidance. Neuron *23*, 139-148.

Ming,G.L., Song,H.J., Berninger,B., Holt,C.E., Tessier-Lavigne,M., and Poo,M.M. (1997). cAMP-dependent growth cone guidance by netrin-1. Neuron *19*, 1225-1235.

Ming,G.L., Wong,S.T., Henley,J., Yuan,X.B., Song,H.J., Spitzer,N.C., and Poo,M.M. (2002). Adaptation in the chemotactic guidance of nerve growth cones. Nature *417*, 411-418.

Mitchison,T.J. and Cramer,L.P. (1996). Actin-based cell motility and cell locomotion. Cell *84*, 371-379.

Miyazaki,N., Furuyama,T., Amasaki,M., Sugimoto,H., Sakai,T., Takeda,N., Kubo,T., and Inagaki,S. (1999). Mouse semaphorin H inhibits neurite outgrowth from sensory neurons. Neurosci. Res. *33*, 269-274.

Mueller,B.K. (1999). Growth cone guidance: first steps towards a deeper understanding. Annu. Rev. Neurosci. *22*, 351-388.

Mullins,R.D. (2000). How WASP-family proteins and the Arp2/3 complex convert intracellular signals into cytoskeletal structures. Curr. Opin. Cell Biol. *12*, 91-96.

Myers,P.Z. and Bastiani,M.J. (1993). Growth cone dynamics during the migration of an identified commissural growth cone. J. Neurosci. *13*, 127-143.

Na,S., Li,B., Grewal,I.S., Enslen,H., Davis,R.J., Hanke,J.H., and Flavell,R.A. (1999). Expression of activated CDC42 induces T cell apoptosis in thymus and peripheral lymph organs via different pathways. Oncogene *18*, 7966-7974.

Nakashiba,T., Ikeda,T., Nishimura,S., Tashiro,K., Honjo,T., Culotti,J.G., and Itohara,S. (2000). Netrin-G1: a novel glycosyl phosphatidylinositol-linked mammalian netrin that is functionally divergent from classical netrins. J. Neurosci. *20*, 6540-6550.

Nakashiba,T., Nishimura,S., Ikeda,T., and Itohara,S. (2002). Complementary expression and neurite outgrowth activity of netrin-G subfamily members. Mech. Dev. *111*, 47-60.

Nelson,P., Christian,C., and Nirenberg,M. (1976). Synapse formation between clonal neuroblastoma X glioma hybrid cells and striated muscle cells. Proc. Natl. Acad. Sci. U. S. A *73*, 123-127.

Newsome,T.P., Schmidt,S., Dietzl,G., Keleman,K., Asling,B., Debant,A., and Dickson,B.J. (2000). Trio combines with dock to regulate Pak activity during photoreceptor axon pathfinding in Drosophila. Cell *101*, 283-294.

Nobes,C.D. and Hall,A. (1995). Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. Cell *81*, 53-62.

O'Connor,T.P., Duerr,J.S., and Bentley,D. (1990). Pioneer growth cone steering decisions mediated by single filopodial contacts in situ. J. Neurosci. *10*, 3935-3946.

Olofsson,B. (1999). Rho guanine dissociation inhibitors: pivotal molecules in cellular signalling. Cell Signal. *11*, 545-554.

Otsuka,A.J., Franco,R., Yang,B., Shim,K.H., Tang,L.Z., Zhang,Y.Y., Boontrakulpoontawee,P., Jeyaprakash,A., Hedgecock,E., Wheaton,V.I., and . (1995). An ankyrin-related gene (unc-44) is necessary for proper axonal guidance in Caenorhabditis elegans. J. Cell Biol. *129*, 1081-1092.

Papakonstanti,E.A. and Stournaras,C. (2002). Association of PI-3 Kinase with PAK1 Leads to Actin Phosphorylation and Cytoskeletal Reorganization. Mol. Biol. Cell *13*, 2946-2962.

Paterson,H.F., Self,A.J., Garrett,M.D., Just,I., Aktories,K., and Hall,A. (1990). Microinjection of recombinant p21rho induces rapid changes in cell morphology. J. Cell Biol. *111*, 1001-1007.

Penzes,P., Johnson,R.C., Alam,M.R., Kambampati,V., Mains,R.E., and Eipper,B.A. (2000). An isoform of kalirin, a brain-specific GDP/GTP exchange factor, is enriched in the postsynaptic density fraction. J. Biol. Chem. *275*, 6395-6403.

Penzes,P., Johnson,R.C., Sattler,R., Zhang,X., Huganir,R.L., Kambampati,V., Mains,R.E., and Eipper,B.A. (2001). The neuronal Rho-GEF Kalirin-7 interacts with PDZ domain-containing proteins and regulates dendritic morphogenesis. Neuron *29*, 229-242.

Penzes,P., Johnson,R.C., Kambampati,V., Mains,R.E., and Eipper,B.A. (2001). Distinct roles for the two Rho GDP/GTP exchange factor domains of kalirin in regulation of neurite growth and neuronal morphology. J. Neurosci. *21*, 8426-8434.

Przyborski,S.A., Knowles,B.B., and Ackerman,S.L. (1998). Embryonic phenotype of Unc5h3 mutant mice suggests chemorepulsion during the formation of the rostral cerebellar boundary. Development *125*, 41-50.

Rajagopalan,S., Vivancos,V., Nicolas,E., and Dickson,B.J. (2000). Selecting a longitudinal pathway: Robo receptors specify the lateral position of axons in the Drosophila CNS. Cell *103*, 1033-1045.

Ramón y Cajal, S. La rétine des vertébrés. IX, 119-258. 1893. La cellule.

Ramón y Cajal, S. Sobre la aparición de las expansiones celulares en la médula embrionaria. 12, 413-419. 1980. Gaceta Sanitaria de Barcelona.

Reale,M.A., Hu,G., Zafar,A.I., Getzenberg,R.H., Levine,S.M., and Fearon,E.R. (1994). Expression and alternative splicing of the deleted in colorectal cancer (DCC) gene in normal and malignant tissues. Cancer Res. *54*, 4493-4501.

Reale,M.A., Reyes-Mugica,M., Pierceall,W.E., Rubinstein,M.C., Hedrick,L., Cohn,S.L., Nakagawara,A., Brodeur,G.M., and Fearon,E.R. (1996). Loss of DCC expression in neuroblastoma is associated with disease dissemination. Clin. Cancer Res. *2*, 1097-1102.

Reyes-Mugica,M., Rieger-Christ,K., Ohgaki,H., Ekstrand,B.C., Helie,M., Kleinman,G., Yahanda,A., Fearon,E.R., Kleihues,P., and Reale,M.A. (1997). Loss of DCC expression and glioma progression. Cancer Res. *57*, 382-386.

Reyes-Mugica,M., Lin,P., Yokota,J., and Reale,M.A. (1998). Status of deleted in colorectal cancer gene expression correlates with neuroblastoma metastasis. Lab Invest *78*, 669-675.

Ridley,A.J., Paterson,H.F., Johnston,C.L., Diekmann,D., and Hall,A. (1992). The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. Cell *70*, 401-410.

Ridley,A.J. and Hall,A. (1992). The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. Cell *70*, 389-399.

Ridley,A.J. (1996). Rho: theme and variations. Curr. Biol. *6*, 1256-1264.

Ridley,A.J. (2001). Rho family proteins: coordinating cell responses. Trends Cell Biol. *11*, 471-477.

Ridley,A.J. (2001). Rho proteins: linking signaling with membrane trafficking. Traffic. *2*, 303-310.

Rodgers,E.E. and Theibert,A.B. (2002). Functions of PI 3-kinase in development of the nervous system. Int. J. Dev. Neurosci. *20*, 187-197.

Rohatgi,R., Nollau,P., Ho,H.Y., Kirschner,M.W., and Mayer,B.J. (2001). Nck and phosphatidylinositol 4,5-bisphosphate synergistically activate actin polymerization through the N-WASP-Arp2/3 pathway. J. Biol. Chem. *276*, 26448-26452.

Rohm,B., Rahim,B., Kleiber,B., Hovatta,I., and Puschel,A.W. (2000). The semaphorin 3A receptor may directly regulate the activity of small GTPases. FEBS Lett. *486*, 68-72.

Roskies,A.L. (1998). Dissecting semaphorin signaling. Neuron *21*, 935-936.

Ruchhoeft,M.L., Ohnuma,S., McNeill,L., Holt,C.E., and Harris,W.A. (1999). The neuronal architecture of Xenopus retinal ganglion cells is sculpted by rho-family GTPases in vivo. J. Neurosci. *19*, 8454-8463.

Sander,E.E., van Delft,S., ten Klooster,J.P., Reid,T., van der Kammen,R.A., Michiels,F., and Collard,J.G. (1998). Matrix-dependent Tiam1/Rac signaling in epithelial cells promotes either cell-cell adhesion or cell migration and is regulated by phosphatidylinositol 3-kinase. J. Cell Biol. *143*, 1385-1398.

Sanders,L.C., Matsumura,F., Bokoch,G.M., and de Lanerolle,P. (1999). Inhibition of myosin light chain kinase by p21-activated kinase. Science *283*, 2083-2085.

Schmidt,A. and Hall,M.N. (1998). Signaling to the actin cytoskeleton. Annu. Rev. Cell Dev. Biol. *14*, 305-338.

Schmitz,A.A., Govek,E.E., Bottner,B., and Van Aelst,L. (2000). Rho GTPases: signaling, migration, and invasion. Exp. Cell Res. *261*, 1-12.

Schmucker,D. and Zipursky,S.L. (2001). Signaling downstream of eph receptors and ephrin ligands. Cell *105*, 701-704.

Schmucker,D. and Zipursky,S.L. (2001). Signaling downstream of Eph receptors and ephrin ligands. Cell *105*, 701-704.

Seeger,M., Tear,G., Ferres-Marco,D., and Goodman,C.S. (1993). Mutations affecting growth cone guidance in Drosophila: genes necessary for guidance toward or away from the midline. Neuron *10*, 409-426.

Sells,M.A., Boyd,J.T., and Chernoff,J. (1999). p21-activated kinase 1 (Pak1) regulates cell motility in mammalian fibroblasts. J. Cell Biol. *145*, 837-849.

Sells,M.A., Pfaff,A., and Chernoff,J. (2000). Temporal and spatial distribution of activated Pak1 in fibroblasts. J. Cell Biol. *151*, 1449-1458.

Serafini,T., Kennedy,T.E., Galko,M.J., Mirzayan,C., Jessell,T.M., and Tessier-Lavigne,M. (1994). The netrins define a family of axon outgrowth-promoting proteins homologous to C. elegans UNC-6. Cell *78*, 409-424.

Serafini,T., Colamarino,S.A., Leonardo,E.D., Wang,H., Beddington,R., Skarnes,W.C., and Tessier-Lavigne,M. (1996). Netrin-1 is required for commissural axon guidance in the developing vertebrate nervous system. Cell *87*, 1001-1014.

Shamah,S.M., Lin,M.Z., Goldberg,J.L., Estrach,S., Sahin,M., Hu,L., Bazalakova,M., Neve,R.L., Corfas,G., Debant,A., and Greenberg,M.E. (2001). EphA receptors regulate growth cone dynamics through the novel guanine nucleotide exchange factor ephexin. Cell *105*, 233-244.

Shekarabi,M., Bourbonniere,M., Dagenais,A., and Nalbantoglu,J. (1997). Transcriptional regulation of amyloid precursor protein during dibutyryl cyclic AMP-induced differentiation of NG108-15 cells. J. Neurochem. *68*, 970-978.

Shekarabi,M. and Kennedy,T.E. (2002). The netrin-1 receptor DCC promotes filopodia formation and cell spreading by activating Cdc42 and Rac1. Mol. Cell Neurosci. *19*, 1-17.

Shirasaki,R., Mirzayan,C., Tessier-Lavigne,M., and Murakami,F. (1996). Guidance of circumferentially growing axons by netrin-dependent and - independent floor plate chemotropism in the vertebrate brain. Neuron *17*, 1079-1088.

Shu,T., Valentino,K.M., Seaman,C., Cooper,H.M., and Richards,L.J. (2000). Expression of the netrin-1 receptor, deleted in colorectal cancer (DCC), is largely confined to projecting neurons in the developing forebrain. J. Comp Neurol. *416*, 201-212.

Song,H., Ming,G., He,Z., Lehmann,M., McKerracher,L., Tessier-Lavigne,M., and Poo,M. (1998). Conversion of neuronal growth cone responses from repulsion to attraction by cyclic nucleotides. Science *281*, 1515-1518.

Song,H. and Poo,M. (2001). The cell biology of neuronal navigation. Nat. Cell Biol. *3*, E81-E88.

Song,H.J., Ming,G.L., and Poo,M.M. (1997). cAMP-induced switching in turning direction of nerve growth cones. Nature *388*, 275-279.

Sperry, R. W. Chemoaffinity in the orderly growth of nerve fiber patterns and connections. 50, 703-710. 1963. Pron. Natl. Acad. Sci. USA.

Stein,E., Savaskan,N.E., Ninnemann,O., Nitsch,R., Zhou,R., and Skutella,T. (1999). A role for the Eph ligand ephrin-A3 in entorhino-hippocampal axon targeting. J. Neurosci. *19*, 8885-8893.

Stein,E., Zou,Y., Poo,M., and Tessier-Lavigne,M. (2001). Binding of DCC by netrin-1 to mediate axon guidance independent of adenosine A2B receptor activation. Science *291*, 1976-1982.

Stein,E. and Tessier-Lavigne,M. (2001). Hierarchical organization of guidance receptors: silencing of netrin attraction by slit through a Robo/DCC receptor complex. Science *291*, 1928-1938.

Stephens,L., Ellson,C., and Hawkins,P. (2002). Roles of PI3Ks in leukocyte chemotaxis and phagocytosis. Curr. Opin. Cell Biol. *14*, 203-213.

Steven,R., Kubiseski,T.J., Zheng,H., Kulkarni,S., Mancillas,J., Ruiz,M.A., Hogue,C.W., Pawson,T., and Culotti,J. (1998). UNC-73 activates the Rac GTPase and is required for cell and growth cone migrations in C. elegans. Cell *92*, 785-795.

Stork, P. J. and Schmitt, J. M. Crosstalk between cAMP and MAP kinase signaling in the regulation of cell proliferation. Trends Cell Biol. 12[6], 258-266. 2002.

Suetsugu,S., Hattori,M., Miki,H., Tezuka,T., Yamamoto,T., Mikoshiba,K., and Takenawa,T. (2002). Sustained Activation of N-WASP through Phosphorylation Is Essential for Neurite Extension. Dev. Cell *3*, 645-658.

Suter,D.M. and Forscher,P. (1998). An emerging link between cytoskeletal dynamics and cell adhesion molecules in growth cone guidance. Curr. Opin. Neurobiol. *8*, 106-116.

Suter,D.M. and Forscher,P. (2000). Substrate-cytoskeletal coupling as a mechanism for the regulation of growth cone motility and guidance. J. Neurobiol. *44*, 97-113.

Suzuki,T., Miki,H., Takenawa,T., and Sasakawa,C. (1998). Neural Wiskott-Aldrich syndrome protein is implicated in the actin- based motility of Shigella flexneri. EMBO J. *17*, 2767-2776.

Swiercz,J.M., Kuner,R., Behrens,J., and Offermanns,S. (2002). Plexin-B1 Directly Interacts with PDZ-RhoGEF/LARG to Regulate RhoA and Growth Cone Morphology. Neuron *35*, 51-63.

Symons,M. (2000). Adhesion signaling: PAK meets Rac on solid ground. Curr. Biol. *10*, R535-R537.

Takenawa,T. and Miki,H. (2001). WASP and WAVE family proteins: key molecules for rapid rearrangement of cortical actin filaments and cell movement. J. Cell Sci. *114*, 1801-1809.

Tamagnone,L., Artigiani,S., Chen,H., He,Z., Ming,G.I., Song,H., Chedotal,A., Winberg,M.L., Goodman,C.S., Poo,M., Tessier-Lavigne,M., and Comoglio,P.M. (1999). Plexins are a large family of receptors for transmembrane, secreted, and GPI-anchored semaphorins in vertebrates. Cell *99*, 71-80.

Tanabe,K., Tachibana,T., Yamashita,T., Che,Y.H., Yoneda,Y., Ochi,T., Tohyama,M., Yoshikawa,H., and Kiyama,H. (2000). The small GTP-binding protein TC10 promotes nerve elongation in neuronal cells, and its expression is induced during nerve regeneration in rats. J. Neurosci. *20*, 4138-4144.

Tanaka,E. and Sabry,J. (1995). Making the connection: cytoskeletal rearrangements during growth cone guidance. Cell *83*, 171-176.

Tanaka,K., Oshimura,M., Kikuchi,R., Seki,M., Hayashi,T., and Miyaki,M. (1991). Suppression of tumorigenicity in human colon carcinoma cells by introduction of normal chromosome 5 or 18. Nature *349*, 340-342.

Taniguchi,M., Yuasa,S., Fujisawa,H., Naruse,I., Saga,S., Mishina,M., and Yagi,T. (1997). Disruption of semaphorin III/D gene causes severe abnormality in peripheral nerve projection. Neuron *19*, 519-530.

Tear,G., Harris,R., Sutaria,S., Kilomanski,K., Goodman,C.S., and Seeger,M.A. (1996). commissureless controls growth cone guidance across the CNS midline in Drosophila and encodes a novel membrane protein. Neuron *16*, 501-514.

Tessier-Lavigne,M., Placzek,M., Lumsden,A.G., Dodd,J., and Jessell,T.M. (1988). Chemotropic guidance of developing axons in the mammalian central nervous system. Nature *336*, 775-778.

Tessier-Lavigne,M. and Goodman,C.S. (1996). The molecular biology of axon guidance. Science *274*, 1123-1133.

Thiagalingam,S., Lengauer,C., Leach,F.S., Schutte,M., Hahn,S.A., Overhauser,J., Willson,J.K., Markowitz,S., Hamilton,S.R., Kern,S.E., Kinzler,K.W., and Vogelstein,B.

(1996). Evaluation of candidate tumour suppressor genes on chromosome 18 in colorectal cancers. Nat. Genet. *13*, 343-346.

Truong,K. and Ikura,M. (2001). The use of FRET imaging microscopy to detect protein-protein interactions and protein conformational changes in vivo. Curr. Opin. Struct. Biol. *11*, 573-578.

Tsai,H.H. and Miller,R.H. (2002). Glial cell migration directed by axon guidance cues. Trends Neurosci. *25*, 173-175.

Van Aelst,L. and D'Souza-Schorey,C. (1997). Rho GTPases and signaling networks. Genes Dev. *11*, 2295-2322.

Vielmetter,J., Kayyem,J.F., Roman,J.M., and Dreyer,W.J. (1994). Neogenin, an avian cell surface protein expressed during terminal neuronal differentiation, is closely related to the human tumor suppressor molecule deleted in colorectal cancer. J. Cell Biol. *127*, 2009-2020.

Vikis,H.G., Li,W., and Guan,K.L. (2002). The plexin-B1/Rac interaction inhibits PAK activation and enhances Sema4D ligand binding. Genes Dev. *16*, 836-845.

Wadsworth,W.G. (2002). Moving around in a worm: netrin UNC-6 and circumferential axon guidance in C. elegans. Trends Neurosci. *25*, 423-429.

Wang,H., Copeland,N.G., Gilbert,D.J., Jenkins,N.A., and Tessier-Lavigne,M. (1999). Netrin-3, a mouse homolog of human NTN2L, is highly expressed in sensory ganglia and shows differential binding to netrin receptors. J. Neurosci. *19*, 4938-4947.

Wear,M.A., Schafer,D.A., and Cooper,J.A. (2000). Actin dynamics: assembly and disassembly of actin networks. Curr. Biol. *10*, R891-R895.

Whitford,K.L., Marillat,V., Stein,E., Goodman,C.S., Tessier-Lavigne,M., Chedotal,A., and Ghosh,A. (2002). Regulation of cortical dendrite development by Slit-Robo interactions. Neuron *33*, 47-61.

Winberg,M.L., Noordermeer,J.N., Tamagnone,L., Comoglio,P.M., Spriggs,M.K., Tessier-Lavigne,M., and Goodman,C.S. (1998). Plexin A is a neuronal semaphorin receptor that controls axon guidance. Cell *95*, 903-916.

Wong,J.T., Wong,S.T., and O'Connor,T.P. (1999). Ectopic semaphorin-1a functions as an attractive guidance cue for developing peripheral neurons. Nat. Neurosci. *2*, 798-803.

Wong,K., Ren,X.R., Huang,Y.Z., Xie,Y., Liu,G., Saito,H., Tang,H., Wen,L., Brady-Kalnay,S.M., Mei,L., Wu,J.Y., Xiong,W.C., and Rao,Y. (2001). Signal transduction in neuronal migration: roles of GTPase activating proteins and the small GTPase Cdc42 in the Slit-Robo pathway. Cell *107*, 209-221.

Wood,W. and Martin,P. (2002). Structures in focus--filopodia. Int. J. Biochem. Cell Biol. *34*, 726-730.

Wu,D., Huang,C.K., and Jiang,H. (2000). Roles of phospholipid signaling in chemoattractant-induced responses. J. Cell Sci. *113 (Pt 17)*, 2935-2940.

Yin,Y., Sanes,J.R., and Miner,J.H. (2000). Identification and expression of mouse netrin-4. Mech. Dev. *96*, 115-119.

Yu,H.H. and Kolodkin,A.L. (1999). Semaphorin signaling: a little less per-plexin. Neuron *22*, 11-14.

Yu,T.W. and Bargmann,C.I. (2001). Dynamic regulation of axon guidance. Nat. Neurosci. *4 Suppl*, 1169-1176.

Yuan,W., Zhou,L., Chen,J.H., Wu,J.Y., Rao,Y., and Ornitz,D.M. (1999). The mouse SLIT family: secreted ligands for ROBO expressed in patterns that suggest a role in morphogenesis and axon guidance. Dev. Biol. *212*, 290-306.

Yuan,X.B., Jin,M., Xu,X., Song,Y.Q., Wu,C.P., Poo,M.M., and Duan,S. (2003). Signalling and crosstalk of Rho GTPases in mediating axon guidance. Nat. Cell Biol. *5*, 38-45.

Yue, Y., Su, J., Cerretti, D.P., Fox, G.M., Jing, S., and Zhou, R. (1999). Selective inhibition of spinal cord neurite outgrowth and cell survival by the Eph family ligand ephrin-A5. J. Neurosci. *19*, 10026-10035.

Zallen, J.A., Yi, B.A., and Bargmann, C.I. (1998). The conserved immunoglobulin superfamily member SAX-3/Robo directs multiple aspects of axon guidance in C. elegans. Cell *92*, 217-227.

Zheng, J.Q., Wan, J.J., and Poo, M.M. (1996). Essential role of filopodia in chemotropic turning of nerve growth cone induced by a glutamate gradient. J. Neurosci. *16*, 1140-1149.

Zheng, J.Q. (2000). Turning of nerve growth cones induced by localized increases in intracellular calcium ions. Nature *403*, 89-93.

Zheng, Y., Zangrilli, D., Cerione, R.A., and Eva, A. (1996). The pleckstrin homology domain mediates transformation by oncogenic dbl through specific intracellular targeting. J. Biol. Chem. *271*, 19017-19020.

Zigmond, S.H. (2000). How WASP regulates actin polymerization. J. Cell Biol. *150*, F117-F120.

Zipkin, I.D., Kindt, R.M., and Kenyon, C.J. (1997). Role of a new Rho family member in cell migration and axon guidance in C. elegans. Cell *90*, 883-894.

Zou, Y., Stoeckli, E., Chen, H., and Tessier-Lavigne, M. (2000). Squeezing axons out of the gray matter: a role for slit and semaphorin proteins from midline and ventral spinal cord. Cell *102*, 363-375.

Zugasti, O., Rul, W., Roux, P., Peyssonnaux, C., Eychene, A., Franke, T.F., Fort, P., and Hibner, U. (2001). Raf-MEK-Erk cascade in anoikis is controlled by Rac1 and Cdc42 via Akt. Mol. Cell Biol. *21*, 6706-6717.