**Signaling Mechanisms that Regulate Cytoskeletal Organization Downstream of Netrin-1 Mediated Axonal Chemoattraction**

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I would like to dedicate this thesis to my son Liam.

This thesis is not much but the beginning of a search for knowledge. You were born out of love while writing it. I hope you grow up to be a loving man, with a thirst for knowledge.

## CONTRIBUTIONS OF AUTHORS

This thesis is a manuscript-based structure. According to the faculty regulations, manuscripts co-authored by others must be accompanied with an explicit statement as to who contributed to such work and to what extend.

### Chapter 2:

I developed the rationale, performed all the experiments and developed the figures described in the chapter two entitled “An unbiased mass spectrometry analysis in search of DCC associated protein complexes downstream of Netrin in commissural neurons”. The mass spectrometry analysis was performed by the Mass Spectrometry Unit at McGill University.

### Chapter 3:

I developed the rationale, performed the experiments and developed the figures for chapter three titled “A complex of DCC and Cool-1/β-Pix, a Cdc42 and Rac specific GEF, is required for netrin-1 dependent filopodia formation by neuronal growth cones and spinal commissural axon chemoattraction to the floor plate.” With the exception of the following:

1. **Dr. K. Adam Baker** performed some of the immunofluorescence staining in Figure 3-1 and contributed to some of the blinded quantifications in Figure 2-4.

2. **Karen Lai Wing Sun** performed the RT-PCR in figure 3-1.
3. **Dr. Masoud Shekarabi** performed the PI3K experiments presented in figure 3-8.
4. **Dr. Arthur Kania** provided the eggs, incubator and facilities to perform the chick electroporation experiments in figures 3-4 through 3-6. He also trained and provided me with invaluable expertise in the procedure.
5. **Dr. Timothy E. Kennedy** co-wrote manuscript.

Additional publications resulting from work during this thesis:

**Appendix I:** "A neuromodulatory role for sonic hedgehog signaling via smoothened: Regulation of commissural axon guidance by inhibiting DCC recruitment to the plasma membrane." Bouchard JF, Rodrigues S, Baker KA, and Kennedy TE. submitted to Development.

I performed the experiments, developed the figure and contributed the text in the Materials and Methods, and Results section corresponding to figure 6. I also co-edited the article. This article was submitted to the journal Development and is currently under review.

**Appendix II:** "Rb/E2F regulates expression of neogenin during neuronal migration." Andrusiak MG, McClellan KA, Dugal-Tessier D, Julian LM, Rodrigues SP, Park DS, Kennedy TE and Slack RS; Molecular and Cellular Biology, 2010.

I trained, provided expertise and contributed to the in vitro culture of spinal cord outgrowth explants experiments included in the paper.

**ABSTRACT**

The development of the nervous system is highly dependent on the differentiation of various neuronal and glial cell populations, efficient targeting of axons, establishment of neuronal connections and continuous branching/pruning and establishment of new connections. These processes are possible through migration, adhesion and cytoskeletal rearrangement processes that are common in most organogenesis. The extracellular matrix and guidance molecules interact with cell surface receptors that transduce signaling mechanisms intracellularly allowing for these cellular responses to take place.

Netrins are bifunctional chemotropic cues that attract or repel different classes of axons by signaling through the netrin receptors Deleted in Colorectal Cancer (DCC) and the UNC5 homologues (UNC5a, b, c and d) during the development of the nervous system. This family of chemotropic molecules is a member of the laminin superfamily. Similar to laminin-integrin signaling, netrin-1's interaction with its receptor DCC results in the activation of the Rho GTPases Rac and Cdc42 and the promotion of a signaling cascade that leads to growth cone cytoskeletal and membrane remodeling. This thesis focused on further identifying the signaling molecules and complexes downstream of DCC that are required for netrin-1 mediated commissural neuron axon chemoattraction.

In the first part of this thesis we have used an unbiased mass spectrometry approach to identify signaling molecules complexed to DCC

in commissural neurons isolated from E12/13 spinal cord. From the mass spectrometry data obtained we chose to characterize Arp2/3 and 14-3-3, both molecules known to play an important role in actin cytoskeleton remodeling. Arp2/3 co-immunoprecipitated with DCC in commissural neuron in a netrin-1-dependent manner. We further established that netrin-1 mediated commissural growth cone expansion and filopodia remodeling is blocked by wiskostatin, a potent chemical inhibitor of N-WASP activity towards the Arp2/3 complex. The treatment of the neurons with wiskostatin also inhibited netrin-dependent externalization of DCC. These results suggest that actin polymerization resulting from N-WASP/Arp2/3 complexes may be required to maintain DCC cell surface recycling during netrin-1 mediated axon guidance. The 14-3-3 epsilon adaptor protein was also identified as a DCC associated protein. However, its association with DCC decreased with the addition of netrin-1. The inhibition of 14-3-3 function using a function-blocking peptide resulted in the collapse of commissural growth cones which could not be recovered by the addition of netrin-1.

In the second part of this thesis we identified β-Pix as a potential GEF downstream of DCC involved in the activation and propagation of Rac-1 and Cdc42 signaling. We found that a β-Pix/Git complex associates with DCC in commissural neurons. β-Pix is highly expressed in the developing spinal cord in various neuronal subpopulations. In commissural neurons β-Pix protein is found in the protruding membranes of the growth cone.

Additionally functional assays using the overexpression of β-Pix mutants showed that β-Pix complexes and association with Pak are important steps in the maintenance of netrin-1 induced changes in growth cone morphology, in commissural axon extension to the midline, and for cortical neuron branching. Using modified GTPase assays we further demonstrated that β-Pix is required for Cdc42 and Rac-1 activation downstream of netrin-1 and is associated with the GTP-bound GTPases. β-Pix may associate with the GTPases by binding to Pak, or alternatively, may bind directly, as has been demonstrated for the related protein α-Pix, resulting in allosteric activation of the GTPase. These results support a novel model, in which β-Pix/Git complexes play an integral part in the activation of Rac-1 and Cdc42 downstream of DCC.

These findings provide novel insight into the signaling events activated by netrin-1 downstream of DCC during commissural axon guidance. They have also identified new questions related to how signal transduction mechanisms activated by netrin-1 regulate the cytoskeleton of the axonal growth cone.

**RESUME**

Le développement du système nerveux dépend en grande proportion en la différentiation de différentes populations de cellules neuronales et gliales, du guidage efficace des axones, de l'établissement de connexions neuronales et de ramification et élagage de ces nouvelles connexions. Ces procès sont possibles grâce à la migration, adhésion et réarrangement du cytosquelette, des mécanismes communs à l'organogénèse. La matrice extracellulaire et les molécules de guidage interagissent avec des récepteurs de surface qui effectuent la transduction des signaux au niveau intracellulaire, permettant l'occurrence de ces réponses cellulaires.

Les Netrines sont des signaux chimiotropiques qui attirent ou bien repoussent différentes classes d'axones, par l'intermédiaire du récepteur Deleted in Colorectal Cancer (DCC) et des récepteurs UNC5 (UNC5a, b, c et d) pendant le dévelopement du système nerveux. Cette famille de molécules chimiotropiques partage une haute homologie avec la famille des laminines. De façon analogue à la signalisation laminine-intégrine, l'intéraction de la Netrine avec son récepteur DCC mène à l'activation des Rho-GTPases Rac-1 et Cdc42 et d'une cascade de signalisation qui mène au remodèlement du cytosquelète et de la membrane du cône de croissance. Cette thèse se concentre sur l'identification plus approfondie des molécules et complexes de signalisation en aval du récepteur DCC

qui sont requis pour la chimio-attraction des axones des neurones comissuraux médiée par la Netrine.

Dans la première partie de cette thèse nous avons utilisé une approche de spectrométrie de masse non biaisée pour identifier des molécules de signalisation complexées au récepteur DCC lié à la Netrine dans des neurones commissuraux isolés de moelle épinière à E12/E13. A partir des données de spectrométrie de masse nous avons choisi de caractériser Arp2/3 et 14-3-3, deux molécules jouant des rôles importants dans le remodelage du cytosquelette d'actine. Arp2/3 a co-immunoprécipité avec DCC de façon dépendante de la Netrine. De plus nous avons démontré que l'expansion du cône de croissance et le remodelage des filopodes commissuraux sont bloqués par la Wiskostatine, un puissant inhibiteur de l'activité de N-WASP envers le complexe Arp2/3. De plus, le traitement aves la wiskostatine a inhibé l'externalisation dépendante de la Netrine du récepteur DCC. Ces résultats suggèrent que la polymérisation de l'actine résultant de complexes N-WASP/Arp2/3 peut être requise pour le maintient du recyclage de DCC à la surface de la cellule pendant le guidage axonal généré par la Netrine. La proteine adaptateur 14-3-3 epsilon a aussi été identifiée comme étant associée à DCC. Néanmoins, son association à DCC décroit suite à l'addition de Netrine. L'inhibition de 14-3-3 par l'utilisation d'un peptide bloqueur a eu comme résultat le collapse des cônes de croissance commissuraux, qui n'ont pas pu être récupérés par l'addition de Netrine.

Dans la deuxième partie de cette thèse nous avons identifié ß-Pix comme un potentiel GEF en aval de DCC, impliqué dans l'activation et la propagation de la signalisation par Rac-1 et Cdc42. Nous avons déterminé qu'un complexe ß-Pix/Git s'associe avec DCC dans les neurones commissuraux. ß-Pix est fortement exprimé dans diverses subpopulations neuronales de la moelle épinière en développement. Dans les neurones commissuraux la protéine ß-Pix se trouve dans les membranes en protrusion du cône de croissance. De plus, des essais fonctionnels utilisant la surexpression de mutants ß-Pix a montré que les complexes ß-Pix et l'association avec Pak sont des pas importants dans le maintient et la morphologie des cônes de croissance, l'extension des axones commissuraux vers la ligne médiane et la ramification des neurones corticaux éllicités par la Netrine. En utilisant des essais de GTPase modifiés nous avons aussi démontré que ß-Pix est requis pour l'activation de Cdc42 et Rac-1 en aval de la Netrine et qu'il est associé avec les GTPases liées à GTP. ß-Pix peut s'associer aux GTPases par l'intermédiaire de Pak ou, en alternative, peut se lier directement, tel que démontré pour la protéine proche $\alpha$-Pix, menant à l'activation allostérique de la GTPase. Ces résultats supportent un nouveau modèle où des complexes ß-Pix/Git pourraient jouer un rôle intégral dans l'activation de Rac-1 et Cdc42 en aval de DCC.

Ces données apportent de nouvelles avenues dans la signalisation en aval de DCC pendant le guidage des axones commissuraux. Ils ont aussi

identifié de nouvelles questions sur comment des mécanismes activés par la netrine-1 régulent le cytosquelette d'actine du cône de croissance.

**LIST OF ABREVIATIONS**

AC: ADF/Cofilin
ADF: Actin depolymerization factor
Arp2/3: Actin related protein 2/3
BDNF: Brain derived neurotrophic factor
BMP: Bone morphogenic proteins
CAM: Cell adhesion molecule
cAMP: Cyclic adenosine monophosphate
cGMP: Cyclic guanosine monophosphate
C. elegans: Caenorhabditis elegans
CN: Commissural neuron
CNS: Central nervous system
Cdc42: Cell division cycle 42 homolog
coIP: Coimmunoprecipitation
Comm: Commissureless
CREB: cAMP response element binding protein
C-terminus: Carboxyl-terminus
DAG: Diacylglycerol
DB domain: DCC-binding domain
D. melanogaster: Drosophila melanogaster
DCC: Deleted in colorectal cancer
DCC-fb: DCC function blocking antibody
DRG neurons: Dorsal root ganglion neurons
EB: Epidermoblasts
ECM: Extracellular matrix
EGF: Epidermal growth factor
Ena/Vasp: Enabled/vasodilator-stimulated phosphoprotein
F-actin: Filamentous actin
FA: Focal adhesion
FAK: Focal adhesion kinase
FBS: Fetal bovine serum
FGF: Fibroblast Growth Factor
FN3: Fibronectin type 3
FZ: Frizzled
FSK: Forskolin
G-actin: globular-actin
GAP: GTPase activating protein
GDF: Growth differentiation factor
GDP: Guanine diphosphate
GDI: Guanine nucleotide dissociation inhibitor
GEF: Guanine nucleotide exchange factor
GFP: green fluorescent protein
GLI: Glioma-associated oncogene
CRIB: Cdc42/Rac interactive-binding
GTP: Guanine triphosphate

GTPase: Guanine triphosphatase
GPI: glycosylphosphatidylinositol
HBSS: Hanks' balanced salt solution
HEK293T: Human embryonic kidney 293T
HS: Heparin sulfate
ICD: Intracellular domain
Ig: Immunoglobulin
IP3: Inositol triphosphate
LH: LIM/Homeodomain gene
LRR: Leucine rich repeats
Mena: Mouse enabled
MN: Motoneuron
NCAM: Neural cell adhesion molecule
NCK: Non-catalytic region of tyrosine kinase adaptor protein
NG108-15: Neuroblastoma glioma 108-15
NgR: Nogo receptor
N-WASP: Neuronal Wiskott-Aldrich syndrome protein
PAK: p21-activated kinase
PBS: Phosphate buffered saline
PC12 cells: Pheochromocytoma cells
PDL: Poly-D-lysine
Pix: Pak-interacting exchange factor
PK substrate: Polylysine substrate
PKA: Protein kinase A
PKC: Protein kinase C
PNS: Peripheral nervous system
PRK: PKC-related kinase
Rac: Ras-related C3 botulinum toxin substrate
Rho: Ras homolog gene family
RGC: Retinal ganglion cell
Robo: Roundabout
RT-PCR : Reverse transcription polymerase chain reaction
SCN: Spinal commissural neurons
SDS: Sodium dodecyl sulfate
SEM: Standard error of mean
SH2 domain: Src homology 2 domain
SH3 domain: Src homology 3 domain
Shh: Sonic hedgehog
SRC: Rous sarcoma oncogene
TAG-1: transient axonal glycoprotein-1
Tsp: Thrombospondin
UNC: Uncoordinated
Wnt: wingless and Int gene
X. laevis: Xenopus laevis

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# CHAPTER 1:

# INTRODUCTION AND LITERATURE REVIEW

## PREFACE

This chapter reviews the established literature and provides an overview of axon guidance and the development of the mammalian spinal cord. It provides an overview of the signaling cascades and regulatory molecules required for the actin cytoskeleton remodeling that takes place during axon guidance, and for the establishment of adhesions and membrane protrusions which enable axon chemoattraction.

## 1.1 Axon Guidance

The nervous system is a complex network of interacting neural circuits that relies on the ability of axons to travel long distances, reach their targets and establish appropriate synaptic connections. The vertebrate spinal cord is an archetype of this circuitry where secreted extracellular guidance cues attract and repel axons and migrating neurons during development.

### 1.1.1 Historical perspective

In his quest to map the central nervous system, Santiago Ramon y Cajal founded the Neuron doctrine and established with very limited technology several neuronal theories that have since been confirmed with substantially more advanced experimental techniques and imaging equipment. Using Golgi staining, Ramon y Cajal traced and mapped individual neurons, establishing them as the basic structural and functional unit of the nervous system. His observations led him to propose the chemotropic model for axonal targeting in the late 19$^{th}$ century (Ramón y Cajal, 1892), which proposes that axon find their targets by responding to guidance molecules along their path. Ross Granville Harrison and Paul Weiss's development of in vitro neuron cultures provided the stage for the development of a new axon guidance model, the stereotropic model suggesting that axons are guided through physical constraints. This model was supported by the observation that axons extended along scratches on a Petri dish (Weiss, 1934), and further supported by experiments

examining neuronal networks grown on spider web substrates (Weiss, 1934). Weiss further proposed that neuronal connections survive when an axon and its target match their electric activity (Weiss, 1941). In the 1940's Roger Sperry carried out experiments in the frog visual system that raised substantial questions about the stereotropic model, and provided an experimental foundation for molecular guidance cues as a mechanism for axon targeting (Sperry, 1945). By severing the optic nerve and rotating the eye in its socket 180 degrees before allowing regeneration to occur, Sperry's established that the regenerating axons still reached their target. However, these connections did not result in the correct behavior. These experiments provided evidence that axon targeting is dependent on chemical recognition rather than functional validation from random connections. Sperry's work served as a starting point from which to begin the search for molecular axon guidance cues.

### 1.1.2 Guidance molecules during development

In both vertebrate and invertebrate organisms the formation of precise connections between neurons depends on the ability of the axonal growth cone to navigate over long distances, acting as a sensor that probes the surrounding environment and responds to the variety of environmental cues it encounters along the way. Such cues, initially proposed by Ramon y Cajal, are either diffusible or cell-surface associated and may act to guide axons at long-range or short-range respectively (Baker et al., 2006;

Kennedy, 2000; Kennedy et al., 1994; Kennedy et al., 2006; Tessier-Lavigne and Goodman, 1996). The plethora of cell surface receptors and their regulated recycling allows for a growth cone to be attracted or repelled by the environmental cues it encounters. Receptors at the cell surface modulate growth cone motility through the regulation of gene expression and remodeling of the actin cytoskeleton. The mechanisms by which the actin cytoskeleton regulates growth cone motility will be further discussed in a subsequent section.

#### 1.1.2.1 The developing spinal cord

The vertebrate nervous system develops at a relatively late stage in embryogenesis. The organogenesis of the gut, lung and liver, are all derived from endodermal tissue. Embryonic mesoderm gives rise to connective tissue and the vascular system, while the ectoderm is the origin of the central and peripheral nervous systems (Altmann and Brivanlou, 2001; Kandel ER et al., 2000). During embryonic development, an ectodermal sheet present along the dorsal midline begins to acquire neural properties and ultimately differentiates to give rise to neural and glial cells. Ectodermal cells that do not differentiate into neural tissue become the epidermis of the skin.

Figure 1-1 illustrates the sequential developmental stages of neural tube formation (Jessell, 2000). Briefly, epidermal cells laterally flank the neural plate, which is derived from undifferentiated ectodermal tissue.

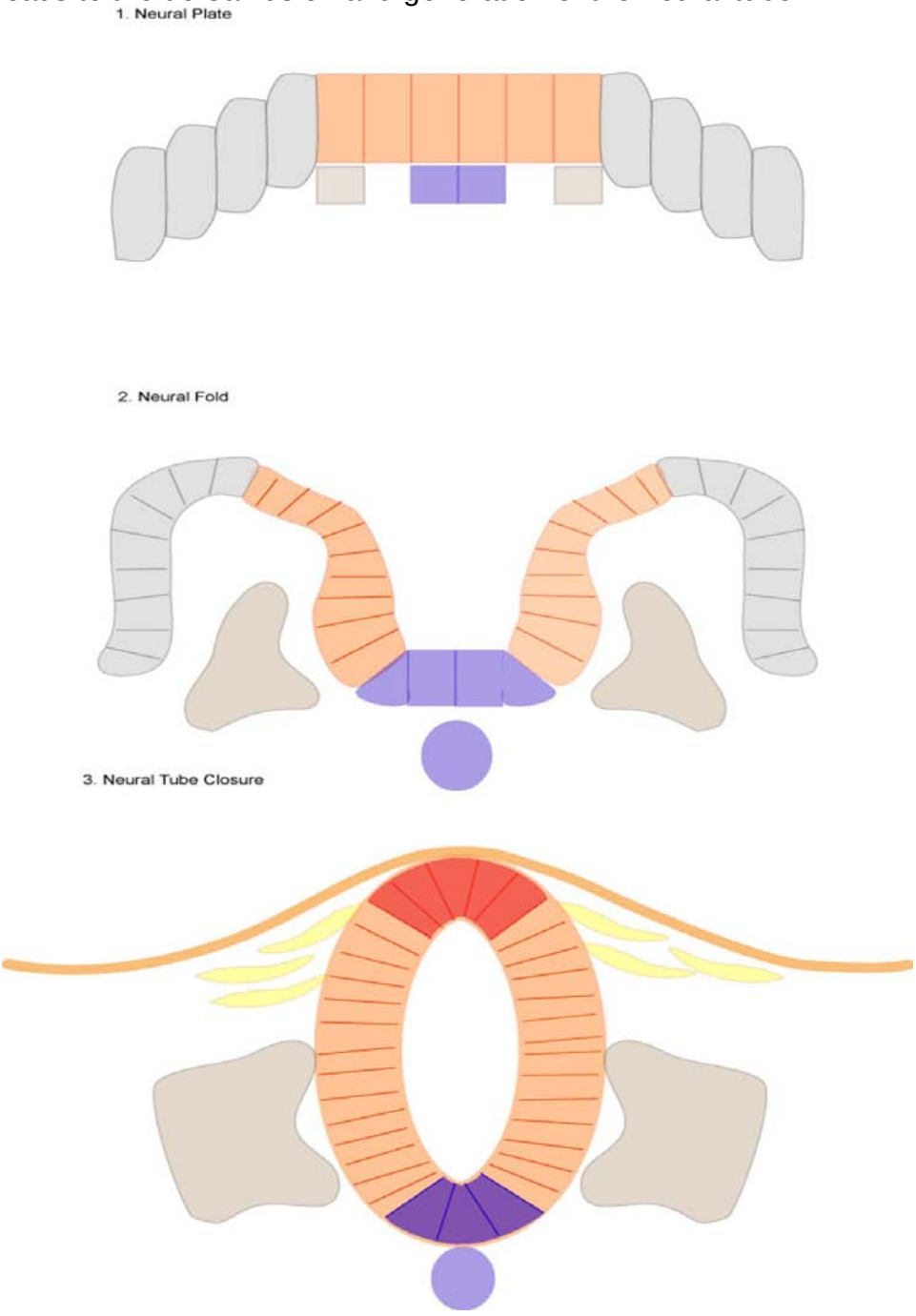
This neural plate lies dorsal to the notochord and paraxial mesoderm that will later differentiate into somites. Migration and differentiation processes along the neural plate give rise to the neural fold. At this stage, floor plate, roof plate and somites begin to differentiate. Neural crest cell migration to the neural dorsal region and roof plate formation give rise to the dorsal fusion of the edges of the neural plate to generate the neural tube (Wilson and Maden, 2005). As the spinal cord matures neuronal subpopulations begin to differentiate, migrate and extend their axons towards their targets. These subpopulations include spinal commissural interneurons, motor neurons and dorsal root ganglion neurons.

The development of the spinal cord is differentially regulated by morphogenic signals along the anterior-posterior axis and the dorsoventral axis (Kandel et al., 2000; Wilson and Maden, 2005). In this short review, we will focus on the development and differentiation of the dorsoventral axis.

Two classes of proteins are thought to regulate the dorsoventral cell patterning of the spinal cord. Sonic Hedgehog (Shh) expression at the ventral midline induces the differentiation of ventral cell types, such as motor neurons. Secretion of members of the Bone Morphogenic Protein (BMP) family by roof plate cells signals dorsal cord patterning. During the initial stages of neural tube formation, Shh is expressed in the axial mesodermal cells that give rise to the notochord. BMPs originate in the

flanking mesoderm that signals to differentiate the dorsal spinal cord. At the neural fold stage, floor plate cells express Shh at the midline and BMPs are expressed at the dorsal tips of the neural folds (Mehler et al., 1997; Wilson and Maden, 2005).

**Figure 1-1. Neural tube differentiation**. The neural plate is the first stage of the neural tube development. Epidermal cells flank undifferentiated ectodermal tissue. Migration and differentiation leads to the development of the neural fold. Neural crest cell migration and roof plate formation then leads to the dorsal fusion and generation of the neural tube.




Adapted from *Jessell, TM. Nat Rev Genet 1, 20-29 (2000)*(Jessell, 2000).

#### 1.1.2.2 BMP regulation of commissural neuron differentiation, ventral migration and axon extension

The differentiation of dorsal progenitor cells into neural crest cells, roof plate cells and dorsal interneurons, results from BMP signaling from the ectodermal cells flanking the neural tube. Once the neural tube is closed secretion of BMP-4, BMP-7, BMP-6 and Gdf-7 by roof plate cells induces the differentiation of various subpopulations of dorsal interneurons (Chizhikov and Millen, 2005; Lee et al., 1998; Liem et al., 1997). Lee et al. demonstrated that Math-1 expressing commissural neuron progenitor cells differentiate into LIM/Homeodomain gene, LH2A/B expressing mature dorsal commissural neurons through the concerted signaling from several BMP family members (Lee et al., 1998). *In vitro* co-cultures of roof plate and commissural neuron explants further demonstrated that the proper orientation of commissural neuron axons toward the ventral midline is initiated by the repellent action of BMPs secreted by roof plate cells (Butler and Dodd, 2003). Using a candidate molecule approach, only BMP-7 was able to re-orient commissural axons as effectively as roof plate explants (Butler and Dodd, 2003). BMP-7 knockout mice also exhibit axon guidance defects in the spinal cord and in vitro roof plate explants from these mice show a significantly reduced ability to repel commissural axons compared to wild type animals (Augsburger et al., 1999).

#### 1.1.2.3 Shh and Netrin: ventral neural tube patterning and commissural chemoattraction

Axon guidance and extension is mediated by the coordinated signaling of short-range and long-range cues (Tessier-Lavigne and Goodman, 1996). Mesodermal cells in the notochord region provide two types of inductive signals: a short-range signal that induces the differentiation of floor plate cells and a long range signal that induces the differentiation of motor neurons and ventral interneurons. These short and long-range morphogenic signals are mediated by the same protein, sonic hedgehog (Shh). Shh mutant mice lack the inductive activity of the notochord, resulting in abrogation of the differentiation of most ventral cell types in the spinal cord (Chiang et al., 1996). Shh is highly expressed in the notochord and, in later stages, by the floor plate cells. The floor plate is a specialized structure formed by glial cells responsible for secreting guidance cues that differentially signal axons and cells to be attracted, and cross the ventral midline or be repelled and migrate away from it. Danforth's short-tailed (Sd) mutant mice lack the floor plate in caudal regions of the neuraxis. In these mice, spinal commissural neurons properly extend their axons to the floor plate in the caudal region and either fail to cross or fail to turn longitudinally toward the ventral funiculus (Bovolenta and Dodd, 1991). Although the gene responsible for this naturally occurring mutation has not been identified, similar phenotypes have been observed in Shh null deficient mice. Gli2 is a zinc-finger-containing transcription factor upstream of Shh signaling. Gli2 knockout

mice exhibit the absence of a floor plate and the accumulation of commissural axons within the floorplate region. Surprisingly these axons still project properly to the ventral midline, suggesting that floor plate cells may not be the sole source of guidance cues that direct axons to the ventral midline.

The search for cues responsible for the guiding of commissural neurons to the ventral midline resulted in the identification of the netrins, a new subgroup of the laminin superfamily. In vitro co-cultures of netrin expressing cells with commissural explants demonstrated that these secreted laminin-like proteins attract commissural axons. In embryonic knockout mice lacking either netrin-1 or the netrin receptor, Deleted in Colorectal Cancer (DCC), exhibit axon path-finding defects such as commissural axon stalling and abnormal trajectories (Fazeli et al., 1997; Serafini et al., 1996). However, commissural axons were typically still able to extend at least half way to the floor plate in the embryonic spinal cord. Furthermore, floorplate explants derived from netrin-1 null mice, although unable to promote commissural axon outgrowth, still induced commissural axon turning, providing evidence for the existence of additional guidance molecules being secreted by floorplate cells. Gli2 -/- and netrin-1 -/- double mutant mice exhibit severely disrupted commissural axon trajectories, supporting the conclusion that both netrin-1 and Shh coordinate commissural axons extension and crossing of the midline (Charron et al., 2003). Novel axon guidance molecules

continue to be identified. The following sections provide a brief review of currently established axon guidance protein families and what is known about their signal transduction.

#### 1.1.2.4 Keeping commissural axons out of the floorplate – Slits and Robo

An interesting dilemma in deciphering the mechanism by which axons cross the floorplate and reach their target arises when one considers the bilateral symmetry of the spinal cord. Commissural axons extend from both sides of the cord and are attracted to the midline, but once they cross the floorplate, they turn and extend longitudinally and are no longer attracted or cross the floorplate again. This suggested that once an axon crosses the floorplate, its intracellular signaling is modified to ignore the cues that once attracted it. Studies using Drosophila identified additional guidance cues that function to keep commissural axons from reentering the floorplate. Slits are large proteins containing Epidermal growth factor (EGF) repeats, Leucine-rich repeats (LRR) and a Laminin G like domain, secreted by the glial cells found at the midline (Dickson and Gilestro, 2006; Rothberg et al., 1990). Growth cones expressing Robo, the Slit receptor, are repelled by Slits. Mammals express 3 Robo homologs in the nervous system, Robo-1, Robo-2 and Rig-1, and three Slits, Slit1-3, while *Drosophila* and *C. elegans* encode only a single Slit (Dickson and Gilestro, 2006). Slits are characterized by a tandem repeat

of four LRR domains, that have been shown by genetic and biochemical analyses to be the domains within Slits that binds to the IgG domains of the Robo receptor (Brose et al., 1999; Kidd et al., 1999). Structure-function studies have demonstrated that this binding mechanism is conserved by all Robo receptors. HS (heparan sulfate) stabilizes the Slit-Robo interaction and is required for the transduction of Slit signaling through the recruitment of effector molecules to the cytosolic domain of the receptor (Hussain et al., 2006). Studies utilizing Drosophila chimeras provided evidence for synergistic function between Slit and netrin receptors in regulating attraction versus repulsion by the ventral midline. Slit mutants, in both Drosophila and mice, exhibit a collapse of the commissural axons onto the midline (Long et al., 2004). Similarly loss-of-function experiments in Drosophila have demonstrated that midline crossing is primarily regulated through Robo 1. Specifically, midline crossing errors are mainly found in the robo mutants but with lower incidence in Robo2 and Robo3 single and double mutants (Rajagopalan et al., 2000; Seeger et al., 1993; Simpson et al., 2000). Interestingly overexpression of any of the three Robos can be sufficient to prevent crossing. Transgenic flies expressing a chimera of Robo's extracellular domain fused to the intracellular domain of Frazzled (Drosophila DCC) expressed in all neurons during development resulted in a phenotype similar to that of the Robo fly knockout, in which axons repeatedly cross the midline. Meanwhile a chimera with the reciprocal domains swapped repelled axons from the midline, similar to the Slit phenotype. Such

studies resulted in the hypothesis that Slit-Robo signaling is necessary to prevent post-crossing axons from re-entering the midline, but only as a result of the persistence of attraction of Netrin signaling through Frazzled (Bashaw and Goodman, 1999). Similar to the expression patterning found in Drosophila, Robo 1 and 2 are also found in low levels in commissural neurons prior to midline crossing and increase as axons extend past the floorplate of mice (Long et al., 2004). Slit and Robo have also been shown to promote proper targeting of axons in the retina, hippocampus and olfactory bulb in mammals (Cho et al., 2007; Erskine et al., 2000; Fouquet et al., 2007; Li et al., 1999; Nguyen Ba-Charvet et al., 1999; Prince et al., 2009).

Although Slit-Robo signaling is best understood for its role in morphogenesis and axon guidance in neuronal development, recent studies have also implicated it in angiogenesis, leukocyte migration, axonal branching, and cell migration (for a recent review refer to (Legg et al., 2008).

#### 1.1.2.5 Semaphorins and Plexins

Semaphorins encompass a large family of bifunctional guidance molecules (Muller et al., 1996; Wolman et al., 2004; Zhou et al., 2008). This protein family was initially identified as a result of the development of growth cone collapse in vitro. These assays led to the identification of collapsin, now understood to be a member of the larger Semaphorin

family. Semaphorins are subdivided into classes 1 through 7; 1, 2 and 5 are expressed only in invertebrate species, while the remaining classes are specific for vertebrates, and a separate class V, consists of viral specific Semaphorins. All Semaphorins encode a sema domain, a 500 aa sequence conserved in all species. Other distinct protein domains include the Ig domain present in many transmembrane Semaphorins, a thrombospondin domain and a basic C-terminus. Semaphorins can be secreted, transmembrane or GPI-linked. Class 1, 4, 5, 6 and 7 Semaphorins are membrane associated (transmembrane or glycosylphosphatidylinositol [GPI] linked), and Semaphorins in classes 2, 3 and V are secreted. Furthermore, some transmembrane Semaphorins have been demonstrated to be cleaved, thereby generating soluble proteins (Zhou et al., 2008). Prominent Semaphorin receptors are the plexin proteins. However, Sema3A is also known to form signaling complexes with the Ig superfamily adhesion protein L1. Interestingly Semaphorins do not bind directly to Plexins or L1, but require neuropilins as a co-receptor (for a more thorough review refer to (Zhou et al., 2008).

Signaling cascades downstream of Semaphorins are best characterized in the context of repellent cell and axon migration (Chabbert-de Ponnat et al., 2005). In the spinal cord, Semaphorin 3A is a key regulator of DRG sensory neurons at the dorsal root entry zone (DREZ) (Dontchev and Letourneau, 2002; Masuda and Shiga, 2005). Semaphorin 4D signaling results in the regulation of integrin-mediated adhesion through the downregulation of Ras-Gap activity and possibly through Rho signaling

(Basile et al., 2005; Basile et al., 2007; Ito et al., 2006; Oinuma et al., 2006). Additionally, transmembrane Semaphorins, such as Sema6 family members, signal bidirectionally. During chick development Sema6D binding to plexinA1 results in reverse signaling that promotes myocardial cell migration, while forward signaling promotes ventricular expansion (Toyofuku et al., 2004). Accumulating evidence has implicated Semaphorins in branching and pruning of neuronal connections (Dent et al., 2004; Skutella and Nitsch, 2001), as well as adhesion that contributes to cancer progression (Chedotal et al., 2005; Klagsbrun and Eichmann, 2005), immune disease (Chabbert-de Ponnat et al., 2005) and angiogenesis (Basile et al., 2006; Klagsbrun and Eichmann, 2005; Sun et al., 2008). Mouse models and genetic analyses have also suggested a role for specific Semas in neurodegenerative diseases such as Parkinson's and schizophrenia (Ding et al., 2008; Eastwood et al., 2003).

#### 1.1.2.6 Ephrins

The ephrin family of proteins was originally identified as repellents that direct retinal axon pathfinding (Davenport et al., 1998). Ephrins and ephrin receptors are divided into two classes based on structural differences: Ephrin-As (A1-A6), are tethered to the plasma membrane via a GPI moiety and Ephrin-Bs (B1-B3) span the plasma membrane and contain a short cytoplasmic tail. Their 14 receptors identified to date have been divided in two sub-groups, the EphAs (EphA1 to A8) and EphBs (EphB1 to B6). Eph-

receptors extracellular domains include a unique N-terminal ephrin-binding domain forming a globular f3-barrel, a cysteine-rich region with 19 conserved cysteines that includes an EGF-like region and two fibronectin type III repeats. The cytoplasmic part contains an uninterrupted tyrosine kinase domain and several protein-protein inter- action modules including SH2-docking sites, a sterile-n-motif (SAM) and a C-terminal PDZ binding motif. Ephrins interactions can occur in a cis- or trans- manner depending on whether they are presented by two opposing cells (and bi-directional signaling is activated) or expressed by the same cell (and bi-directional signaling is inhibited) (Reber et al., 2007). Cis interactions have been shown to inhibit trans interactions (Carvalho et al., 2006). Due to their role in bi-directional signal transduction, Ephrins have recently been a focus in the cancer field as a potential therapeutic target (Genander and Frisen, 2010; Pasquale, 2010). Bi-directional signaling by Ephrins has also been implicated in synaptic function (Aoto and Chen, 2007), bone homeostasis (Edwards and Mundy, 2008; Kwan Tat et al., 2008), cell adhesion (Puschmann and Turnley, 2010; Woo et al., 2009) and in insulin secretion (Konstantinova et al., 2007). Ephrin-mediated axon guidance results from graded expression by an array of cells in the target zone of an innervating axon. In addition to directing axonal pathfinding, Ephrins also contribute to epithelial cell-cell contact modulation by regulating both adherens and tight junctions (Lee et al., 2008). Ephrins have also been implicated in cross-talk signaling with other receptor tyrosine kinases. An example of this is the FGF receptor induced tyrosine phosphorylation of Ephrin-B1 during

Xenopus blastomere dissociation (Chong et al., 2000). Activated FGF receptor associates directly with Ephrin-B1 in a cis manner, inhibiting ephrin-B1 from inducing blastomere dissociation (Chong et al., 2000). More recently, these proteins were identified as substrates for cell surface proteases (Lin et al., 2008; Mancia and Shapiro, 2005). Ephrin ligand can be proteolytically released from its membrane tether by a complex on the opposing cell composed of the ephrin receptor and an ADAM metalloprotease (Mancia and Shapiro, 2005). Similarly, MMP-2/MMP-9-specific inhibition or cleavage-resistant mutations in the ectodomain of EphB2 prevented EphB2-mediated cell-cell repulsion in HEK293 cells, and blocked ephrin-B1-induced growth cone withdrawal in cultured hippocampal neurons, suggesting matrix metalloproteinases (MMPs) play an important regulatory role in repulsive EphB2 signaling (Lin et al., 2008).Such a discovery explains how the Eph-ephrin complex may influence cell-cell repulsion. Some of the signals activated upon Eph-ephrin binding produce changes in the cytoskeleton that enable the two cells to pull apart after activation of the metalloprotease and cleavage of ephrin (Pasquale, 2000).

#### 1.1.2.7 Morphogens turn into guidance cues, BMPs, Shh and Wnt

Following neuronal differentiation, morphogens such as BMPs and Shh function as axon guidance cues. BMPs, Shh and Wnts sequentially contribute to directing commissural neuron extension along their trajectory

that ultimately takes them to the brain. As such, gradients of morphogen expression that are generated during early development have a subsequent role at a later stage, acting as long distance axon chemo-attractants or repellents (Charron and Tessier-Lavigne, 2007).

The Wnt family of morphogens is best known for its role in the regulation of anterior-posterior patterning of the spinal cord. Wnt signals thorough Fz (Frizzled) and disheveled to mobilize β-catenin, which enters the nucleus and regulates transcription of specific genes (Huelsken and Behrens, 2002). Wnt's role in axon guidance is best exemplified by studies in *C. elegans*. Due to the small number of Wnts and their receptors expressed in *C. elegans* (five Wnts and four Frizzled receptors) compared to mammals (19 Wnts and 12 Frizzled receptors in the mouse), *C. elegans* has proven to be an ideal organism to study Wnt function in regulation of anterioposterior axon guidance and cell migration (Pan et al., 2006). All five Wnts function in neuronal migration, with significant functional overlap and a subset of these Wnts also controls the anterior guidance of growth cones. Moreover, genetic evidence indicates that at least one of the Wnts functions as a repulsive cue, an effect that is mediated by Frizzled receptors (Pan et al., 2006). Similarly, in mammals, ascending commissural neurons that have crossed the midline are attracted to high anterior concentrations of Wnt4 by Fz3 (Lyuksyutova et al., 2003). These data support the emerging concept in the axon guidance field that morphogens that regulate early patterning events are reused later as axon

guidance cues.

The definitive proof that Shh is necessary to direct the turning of commissural growth cones at the ipsilateral edge of the floor plate was provided by the generation of a mouse conditional mutant. The restricted expression of Wnt1 in the dorsal portion of the spinal cord (Charron et al., 2003) was used to selectively inactivate Smoothened (Smo) in commissural neurons using the Cre/loxP recombinase system driven by the Wnt1 promoter (Charron et al., 2003; Danielian et al., 1998). In the resulting mice, Smo-deficient commissural axons grew towards the floor plate in a defasciculated manner, invading the motor columns. Taken together, these results demonstrate that Shh signalling through Smo contributes to directing commissural axons to the floor plate at the ventral midline of the neural tube.

The Shh family of proteins binds to seven-pass transmembrane proteins Patched 1 (Ptc1) and Patched 2 (Ptc2). Under basal conditions, Ptc associates with and sequesters the activity of a G-protein associated receptor-like protein, Smo. In response to binding Shh, Ptc releases Smo, which then activates a Gi subunit to inhibit cAMP production within the cell. Subsequent transcription of Shh downstream targets requires the activity of the GLI family of zinc finger transcription factors, which are in turn negatively regulated by the cyclic AMP-dependent protein kinase A (PKA) (Charron and Tessier-Lavigne, 2007).

Once the growth cone has crossed the floorplate it ignores the previously attractive cues secreted by the floorplate. The regulation of cAMP levels in the growth cone responsible for receptor externalization is one hypothesis put forward in appendix 2 to explain response modulation (unpublished data). This proposal would be consistent with recent data showing that enhancing protein kinase A activity in pre-crossing axons diminished Shh-induced Semaphorin repulsion and caused profound midline stalling and overshooting/wandering of post-crossing axons (Parra and Zou, 2010).

In the model proposed by appendix 2 data, the increased levels of PKA activity resulting from Shh release of Ptc signaling may lead to the externalization of DCC through the increased PKA activity, leading to an amplification of the axonal chemo-attractive response to the floorplate (unpublished data; appendix 2).

#### 1.1.2.8 Laminins and Integrins – enabling adhesion during axon outgrowth and guidance

As axonal growth cones extend in response to environmental cues they activate a process of matrix degradation, followed by process extension, the formation of cell-matrix or cell-cell adhesions, and retraction of their trailing edges, which allows them to navigate through the extracellular environment (Henke-Fahle et al., 2001; Serpe and O'Connor, 2006; Zimmermann and Dours-Zimmermann, 2008; Zisman et al., 2007). The mechanisms underlying these processes have been described in most

detail in systems such as leukocyte and epithelial cell migration where cell motility involves either membrane recycling through an amoeboid mechanism or cytoskeletal remodeling. During growth cone extension, similar intracellular signaling mechanisms take place, but will be discussed in greater detail in a later section.

The formation of sites of cell-cell and cell-substrate adhesion plays a crucial role during cell motility. Integrin signaling has been established in varying systems as a hallmark of the signal transduction mechanisms underlying adhesion. The extracellular environment in the CNS includes proteoglycans and glycoproteins with a small amount of fibrous glycoproteins such as collagen and fibronectins (Sobeih and Corfas, 2002). Several forms of laminins are also present and appear to contribute during development, differentiation and cell migration. Transgenic mice from which specific laminin genes have been knocked out develop congenital dystrophy and epidermolysis bullosa, demonstrating their functional significance (Ryan et al., 1996).

Laminins are a large group of flexible proteins consisting of three long polypeptide chains, which are connected by disulfide bonds. The prototype of this family of basement glycoproteins is laminin-1, which forms a large cross-like multidomain heterotrimer assembled from one $\alpha$, one $\beta$, and one $\gamma$ chain. Laminins contain multiple functional domains, which facilitate its interaction with type IV collagen, heparan sulfate, entactin and laminin

receptors of the integrin family (Durbeej, 2010). Recent studies have proposed that laminin $\gamma$1 and $\gamma$3 short arms forms a basement membrane complex with netrin-4, the most recently described member of the netrin family (Schneiders et al., 2007). However, the strongest evidence for a functional role for laminins during neural development is in the peripheral nervous system. Specifically, laminin-integrin signaling regulates DRG sensory neuron neurite extension in vitro on patterned substrates (Zhu et al., 2010). Laminin-1 is highly enriched in the peripheral nerve system and is a potent promoter of axon extension both in vitro and in vivo (Yu et al., 2007). Mutations in laminin subunits expressed in the PNS and in skeleton muscle may cause peripheral neuropathies and muscular dystrophy in both humans and mice. Schwann cell proliferation and differentiation is dependent on laminins and the knockout of laminins such as laminin-5 leads to Schwann cell apoptosis (Chen and Strickland, 2003; Podratz et al., 2001; Yu et al., 2005; Yu et al., 2007). In the CNS laminins play an important role in axon regeneration (Grimpe et al., 2002), cell survival (Chen and Strickland, 1997), plasticity (Tian et al., 1997), and potentiation (Nakagami et al., 2000). Laminin-2 substrates dramatically enhance myelin membrane formation (Buttery and ffrench-Constant, 1999) and Laminin-2 deficient mice have quantitative and morphologic defects in CNS myelin (Chun et al., 2003).

Integrins also interact with ligands outside of the laminin family (Milner and Campbell, 2002), and this has been suggested to included binding netrin-

1. During development of the pancreas, netrin-1 is thought to engage $\alpha6\beta4$ and $\alpha3\beta1$ integrins through a bindings site located at the c-terminus of netrin-1 (Yebra et al., 2003). The netrin-1-integrin complexes is proposed to promote cell adhesion and migration in response to the growth factor HGF (Hebrok and Reichardt, 2004). Additionally, studies in *C. elegans* demonstrated that certain $\alpha-$ and $\beta$-integrins regulate repellent responses by axons to Slits secreted from the midline (Stevens and Jacobs, 2002). These studies highlight the importance of the cross-talk between ECM substrate and intracellular signalling cascades as exemplified by netrin, Slit and Ephrin signaling connection, with integrin dependent cytoskeletal remodeling.

### 1.1.3 Netrins and their receptors

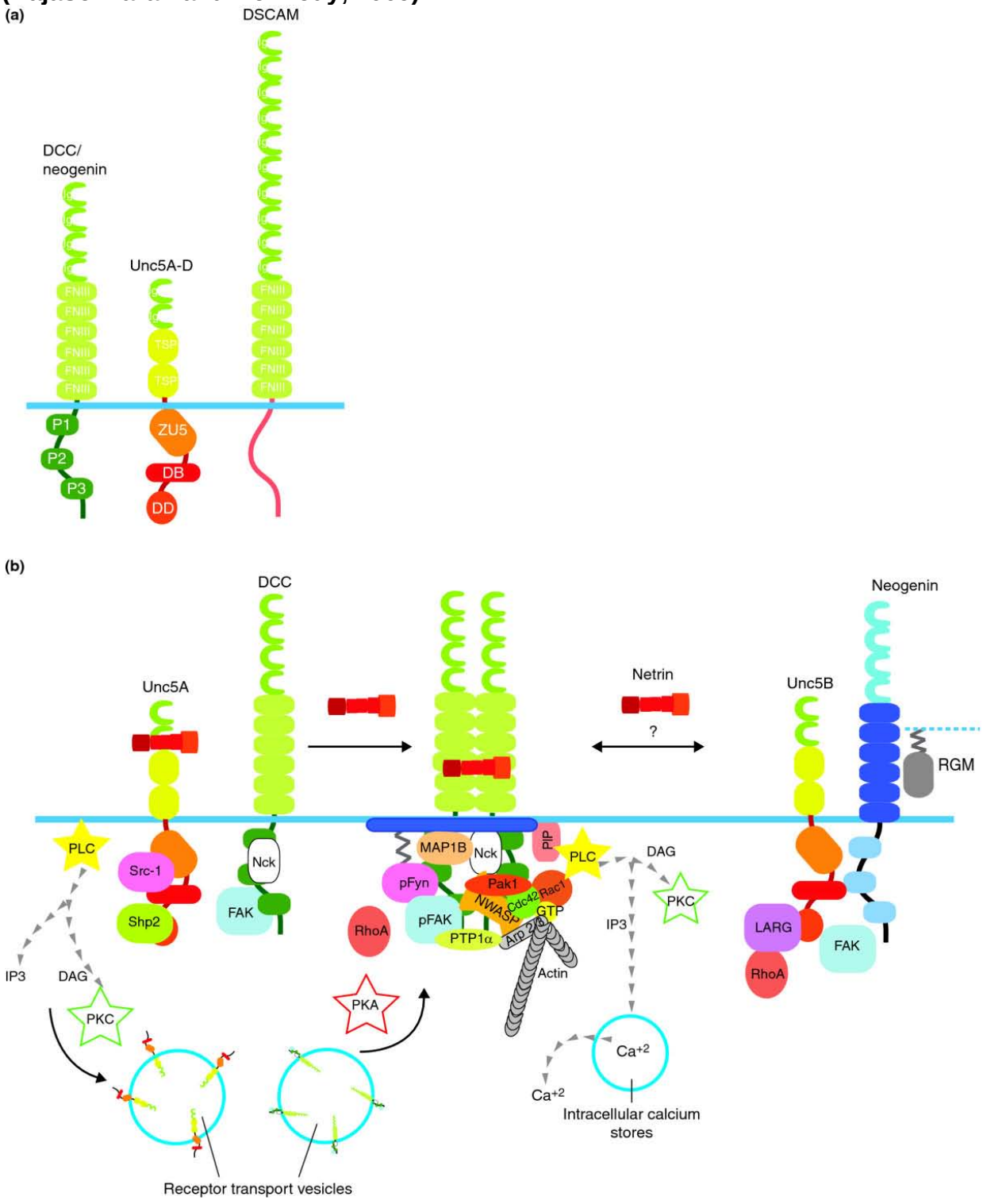
This family of small (~75 kDa) bifunctional guidance molecules is composed of 6 family members in vertebrates, netrin 1-4 and netrins G1 and G2 (Manitt and Kennedy, 2002; Rajasekharan and Kennedy, 2009). Netrins 1-4 are secreted proteins while the G-members of the family are GPI linked to the plasma membrane (Manitt and Kennedy, 2002). In the developing chick spinal cord netrin-1 and 2 are highly expressed in a gradient with its apex emanating from floor plate cells at the ventral midline (Kennedy et al., 1994; Kennedy et al., 2006).

Domains VI and V of Netrins are homologous to the amino terminal VI and V domains of laminin's. Their domain structure includes a VI and a V domain, and a charged c-terminus domain that shares no homology with laminins but is thought to be required for netrin association with heparin (Kappler et al., 2000). The c-terminus is also required for multimerization of the netrin molecules possibly through its association with proteoglycans (Kappler et al., 2000).

Netrin functions as a chemoattractant or chemorepellent depending on the receptor presented at the growth cone's cell surface. Deleted in colorectal cancer (DCC) is required for chemoattraction while Unc5 homologues mediate chemorepellent responses to netrin by axonal growth cones. Additional netrin receptors, which have not been as well characterized, include Neogenin, a paralogue of DCC and a possible association with α6β4 and α3β1 integrins. Additionally, recent studies have proposed that DsCAM, which is also a cell adhesion molecule and member of the immunoglobulin superfamily, signals independently of DCC to signal chemoattraction in response to netrin-1 (Andrews et al., 2008; Ly et al., 2008). This thesis focuses entirely on the signaling mechanisms underlying netrin-1 mediated chemoattraction, and primarily responses evoked by DCC. The function of the Unc5 homologue family of netrin receptors has been recently reviewed (Freitas et al., 2008; Moore et al., 2007). The current understanding of Neogenin function has also been recently reviewed (De Vries and Cooper, 2008). Figure 1-2 provides a

model that summarizes our current understanding of the intracellular signals activated downstream of netrin receptors and their functional responses.

**Figure 1-2. Netrin receptors and their signaling pathways; (Rajasekharan and Kennedy, 2009)**




**A) Netrin receptors identified to date. B) Immediate intracellular signaling partners to DCC/Unc5 and Neogenin and signaling cascades upon netrin-1 binding.**

#### 1.1.3.1 Netrins during spinal cord development

Members of the netrin family were initially identified in *C. elegans* and subsequently biochemically characterized in vertebrates. *Unc-6*, from *C. elegans*, was the first netrin to be identified (Hedgecock et al., 1990; Ishii et al., 1992) based on a genetic screen for mutations that disrupt neural development. *Unc-5* and *unc-40,* the *c. elegans* homologues of the vertebrate Unc-5 and DCC, mutants were identified in the same screen, and intriguingly exhibited defects that were a subset of those found in Unc-6 mutants. Most notably, all of these mutants exhibited defects in circumferential migratory paths taken by cells and axons. Unc-5 mutants primarily exhibiting defects in ventral to dorsal migration, Unc-40 mutants having defects in dorsal to ventral migration, and Unc-6 mutants exhibiting defects in both (Hedgecock et al., 1990). In an independent series of studies, two vertebrate orthologs were purified from homogenates of embryonic chick brain, based on an assay for commissural axon outgrowth from explants of embryonic spinal cord. The two proteins identified and cloned were named netrin-1 and netrin-2 (Serafini et al., 1994). In situ hybridization studies revealed expression of netrin-1 by floor plate cells, and at a lower level by the ventral neural epithelial cells that surround the floor plate (Kennedy et al., 1994). Analysis of the distribution of netrin protein revealed a graded distribution in the embryonic spinal cord, with its apex at the floor plate (Kennedy et al., 2006). These results supported the idea that a gradient of increasing netrin expression guides

commissural axons ventrally to the floor plate. *In vitro* functional analyses using purified recombinant netrin-1, demonstrated that soluble netrin-1 functions as a diffusible chemoattractant for commissural neurons (Kennedy et al., 1994; Serafini et al., 1994). Functional studies, carried out in vitro, using either floorplate explants or Cos cells secreting recombinant netrin-1, demonstrated that netrin-1 repelled the axons of trochlear motor neurons (Colamarino and Tessier-Lavigne, 1995). Such experiments raised the possibility, later on confirmed, that receptors orthologous to C. elegans Unc40 and Unc5 are present in the vertebrate spinal cord. The identification of Netrin-A and Netrin-B in drosophila using pcr-amplification followed soon after the identification of netrins and netrin receptors in vertebrates (Harris et al., 1996). The phenotypes found in drosophila knockouts of *frazzled* (drosophila ortholog of DCC) and both netrins were consistent with the loss of function phenotypes seen in *C. elegans* and the mouse (Deiner and Sretavan, 1999; Harris et al., 1996; Hedgecock et al., 1990). These studies solidified the concept that netrins are a highly conserved family of secreted chemotropic cues that direct axon extension in a wide range of bilaterally symmetric animal species.

*C. elegans Unc6* and *Unc40* knockout studies identified a role for netrins in axon guidance, and also indicate that they direct cell migration in the neural tube (Hedgecock et al., 1990). More recent studies have indicated that the gradient of netrin protein present in the embryonic mammalian spinal cord (Kennedy et al., 2006) directs oligodendrocyte precursor cell

migration away from the ventral midline toward the dorsal regions of the spinal cord (Jarjour et al., 2003). Interestingly, the chemorepellant function of OPCs is antagonized by PDGF's chemoattraction (Tsai et al., 2003). Netrin-1 also has a role in directing initial deflection of the axons of some classes of motor neurons away from the midline, and also antagonizes the chemoattraction of these axons to Semaphorin-D (Varela-Echavarria et al., 1997).

*Netrin structure-function studies*

The first structure-function analysis of the netrin family was carried out in C. elegans. Mutation of Unc-6 demonstrated the existence of four domains responsible for four separable contributions to dorsoventral migration in the developing worm (Ren et al., 1999). Domain V of Unc-6 is composed of three tandem arrays of cysteine-rich epidermal growth factor (EGF) repeats named V-1, V-2 and V-3. Deletions in the C-terminal and/or the V-3 domain resulted in dorsal and ventral migration defects. Other studies have hypothesized that the C-terminal domain, which contains many basic amino acid residues, may bind to negatively charged sugars associated with proteoglycans in the extracellular milieu, such as heparin sulfate proteoglycans and (Kappler et al., 2000). There is also some evidence that netrin signaling is potentiated by multimerization, which is hypothesized to cross-link netrin receptors to activate signaling (Geisbrecht et al., 2003). Mutations in domain VI resulted selectively in ventral migration defects, suggesting that domain VI contains the binding site for the Unc-40

receptor. In contrast, defects in ventral to dorsal migration resulted from mutations in the V-2 domain. This domain was therefore proposed as a potential binding site for Unc5. In vitro assays using a recombinant human Netrin fragment identified direct binding between fifth fibronectin type III repeat of the extracellular domain of DCC and soluble netrin (Bennett et al., 1997). Additionally, the interaction between DCC-Ig and heparan sulfate/heparin, both on the surface of cells and immobilized on plastic, was blocked by the same anti-DCC antibody that blocks netrin-1-dependent commissural axon outgrowth. These studies provided evidence that a requirement for heparin in DCC signaling, was mediated through netrin and not through a direct interaction of heparin with DCC (Bennett et al., 1997).

#### 1.1.3.2 DCC receptor signaling during spinal cord development

DCC mediates chemoattraction to a gradient of netrin-1. DCC was initially identified as a human gene mutation associated with colorectal cancer study, and proposed to function as a tumour suppressor (Fearon et al., 1990).

Critically, the *C. elegans* studies describing the requirement for DCC's ortholog *Unc-40* in *Unc-6* mediated axon guidance and cell migration were the first suggestion that DCC might play an important role in the CNS development. Subsequent analyzes carried out in the developing rat spinal

cord provided strong evidence that DCC is a receptor for netrin-1, and is required for chemoattractant responses made by commissural neurons to netrin-1 in the embryonic spinal cord (Keino-Masu et al., 1996). Treatment of dorsal spinal cord explants with a DCC function-blocking antibody inhibited netrin-1-induced commissural axon outgrowth (Keino-Masu et al., 1996), and knocking DCC out of mice pheno-copied the defects in commissural axon guidance found in netrin-1 knockouts (Fazeli et al., 1997; Serafini et al., 1996). Similarly, analysis of the drosophila DCC ortholog, *Frazzled*, revealed expression in the embryonic CNS by motor axons in the peripheral nervous system as well as by cells in the epithelium of the gut and epidermis (Kolodziej et al., 1996). Furthermore, *Frazzled* null flies mimicked the phenotype caused by deletion of both drosophila netrin genes, including CNS axon guidance defects and motor axon targeting defects in the periphery.

In mammals, DCC transcripts are present at very low levels in most adult tissue, however its highest levels are found in neural tissue (Cooper et al., 1995). DCC–null mice die within a few hours of birth due to defects in nervous system development. However, defects are not confined to the CNS midline, but are also present in retinal axon guidance to the hypothalamus and respiratory defects are also observed. Interestingly, no defects have been documented in the gut of homozygous deficient mice (Fazeli et al., 1997), in spite of the association of DCC with colorectal cancer in humans. Behavioral defects such as inability of suckling are also

noticed in the first few hours after birth suggesting a role in complex neuronal circuitry (Fazeli et al., 1997).

*DCC structure-function studies*

Molecular cloning of Unc-40 revealed that it is a single-pass transmembrane receptor consisting of an extracellular domain with six fibronectin III (FN3) repeats and four Immunoglobulin (Ig) repeats, a transmembrane region and an intracellular region composed of 3 domains that have been named P1, P2 and P3 (Kolodziej, 1997). In vitro pull-down assays identified the fifth FN3 domain as a potential binding site for Netrin-1 (Geisbrecht et al., 2003). Direct binding between DCC and Netrin-1 was confirmed by in vitro studies using a purified DCC- ec-FC construct (Geisbrecht et al., 2003), however the specific binding site has not yet been identified. Studies using a metalloproteinase inhibitor also demonstrated that Netrin-DCC dependent axon outgrowth is regulated by metalloproteinase-dependent cleavage of the ectodomain of DCC (Galko and Tessier-Lavigne, 2000). Although some mammalian signaling studies demonstrated that the DCC P3 domain was required for netrin-1 signal transduction during axon outgrowth, a thorough structure-function characterization of DCC has not been done in mammals, but a mutagenesis analysis of the DCC orthologue *Frazzled* has been carried out in drosophila (Garbe et al., 2007). Unlike what had been proposed for mammalian DCC (Kolodziej, 1997), deletion of any of the individual P-domains was not sufficient to prevent commissure formation in vivo.

However, a drosophila mutant with a deletion of the P3 domain did result in a subpopulation of commissural axons being unable to cross the midline. Furthermore, deletion of the P1 domain increased aberrant targeting of axons; however midline commissures were still intact. These experiments highlighted the importance of P1 and P3 domains' for Frazzled signaling, but demonstrated that none of these deletions were sufficient to phenocopy the phenotype found in Frazzled loss of function mutants.

*Netrin-DCC intracellular signaling cascades*

The intracellular domain of DCC contains several highly conserved putative tyrosine phosphorylation sites in its P3 domain and many proline-rich regions in the P2 domain, including four SH3- binding motifs (Li et al., 2004). Over the past 5 years, some of the immediate events that take place upon ligand binding to DCC have been identified. Focal Adhesion Kinase (Fak) and the adaptor protein Nck are constitutively bound to the receptor (Li et al., 2004; Li et al., 2002a; Ren et al., 2004). Netrin-1 binding DCC activates FAK and recruits the src family member Fyn to the intracellular domain of DCC (Li et al., 2004; Meriane et al., 2004; Ren et al., 2004). These kinases initiate a cascade of tyrosine phosphorylation events, including the phosphorylation of DCC itself, which lead to the activation of Rho GTPases Rac-1 and Cdc42 and the activation of the MAPK pathway (Forcet et al., 2002; Li et al., 2002b; Shekarabi and Kennedy, 2002).

The association of Nck with DCC is mediated through a proline-rich region in the P2 domain of DCC and the second SH3 domain of Nck (Li et al., 2002a). The interaction of Nck with DCC serves as a foundation for the activation and recruitment of Rac-1 and Cdc42 and has been demonstrated to be required for netrin-dependent axon outgrowth (Li et al., 2002b; Shekarabi and Kennedy, 2002; Shekarabi et al., 2005). Evidence has been provided that the recruitment of the receptor complex to a lipid-raft like domain is required for transduction of netrin-1 signaling (Guirland et al., 2004; Herincs et al., 2005). There is also a report that DCC dependent cell death signaling requires DCC localization to lipid rafts (Furne et al., 2006). However, another study suggested that DCC signaling takes place in a soluble non-lipid raft compartment (Petrie et al., 2009) and that netrin accumulation at these lipid-rich compartments has no effect on the targeting of the receptor to rafts. These experiments were however performed using whole mouse brain homogenates where netrin is also signaling through Unc5H and different mechanisms could be occurring, so it remains unclear whether lipid raft localization of the DCC receptor signaling complex is a requirement for DCC dependent netrin-1 signaling.

In commissural neurons the activation of the Rho GTPases Rac-1 and Cdc42 results in the recruitment and activation of the serine/threonine kinase, Pak-1 and also recruitment of the scaffold N-Wasp (Shekarabi et al., 2005). The activation of this signal transduction complex downstream

of DCC results in the remodeling of growth cone morphology, which is thought to underlie the capacity to direct axon extension. The machinery responsible for netrin-mediated cytoskeletal dynamics is discussed further in section 1.2, Axon Guidance and the Actin Cytoskeleton.

*Modulation of DCC Signaling*

DCC signaling is modulated by $Ca^{2+}$ influx and by the level of intracellular cAMP and also cGMP. Although netrin does not regulate PKA directly (Bouchard et al., 2004), increased levels of cAMP lead to PKA activation which triggers the recruitment of DCC to the plasma membrane and amplification of axon outgrowth responses to netrin-1 (Bouchard et al., 2008; Bouchard et al., 2004). Increased intracellular Ca2+ levels are also required for netrin mediated growth cone turning (Hong et al., 2000; Ming et al., 2002). In fact, addition of netrin-1 to Xenopus neurons leads to production of phosphoinositides by recruitment of phosphatidylinositol transfer protein-$\alpha$ (Xie et al., 2005), and activation of phosphatidylinositol-3 kinase (Ming et al., 1999). The breakdown of phosphoinositides by phospholipase C into IP3 and diacylglycerol [DAG, (Ming et al., 1999)] then results in activation of protein kinase C which has also been shown to modulate netrin-1 response (Bartoe et al., 2006).

#### 1.1.3.3 Netrin signaling in systems other than the CNS

Netrin knockout mice exhibit massive deficits in several CNS commissures, including the ventral spinal commissure, the corpus callosum and the hippocampal commissure (Serafini et al., 1996). Essentially identical defects are present in DCC null mice (Fazeli et al., 1997). These defects demonstrate that netrin-1 and DCC are essential for normal development of CNS; however the defects found in netrin-1 and DCC knockout mice are not confined to the neural tube. Recent studies have demonstrated functions for netrins in cell-cell and cell-matrix adhesion that contribute to the development of lung, mammary gland, angiogenesis, and lymphangiogenesis (Dalvin et al., 2003; Liu et al., 2004b; Lu et al., 2004; Srinivasan et al., 2003; Yebra et al., 2003).

In some ways the vascular system resembles the nervous system. Specialized endothelial cells, called vascular tip cells, are found at the ends of developing veins and arteries, also navigate through the embryo and are guided to their appropriate targets, much like axonal growth cones. Treatment of endothelial tip cells with netrin-1 resulted in tip cell filopodial retraction, and this effect was abolished in Unc5b-deficient mice, suggesting that netrin-1 mediates repulsive guidance of capillary tip cells through UNC5B signaling (Eichmann et al., 2005; Lu et al., 2004). In contrast to this repellent response, treatment of netrin in chick embryonic endothelial tissue induced vascular sprouting, suggesting that netrin may

be a bifunctional attractant and repellent for the developing vasculature, much as it is for axons (Bouvree et al., 2008).

Further evidence for netrin influencing morphogenesis arises from studies addressing its role in the development of the mammary gland, lung, and pancreas (Dalvin et al., 2003; Liu et al., 2004; Lu et al., 2004; Srinivasan et al., 2003; Yebra et al., 2003). Similar to its expression at the tips of the vascular capillaries (Lu et al., 2004; Park et al., 2004), netrin is also present at the tips of the extending mammary luminal epithelial cells, while neogenin is expressed by adjacent cap cells (Srinivasan et al., 2003). Disruption of netrin-neogenin signaling results in enlarged terminal end buds and disorganization of the cap cells, suggesting that the interaction between netrin-1 and neogenin stabilizes the organization of the bud, at least partly through netrin-1 and neogenin promoting adhesion between the cell layers. During the branching morphogenesis of the developing lung, netrin-1 and netrin-4 are similarly enriched in the basement membrane of proximal tubules while distal cells express DCC and Unc5 (Liu et al., 2004). In vitro assays provided evidence that netrin-4 treatment inhibits tubular budding, suggesting a role for netrin as a regulator of proximal budding while at distal sites its absence allows for further branching.

Netrin's adhesive properties are further supported by analysis of paranodal junctions in netrin-1 and dcc null mice. The paranode is a highly specialized junctional connection that flanks the node of Ranvier and is

made between oligodendrocytes and axons. In the absence of either netrin-1 or dcc oligodendroglial paranodes are able to establish initial contacts but with time become disorganized, indicating that netrin-1 is required to maintain the adhesive connection formed between the oligodendroglial paranodal loops and the axon (Jarjour et al., 2008).

It remains to be determined how the mechanisms underlying the role of netrin regulating axon guidance and cell motility are related to the signal transduction activated by netrin when it promotes adhesion. It is very possible that these are not such distinguishable pathways as originally though.

## 1.2 Axon guidance and the actin cytoskeleton - adhesion, extension, turning and remodeling

The axon is a uniquely specialized extension of neurons. However, the axonal shaft and the growth cone leading the way are fundamentally elaborate membrane protrusions with substantial similarity to those found at the leading and rear ends of other types of motile cells. Much research has focused on the cytoskeletal and morphological changes required at the leading edge of a growth cone to enable the axon to be attracted or repelled relative to various guidance cues. It is generally accepted that chemotropism is dependent on regulation of the cytoskeletal and microtubule machinery, and that the mechanisms at work in axonal growth cones overlap substantially with signaling mechanisms activated

in other types of migrating cells. In this section I will discuss models for how cytoskeletal dynamics contribute to axon chemoattraction.

### 1.2.1 Evidence axon guidance depends on the regulation of the actin cytoskeleton

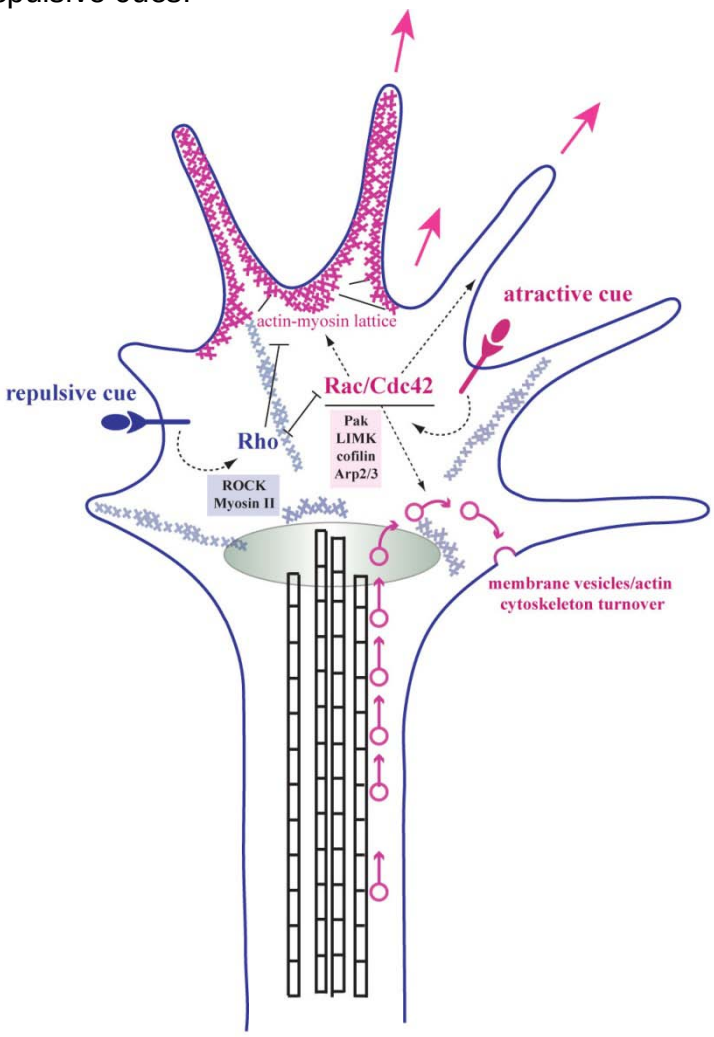
The growth cone engages its cytoskeleton to drive forward and turn, continuously progressing through three stages of advance that are influenced by environmental factors: protrusion, engorgement and consolidation (Dent et al., 2011). Membrane protrusions at the leading edge, such as filopodia and lamelipodia, sense the surrounding environment and establish short-term and high-turnover adhesions to generate the traction required for the forward movement (see Figure 4-4). The following sections will describe in more detail these physical and biochemical mechanisms.

Biochemical signals for growth cone steering initiate at the tips of filopodia when axon guidance receptors encounter and establish a physical bond with their ligand. This interaction results in the recruitment of signaling molecules that directly or through the action of their effectors regulate the actin cytoskeleton. Section 1.1.4 discusses how Rho GTPases, key regulators of cellular movement, are activated during growth cone guidance.

### 1.2.2 Cytoskeletal dynamics – Actin/Microtubule interactions and regulators

Cellular movement requires a complex interaction between the actin cytoskeleton and microtubule dynamics. Microtubule steering is possible through spatio-temporal guidance provided by the constant remodeling of the actin cytoskeletal. Actin structures function as a barrier and their local breakdown and constant remodeling allows for MT intrusion into the periphery resulting in growth cone turning (Figure 1-3).

**Figure 1-3. Growth cone cytoskeleton dynamics during turning.** Actin remodeling at the growth cone is dependent on the balanced cycling between active and inactive Rho GTPases in varying subcellular compartments. This regulation is initiated with the binding of attractive and repulsive cues.




The movement of an axonal growth cone can be divided into 3 distinct stepwise processes: protrusion, engorgement and consolidation. During protrusion, adhesions generated by receptor-ligand binding anchor the growth cone to the substrate and locally stabilized the retrograde flow of filamentous (F)-actin. The continued polymerization of actin causes the filopodia and lamellipodia to protrude forward. Engorgement follows with the severing and recycling of the actin filaments to the central domain of

the growth cone. F-actin arcs reorient toward the region of growth, which allows MTs to invade the transition and peripheral regions of the growth cone. Consolidation of the new central domain occurs when the growth cone neck forms a new segment of the axon shaft. This compression is possible through the action of myosin II-containing actin arcs on MTs (Geraldo and Gordon-Weeks, 2009).

#### 1.2.2.1 Actin and MT polimerization

Actin filaments are polarized polymers generated from the addition of ATP-bound glomerulus (G)-actin monomers to the plus end. De novo actin assembly has been visualized in Aplysia growth cones by brief treatment with cytochalasin D (Sahly et al., 2006). Cytochalasin D “freezes” actin filament growth by binding to the barbed-end of the actin filament and thereby indirectly promoting their depolymerization. Following wash-out of Cytochalasin D, F-actin assembly resumes at the leading edge of the membrane (Sahly et al., 2006). Such experiments allowed for the direct visualization of F-actin treadmilling and led to the identification of actin nucleators.

Actin nucleators promote de novo actin filament assembly. The nucleator family can be subdivided into 4 classes: the Arp2/3 complex, formin proteins, Spir proteins and cofilin. These subclasses nucleate F-actin through different mechanisms and are located in separate micro-domains

of the growth cone (Gungabissoon and Bamburg, 2003). All of these proteins can localize and interact with the leading edge membrane either directly or indirectly through protein-lipid interactions (Pollard, 2007). I briefly summarize each of these subclasses below but will provide greater detail on the regulation of the Arp2/3 complex and its possible involvement in netrin signalling in chapter 2.

#### 1.2.2.1.1 Arp2/3 complex

The Actin related protein (Arp) 2/3 complex was the first actin nucleator to be discovered (Kelleher et al., 1995). Arp 2/3 is a multiprotein complex composed of 7 protein subunits. Arp2 and Arp3, are stabilized in an inactive state by five other subunits, ARPC1 (for the 40-kDa subunit), ARPC2 (35-kDa subunit), ARPC3 (21-kDa subunit), ARPC4 (20-kDa subunit), and ARPC5 (16-kDa subunit). All subunits are conserved throughout most eukaryotes in addition to at least one gene for formin, another actin filament-nucleating protein. Arp2/3 initiates actin filament branches from the sides of existing mother actin filaments. Briefly, WASP proteins are activated downstream of Cdc42. This causes an allosteric change that allows the VCA domain of WASP to bind and activate Arp2/3. Dimerization of WASP by proteins containing F-BAR [Fes-Cdc42-interacting protein 4 (CIP4) homology (FCH) bin-amphiphysin-Rvs] and SH3 (Src homology 3) domains causes hyperactivation of Arp2/3 by allowing two VCA domains to interact with each Arp2/3 complex.

Clustering of WASP by phosphatidyli-nositol biphosphate (PIP2) brings VCA domains into close proximity and also leads to hyperactivation of Arp2/3. Together, these inputs stimulate the polymerization of a new actin filament along the side of an existing filament at a fixed 70° angle. Cortactin, a scaffold that competes with NWASP for Arp2/3 binding, associates with Arp2/3 through the N-terminal acidic (NtA) region and can stabilize the actin branchpoint as well as displace WASP and WAVE family proteins. Coronin 1B and the phosphatase Slingshot replace Arp2/3 from the branchpoint. Coronin 1B–containing branches are positioned at variable angles from the existing actin filament compared with Arp2/3-containing branches. Branches containing Coronin 1B and Slingshot are unstable and lead to the disassembly of the branched actin filament (Cai et al., 2007).

The Arp2/3 complex has been implicated in axon pathfinding *in vivo*. In Drosophila, the Arp2/3 complex and its regulators WAVE/Scar and Kette are required for proper axon pathfinding (Zallen et al., 2002), and in *C. elegans*, the Arp2/3 regulators WAVE-1/WAVE, WASP-1/WASP, GEX-2/Sra-1, and GEX-3/Kette are required for proper axon guidance (Shakir et al., 2008). Even though some initial studies in cultured hippocampal neurons showed that inhibition of Arp2/3 activity resulted in increased axon length with little effect on growth cone filopodia or morphology (Pinyol et al., 2007; Strasser et al., 2004); in these same experiments they demonstrated Arp2/3 was required for axon turning. Additionally, the

Arp2/3 complex appears to be required for growth cone morphology and filopodia formation *in vivo (C. elegans)*, and it is specifically required for the rate of filopodia initiation but not filopodial stability or length (Norris et al., 2009).

#### 1.2.2.1.2 Other actin nucleators

Formins, unlike the Arp2/3 complex, nucleate at the barbed end of the growing actin filaments. These proteins bind two actin monomers and stabilize the dimers. Their interaction with and stabilization of profilin-actin complexes is thought to accelerate the assembly process during bundle formation (Paul and Pollard, 2009). Spir proteins bind four actin monomers forming a longitudinal tetramer (Quinlan et al., 2005). Like Arp2/3 these complexes occur and remain at the pointed ends of filaments. These proteins are thought to have a role regulating vesicle trafficking during actin assembly (Kerkhoff et al., 2001).

#### 1.2.2.1.3 ADF/Cofilin

Actin depolymerization factors (ADF)/cofilins are known for being key regulators of rapid actin turnover at the leading edge of extending membranes. ADF and cofilins association with G-actin and F-actin differs however, they colocalize in growth cones at the tips of F-actin and their overexpression leads to neurite outgrowth (Bamburg and Bray, 1987; Kuhn et al., 2000; Meberg and Bamburg, 2000). ADF/cofilin activity is

regulated through reversible phosphorylation by LIM kinases and slingshot phosphatases. LIM kinases and ADF/cofilin phosphatases are activated by Rho GTPases activity such as Rho, Rac and Cdc42 at the local level. ADF/cofilins function by depolymerizing actin filaments by either increasing the rate of dissociation of ADP-actin from the pointed end of actin filaments and/or increasing filament end severing (Carlier et al., 1997; Maciver, 1998). Actin filaments turn over at a rate 100– 200-fold faster in vivo than in vitro, the difference requiring ADF/cofilin proteins (Zigmond, 1993). Recent evidence suggests cofilin can also stimulate actin assembly by de novo nucleation dependent on concentration levels; low concentrations favor severing, whereas high concentrations favor nucleation (Andrianantoandro and Pollard, 2006). Yeast association/dissociation studies demonstrated that cofilin can rapidly dissociate the Arp2/3 complex from branches by direct competition for binding sites on the actin filament and by propagation of structural changes in the actin filament that reduce Arp2/3 affinity (Chan et al., 2009).

#### 1.2.2.1.4 Nucleation regulation – the WASP/VASP family

Members of the Wiskott-Aldrich syndrome protein (WASP) family are multidomain nucleation-promoting factors that organize signaling complexes to activate Arp2/3 in a temporally restricted and spatially localized manner (Soderling and Scott, 2006). This family contains two categories of proteins: WASP and neuronal WASP (N-WASP), and Wiskott-Aldrich verprolin homologous proteins (WAVE) 1, 2, and 3. Members of the WASP and WAVE family contain a conserved verprolin-cofilin-acidic region (VCA) domain at the C terminus, which binds to and activates Arp2/3 (Panchal et al., 2003).

Rho-family GTPases (Cdc42 and Rac) cooperate with phosphatidylinositol 4,5-bis-phosphate (PIP2) to overcome N-WASP autoinhibition by binding its GTPase-binding domain (GBD) and displacing VCA, which can then interact with actin and Arp2/3 complex (Leung and Rosen, 2005). In contrast to the autoinhibited WASp family, full-length Scar/WAVE proteins are constitutively active (Machesky et al., 1999). *C. elegans* studies have suggested that WVE-1/WAVE and CED-10/Rac act in parallel to a pathway that includes WSP-1/WASP and MIG-2/RhoG (Shakir et al., 2008). Accumulating evidence shows that WAVE/WASP family members localize to different subcellular compartments (filopodia, lamelipodia, vesicle recycling compartments and central actin-microtubule zone) within the growth cone suggesting different regulators of Arp2/3 for specific actin structures (Nozumi et al., 2003; Pollard, 2007). Shekarabi et al.

demonstrated that N-WASP is recruited to complex with DCC in commissural neurons, and that this is required for netrin-1 induced membrane expansion and filopodia formation in the growth cone (Shekarabi et al., 2005). Interestingly, loss of WAVE function inhibited axon growth, whereas overexpression of a membrane-tethered WAVE mutant partially rescued axon growth in Rac1-knock-out neurons (Tahirovic et al., 2010).

#### 1.2.2.1.5 Actin capping proteins

Capping protein (CP) binds to the barbed ends of actin filaments with a low nanomolar range affinity and caps the filament end by preventing the addition or loss of actin subunits. CP binding prevents prolonged polymerization of an actin filament, leading to the formation of short filaments that are necessary to provide force to push the cell membrane forward. CP regulates the actin monomers cytoplasmic pool by preventing excessive addition of monomeric actin to barbed ends. Inhibition of CP is important for the formation of the long unbranched actin filaments in filopodia, based on the action of formins and VASP family proteins (Chesarone et al., 2010). The knockout of CP in mammalian cultures results in the loss of lamellipodia disrupting cell motility (Mejillano et al., 2004) while CP null drosophila die at early larvae stages (Hopmann et al., 1996) suggesting a crucial role in cell motility during development.

### 1.2.3 Rho GTPases downstream of axon guidance cues

#### 1.2.3.1 Rho GTPases: how they work

The Rho family of small guanosine triphosphate GTP-binding proteins (Rho-GTPases) is pivotal in the regulation of the actin organization, nucleation and extension and adhesion turnover events during cell migration, axon extension or growth cone turning or even protein trafficking and cell phagocytosis. Rho proteins cycle between an active GTP-bound form, and an inactive GDP-bound form. Regulators of GTP loading and hydrolysis of Rho-GTPases include guanine-nucleotide-exchange factors (GEFs) and GTP-ase-activating proteins (GAPs) respectively (Ridley, 2001b). Furthermore, guanine nucleotide dissociation inhibitors (GDIs) bind to the GDP-bound forms to prevent nucleotide dissociation thereby keeping GTPases inactivated (Ridley, 2001a). The Rho GTPase family is comprised of more than 20 members, of which RhoA/B, Rac1/2 and Cdc42 have been well characterized as key modulators of the dynamics of actin remodeling (Ridley, 2001b).

Each of these three Rho GTPases have distinct effects on the actin cytoskeleton. Experiments with fibroblasts elucidated their distinct roles: Rac activity is responsible for the formation of large lamellipodia and membrane ruffles, while Cdc42 induces filopodia formation during cell migration and Rho activity promotes maturation of focal adhesions and formation of stress fibers (Schmitz et al., 2000). The major actin nucleation targets of Rac and Cdc42 are the WASP/WAVE family of proteins. Pak

and PI5K are additional important Rac downstream effectors, which play a role in actin reorganization at the leading edge of lamellipodia. PI5K activation leads to actin filament uncapping (Tolias et al., 2000). Pak, a serine/threonine kinase, phosphorylates and activates LIM kinase, which inactivates actin depolymerization proteins ADF/cofilin downstream of Rac and Cdc42 (Edwards et al., 1999). Several reports have suggested that Cdc42 may also participate in the reorientation of the microtubule-organizing center (MTOC) and the golgi during directional cell migration (Etienne-Manneville and Hall, 2001; Kaverina et al., 2002; Nobes and Hall, 1999). A Par6, Par3, αPKC complex mediates this event (Etienne-Manneville and Hall, 2003). The main downstream effectors of RhoA during actin remodeling are mDia and ROCK. Rho kinase 1 and 2 (ROCK1/2) act on various targets, including LIM kinase, ERM proteins (ezrin/radixin/moesin) and others, all of which enhance actin polymerization (Geiger and Bershadsky, 2001). ROCK regulates cell contractibility by acting on myosin-II regulatory light chain (MLC) dynamics (Geiger and Bershadsky, 2001). Dia molecules are activated by Rho-GTP, and then interact with profilin, src and IRSp53 through an exposed FH (formin homology) domain (Tominaga et al., 2000; Watanabe et al., 1999).

#### 1.2.3.2 Rho GTPases in axon guidance

A current paradigm for the role of Rho GTPases in axonal guidance suggests that attractive cues promote actin polymerization in the region of the growth cone that is proximal to the source of attractants, by activating

Rac1 and Cdc42 to generate an attractive response. In contrast, repulsive cues decrease actin polymerization in the proximal region of the growth cone by activating RhoA and generating growth cone collapse to mediate axon repulsion (Hall, 1998; Mueller, 1999). Further evidence suggests that the roles of these small GTPases can be to some extent interchanged, possibly depending on the cell type, or on which of the intracellular events are being analyzed. For example, in chick DRG neurons Sema3D-induced growth cone collapse is mediated by Rac1 (Jin and Strittmatter, 1997) and similarly, Rac1 activity is required for Ephrin-A2-mediated endocytosis of the growth cone plasma membrane during growth cone collapse in chick retinal cells and sensory neurons (Jurney et al., 2002). Such evidence could be explained by local activation of each GTPases as it occurs during cell migration where each has a role in both the extension of membranes at the leading edge and retraction and dissociation of adhesions at the trailing end. The exact role of each of these GTPases in axonal growth cones during axon extension and turning remains to be determined. Is it the case that Cdc42 activity is required for the initial response to a cue, followed by directed filopodial extensions or that Rac-1 is required for extension of lamellipodia or adhesion turnover or even receptor localization at the cell surface? Is RhoA required for the establishment of stress fibers or the dissociation of adhesions at the trailing/retracting side of the growth cone? Answers to these questions remain elusive and can vary depending on the cell type and model system addressed. Without a doubt, it is clear that they each play crucial roles in axon guidance. Rac1-

deficient cerebellar granule neurons isolated from a conditional Rac-1 knock-out mouse, which do not express other Rac isoforms, showed impaired neuronal migration and axon formation both in vivo and in vitro. In addition, Rac1 ablation disrupted lamellipodia formation in growth cones (Tahirovic et al., 2010). Additionally, Rac1/Mtl double mutants in drosophila exhibit misguided axons and the extension of axons beyond their synaptic targets (Hakeda-Suzuki et al., 2002). Constitutively active (CA) or dominant negative (DN) Cdc42 causes axon fasciculation and midline crossing errors in Drosophila CNS interneurons (Kim et al., 2002), and CA Cdc42 and DN RhoA cause midline crossing errors in the Drosophila CNS (Fritz and VanBerkum, 2002). In the mammals we have already discussed the crucial role of a sequential activation of Rac-1 and Cdc42 downstream of netrin-1-DCC signaling (Shekarabi and Kennedy, 2002). Such experiments suggest that a balanced regulation of all of these GTPases ensures appropriate axon outgrowth, targeting and the formation of appropriate connections during axon guidance. Numerous studies are now investigating the role of various GEFs and GAPs that regulate these GTPases in such settings.

### 1.2.4 GEFs in axon guidance

#### 1.2.4.1 GEF families

The activation of Rho GTPases in response to upstream signaling is catalyzed by guanine nucleotide exchange factors (GEFs), which convert Rho/Rac/Cdc42 from an inactive GDP-bound form to the active GTP-

bound form. Upon extracellular activation of cell surface receptors, GEFs engage with Rho GTPases and form macromolecular complexes with scaffolds and/or kinases. The activated Rho GTPase transduces the signal to downstream effectors generating a specific cellular response, i.e. lamellipodia formation, filopodia extension, vesicle budding and trafficking, etc. Signaling specificity of RhoGTPases has been postulated to be dependent on the specific GEF/RhoGTPase and effector molecule complexes (Cerione, 2004).

The GEF superfamily is subdivided into two sub-families: the DH/PH tandem domain containing GEFs, called Dbl (Diffuse B-cell lymphoma) family GEFs, and the DOCK family GEFs, containing DOCK180 homology domains DHR1 and DHR2 (Cote and Vuori, 2007; Zheng, 2001).

The Dbl protein was discovered in 1985 as an oncogene in NIH 3T3 cells transformed with DNA from human B- cell lymphoma (Eva and Aaronson, 1985; Schmidt and Hall, 2002). Dbl was identified based on its sequence similarity to a ~180 amino acid sequence of the yeast Cdc24p, which had been genetically characterized as an upstream activator of Cdc42p and later shown to be required for Cdc42 GEF activity in vitro (Hart et al., 1994). Since then, more than 80 DH–PH containing GEFs for Rho family GTPases have been identified, all of them sharing a homologous DH/PH domain but differing in additional regulatory signaling domains (i.e. SH3,

CH, AI, CR…) (Rossman et al., 2002; Schmidt and Hall, 2002; Venter et al., 2001).

Crystallographic and NMR analysis of the DH domains of βPix, Sos1, Trio (DH1), and Tiam-1 reveal a highly related three-dimensional structure that is composed of a flattened, elongated bundle of 11 α-helices (Aghazadeh et al. 1998; Liu et al. 1998; Soisson et al. 1998; Worthylake et al. 2000). Two of these helices, CR1 and CR3, are exposed on the surface of the DH domain and participate in the formation of the GTPase interaction pocket. The DH domain directly interacts with Rho GTPases, resulting in the dissociation of GDP from the Rho GTPases. The nucleotide-depleted GTPase subsequently binds to cytosolic GTP forming a GTP-bound activated GTPase, which then transduces signals to downstream effectors.

The Dock family of GEFs lacks the DH domain. This atypical family of GEFs is smaller than the Dbl-family with only a few dozen identified to date. The mammalian DOCK 180 was initially identified as a 180 kDa protein interacting with the proto-oncogene c-Crk and involved in cytoskeleton organization (Cote and Vuori, 2002; Hasegawa et al., 1996). The DHR1domain in Dock family members always precedes the DHR2 domain. The DHR2 domain associates with Cdc42 or Rac and catalyses the GDP/GTP nucleotide exchange activity of the GTPases. Interestingly, this family of GEFs can only activate Cdc42 and Rac and not Rho and is conserved in plants and animals but not in yeast, while the Dbl family can

activate all of the GTPases and is ubiquitous in all species but not plants (Schultz et al., 1998).

#### 1.2.4.2 Regulation of GEFs

Although not consistent throughout all GEFs, a few regulatory models have emerged. GEFs can be regulated through the relief of intramolecular inhibitory sequences, by protein–protein interactions, by altering intracellular location, and by down-regulation of GEF activity through degradation.

Dbl, Vav, Tiam1, and Ect2, all contain an N-terminal intramolecular regulatory sequence (Katzav et al., 1991; Miki et al., 1993; Ron et al., 1989; van Leeuwen et al., 1995). The removal of these N-terminal sequences leads to constitutive activation when the protein is expressed in vivo. Similarly, the PH domain of Vav, Dbl and Sos-1 regulates their GEF activity (Das et al., 2000; Russo et al., 2001). It has been postulated that intramolecular autoinhibition mechanisms are relieved by phosphorylation or by binding to other proteins, however in most cases this remains to be confirmed. Vav is usually in its autoinhibitory conformation where the N-terminal sequences folds into an α-helical structure and binds to the active site of the DH domain, blocking the access of Rac1. Src family tyrosine kinases after cytokine or adhesion receptor activation phosphorylate

Tyr174, releasing the constraint on the DH domain and allowing Rac1 access.

Protein-protein interactions can also regulate GEF activity and impact intracellular location. An example is the interaction of a GEFs' PH domain with phosphatidylinositol phospholipids. Treatment of mammalian cells with the PI3K inhibitor wortmannin inhibited the activation of Rac1 downstream of growth factor receptor activation (Hawkins et al., 1995; Van Aelst and D'Souza-Schorey, 1997). Vav and Sos1, showed enhanced GEF activity towards Rac1 upon PtdIns(3,4,5)P3 binding to the PH domain and decreased substrate binding and GEF activity of the DH domain upon PtdIns(4,5)P2 binding (Das et al., 2000). $\alpha$-Pix direct association with the p85 regulatory subunit of PI3-kinase leads to its activation downstream of PDGF and Ephrin-B2. Synthetic phosphoinositide and membrane targeted PI3-kinase increased $\alpha$-Pix activity in vivo (Yoshii et al., 1999).

Another example of GEF regulation is the downregulation of β-Pix by degradation through EGF activity. β-Pix protein is recruited by activated Cdc42 in response to EGF stimulation, and also associates with c-Cbl, inhibiting the binding of c- Cbl to the EGF receptor and inhibiting receptor downregulation. Pix degradation increased after Cdc42 overexpression and EGF receptor stimulation. β-PIX degradation releases c-Cbl, which in turn mediates the degradation of the EGF receptor, generating a negative regulatory loop for receptor signaling at the cell surface (Schmidt et al., 2006).

Overall, the biochemical mechanisms controlling the spatial and temporal regulation of GEFs remain poorly understood. In addition to GEF activation, concomitant upstream signals are probably required to suppress the negative regulation imposed on Rho GTPase substrates by RhoGAPs and RhoGDIs. Proposed models suggest that GEFs are the component in the RhoGTPase signaling complex which assemble the specific Rho/Cdc42/Rac signaling complex and direct the GTPase to select the specific downstream effector (Sinha and Yang, 2008). This model does not however cover cases where more than one GEF may interact with the same GTPase, even in response to activation of the same receptor. This issue arises in the case of Trio, Dock180 and β-Pix proposed in chapter 3 of this thesis, which all seem to have similar functions downstream of DCC. Precise temporal and spatial regulation within the cell may contribute to distinguishing different functions in this case.

#### 1.2.4.3 GEFs in axon guidance

Although it is clear that RhoGTPases play a crucial role in the cytoskeletal remodeling that takes place during GC chemoattraction and repulsion, exactly how the GTPases are regulated to direct growth cone motility remains unclear. Nevertheless, a few Rac-1, Cdc42 and Rho GEFs have been identified so far; many of them identified as candidates from their already known function as RhoGEFs in other systems.

Trio was one of the initial GEFs indentified to have a role in axon guidance. Trio and Dock were shown initially to regulate Pak activity during photoreceptor axon path-finding and to regulate Rac dependent motor axon patterning downstream of Notch in drosophila (Newsome et al., 2000; Song and Giniger, 2011). Additionally, genetic studies in drosophila have demonstrated its involvement in axon path-finding at the CNS midline. Mutations in Abl and trio dominantly enhanced fra and Netrin mutant CNS phenotypes, and fra;Abl and fra;trio double mutants displayed a dramatic loss of axons in a majority of commissures (Forsthoefel et al., 2005). In mammals, some evidence points to Trio's GEF domain1 involvement in the Rac dependent activation of RhoG in the NGF pathway for the establishment of neurite outgrowth (Estrach et al., 2002). Additionally, Trio appears to be required for Netrin-1 dependent embryonic spinal commissural axon outgrowth, in in vitro explants assays carried out in collagen gels (Briancon-Marjollet et al., 2008). Analysis of Trio knockout mice demonstrated skeletal muscle deformities and aberrant organization of the hippocampus and olfactory bulb (O'Brien et al., 2000). This study also demonstrated that Trio function is essential during late embryonic development as genotype analysis indicated that trio knockout embryos died between embryonic day 15.5 and birth. Analysis of Trio -/- commissural axon projections in the rat spinal cord demonstrated a minor phenotype where axon defasciculation occurred prior to crossing of the midline, however the majority of axons still crossed and did not stall as is

the case in DCC deficient commissural axons (Briancon-Marjollet et al., 2008). Studies on Trio expression in the developing rat brain demonstrated 4 novel shorter isoforms of Trio expressed in rat cortex and cerebellum (Ma et al., 2005; McPherson et al., 2005) in post-natal development. It is possible that the redundancy of Trio isoforms or their involvement in the activation of different GTPases or at different stages of development may explain the relatively minor axon guidance defects seen in Trio knockout mice.

Son of sevenless (Sos), a Ras and Rac GEF, also functions to regulate Rac activity required for proper Slit signaling at the drosophila midline. Sos forms a complex with Dock and Robo at the plasma membrane, which results in activation of Rac1. Its Ras GEF function was not shown to be required for Robo signaling at the midline (Yang and Bashaw, 2006).

LARG is another Dbl-family GEF identified as a regulator of RhoGTPase RhoA in axon guidance but downstream of Plexins. The Semaphorin 4D receptor Plexin-B1 and Plexin-B2 can directly interacts with LARG through LARG's PDZ domain leading to the stimulation of RhoA activity and cytoskeleton reorganization (Aurandt et al., 2002; Perrot et al., 2002). A study examining the characterization of PDZ-RhoGEF activity revealed that PDZ-RhoGEF and LARG can form homo- and hetero-oligomers, whereas p115RhoGEF can only homo-oligomerize, through their unique C-terminal regions (Chikumi et al., 2004). Deletion of the C-terminal tail of

PDZ-RhoGEF had no significant effect on the GEF catalytic activity towards Rho in vitro, while it resulted in a drastic increase in its ability to increase GTP-bound Rho in vivo. Such results suggest that the cooperation of other molecules may be required for its GEF function towards Rho. Ephexin-1 has been identified as a Rho GEF that can directly interact with EphA4, become phosphorylated and lead to EphA dependent growth cone collapse. (Sahin et al., 2005).

GEFs may also impact on axon guidance not specifically through the activation of actin polymerization but through other intracellular signals such as the regulation of receptor trafficking to the cell surface. Ephrin binding to Ephs triggers Vav-dependent endocytosis of the ligand-receptor complex, thus converting an initially adhesive interaction into a repulsive event. In the absence of Vav proteins, ephrin-Eph internalization is blocked, leading to defects in GC collapse in vitro and significant defects in axonal projections in vivo (Cowan et al., 2005).

Dock180 has been the only member of the Dock family of GEFs that has been identified as having a potential role in axon guidance, although the evidence remains limited. Mutants of Elmo and Dock180 suppress NGF- and RhoG-induced neurite outgrowth in PC12 cells and a RhoG-Elmo-Dock180 complex activates Rac1 during integrin mediated spreading (Katoh and Negishi, 2003). The strongest evidence has been provided by the Rao lab, who showed that axon outgrowth and axon attraction induced

by netrin-1 were inhibited after DOCK180 knockdown in vertebrate neurons and that netrin-1 induced Rac activation was decreased with the overexpression of Dock180 mutants in in vitro GTPase assays using 293 cells (Li et al., 2008). It remains unclear however its true role in midline guidance since the analysis of Dock180 deficient mice demonstrated a dramatic reduction of all skeletal muscle that could be attributed to a strong deficiency in myoblast fusion but no apparent defects were reported in the CNS and these mice were viable after birth (Laurin et al., 2008). Further studies will be required to further elucidate the role of these GEFs downstream of guidance cues.

#### 1.2.4.4 β-Pix, an additional candidate

Although Dock180 and Trio have been proposed as Rac1 regulators downstream of DCC during Netrin mediated axonal chemoattraction, their role downstream of DCC is still unclear considering their modest knockout guidance phenotype in vivo. The fact that neither of these GEFs up-regulate Cdc42, which takes place downstream of DCC and is implicated sequentially upstream of Rac1 activation (Shekarabi and Kennedy, 2002) also raises the possibility that alternative GEFs are activated during DCC signaling. Chapter 3 provides evidence that an alternative GEF, β-Pix, participates in the complex up-regulation of Cdc42 and Rac1 downstream of DCC to promote chemoattraction in response to netrin-1.

The Pak-interacting exchange factor (Pix) family of homologous GEFs was initially identified for its ability to associate with Pak family kinases through their SH3 domain, and to promote the translocation of Pak complexed to RhoGTPases Rac/Cdc42 to focal adhesions (Bagrodia et al., 1999; Bagrodia et al., 1998; Manser et al., 1998).

Manser et al. 1998 initially identified two Pak-binding proteins that contain Src-homology 3 (SH3), Dbl-homology (DH) and pleckstrin-homology (PH) domains, which they named α-Pix and β-Pix. These two closely related proteins are encoded by the genes ARHGEF6 (αPix) and ARHGEF7 (βPix), respectively. α-Pix also contains a region homologous to the calponin-related domains of Vav and IQGAP (Castresana and Saraste, 1995). In parallel Bragodia et al. in a Pak3 yeast-two-hybrid screen identified p50Cool-1 (for: cloned-out of library), along with a splice variant, p85Cool-1, and a closely related protein, Cool-2, which are identical to β-Pix and α-Pix, respectively. Since then β-Pix splice variants (βPix-a/β1Pix, βPix-d/β2Pix, βPix-b, βPix-bL, βPix-c) were cloned from mouse and rat brain and found to be highly expressed during the period of active neurogenesis in the cerebral cortex and cerebellum suggesting a role in neuronal cell migration (Kim et al., 2000; Kim and Park, 2001; Rhee et al., 2004). Interestingly, betaPix-d lacks the leucine zipper domain that is present in the other Pix isoforms (Kim and Park, 2001). The leucine zipper is important for the homo-dimerization of Pix proteins and their promotion

of cytoskeletal remodeling such as membrane ruffles (Kim et al., 2001). It will be interesting to dissect out the role of the various isoforms in the CNS during development.

αPix and βPix-bL are distinguished from most βPix forms by the presence of a calponin homology (CH) domain. Although the function of this single CH domain is mainly unknown, the αPix CH domain seems to be required for proper targeting of αPix to the plasma membrane as well as interaction with one of its binding partners β-parvin (Rosenberger and Kutsche, 2006). Additionally, a mutation in ARHGEF6 leading to an αPix protein lacking 28 amino acids in the CH domain was identified in patients with X-linked non-specific mental retardation (Kutsche et al., 2000).

Unlike some of the other Dbl-homology GEFs previously identified, the Pix family shares the ability of directly associating with the upstream RhoGTPases Rac/Cdc42 and activating their downstream effector Pak (Bagrodia and Cerione, 1999). The localization of Pak by β-Pix is mediated by its interaction with Git1 (G protein-coupled receptor kinase-interacting protein) (Webb et al., 2005) and the Pix–Git complex functions as a linker for other signaling components, such as the focal adhesion protein Paxillin, thus relocalizing the entire complex to focal adhesions (Premont et al., 2004). These complexes have been implicated in the localization and activation of Pak and Rac1 not only in motile cells but also in the promotion of receptor recycling (Flanders et al., 2003; Jozic et al.,

2005; Wu et al., 2003) and synaptic structure formation (Audebert et al., 2004; Lahuna et al., 2005; Zhang et al., 2003). Pak interaction with β-Pix is mediated through a high affinity and unconventional SH3-motif interaction (Hoelz et al., 2006; Li et al., 2006; Manser et al., 1998). Additionally, it appears that when Pak is activated, Ser-199 in the Pix-binding site is phosphorylated. This phosphorylation is sufficient to reduce the affinity for Pix 6-fold (Mott et al., 2005). It has been proposed that β-Pix competes out the inhibitory activity of p50Cool/Pix toward Pak1 and that α-Pix can directly activate Pak1 dependently and independently of its GEF activity (Daniels et al., 1999).

Dimerization of αPix enables it to activate Rac, whereas dissociation of the dimer into monomers allows αPix to function as a GEF for Rac as well as Cdc42 (Feng et al., 2004). Moreover, given the ability of αPix to bind to activated forms of Rac1 and Cdc42, it has been demonstrated that the binding of activated Cdc42 to the αPix dimer significantly increased its Rac-specific GEF activity (Baird et al., 2005). This Cdc42-Rac activation cascade is consistent with previous evidence supporting the sequential activation of Cdc42 and Rac downstream of DCC (Shekarabi and Kennedy, 2002)(add Shekarabi et al 2005).

β-Pix can also be regulated by phosphorylation events. Endothelin-1 (ET-1) regulates the focal complex by PKA mediated phosphorylation of β-Pix

at the Ser-516 and Thr-526 sites, which are highly conserved in Pix proteins, suggesting an important functional role for these residues. Wild type β-PIX when overexpressed, enhanced ET-1 dependant activation of Cdc42 while β-Pix mutants lacking either GEF activity (DH domain) or PAK binding activity (SH3 domain), or mutated at the PKA phosphorylation sites could not mimic this activity. Additionally, NGF induced neurite extension is dependent on the phosphorylation of these β-Pix sites (Chahdi et al., 2005; Shin et al., 2002). β-Pix is also phosphorylated on tyrosine 442 in response to growth factors and/or in a Src/FAK-dependent manner (Feng et al., 2006). Phosphorylation of Tyr-442 activates its Cdc42-GEF activity and promotes the formation of a Cdc42-Cool-1-Cbl complex, which regulates the timing of EGF receptor (EGFR) degradation and is essential for Src-induced cellular transformation. It remains to be seen if similar phosphorylation events regulate receptor recycling in other systems. Taken together, current data suggest that depending on its phosphorylation status, βPIX may function either as a scaffold, a GEF, or both to coordinate the activation of Cdc42 and Rac at the leading edge.

An alternative way in which Pix activity can be regulated is through localization and targeting of macromolecular complexes to the cell membrane. An α-Pix-Git2 complex controls neutrophil directional chemotaxis by regulating the localization of the PI3-kinase effector AKT and PTEN in neutrophils exposed to chemoattractants (Mazaki et al., 2006). αPix also forms a complex with the regulatory p85 subunit of PI3-

kinase, and PI3-kinase enhances the GEF activity of αPix, thus lending further support to the existence of cross-talk between the Pix-Git complex and PI3-kinase signaling pathways. In this study α-Pix was found to be activated downstream of platelet-derived growth factor (PDGF) receptor EphB2 receptor, and integrins through its direct interaction with Pak, Nck and/or direct association with the p85 regulatory subunit of PI3-kinase (Yoshii et al., 1999). Finally, extensive data supports a role for Git-Pix-Pak complexes in neuronal morphogenesis. Consistent with other cellular models, in NGF-stimulated PC12 cells, PAK and βPIX activate Rac1 leading to lamellipodia formation (Obermeier et al., 1998). Neurite extension in response to βFGF is repressed when two major ERK/PAK phosphorylation sites (Ser525, Thr526) in βPIX are mutated (Shin et al., 2002). Furthermore, in chick embryonic retinal neurons the overexpression of GIT1 fragments predicted to competitively sequester Pix led to the inhibition of neurite extension (Albertinazzi et al., 2003).

Pix-Git complexes also have a role in spine morphogenesis and synaptic plasticity. Spine formation is inhibited by the overexpression of βPIX or mutant GIT1, which cannot bind βPix (Zhang et al., 2003). FRET analysis showed activated Rac normally localizes selectively to the base of dendritic protrusions; however, it is detected throughout the elongated protrusions observed upon overexpression of βPix (Zhang et al., 2005). Such data suggest a role for Pix/Pak/Rac complexes in the extension of dendritic protrusions that is regulated by the competitive association and sequestering of Pix through Git binding. Direct interaction with pre- and

post-synaptic proteins such as Shank, Piccolo and liprin-$\alpha$ further supports a potential role for these complexes at synaptic membranes (Kim et al., 2003; Ko et al., 2003; Park et al., 2003). Additionally, in hippocampal neurons activation of ephrinB leads to phosphorylation of GIT1 on Tyr392. This event creates a docking site for the SH2-SH3 adaptor Grb4, which binds to the tail of activated ephrinB. Activated ephrinB thus forms a ternary complex with Grb4 and GIT1. Interfering with the formation of the ephrinB-Grb4-GIT1 complex blocks reverse signaling and perturbs normal spine morphogenesis and synapse formation (Segura et al., 2007). The role of specific Pix isoforms and of Git2 at the synapse remains to be established.

## 1.3 Concluding Remarks

The continued search for an understanding of the intracellular signals required during axon guidance is crucial for the advancement in therapies for degenerative diseases and regenerative growth. Additionally these signals also appear consistently in the adult brain during plasticity and in the establishment of synaptic connections. It will be important to unravel how some of the components already identified downstream of netrin-1 are regulated in time and localization and what the differences are in their regulation between different cell types. In the following chapters additional signaling molecules are identified downstream of netrin-1 and a new

model is proposed for the regulation of RhoGTPases downstream of netrin-1 during commissural neuron chemoattraction.

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# CHAPTER 2:

# AN UNBIASED MASS SPECTROMETRY ANALYSIS FOR DCC ASSOCIATED PROTEINS DOWNSTREAM OF NETRIN IN COMMISSURAL NEURONS

## PREFACE

Until relatively recently, little was known of the signaling mechanisms regulated downstream of DCC that promote netrin-1-mediated axon guidance. The Kennedy laboratory, and others, have provided evidence that netrin-1 signaling through DCC induces filopodia formation and growth cone expansion by activating the RhoGTPases Cdc42 and Rac1 and their downstream effector the serine-threonine kinase Pak1. In order to identify additional cytoskeletal signaling regulators downstream of DCC we performed a mass spectrometry analysis on proteins immunoprecipitated with DCC from commissural neuron lysates. The function of two of the proteins isolated from this unbiased mass spectrometry screen, Arp2/3 and 14-3-3, were characterized further using function blocking experiments. The results obtained provide further insight into the intracellular signaling mechanisms downstream of DCC that regulate the remodeling of the actin cytoskeleton.

**ABSTRACT**

The chemotropic guidance cue netrin-1 attracts or repels different classes of neurons during the development of the central nervous system. In the developing spinal cord netrin-1 is one of the guidance cues responsible for the commissural axon extension to the floorplate. Previous studies demonstrated that the attractive response to netrin-1 is dependent on the activation of the Rho GTPases Rac and Cdc42 in commissural growth cones downstream of the netrin receptor DCC. In this study we used an unbiased proteomics approach to identify additional candidate proteins that might contribute to regulating actin remodeling in axonal growth cones in response to netrin-1. Arp2/3 and 14-3-3 were two of the protein hits identified in this analysis. Arp2/3 association with DCC was confirmed and increased upon netrin treatment. Similarly 14-3-3 association with DCC was confirmed, however, in contrast to Arp2/3, upon netrin treatment 14-3-3- binding decreased. Function blocking analysis using a chemical and peptide function blocking approach provided evidence that both Arp2/3 and 14-3-3 are required for netrin mediated commissural growth cone expansion and filopodia formation. Additionally, treatment of commissural neurons with netrin-1 was not sufficient to rescue the loss of cell surface DCC when cells were co-treated with wiskostatin, a chemical inhibitor of N-Wasp mediated Arp2/3 activity. These studies provide insight into novel mechanisms regulating the response to netrin-1 in commissural neurons.

## INTRODUCTION

Growth cones undergo continuous remodeling of the actin cytoskeleton during axon guidance. Actin remodeling includes the expansion of the growth cone by protruding membranes, extension of filopodia for directional sensing of the extracellular environment and the formation of high turnover and stable adhesions required for the forward propulsion of the axon shaft.

Early studies carried out using *in vitro* cultures of Xenopus neurons and genetic analysis carried out in *C. elegans* demonstrated that F-actin is the primary cytoskeletal element that maintains growth cone shape and is essential for proper axon guidance, whereas microtubules (MTs) are essential for giving the axon structure and serve an important function in axon elongation (Chien et al., 1993; Dent and Gertler, 2003; Yamada et al., 1970; Yamada and Wessells, 1971). Subsequent studies (Bray et al., 1978) demonstrated that both MTs and the actin cytoskeleton are essential for growth cone steering (Geraldo and Gordon-Weeks, 2009). Application of MT-depolymerising or MT-stabilizing compounds resulted in the inability of growth cones to turn at sharp substrate borders in culture (Buck and Zheng, 2002; Challacombe et al., 1997; Williamson et al., 1996). Similarly, filopodial F-actin depolymerisation inhibits microtubule extension and growth cone turning toward the depolymerized side (Zhou et al., 2002). Examining the motility of the large growth cones elaborated

by neurons from the marine mollusk *Aplysia* revealed that treatment with low concentrations of cytochalasin B, which selectively disrupts F-actin, revealed the loss of growth cone filopodia, lamelipodia containing an abnormal number microtubules and an inability to form neurites (Burnette et al., 2007; Dent et al., 2007). These studies identified an essential role for F-actin underlying growth cone morphology and motility.

Although MTs form the core cytoskeletal element of the axon shaft and actin fibers encompass most of the peripheral growth cone (GC), some MTs do extend out to the periphery of the GC and interact with newly formed actin filaments at the tips of filopodia (Schaefer et al., 2002). These findings underscore the importance of the interaction of MT and F-actin during growth cone steering. It remains unclear how these cytoskeletal filament interactions occur, how they are regulated, and whether they are preserved between cytoskeletal structures such as growth cone filopodia, lamelipodia and dentritic filopodia and spines.

The Arp2/3 complex is a seven-member protein complex that nucleates the formation of new actin filaments from the sides of pre-existing actin filaments. The complex was originally identified as a profilin-binding complex and contains two actin-like proteins, Arp2 and Arp3, plus five additional proteins (Machesky and Gould, 1999; Zigmond, 1998). Unlike previously known F-actin nucleators, the Arp2/3 complex caps not the barbed end of the actin filament but rather its pointed end, leaving the new

barbed end free, thereby enhancing polymerization *in vivo*. The Arp2/3 complex remains attached to the pointed end, blocking both polymerization and depolymerization (Gournier et al., 2001). Arp2/3 is structurally and functionally well conserved in a variety of species, from yeast, to Xenopus, to humans. It is found at the leading edge of motile cells, enriched in lamellipodia. It is therefore not surprising that it plays a crucial role at protrusions of growth cones. Several studies have demonstrated a role for Arp2/3 complexes with N-Wasp and Mena in neuritogenesis in hippocampal neurons and dendritic remodeling downstream of nerve growth factor (Banzai et al., 2000; Goldberg et al., 2000). Despite conflicting data regarding the involvement of Arp2/3 in growth cone motility (Strasser et al., 2004), it is clear that axon guidance and filopodia formation are processes highly dependent on Arp2/3 activity (Korobova and Svitkina, 2008). Genetic studies in *C.elegans* have further demonstrated that Arp2/3, forming a complex with members of the WAVE/WASP family, regulates lamellipodia and filopodia formation during axon guidance (Norris et al., 2009; Shakir et al., 2008).

14-3-3 proteins regulate actin dynamics through binding and stabilization of inactive phospho-cofilin. Expression of 14-3-3ζ in cells results in increased levels of phospho-cofilin (Gohla and Bokoch, 2002). It has been suggested that 14-3-3 proteins protect cofilin from dephosphorylation by decreasing the availability of inactive cofilin, thereby affecting the rate of cofilin phosphocycling. Alternatively, 14-3-3 may act as a chaperone to

bring cofilin in proximity with its other regulatory molecules, phosphatases LIMK/TESK or Slingshot (SSH). The nine different isoforms of 14-3-3 can form homo or hetero-dimers and are highly conserved throughout several species. These scaffolds are regulators of not only the F-actin regulatory machinery but also influence other crucial cellular pathways, such as apoptosis, gene expression and vesicle trafficking.

In this study we identify Arp2/3 and 14-3-3ζ as DCC associated molecules by performing a non-biased mass spectrometry analysis of proteins isolated from a DCC immunoprecipitation from commissural neuron lysates. We confirm that upon treatment of embryonic rat commissural neurons with netrin-1, Arp2/3 association with DCC increased while 14-3-3 association decreased. We provide evidence that both Arp2/3 and 14-3-3 function are required for netrin-1 mediated growth cone expansion and filopodia formation in commissural neurons, perhaps through separate pathways. Importantly, these findings implicate the function of two additional F-actin regulatory proteins as downstream components of DCC signaling in the growth cones of embryonic spinal commissural neurons.

## MATERIALS AND METHODS

*Reagents and Cell Culture:* Embryonic rat spinal commissural neurons were cultured as described (Bouchard et al., 2004). Briefly, dorsal halves of the spinal cord of embryonic day 13 (E13; E0, vaginal plug) rat embryos were microdissected, dissociated for 30 min at 37°C in $Ca^{2+}$/$Mg^{2+}$- free HBSS (Invitrogen), followed by trituration with a flame-polished Pasteur pipette, and cultured in Neurobasal (Invitrogen) with 10% heat-inactivated fetal bovine serum with 100 U/ml penicillin and 100 U/ml streptomycin. After 24 h, the medium was changed to Neurobasal supplemented with 2% B-27 (Invitrogen), 2 mM glutamine, and penicillin/streptomycin. Tissue culture dishes were coated with 10 µg/ml poly-D-lysine (PK; Sigma, St. Louis, MO) at RT for 1h. Recombinant netrin-1 protein was purified from a HEK 293-EBNA cell line secreting netrin-1, as described (Serafini et al., 1994; Shirasaki et al., 1996). For R18 experiments E13 commissural neurons were transduced with recombinant HSV-WLRL-GFP or HSV-R18-GFP and plated overnight in Neurobasal (Invitrogen) with 10% heat-inactivated fetal bovine serum with 100 U/ml penicillin and 100 U/ml streptomycin.

The following reagents were used: R18 and WLRL expression vectors were constructed by synthesizing oligos encoding the peptide sequence (R18: ccccactgtgtcccccgagatctttcgtggttagatttagaagcaaatatgtgtttaccc WLRL: ccccactgtgtcccccgagatctttcgtggttaaggttagaagcaaatatgtgtttaccc),

ligating into pCS2+ EGFP and then subcloning R18 EGFP and WLRL EGFP into pHSVPrPUC. Recombinant herpes simplex virus was produced by transfecting pHSVPrPUC plasmids into 2-2 Vero cells that were superinfected with 5dl 1.2 herpes simplex virus (HSV) helper virus 1 d later. Recombinant virus was amplified through three passages and stored at −80°C as described previously (Neve et al., 1997; Kent et al., 2010). Wiskostatin was obtained from Calbiochem. The following antibodies were used: anti-p34-Arc/ARP2 (polyclonal; Upstate Signaling), anti-pan-14-3-3 (polyclonal; Santa Cruz), anti-DCCin (monoclonal; PharMingen, Canada).

*F-actin staining, and quantification:* Filamentous actin was visualized using Alexa488 or Alexa 546-phalloidin (Sigma). Filopodia number and growth cone surface area were quantified as described (Shekarabi & Kennedy 2002). For quantifications, photomicrographs were taken using a Carl Zeiss Axiovert microsope, phase-contrast optics, a 20× objective lens, Magnafire CCD camera (Optronics, CA), and analyzed using ImageJ analyses software.

*2-Dimensional Liquid Chromatography Mass Spectrometry*: Following SDS-PAGE protein separation and band isolation, bands were tryptically digested. Mass spectrometry analysis was carried out on a quadrupole time-of-flight microinstrument (QTOF; Waters Corporation, Milford, MA). QTOF micro tandem mass spectrometry analysis utilized data-directed

acquisition of the most intense multiply-charged ion from each survey scan while using dynamic ion exclusion of previously selected precursor ions for 90 seconds. Qtrap 4000 tandem mass spectrometry analysis was done using Information Dependent Acquisistion of the three most intense multiply charged ions from each survey scan with dynamic exclusion of 90 sec. Peaklists for database searching were generated using Mascot Distiller 2.0.0 software (Matrixscience, Boston, MA). Tryptic peptide identifications were carried out by searching a National Center for Biotechnology Information non-redudant (NCBInr; ftp://ftp.ncbi.nih.gov/blast/db/FASTA/nr.gz) protein sequence database using Mascot Distiller. A program developed in-house (CellMapBase) was used to cluster and group peptides and proteins to generate a non-redundant minimal list of identified proteins. Proteins with significant hits ($p<0.05$) were analyzed for each tissue sample. Only proteins that were detected in 2 of the 3 samples were documented as positive identifications.

*Co-Immunoprecipitation:* For coimmunoprecipitation (coIP) analyses commissural neurons were treated with 200 ng/ml netrin-1 for 5 min and then lysed in ice-cold 1% Triton X-100 lysis buffer with protease inhibitors on ice. Immunoprecipitations were performed using 1 µg of anti-$DCC_{IN}$, on a rocking platform at 4°C for 1h. Protein A/G beads were added and allowed to incubate for 30 min. Beads and associated proteins were then pelleted and washed three times in lysis buffer with protease inhibitors.

Proteins were eluted from the beads using 10 ml of Laemmli buffer and characterized by Western blot analysis.

*Biotinylation assays:* Biotinylation assays were performed as previously described (Bouchard et al., 2004). Briefly, E13 dorsal spinal cords were dissociated, and commissural neurons were plated and cultured for 6 d at a density of ~2,000,000 cells per 100 mm PDL-coated tissue culture dish. On day 6, cells were left untreated or treated with wiskostatin for 15 min, followed by the addition of 50 ng/ml netrin-1 or vehicle to the culture media for 15 min. Neurons were exposed for 15 min to 10 µM FSK as an additional control. Cells were then washed with ice-cold PBS containing 0.1 mM calcium chloride and 1 mM magnesium chloride, pH 7.4, to halt protein trafficking. Surface biotinylation was performed by adding EZ-Link Sulfo-NHS-LC-biotin (Pierce), 5 ml per plate at 0.5 mg/ml in PBS at 4°C for 30 min, removed, and the reaction was quenched by the addition of 5 ml of 10 mM ice-cold glycine in PBS at 4°C for two 10 min periods. Subsequently, cells were washed twice with 5 ml of ice-cold PBS and lysed with RIPA buffer. Biotinylated proteins were precipitated with streptavidin–agarose (Pierce) and analyzed by Western blot.

## RESULTS

*Isolation and identification of DCC associated proteins in commissural neurons by mass spectrometry*

With the goal of identifying candidate proteins that function downstream of DCC in embryonic spinal commissural neurons, we employed two dimensional liquid-chromatography mass spectrometry analysis of proteins co-immunoprecipitating with DCC from lysates of netrin-1 stimulated embryonic spinal commissural neurons. Cultures of E13 rat spinal commissural neurons were grown for 2 days *in vitro* prior to treatment with netrin-1 for 15 minutes. Commissural neurons were lysed and proteins immunoprecipitated from 1mg of lysate using a monoclonal DCC antibody (BD Pharmingen; Lane 3 of Figure 2-1A). As a control 1mg of the same lysate was incubated with Protein A/G beads alone (Lane 2 of Figure 2-1A). 20 μg of protein from the whole cell lysate was loaded as a control (Lane 4 of Figure 2-1A). DCC associated proteins were separated by SDS-PAGE and 10 clear, well-defined bands of varying sizes not visible in the beads-only lane were isolated and analyzed by 2DLC-MS (Figure 2-1A). A Coomassie blue-stained gel is included to show the relative amount of protein present in each band (Figure 2-1A). This process was repeated and only proteins with 1 or more peptide hits that were present in both mass spectrometry runs were considered for further analysis. A short sample list of peptide hits can be found in Figure 2-1B. A

wide range of proteins with varying functions in cellular homeostasis was found; from actin associated proteins, such as those described hereafter to components of the ribosome (Figure 2-1B). Consistent with these findings, recent evidence has argued that the intracellular domain of DCC interacts directly with the ribosome (Tcherkezian et al., 2010), a finding that extends previous reports that DCC plays an important role regulating local translation in axonal growth cones (Leung et al., 2006; Lin and Holt, 2007, 2008a, b; Merianda et al., 2009). These results further validate our mass spectrometry screen where a high number of ribosomal protein translation machinery components such as, ribosomal subunit 2 and subunit 3 and even elongation factor Tu (EF-Tu), which hydrolyzes guanosine triphosphate (GTP) and releases tRNA in response to codon recognition (Figure 2-1B).

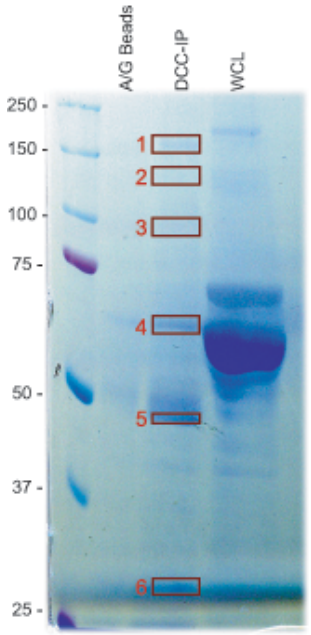
MAPK signaling components such as MAPKK (Figure 2-1B) and MAPKKK (not shown) were also consistently identified in our mass spectrometry runs. This result is consistent with a previous report that MAPK signaling components associate with DCC in commissural neurons to promote axon outgrowth and guidance (Forcet et al., 2002).

In order to validate proteins identified by mass spectrometry as potentially interacting with DCC, we probed DCC immunoprecipitates from embryonic commissural neuron homogenates using specific antibodies and western blot analysis. Short single hit peptides such as the predicted Intersectin-2 peptide hit in band 2 were confirmed to be non-specific by western blot analysis of a DCC immunoprecipitation using an Intersectin-2 specific

antibody (data not shown). Such results further highlight an additional drawback of utilizing mass spectrometry as a single method for protein identification. Not all peptides resulting from the digestion of a protein can be observed or correctly identified with MS analysis, especially those with diverse or unexpected post-translational modifications (Han et al., 2008). For this reason, only proteins with two or more significant ($p<0.05$) peptide hits in the two separate experiments were considered for further analysis.

**Figure 2-1. Mass spectrometry analyses of DCC associated proteins** A) SDS-PAGE gel stained with commassie blue outlining the bands isolated for mass spectrometry. Lane 1 – E13 commissural lysates incubated with protein A/G beads alone; Lane 2 – DCC immunoprecipitates of E13 commissural lysates; Lane 3 – E13 commissural whole cell lysates. B) Table of sample of peptides isolated in the mass spectrometry screen.

A




B

| *Protein description* | *Peptides* | *Band* |
|---|---|---|
| ***Cytoskeleton associated hits*** | | |
| Ssh1 protein | AQEIETRLR | |
| | KPDVSGSGAGAAPEPPASLLEPSRETSK | 1 |
| Actin-related protein complex 3, mu 2 subunit | LNQQKLPSLK | 2 |
| Mitogen-activated protein kinase kinase kinase | MEPGRGAGPAGMAEPR | 3 |
| 14-3-3 protein epsilon isoform | EAAENSLVAYK | 6 |
| ***Ribosomal translation associated hits*** | | |
| Ribosomal protein S2 | GCTATLGNFAK | 1 |
| RPS2 protein | KLLMMAGIDDCYTSAR | 1 |
| ribosomal protein S3 | ELAEDGYSGVEVR | 2 |
| | GLCAIAQAESLR | |
| factor Tu GTP binding domain protein 2 | IAVEPVNPSELPK | 1 |

*14-3-3 epsilon association with DCC in commissurals decreases with Netrin treatment*

ADF/cofilin (AC) is responsible for rapid F-actin turnover in various cell types. In neurons ADF and cofilin are highly co-localized with F-actin in growth cones and are necessary for neurite outgrowth. The activity of these F-actin-capping proteins is tightly regulated by LIM kinases and SSH phosphatases. 14-3-3ζ scaffolding proteins regulate the level of AC activation by SSH by associating with the phosphoserine in AC and protecting it from dephosphorylation, thereby holding it in its inactive form (Gohla and Bokoch, 2002). Phosphorylated AC has lower affinity for G and F-actin (Ressad et al., 1998). The balance in levels of phosphorylated and non-phosphorylated AC maintains the rapid turnover of F-actin at the forefront of the growth cone. Furthermore recent evidence suggests that gradients of NGF and netrin-1 locally activate AC to promote actin polymerization and subsequently promote growth cone turning up a gradient of a chemoattractant (Marsick et al., 2010). Using mass spectrometry we identified 14-3-3ζ as a candidate novel DCC interacting protein (Figure 2-1B). To validate 14-3-3 as a DCC interactor, DCC immunoprecipitation was repeated from commissural neuron lysates and a western blot probed with a 14-3-3 pan antibody (Figure 2-2A). In agreement with our mass spectrometry data we found that 14-3-3 associated with DCC in commissural neurons, however surprisingly this association was decreased with the addition of netrin-1 (Figure 2-2A). This finding suggested that 14-3-3 might be negatively regulating AC by

keeping it in its phosphorylated state and upon netrin-1 binding to DCC 14-3-3 is released from the complex and AC can locally promote actin polymerization.

**Figure 2-2.Validation of mass spectrometry analyses results.** A) Western blot analyses of DCC immunoprecipitations from E13 spinal cord commissural lysates treated and untreated with 200mg/ml netrin-1 for 5 minutes. Western blot using a pan-14-3-3 antibody confirms a decrease in association of 14-3-3 with DCC complexes. B) Western blot analyses to confirm association of Arp2/3 with DCC seen by mass spectrometry. Western blot using an anti-p34 Arp2 antibody on DCC immunoprecipitates from E13 commissural lysates shows an increase in association with netrin-1 treatment.

A
IP
Netrin
Control
DCC
-
-
+
IgG
IP
pan 14-3-3
Input
pan 14-3-3
B
Netrin (200ng/ml) 5'
A/G beads
(+)
IP
(-)
(+)
50kDa
25kDa
p34 – Arp2/3
IgG
DCC IP
WB: p34- Arp2/3
Input
WB: p34- Arp2/3

*Application of netrin-1 is insufficient to rescue growth cone collapse induced by blocking 14-3-3 function*

To test the hypothesis that 14-3-3 activity might negatively regulate cofilin mediated actin depolymerization, commissural neurons were infected in culture with an RFP-tagged HSV encoding a peptide (R18) that blocks 14-3-3 function. The R18 peptide binds to the same amphipathic groove on the surface of 14-3-3 as Ser/Thr phosphorylated peptides. R18 was initially identified through a screening of phage display libraries. This potent 14-3-3 peptide antagonist (PHCVPRDLSWLDLEANMCLP) was shown to inhibit the formation of the 14-3-3-Raf-1 complex, reduce Raf-1 dependent transcriptional activity in cells and block the activation of ExoS by 14-3-3 (Wang et al., 1999). The function of R18 as a potent 14-3-3 peptide antagonist has been further confirmed by blocking 14-3-3 association with p27Kip1 and affecting 14-3-3 dependent recruitment of p27Kip1 to the cytoplasm (Fujita et al., 2003). This was used to demonstrate a role for 14-3-3's in apoptosis (Dong et al., 2008) and to establish 14-3-3 proteins as inhibitors of IGF-I-induced cardiac fibroblast proliferation via a PI3-K-dependent NFAT signalling pathway (Qi et al., 2010). The peptide binds to all the 14-3-3 isoforms with equal affinities and it has also been shown to inhibit 14-3-3 binding to both phosphorylated and non-phosphorylated downstream effectors.

Dr. Alyson Fournier's laboratory generated and provided us with a recombinant R18-green fluorescent protein expressing herpes simplex

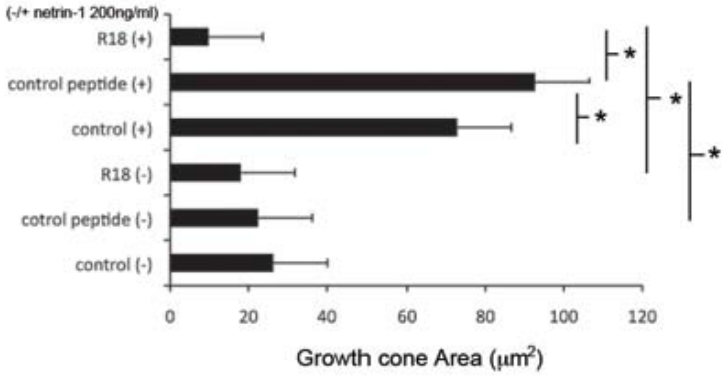
virus (HSV-R18-GFP) and a control HSV-WLRL-GFP virus that does not bind to 14-3-3 because of a substitution in the core-binding motif (WLDL to WLRL) (Wang et al., 1999). Cultured embryonic rat spinal commissural neurons were infected for 2 hours. Following infection commissural neuron culture media was changed and cells were incubated overnight. Infected commissural neurons were then treated with 200 ng/ml of netrin-1 for 15 minutes, fixed and immunostained with Phalloidin-546 for analysis of growth cone expansion, including measurement of growth cone surface area and the number of filopodia per growth cone. The surface area of the growth cones of cultured embryonic rat spinal commissural neurons infected with either the R18 peptide or control peptide was not significantly changed under basal conditions (Figure 2-3A-B).

Application of netrin for 15 minutes was sufficient to induce growth cone spreading in non-treated commissural neurons by 2-fold (Figure 2-3A-B). Similarly, the growth cones of commissural neurons that were infected with virus carrying the control peptide expanded by more than 2-fold (Figure 2-3A-B). In contrast, commissural neurons expressing the R18 peptide had either growth cones that had collapsed to sizes similar to basal conditions or even collapsed completely (Figure 2-3A-B).

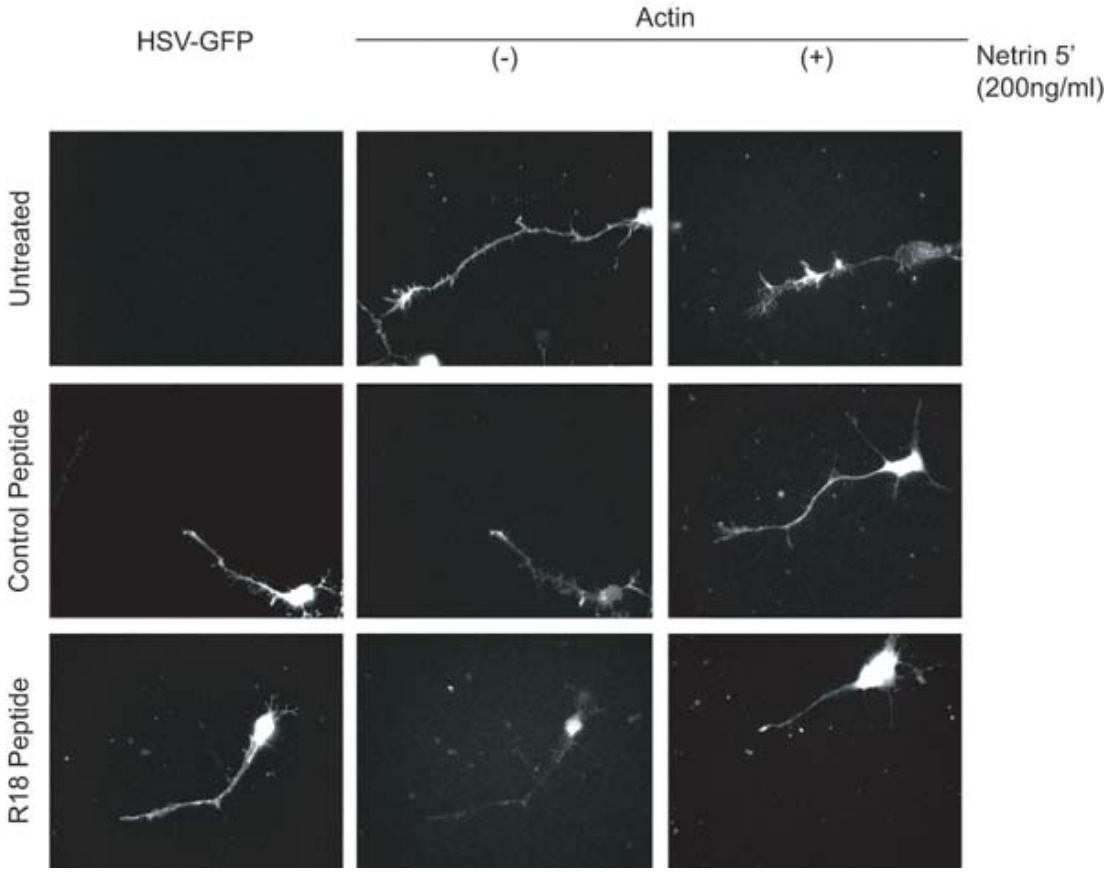
These findings provide evidence that 14-3-3 function is required downstream of netrin-1 in order to induce and/or maintain growth cone expansion.

**Figure 2-3. 14-3-3 function blocking peptide inhibits netrin mediated growth cone expansion.** A) Quantification of growth cone area of E13 commissural neurons after 2 hour infection with R18 peptide containing virus and/or 15 minute treatment with netrin-1 (200ng/ml). n>10 from 3 separate experiments; mean ± SEM; $p<0.05$. B) E13 commissural neurons infected with HSV-GFP alone (top lane), HSV-GFP-control peptide (middle lane) or HSV-GFP-R18 peptide (bottom lane). Column 1 shows green channel and columns 2 and 3 shows the red channel for phalloidin-546 staining. Neurons in column 3 were treated with netrin-1 (200mg/ml) for 15 minutes. Images were captured with a 20x objective lens.

A


(-/+ netrin-1 200ng/ml)
R18 (+)
control peptide (+)
control (+)
R18 (-)
cotrol peptide (-)
control (-)
0
20
40
60
80
100
120
Growth cone Area (μm²)
*
*
*
*

B


HSV-GFP
Actin
(-)
(+)
Netrin 5'
(200ng/ml)
Untreated
Control Peptide
R18 Peptide

*Netrin-1 increases the association of Arp2/3 with DCC in commissural neurons in vitro*

The Arp2/3 complex has been implicated in axon pathfinding *in vivo* in *Drosophila* and *C. elegans* (Shakir et al., 2008; Zallen et al., 2002). In both species, Arp2/3, and members of the WAVE/Scar complex that regulate Arp2/3, are required for appropriate axon guidance during development (Shakir et al., 2008; Zallen et al., 2002). Recent studies in *C. elegans* have revealed that Arp2/3 mutants display reduced numbers of growth cone filopodia and also reduced growth cone size (Norris et al., 2009).

To validate the possible association of Arp2/3 with DCC in commissural neurons identified during the mass spectrometry analysis (Figure 1A-B) we repeated a DCC immunoprecipitation from commissural neuron lysates followed by a western blot using an antibody against p34/Arc2, subunit 2 of the Arp2/3 protein complex (Figure 2-2B). Arc2 was readily detected by co-immunoprecipitation in association with DCC in basal culture conditions, without the addition of exogenous netrin-1. Fifteen minutes following the addition of 200 ng/ml netrin-1, an increase in Arc2 associated with DCC was detected. The findings presented, in addition to the mass spectrometry results, are consistent with Arc2 being recruited into a complex with the intracellular domain of DCC in response to netrin-1.

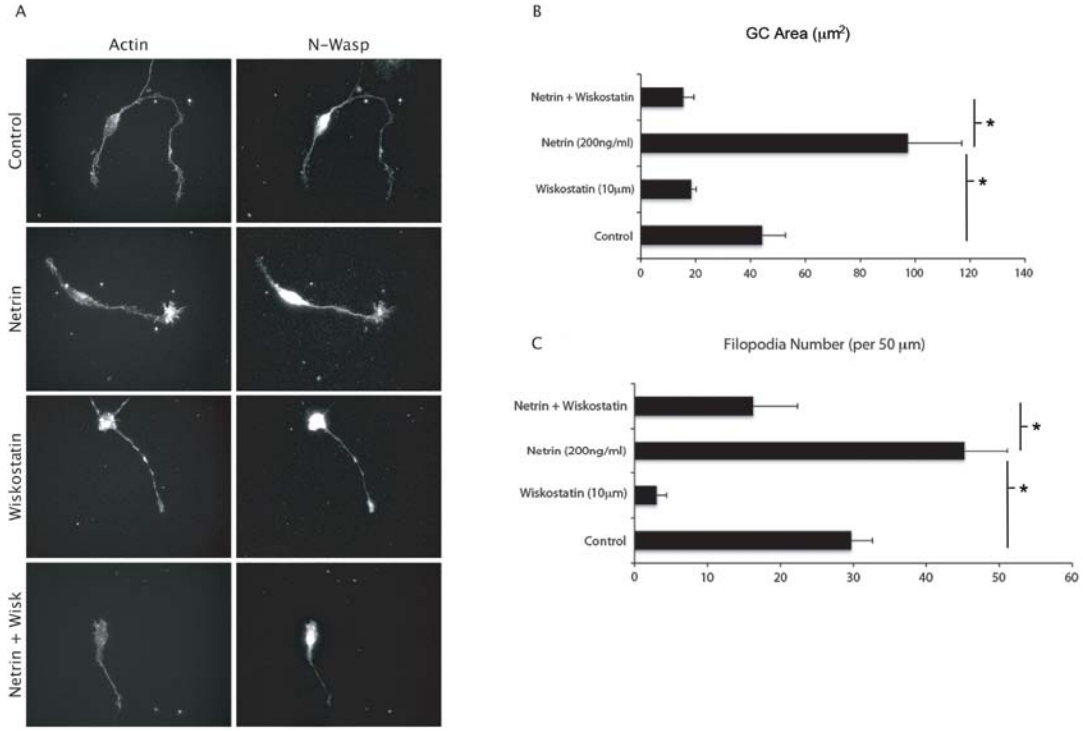
*Wiskostatin treatment inhibits netrin-mediated growth cone remodeling*

The Arp2/3 complex can be activated by WASp/Scar/WAVE family members, Listeria ActA, cortactin, budding yeast Abp1 and Pan1, fission yeast myosin-I's, and Dictyostelium CARMIL proteins (Duncan et al., 2001; Goode et al., 2001; Higgs and Pollard, 2001; Jung et al., 2001). Most of these proteins bind Arp2/3 via a conserved acidic domain. The binding of Arp2/3 to the side of a mother actin filament also activates the Arp2/3 complex, in a manner cooperative with activation by WASps (Higgs and Pollard, 2001). The Kennedy laboratory previously reported an increased association of N-Wasp and DCC in response to netrin-1 (Shekarabi et al., 2005). The finding presented in this study suggests a model in which N-Wasp might provide a component of a link between DCC and polymerized actin at the tips of newly formed filopodia. We then hypothesized that N-Wasp might activate Arp2/3 downstream of netrin-1 in commissural neurons to promote the polymerization of actin required for filopodia formation and growth cone expansion that occurs in response to application of netrin-1.

To test this hypothesis we used Wiskostatin, a chemical inhibitor of N-Wasp dependent Arp2/3 activation. Wiskostatin is a selective, reversible inhibitor of N-WASP that functions by stabilizing N-Wasp in its autoinhibited conformation, thereby preventing the activation of Arp2/3.

Treatment of cultured commissural neurons with 10μM Wiskostatin for 15 minutes resulted in growth cone collapse (Figure 2-4A), providing evidence that constitutive activation of N-WASP and its effectors, like Arp2/3, are required to maintain growth cone morphology. Adding netrin-1 and Wiskostatin for 15 minutes to the cultures did not rescue the Wiskostatin dependent growth cone and filopodia collapse (Figure 2-4A-C). Quantification of growth cone area demonstrated that the 2-fold increase promoted by netrin-1 was blocked by Wiskostatin treatment (Figure 2-4B). Interestingly although growth cones collapsed completely when treated with Wiskostatin, the localization of N-Wasp (Figure 2-4A), and DCC (not shown) were not affected, but were still found at the tip of the collapsed growth cones. It is possible that recruitment of these proteins to the growth cone is not dependent on Arp2/3 activation by N-Wasp or alternatively these proteins upon wiskostatin treatment were "trapped" in their subcellular compartment.

**Figure 2-4. Netrin mediated filopodia extension is blocked by wiskostatin treatment.** A) Representative examples of commissural neuron growth cone morphologies treated with wiskostatin and/or netrin-1 or left untreated. The left column shows actin through phalloidin-A488 staining and the right column depicts N-Wasp immunostaining. Images were taken using a 20x objective. B-C) Quantification of growth cone area and filopodia number per 50 μm of axon in all conditions (n≥25 per condition; error bars indicate SEM; $p<0.05$).


A
Actin
N-Wasp
Control
Netrin
Wiskostatin
Netrin + Wisk
B
GC Area (μm²)
Netrin + Wiskostatin
Netrin (200ng/ml)
Wiskostatin (10μm)
Control
0
20
40
60
80
100
120
140
*
*
C
Filopodia Number (per 50 μm)
Netrin + Wiskostatin
Netrin (200ng/ml)
Wiskostatin (10μm)
Control
0
10
20
30
40
50
60
*
*

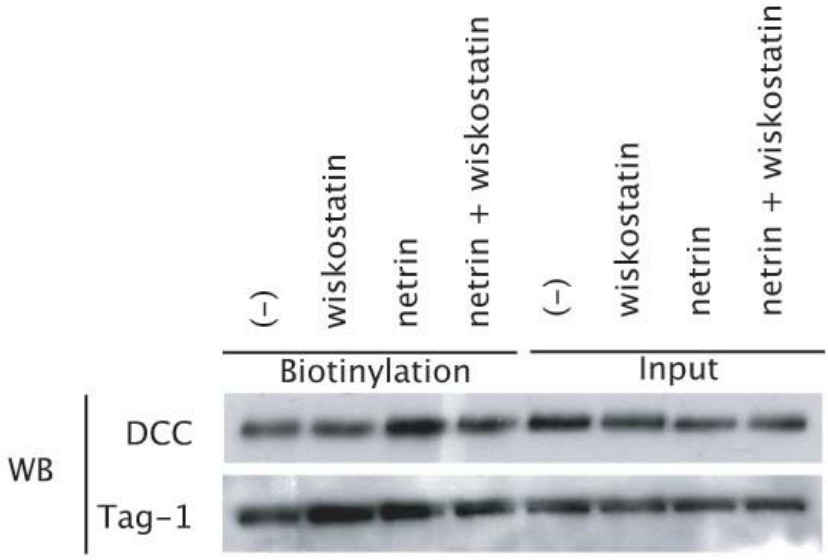
*DCC externalization is dependent on N-Wasp activation of Arp2/3DCC externalization is dependent on N-Wasp activation of Arp2/3*

Receptor recycling to the cell membrane occurs as a result of the coordination of endocytic and exocytic vesicles cycling in and out of the plasma membrane. This process is facilitated by loosening of a mesh of cortical F-actin located under the plasma membrane at the leading edge of a growth cone, or the protruding membrane of a motile cell, depending on the cell type.

The capacity of DCC to promote actin remodeling in growth cones is enhanced by DCC recruitment to the plasma membrane through a PKA dependent pathway (Bouchard et al., 2004). Additionally, the disruption of cortical F-actin by inhibiting RhoA activity in commissural neurons increases the levels of DCC at the plasma membrane (Moore et al., 2008). These findings are consistent with previous studies implicating Rho signaling in recycling (Gasman et al., 2003; Qualmann and Mellor, 2003; Stirling et al., 2009). Similarly, Cdc42 has been established as a dual regulator of polarity and vesicle trafficking in secretory, epithelial and other cell types (Harris and Tepass, 2010a). Cdc42 was shown to promote vesicle formation at the Golgi by regulating actin dynamics through Wasp and Arp2/3 (Luna et al., 2002; Matas et al., 2004). Cdc42 promotes actin assembly early in vesicle formation, but subsequently dissociates from the vesicle as cargo molecules become more concentrated, thus allowing dynein recruitment and vesicle movement (Chen et al., 2005).

We decided to test whether DCC recruitment to the plasma membrane was up-regulated after netrin-1 treatment through a similar mechanism. Embryonic rat spinal commissural neurons were cultured and treated with netrin-1 for 15 minutes. Cell surface proteins were then biotinylated and isolated as previously described (Bouchard et al., 2004). Cell surface DCC increased as previously shown with the application of netrin for 15 minutes (Figure 2-5). Consistent with previous data netrin-1 treatment did not affect levels of cell surface Tag-1. Interestingly, the application of 10μM Wiskostatin decreased netrin-1-induced externalization of the DCC (Figure 2-5). We have also considered some evidence that Wiskostatin can negatively impact membrane transport non-selectively by decreasing overall cellular ATP levels (Guerriero and Weisz, 2007). However, this study also showed that 10μM of Wiskostatin decreased ATP levels by only 20% following 1 hour of treatment, while in our case cells were treated for 15 minutes and a significant decrease in biotinylated DCC was observed. This would mean that within the 15 minute treatment period of wiskostatin there would not have been a significant impact on total cell ATP levels. Additionally, in our study the cell surface levels of Tag-1 remaining unaffected, indicating specificity on cell surface levels of DCC. We can therefore conclude that the effect seen from the treatment of wiskostatin on netrin-1 induced DCC externalization and cytoskeletal changes is likely to be due to its direct effect on N-Wasp/Arp2/3 complexes.

**Figure 2-5. Netrin-mediated increase in DCC membrane recruitment is dependent on N-Wasp/Arp2/3 complexes.** E13 commissural neuron 2DIV cultures were treated with wiskostatin and/or netrin-1 (200mg/ml) for 15 minutes or left untreated. Biotinylated proteins were isolated as previously described (Bouchard et al., 2004) and separated by SDS-page electrophoresis. Western blot analyses was carried out using monoclonal antibodies against DCC and Tag-1. Tag-1 protein surface levels remained consistent throughout conditions while an increase in biotinylated DCC was observed in netrin-1 treated neuron lysates. Netrin-mediated increase of biotinylated DCC protein levels was lowered with the addition of wiskostatin.




## DISCUSSION

A mass spectrometry study was performed with the aim of identifying novel downstream effectors of netrin-1 mediated remodeling of F-actin in

the axonal growth cones of embryonic rat spinal commissural neurons. Proteins were isolated as part of a complex that co-immunoprecipitated with DCC from lysates E13 rat spinal commissural neurons grown for 2 days *in vitro* and treated with netrin-1 for 15 min. The subsequent validation of candidates identified by this screen identified 14-3-3 family members and Arp2/3 as two DCC associated proteins. Both are proteins known to influence the organization of F-actin, but interestingly they function through largely distinct pathways.

We validated the association of 14-3-3 with a DCC containing protein complex in commissural neurons, however this association decreased with the addition of netrin-1. Functional studies using a 14-3-3 peptide antagonist demonstrated that 14-3-3 function is required for netrin induced growth cone expansion. The results of these experiments provide evidence that if 14-3-3 is acting directly on AC downstream of netrin-1, it is not acting to downregulate, but rather to promote F-actin polymerization. An alternative possibility is that blocking 14-3-3 function may disrupt the homeostasis of F-actin turnover and as a result trigger process collapse. It is also possible that 14-3-3 may not be directly acting on AC downstream of DCC and instead it could be required in an alternative pathway. 14-3-3 functions through association with phosphorylated Ser-Thr sequences and maintains these substrates in a phosphorylated conformation. The intracellular domain of DCC is highly phosphorylated at Ser-Thr residues (Meriane et al., 2004). The role of Ser/Thr phosphorylation of DCC is still unknown, however, the interaction of DCC with the mitogen-activated

protein kinases ERK1/2 and MEK1/2 and their contribution to netrin-1 signaling in axon outgrowth and guidance suggest that ERK1/2 and MEK1/2 could be potential DCC Ser/Thr kinase candidates (Forcet et al., 2002). The DCC intracellular domain contains several sequences that fall into the 14-3-3 $Rx_{1-2}Sx_{2-3}S$ binding motif (data not shown) where x stands for any amino acid and at least one of the Ser is phosphorylated. 14-3-3 may directly bind to the DCC intracellular domain through one of these sites. Once netrin-1 binds to DCC, a conformational change may occur that would release 14-3-3 and allow for these Ser sequences to be exposed for phosphorylation and association with downstream effectors.

We also validated Arp2/3 subunit 2, p34, as a component of a DCC complex from a peptide hit identified by mass spectrometry. DCC co-IPs from netrin-1 treated rat spinal commissural neurons confirmed an increased association between DCC and p34 in netrin treated commissural neurons. The Arp2/3 complex can be activated by WASp/Scar/WAVE family members, Listeria ActA, cortactin, budding yeast Abp1 and Pan1, fission yeast myosin-I's, and Dictyostelium CARMIL proteins (Duncan et al., 2001; Goode et al., 2001; Higgs and Pollard, 2001; Jung et al., 2001). Consistent with this, a previous study in the Kennedy lab demonstrated that an association of N-Wasp with DCC increases in embryonic rat spinal commissural neurons in response to netrin-1 (Shekarabi et al., 2005). We tested whether N-Wasp might activate Arp2/3 downstream of netrin-1 in commissural neurons to promote the polymerization of actin required for filopodia formation and

growth cone expansion that occurs in response to netrin-1. Wiskostatin, a chemical that functions by stabilizing N-Wasp in its autoinhibited conformation, thereby preventing the activation of Arp2/3, blocked netrin mediated growth cone expansion and filopodia formation in commissural neurons. Interestingly, N-Wasp and DCC were still found at the growth cone suggesting their sub-cellular localization is independent of N-Wasp activation of Arp2/3. Netrin-1 increases cell surface DCC, without increasing the intracellular concentration of cAMP or activating PKA (Bouchard et al., 2004). However, activation of PKA recruits DCC containing cargo vesicles to the plasma membrane, and as a result potentiates axon outgrowth in response to netrin-1, as demonstrated by Bouchard *et al.* using biotinylation and cell surface immunofluorescence experiments. Disruption of cortical F-actin by inhibition of RhoA in embryonic rat spinal commissural neurons similarly recruits DCC to the plasma membrane (Moore et al., 2008). These findings are consistent with previous studies implicating Rho signaling in recycling (Gasman et al., 2003; Qualmann and Mellor, 2003; Stirling et al., 2009). In other cell models Cdc42 promotes vesicle formation at the Golgi by regulating actin dynamics through Wasp and Arp2/3 (Luna et al., 2002; Matas et al., 2004). Biotinylation experiments showed cell surface DCC levels increase with netrin-1 treatment consistent with Bouchard's previous findings. This netrin-1 mediated increase in cell surface DCC was blocked with Wiskostatin treatment. These results suggest that N-Wasp mediated

activation of Arp2/3 is required for Netrin induced externalization of DCC containing vesicles.

On the basis of these results, we propose a model where netrin-1 binding DCC both promotes activation of local F-actin polymerization, while in parallel the recruitment of actin regulatory proteins such as N-Wasp and Arp2/3 may contribute to a positive feedback mechanism by recruiting DCC containing vesicles to the cell surface. Netrin-1 mediated activation of Cdc42 may then result in N-Wasp activation of Arp2/3 resulting in the polymerization of filamentous actin and the formation of new filopodia. In parallel, Cdc42/N-Wasp dependent Arp2/3 activation may lead to the formation of DCC containing vesicles promoting DCC transport to the cell surface for amplification of the chemoattractive signal toward the netrin gradient. The positive feedback resulting from this loop would locally amplify the response to netrin-1 and contribute to the capacity of a growth cone to turn up a gradient of netrin-1.

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# CHAPTER 3:

**A complex of DCC and Cool-1/β-Pix, a Cdc42 and Rac1 specific GEF, is required for netrin-1 dependent filopodia formation by neuronal growth cones and spinal commissural axon chemoattraction to the floor plate.**

## PREFACE

The Kennedy lab has previously reported that activation of the RhoGTPases Cdc42 and Rac1, and recruitment of their downstream effectors p21-activated kinase (Pak-1) and N-WASP, is required for the netrin-1 receptor DCC to regulate growth cone morphology. In this chapter I identified the beta Pak-interacting exchange factor (β-Pix) as a potential GEF and regulator of Cdc42/Rac1 and Pak activation downstream of netrin-1 in commissural neurons.

**ABSTRACT**

The netrin-1 receptor deleted in colorectal cancer (DCC) is required for commissural axon extension toward the floor plate in the developing spinal cord. We have previously reported that activation of the RhoGTPases Cdc42 and Rac1, and recruitment of their downstream effectors p21-activated kinase (Pak-1) and N-WASP, is required for the netrin-1 receptor DCC to regulate growth cone morphology. Previous findings have suggested that activation of Rac1 by netrin-1 is downstream of Cdc42 activation in commissural neurons. Furthermore, netrin-1 induced a DCC dependent increase in Cdc42 binding the non-hydrolyzable GTP analogue GDPgammaS, consistent with DCC activating a Cdc42 specific GEF. Here we report that a protein complex of the Rac and Cdc42 specific GEF, Cool-1/β-Pix and the ArfGAP Git-2, constitutively associates with DCC in commissural neurons. We show that Cool-1/β-Pix multimerization and association with p21-activated kinase (Pak-1) is required for netrin mediated filopodia formation, maintenance and growth cone expansion, and demonstrate that a functional Cool-1/β-Pix protein is required for axon extension toward the midline. Our findings indicate that DCC induced changes in growth cone morphology and regulation of axonal actin dynamics require the Rac and Cdc42 specific GEF Cool-1/β-Pix.

## INTRODUCTION

The formation of a complex network of neural circuits during development relies on the ability of axons to travel long distances, reach their targets, and establish appropriate synaptic connections. The vertebrate spinal cord is a prototypical example of the capacity of gradients of secreted extracellular cues to direct cell and axon migration during development. Netrin-1 was initially identified as a chemoattractant for embryonic commissural axons extending from the dorsal spinal cord to the ventral midline (Serafini et al., 1996; Serafini et al., 1994). It is a ~70kDa secreted protein composed of domains homologous to the V and VI domains of laminins (Kappler et al., 2000; Koch et al., 2000; Serafini et al., 1994). It is now clear that netrin function is not restricted to the CNS but contributes to the development of multiple tissues, including muscle, mammary gland, vasculature, and lung (Dalvin et al., 2003; Fitzgerald et al., 2006; Hinck, 2004; Liu et al., 2004b), Deleted in colorectal cancer (DCC), a receptor required for chemoattractant responses to netrin-1, is an ~185 KDa transmembrane protein and member of immunoglobulin superfamily. Genetic studies first identified a requirement for UNC-40, the *C. elegans* homologue of DCC, in cell and axon migration directed toward UNC-6, the C. elegans homologue of netrin-1 (Hedgecock et al., 1990; Zallen et al., 1999). Subsequent analysis of DCC knockout mice revealed deficits in the formation of midline CNS commissures, including the ventral spinal

commissure, similar to the phenotype found in netrin-1 knockout mice (Chan et al., 1996; Deiner et al., 1997; Deiner and Sretavan, 1999; Fazeli et al., 1997; Kolodziej et al., 1996; Livesey and Hunt, 1997).

Fyn, a member of the src family of tyrosine kinases member, and FAK, a tyrosine kinase involved in cytoskeletal rearrangement and the formation and maintenance of focal adhesions, have been implicated in the initial activation of netrin signalling downstream of DCC. Fyn and Fak directly phosphorylate the intracellular domain of DCC, events required for netrin dependent axon outgrowth and turning (Li et al., 2004; Liu et al., 2004a; Ren et al., 2004). Upon netrin-1 binding to DCC, the Rho GTPases Rac1 and Cdc42 are recruited and activated, and their function is required for netrin-dependent growth cone expansion and filopodia formation in commissural axons (Li et al., 2002a; Shekarabi and Kennedy, 2002; Shekarabi et al., 2005). The identity of the downstream effectors required for activation of RhoGTPases downstream of DCC remains unclear.

Pak-1, p21-activated protein kinase 1, is a cytoplasmic serine/threonine kinase that is recruited to the intracellular domain of DCC by netrin-1 (Shekarabi et al., 2005). Pak-1 binds specifically to GTP-bound, but not GDP-bound Cdc42 and Rac1. Binding Rac1 or Cdc42 induces a conformational change in Pak-1 that exposes its Pak-interacting exchange factor (Pix) binding region allowing it to associate with Pix. Importantly, Pix forms multimeric complexes with the Arf-GAP family of proteins, Git-1 and

2 which are required for Pak-1 autophosphorylation and kinase activation (Chong et al., 2001; Daniels et al., 1999; Mott et al., 2005), and are essential for formation of focal contacts, cell migration and the actin cytoskeletal turnover required during cell motility (Jones and Katan, 2007; Kim et al., 2001; Lamorte et al., 2003; Lee et al., 2004). β-Pix, also known as Cool-1, is a GEF that contains SH3, Dbl homology (DH), and pleckstrin homology (PH) domains. These GEFs were originally identified for their ability to associate with Pak family kinases through their SH3 domain, and to promote the translocation of Pak complexed to RhoGTPases Rac/Cdc42 to focal adhesions (Stofega et al., 2004).

Members of the Pak, Pix and Git families are ubiquitously expressed and are highly enriched in the CNS during development (Kim et al., 2000). Here we report a role for β-Pix-Git2-Pak1 complexes signalling downstream of netrin-1 in commissural neurons. We demonstrate that DCC associates with β-Pix-Git2 complexes constitutively in these cells and that β-Pix is enriched in the axonal growth cones of commissural neurons. We show that the activation of Rho GTPases Rac1 and Cdc42 downstream of netrin-1 is dependent on β-Pix function. Mutations disrupting Pak1 interaction or the formation of β-Pix-Git2 multimers or β-Pix homodimers inhibit netrin-1 induced growth cone expansion and filopodia formation, and commissural axon extension to the floorplate requires the interaction of β-Pix with Pak1 and Git2 proteins. These

findings provide evidence that β-Pix is required for netrin-1 dependent cytoskeletal remodelling during axon guidance.

## MATERIALS AND METHODS

*Reagents and Cell Culture:* Embryonic rat spinal commissural neurons were cultured as described (Bouchard et al., 2004). Briefly, dorsal halves of the spinal cord of embryonic day 13 (E13; E0, vaginal plug) rat embryos were microdissected, dissociated for 30 min at 37°C in $Ca^{2+}$/$Mg^{2+}$- free HBSS (Invitrogen), followed by trituration with a flame-polished Pasteur pipette, and cultured in Neurobasal (Invitrogen) with 10% heat-inactivated fetal bovine serum with 100 U/ml penicillin and 100 U/ml streptomycin. After 24 h, the medium was changed to Neurobasal supplemented with 2% B-27 (Invitrogen), 2 mM glutamine, and penicillin/streptomycin. Tissue culture dishes were coated with 10 µg/ml poly-D-lysine (PK; Sigma, St. Louis, MO) at RT for 1h. In experiments that used netrin-1 as a substrate, the coverslips were coated with PK, washed, and then coated with 5 µg/ml netrin-1 protein at 37°C overnight. Recombinant netrin-1 protein was purified from a HEK 293-EBNA cell line secreting netrin-1, as described (Serafini et al., 1994; Shirasaki et al., 1996). For axon outgrowth assays, dorsal spinal cord explants were dissected from E13 rat embryos and cultured in three-dimensional collagen gels as described (Serafini et al., 1994; Tessier-Lavigne et al., 1988) with 3-4 explants per gel. Explants were cultured at 37oC 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.

For biochemical analysis of proteins, the dissociated neurons were cultured at 3 x $10^6$ cells per 100 mm dish. For immunostaining, the neurons were plated on 13 mm glass coverslips (Carolina Biological Supply, Burlington, NC) at 1 x $10^4$ cells per coverslip. Cells were then either lysed or fixed and immunostained. Filopodia number and growth cone surface area were quantified as described previously (Shekarabi and Kennedy, 2002). Statistical significance of differences between means was evaluated by paired t-test. 293T cells were cultured in DMEM (Invitrogen) with 10% heat-inactivated fetal bovine serum, and 100 U/ml penicillin and 100 U/ml streptomycin.

The following monoclonal antibodies were used for immunofluorescence and western blot analysis: anti-human DCC (BD Pharmingen); anti-β-Pix (BD Transductions); anti-p95PKL/GIT (BD Transductions); anti-TAG-1 (4D7; Hybridoma Bank [HB]); anti-Islet-1 (40.2D6; HB); anti-LIM 2 (4F2; HB). Additionally a rabbit polyclonal antibody anti-β-Pix (Chemicon International, Inc.) was used for immunofluorescence and western blot analysis.

*cDNA constructs and microRNAs:* β-Pix cDNAs were kindly provided by the Manser lab. The characterization of the plasmids has been previously

described in Koh, Manser et al. 2001 and Manser, et al. 1997 (Koh et al., 2001; Manser et al., 1997). Briefly, cDNAs were cloned into the pXJ-Flag vector or the pXJ-GST vector for mammalian cell expression (Manser et al., 1997). The N-terminal deletion mutant, pXJ- Flag-ΔN80β1PIX, was generated by splicing a PCR fragment covering 241 bp to the KpnI site (962 bp) of β1PIX to vector containing DNA fragment C-terminal to the KpnI site. The C- terminal truncation mutant pXJ-Flag-β1PIX1-459 was generated by splicing a PCR fragment containing the truncated C terminus to the N terminus of β1PIX at the internal KpnI site. pXJ-GST-β1PIX was also constructed by PCR cloning.

A shRNA with a microRNA stem (shRNAmir) was designed against target sequences for β-Pix using Invitrogen's RNAi Designer. The sequence picked targets β-Pix rat mRNA starting at nucleotide 1170 (CCAGTGTCAAGAAGTACGAAA). Oligonucleotides encoding the miRNA sequences were ligated into a pcDNA6.2-GW/EmGFP-miR vector (Invitrogen) and lentivirus was produced as described previously (Thomas et al., 2009). Briefly, the EmGFP-miR cassette was then amplified by PCR and subcloned into the pRRLsinPPT vector, and VSVG pseudotyped virus was produced in HEK-293T cells. Virus particles were purified by ultracentrifugation and titered in HEK-293T cells.

*GST-CRIB pull-down assays:* 293T cells were cultured at 0.5 million cells in confluency and transfected using Lipofectamine reagent according to the manufacturers protocol (Invitrogen). One day later, cells were serum

starved for 4 hrs and stimulated with 200ng/ml netrin-1 for 5 min. Cells were chilled on ice, washed with ice-cold phosphate-buffered saline (PBS) and lysed in buffer containing 0.5% NP-40, 50 mM Tris pH 7.4, 150 mM NaCl, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml aprotinin, 1 μg/ml leupeptin and 20 μg of recombinant GST–CRIB. Lysates were then incubated with glutathione–agarose beads (Sigma) for 60 min at 4°C, washed with lysis buffer and eluted with SDS sample buffer. Bound Rac1 and Cdc42 was analyzed by Western blotting using a monoclonal anti-Rac1 antibody (Transduction Laboratories, Lexington, KY) and a polyclonal anti-Cdc42 (SC-87; Santa Cruz Biotechnology, Santa Cruz, CA). Whole-cell lysates were also analyzed for the presence of Rac1 and Cdc42 for normalization.

*Immunofluorescence:* Commissural neuron cultures were dissociated and cultured as described above. After netrin-1 treatment cells were fixed in 4% formaldehyde/0.1% gluteraldehyde (fresh at 37°C) for 30-60s then washed with PBS and blocked for 1 hr at RT with 3%BSA/3%HS/0.1% Triton/PBS. Cells were incubated overnight at 4°C with primary antibodies previously diluted in block buffer. Cells were then washed in PBS 3x at RT for 5min and incubated with Alexa fluorescently labelled antibodies diluted in block buffer for 30 min at RT. Antibodies were washed with PBS 3x for 5 min at RT and coverslips were then mounted on glass slides using Gelmount (Biomeda corp., Foster City, USA). Controls for staining was

established by using only primary antibody or the Alexa fluorescently labelled secondary antibodies.

*In Ovo Electroporation:* In ovo electroporation (Momose et al., 1999) of expression plasmids was performed at stage 18. Electroporation frequencies varied between 5% and 30% of LMC neurons electroporated, depending on the construct used and DNA concentration.

*Chick open-book assay:* Spinal cord from chick embryos electroporated with cDNA constructs were microdissected between stages 24-26 and fixed in 4% paraformaldehyde for 1 hr at 4°C. Whole cords were then blocked in 3% BSA/3%HS/0.3% Triton/PBS blocking buffer. Primary antibodies were diluted in block buffer and cords were incubated with primary antibody solution overnight. The tissue was washed 3x with PBS at RT for 15 minutes and incubated with secondary Alexa tagged antibodies for 1 hr at RT followed by 3 washes of PBS at RT. Spinal cords were then set up as an open book on slides and mounted using Gelmount (Biomeda corp., Foster City, USA). Confocal z-stack images were acquired and electroporated interneuron axon extension was quantified using ImageJ (NIH, USA).

*Co-Immunoprecipitation:* For coimmunoprecipitation (coIP) analyses commissural neurons were treated with 200 ng/ml netrin-1 for 5 min and

then lysed in ice-cold 1% Triton X-100 lysis buffer with protease inhibitors on ice. Immunoprecipitations were performed using 1 µg of anti-$DCC_{IN}$, β-Pix or Git-2 antibodies on a rocking platform at 4°C for 1h. Protein A/G beads were added and allowed to incubate for 30 min. Beads and associated proteins were then pelleted and washed three times in lysis buffer with protease inhibitors. Proteins were eluted from the beads using 10 ml of Laemmli buffer and characterized by Western blot analysis.

*RT-PCR:* Total RNA was extracted from rat embryo heads and spinal cords, and 2 DIV E14 commissural neuron cultures using TRIzol® (Invitrogen Life Technologies, Burlington, Ontario). RT-PCR was performed with 0.5 mg of total RNA per reaction using the QIAGEN® OneStep RT-PCR Kit (Qiagen, Mississauga, Ontario). The primers for the amplification of β-PIX were annealed at 55$^{o}$C, and their sequences were 5'TGCCTACTGTGCCAACC3' and 3'AGATGAAACCGCTCATCC5'. 25ml of the RT-PCR products were separated on a 1% agarose gel containing ethidium bromide and visualized by UV light.

## RESULTS

*Embryonic spinal commissural neurons express β-Pix*

As an initial step toward investigating the possible relevance of β-Pix in netrin-1 signaling, we carried out an analysis of expression during neural

development. Using *in situ* hybridization analysis, previous studies have demonstrated expression of four different isoforms of β-Pix in the developing mouse brain and spinal cord (Kim et al., 2000; Kim and Park, 2001). We confirmed β-Pix expression in RNA isolated from P8 rat brain, E13 rat spinal cord, and cultured embryonic spinal commissural neurons using rt-PCR (Figure 3-1A). Appropriately, no expression of β-Pix mRNA was detected in tissue isolated from bladder or oesophagus.

To investigate β-Pix protein expression, embryonic rat spinal commissural neurons were cultured for 2 days in vitro, as described (Moore and Kennedy, 2008). Using western blot analysis, all previously identified isoforms of β-Pix, βPix-a/β1Pix, βPix-d/β2Pix, βPix-b, βPix-bL, βPix-c, were readily detected in whole cell homogenates of cultured commissural neurons (Figure 3-1B).

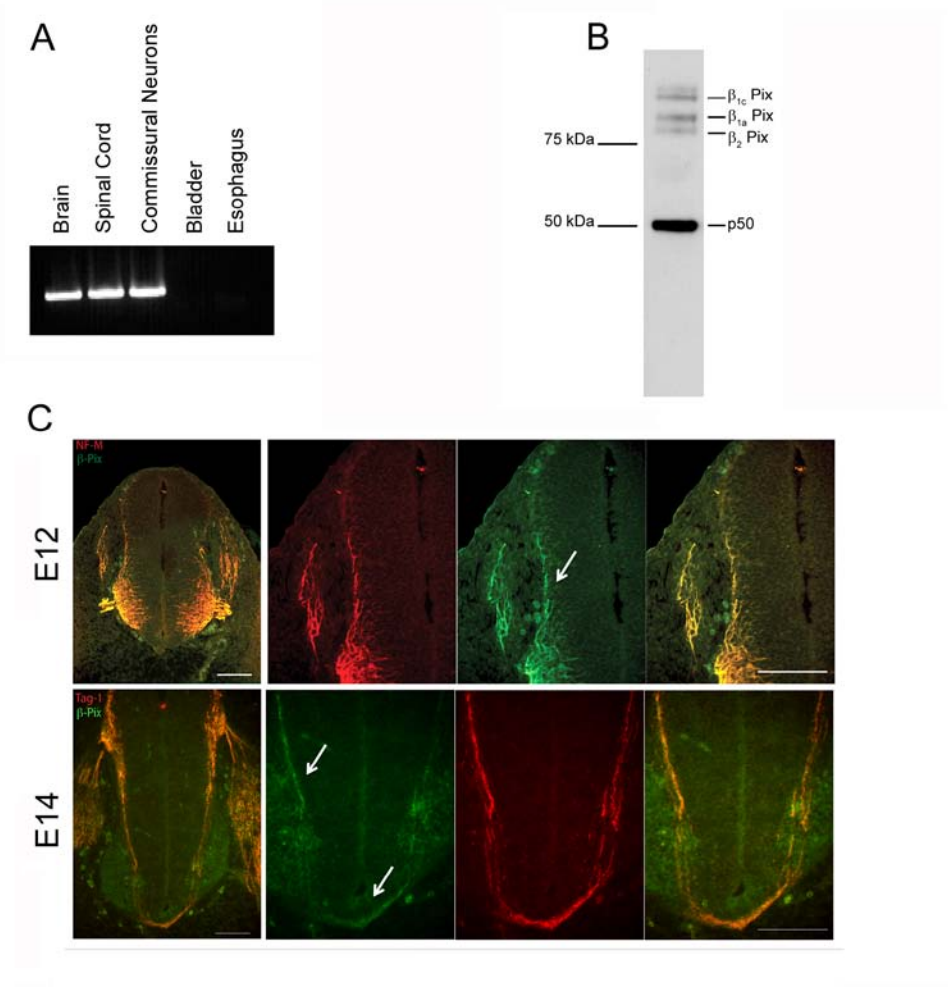
The distribution of β-Pix protein was assessed immunohistochemically in sections of embryonic day 12 (E12) rat spinal cord, an age at which commissural axons are extending to the floorplate. Sections were co-stained with an antibody against neurofilament M (NFM) to visualize axons (Figure 3-1C). To determine if β-Pix was localized to the axons of commissural neurons, embryonic day E13 spinal cord wholemounts were co-immunostained for β-Pix and Tag-1, a commissural neuron marker (Figure 3-1C). At stage E13, β-Pix immunoreactivity was detected throughout the embryonic spinal cord, but enriched in TAG1 positive

commissural interneurons and in motoneurons located in the nascent ventral horn, as well as strong reactivity detected in floorplate cells, roof plate cells and DRGs (data not shown). These findings are consistent with widespread expression of β-Pix, including readily detectable β-Pix protein in commissural axons as they extend toward the ventral midline.

**Figure 3-1. β-Pix proteins expression in the developing spinal cord.**

A) Detection of β-Pix transcripts by reverse transcriptase-PCR. β-Pix is expressed by many cell types, including commissural neurons, in E13 rat spinal cord dorsal tissue, commissural neuron (CN) dissociated 2 DIV cultures and in whole brain tissue from E13 rat, but not in oesophagus or bladder of adult rat. B) Multiple β-Pix protein isoforms are expressed by commissural neurons (2 DIV) isolated from E13 rat dorsal spinal cord. Commissural neurons were isolated and cultured as described in materials and methods followed by SDS-PAGE electrophoresis protein separation and western blotting. Two different antibodies (mouse monoclonal and a rabbit polyclonal) against β-Pix were used for western blotting in these and following experiments. C) β-Pix protein is detected in interneuron axons as they extend to the floorplate. 18 μm sections of E12 rat spinal cord were immunostained for NF-M (chicken polyclonal antibody) or Tag-1 (mouse IgM antibody) in red and β-Pix (mouse monoclonal antibody) in green.

Images were acquired using a Magnafire CCD camera on an Axiovert Zeiss microscope, 20x and 40x objectives. Scale bars = 100μm.


A
Brain
Spinal Cord
Commissural Neurons
Bladder
Esophagus
B
75 kDa
50 kDa
β1c Pix
β1a Pix
β2 Pix
p50
C
E12
E14
NF-M
β-Pix
Tag-1
β-Pix

*Regulated association of a β-Pix-Git2 complex with DCC in commissural neurons*

To investigate the possibility that a β-Pix/Git-2 complex acts downstream of DCC to activate Pak we began by testing whether such a complex associates with the DCC receptor in commissural neurons *in vitro*. DCC was immunoprecipitated from commissural neuron lysates using a monoclonal antibody against the intracellular domain of DCC. Protein complexes were separated by SDS-PAGE and assayed for β-Pix using western blots. All β-Pix isoforms co-immunoprecipitated with DCC at roughly equivalent levels. Similarly, following immunoprecipitation with antibodies against β-Pix, DCC was readily detected (Figure 3-2A, left panel).

When commissural cultures were treated with 200 ng/ml of netrin-1 for 5 minutes, increased levels of β-Pix protein were detected to co-immunoprecipitate with DCC (Figure 3-2A, right panel). These results provide evidence that a complex including β-Pix/Git-2 and DCC can be readily detected in commissural neurons cultured in vitro and that there may be recruitment of a β-Pix/Git-2 complex to DCC in response to netrin-1. Upon netrin-1 binding, tyrosine kinases such as FAK and the SFK fyn are recruited to DCC and activated. A likely possibility is that recruitment and activation of a β-Pix/Git2 complex downstream of DCC may be dependent on upstream activation of FAK and SFK signalling, as demonstrated in other cell types (Brown et al., 2005).

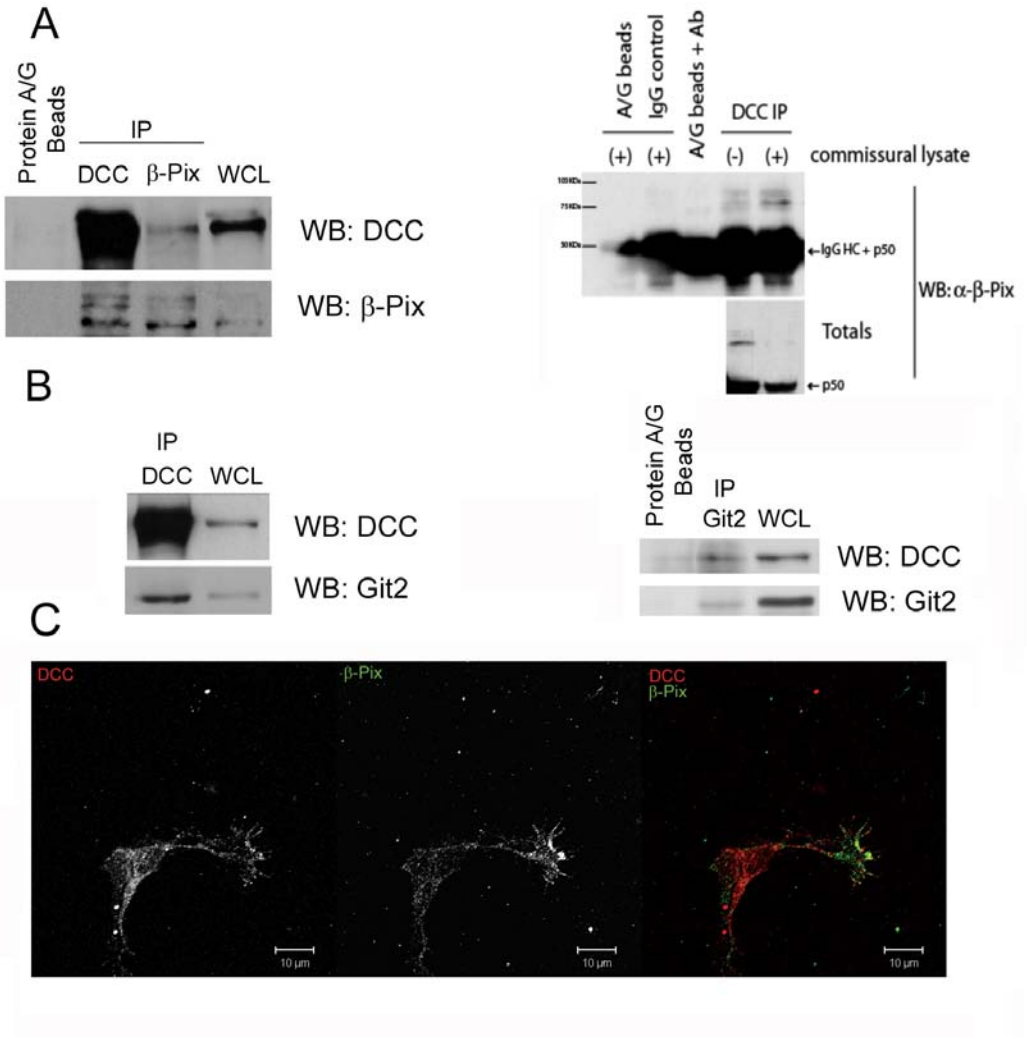
*DCC and β-Pix are enriched in commissural neuron growth cones*

The association of β-Pix/Git-2 complexes with DCC in commissural neurons led us to investigate the subcellular localization of these complexes by immunoflorescence in commissural cultures grown on poly-D-lysine or a substrate of netrin-1 (5 mg/ml). In either condition, β-Pix immunoreactivity was detected associated with the cell body and axon of commissural neurons. In the growth cone itself, β-Pix was detected in both the central and peripheral domains, but was particularly enriched at the leading edge of the growth cone along with punctate DCC immunoreactivity (Figure 3-2C).

**Figure 3-2: DCC associates with a β-Pix/Git-2 complex in commissural neurons.**

A&B) Commissural neurons were cultured for 2DIV and whole cell lysates isolated using 1% Triton lysis buffer. DCC and Pix immunoprecipitations were performed using 1mg of either antibody (DCC – intracellular region specific and β-Pix mouse monoclonal antibodies or β-Pix and Git-2 rabbit polyclonal antibody). Immunoprecipitated proteins and whole cell lysates were separated by SDS-PAGE and immuno-blotted using antibodies against DCC, Pix or Git2. B) β-Pix association with DCC is increased in netrin-1 treated commissural neurons. 2DIV commissural neuron cultures were either left untreated or treated with 200ng/ml netrin-1 for 5 minutes

prior to cell lysis and immunoprecipitation as described above. C) DCC and β-Pix proteins are enriched at the leading edges of commissural growth cones. Commissural neurons were cultured on netrin-1 (2mg/ml) coated coverslips. After 2DIV cultures were fixed with 4% PFA and immuno-stained against DCC (goat polyclonal) or β-Pix (mouse monoclonal). Images were acquired using a Zeiss-confocal microscope, 63x objective. Scale bars = 100μm.



*Netrin induced filopodia formation and growth cone expansion require β-Pix dimerization and association with Pak-1*

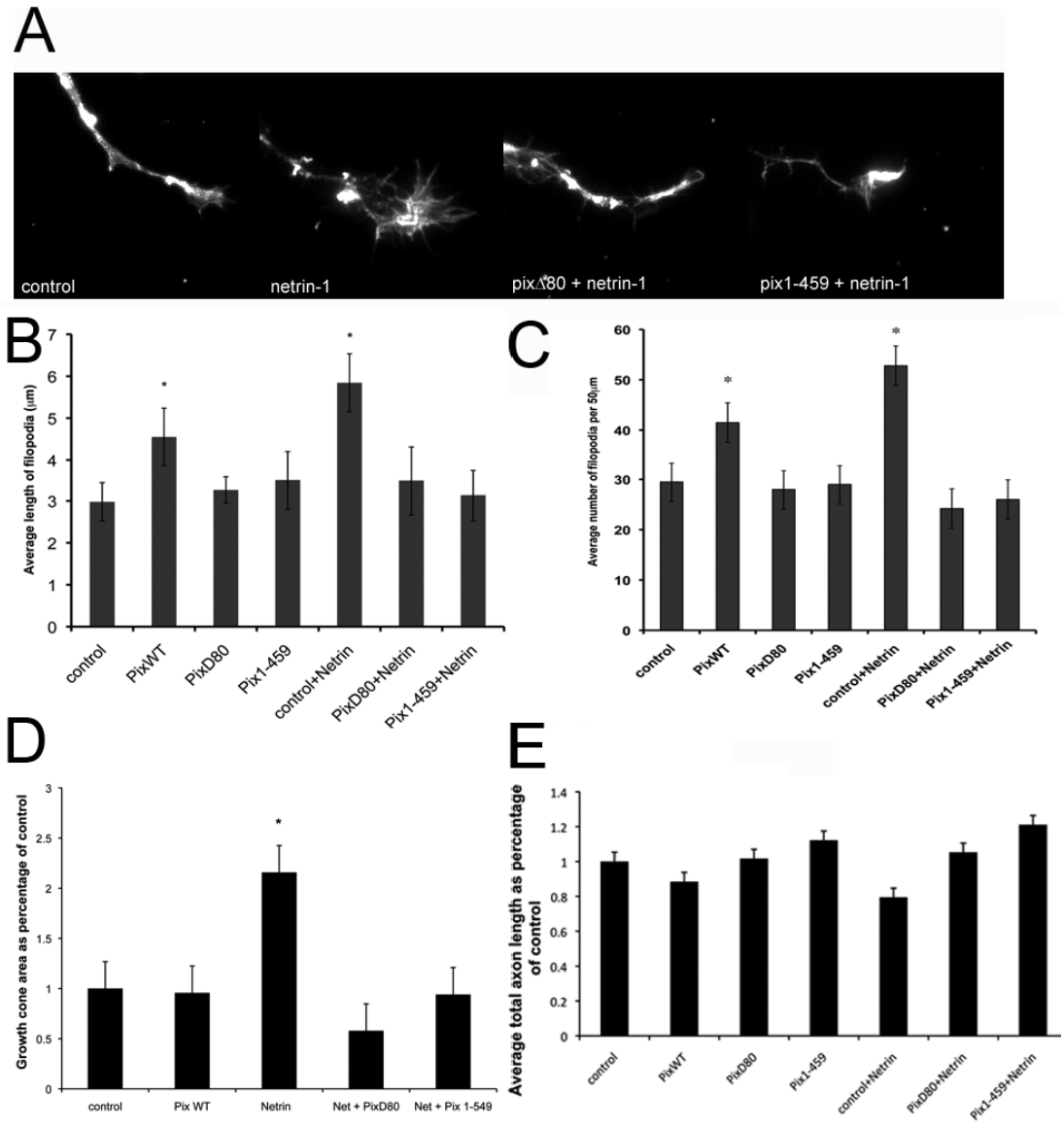
To examine the functional role of β-Pix/Git-2/DCC complexes in commissural neurons we ectopically-expressed functional mutants of β-Pix in cultured commissural neurons. Bath application of netrin-1 to cultured commissural neurons induces growth cone expansion and filopodia formation (Shekarabi and Kennedy, 2002). Netrin-1 activates Cdc42, Rac1, and Pak1 in these cells. Furthermore, netrin-1 induced changes in growth cone morphology require Cdc42 and Rac1, as dominant negative mutants blocked netrin-1 induced changes in growth cones (Shekarabi et al., 2005). Consistent with these previous findings, we detected an increase in filopodia number and length and commissural neuron growth cone expansion within 5 min of bath application of netrin-1 (Figure 3-3A-D). Ectopic expression of wild type β-Pix in commissural neurons was sufficient to induce a partial increase, ~50%, in the number and length of filopodia, but did not significantly alter total growth cone surface area (Figure 3-3A-D). These findings suggest that while β-Pix appears to contribute to the reorganization of F-actin required to increase the number of filopodia, the engagement of additional cytoskeletal machinery is required for membrane extension and growth cone expansion.

Ectopic expression in commissural neurons of a β-Pix mutant that is unable to associate with Pak1 (PixΔ80) inhibited the netrin-1 induced increase in the number and length of filopodia, and growth cone expansion (Figure 3-3A-D). Similarly, expression of a β-Pix mutant that can no longer form multimers with Git-2 proteins significantly disrupted netrin-1 function (Figure 3-3A-D). Total axonal length was not altered upon expression of either of these mutants, nor when neurons were cultured overnight on a substrate of netrin-1, consistent with previous results (Shekarabi et al., 2005). These findings provide evidence that the association of Pak and GIT-2 with β-Pix is required for netrin-1 signaling.

**Figure 3-3. β-Pix proteins that cannot form multimers or bind Pak inhibit netrin-1 induced cytoskeletal rearrangements in commissural neuron growth cones.**

A-E) Commissural neurons co-transfected with GFP alone or GFP and either a β-Pix mutant that is unable to associate with Pak1 (PixD80) or with a β–Pix mutant lacking its coiled coil region rendering it unable to form multimers (Pix1-459). Transfected neurons were then cultured for 2DIV and stimulated with 200ng/ml of netrin-1 for 15 min. F-actin was visualized using Phalloidin-Alexa546. Images were captured by confocal microscopy (63x objective) and the number of filopodia per 50μm of axon were counted using ImageJ. (* = $p < 0.01$) A) Representative images of transfected commissural neurons. B-C) Overexpression of dominant

negative mutants significantly inhibited the netrin-1 induced increases in filopodia formation and filopodia length. Overexpression of a Pix WT construct was sufficient to promote a significant increase in both filopodia number and filopodia length. D) GC expansion was quantified by measuring GC area using ImageJ. Overexpression of either of the β-Pix mutants described above blocks netrin-1 dependent GC expansion. E) No significant changes in total axon length were observed between commissural neurons plated on netrin-1 or PDL substrates overnight nor between β-Pix mutant expressing neurons vs control. Axon length was also measured using ImageJ.


A
control
netrin-1
pixΔ80 + netrin-1
pix1-459 + netrin-1
B
Average length of filopodia (μm)
control
PixWT
PixD80
Pix1-459
control+Netrin
PixD80+Netrin
Pix1-459+Netrin
C
Average number of filopodia per 50μm
control
PixWT
PixD80
Pix1-459
control+Netrin
PixD80+Netrin
Pix1-459+Netrin
D
Growth cone area as percentage of control
control
Pix WT
Netrin
Net + PixD80
Net + Pix 1-549
E
Average total axon length as percentage of control
control
PixWT
PixD80
Pix1-459
control+Netrin
PixD80+Netrin
Pix1-459+Netrin

*Commissural axon extension toward the floor plate requires β-Pix to associate with Pak-1*

Since β-Pix protein complexes were required for netrin-1 function *in vitro* we decided to investigate their role in netrin-1 function *in vivo*. To do so we used the capacity to electroporate cDNAs into the embryonic chicken spinal cord (Momose et al., 1999). cDNAs encoding the β-Pix mutants (PixΔ80 and Pix1-459) or an empty vector (pCEP4) were electroporated *in ovo* in conjunction with pEGFP-N1, which expresses GFP, into the lumen of 3 day-old chick embryo spinal cord (stage 14-16). Chick embryos were dissected 48 hrs post-electroporation (stage 24-26) and spinal cords dissected. The dorsal midline of the neural tube was cut and the spinal cords placed as "open-books" for analysis of axon extension (Figure 3-4A). Only axons extending from the most dorsal part of the cord that could be clearly followed all the way to the growth cone were quantified. GFP expressing neurons, with axons extending normally to the floor plate, were readily detected. Ectopic-expression of the β-Pix mutant that is unable to interact with Pak (PixΔ80) resulted in a significant decrease in axon extension toward the floorplate when compared to control neurons expressing GFP alone (six-fold decrease) (Figure 3-5A-B).

Visualization of transverse sections of the spinal cords demonstrated that these axons were shorter and extended in random directions (Figure 3-4B). GFP positive axons did not cross the midline to the same extent in PixΔ80 electroporated cords, as was detected in spinal cords

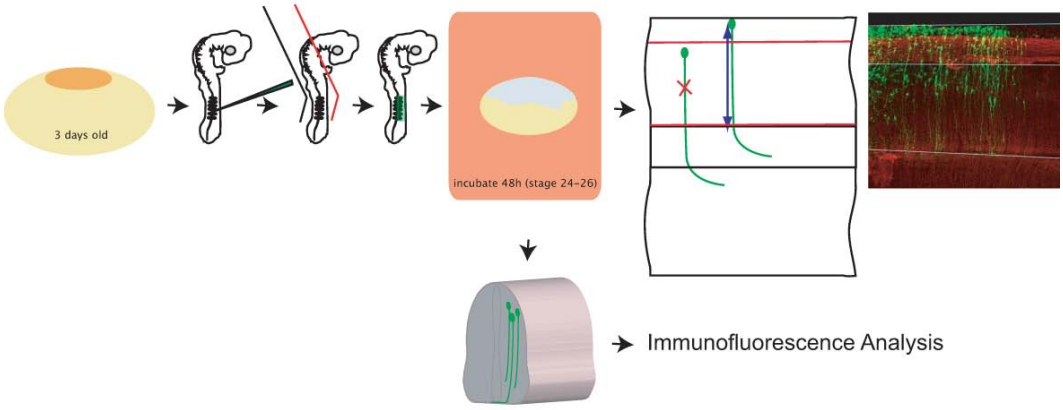
electroporated with GFP alone (Figure 3-4B). Similar results were observed as a result of electroporating the β-Pix mutant that is unable to form multimeric complexes (Figure 3-5A-B). Notably, when these same mutants were expressed in commissural neurons grown in dispersed cell culture, axons exhibited no significant difference in length between control and mutant expressing cells. This finding provides evidence that the expression of the mutants does not result in a non-specific deficit in the capacity of these cells to extend an axon, and supports the conclusion that the defects found in the spinal cord reflect a defect in appropriate axon guidance (Figure 3-3E).

Furthermore, we observed no effects on the differentiation of different interneuron subpopulations when either of the mutants were over-expressed (Figure 3-6A-B). Motor neuron marker Islet-1 and interneuron marker such as Pax-2 maintained their distribution when compared to control electroporated chick cords or the non-electroporated side of the spinal cord (Figure 3-6A-B). These results in addition to our previous in vitro growth cone assays demonstrate that β-Pix complexes with Pak and that β-Pix/Git-2 multimers are required for proper commissural axon extension in the spinal cord in response to Netrin-1.

**Figure 3-4. Analyses of β-Pix mutants effect in the developing SC in vivo using electroporation of the chick spinal cord.** A) The spinal cords of 3 day-old chick embryos were co-electroporated with either of the β-Pix mutants or the corresponding empty vector with pEGFP-N1 (3:1).

Eggs were then incubated for 24-48 hours. GFP expressing neurons were easily detectable following 24 hrs and commissural axons extended normally toward the floorplate. Spinal cord open-book preparations were used 48h post-electroporation to quantify axon extension. Only axons extending from cell bodies located in the dorsal most third of the spinal cord were measured. Additionally, only axons that could be visualized continuously from cell body to growth cone were measured. B) Transverse sections of electroporated chick SC immunostained against NF-M and depicting pEGFP expressing axons as they extend toward the floorplate.

A


3 days old
incubate 48h (stage 24-26)
Immunofluorescence Analysis

B

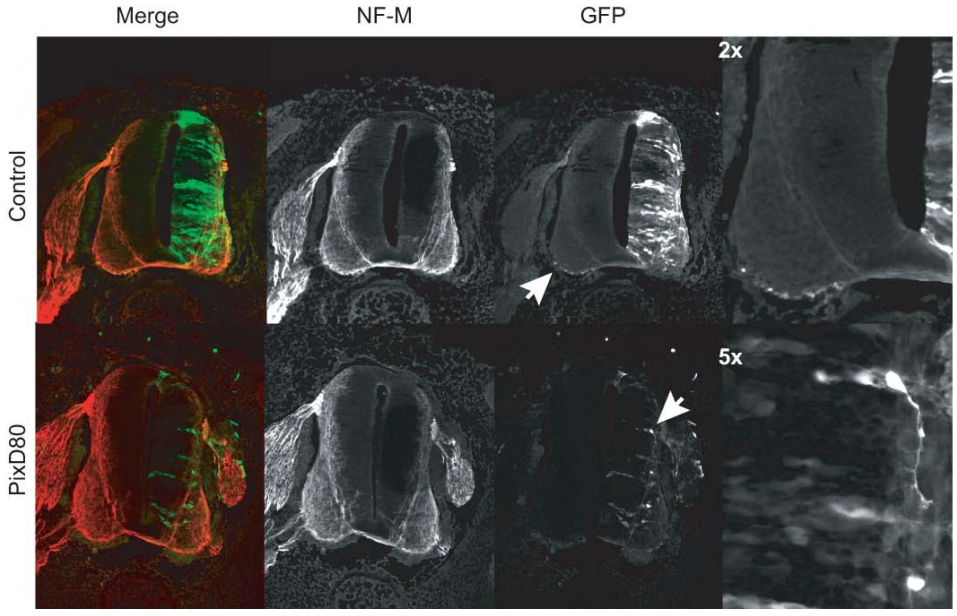
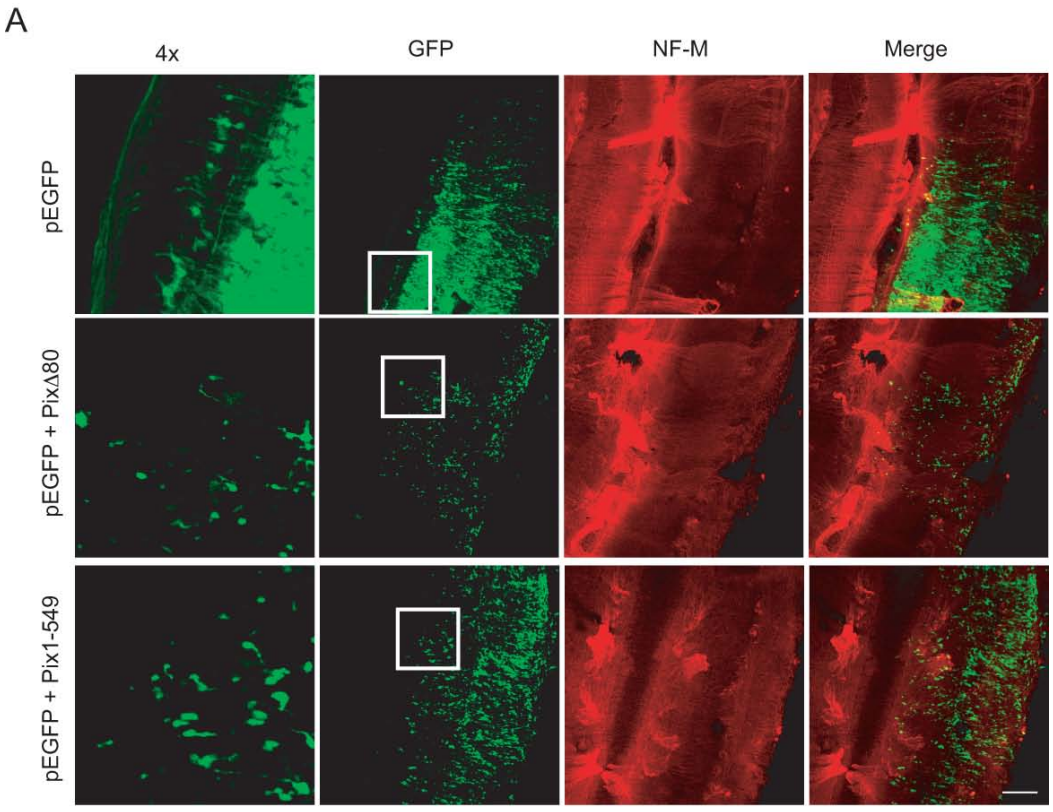

Merge
NF-M
GFP
2x
5x
Control
PixD80

**Figure 3-5. Commissural axon extension toward the floor plate in the embryonic spinal cord is dependent on β-Pix association with Pak-1 and its coiled-coil region.** A) Sample images of "open-book" electroporated chick SC. B) Axon lengths were significantly reduced by expression of either of the β-Pix mutants. Confocal images were aquired using a 10x objective. Scale bars = 100μm. (** = p > 0.001).


A
4x
GFP
NF-M
Merge
pEGFP
pEGFP + PixΔ80
pEGFP + Pix1-549

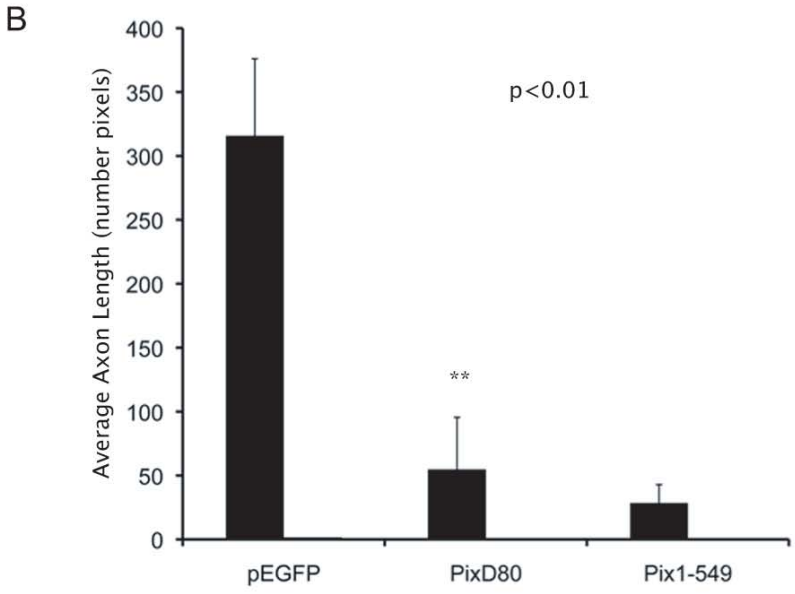
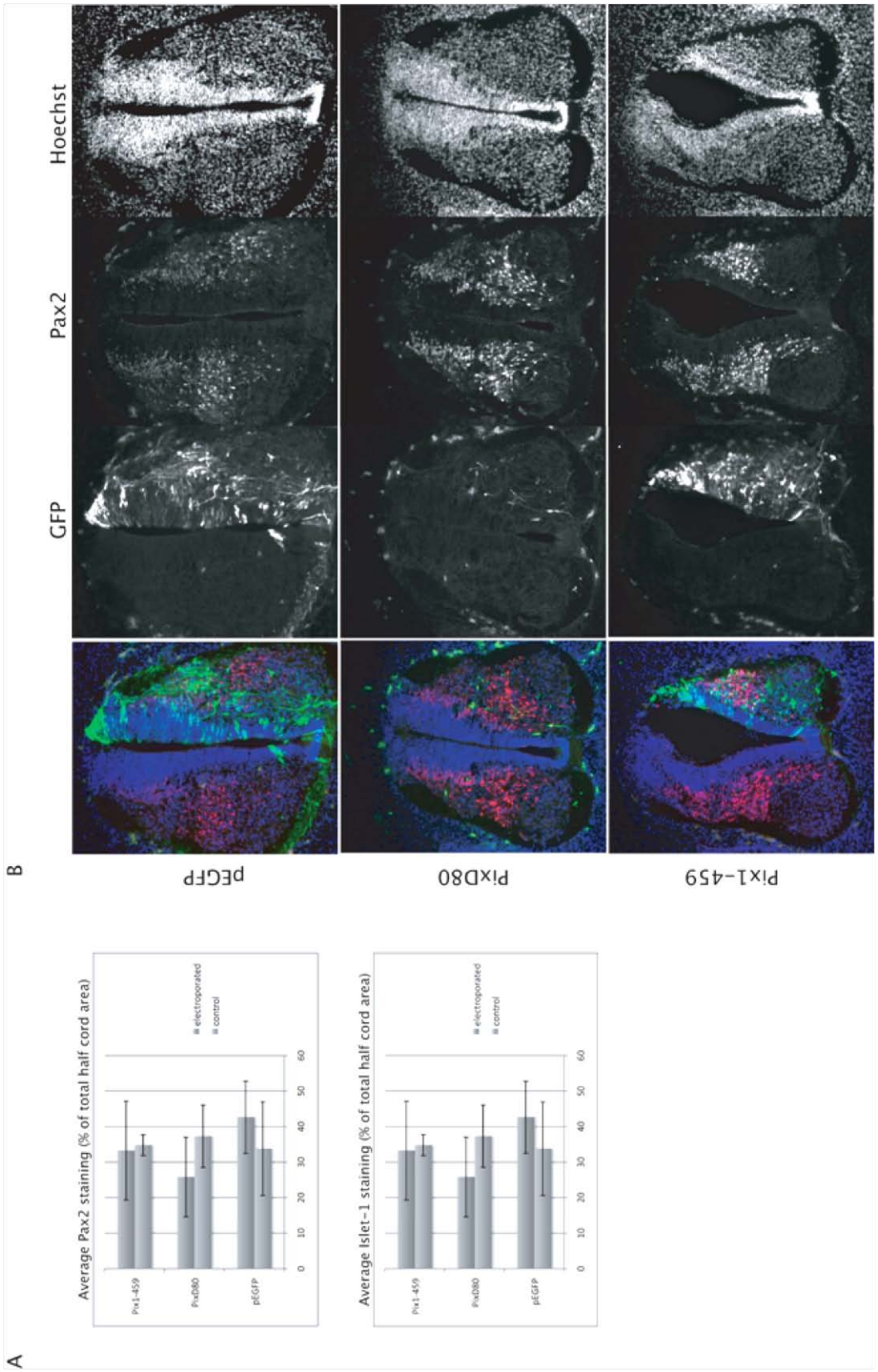

B
Average Axon Length (number pixels)
400
350
300
250
200
150
100
50
0
p<0.01
**
pEGFP
PixD80
Pix1-549

**Figure3-6. Differentiation and morphology of interneurons is not affected by electroporation.** A) Quantification of average Pax2 and Islet-1 staining using densitometry. Error bars depict standard deviation. B) Sample images of sections of embryonic chick spinal cord of 18μm thickness that were immunostained for Pax2 (mouse monoclonal) and Islet-1 (not shown) and nuclei labeled using Hoechst. Images were acquired using a Magnafire CCD camera on an Axiovert Zeiss microscope, 20x objective and 2x zoom. Scale bars = 100μm.


A
Average Pax2 staining (% of total half cord area)
Average Islet-1 staining (% of total half cord area)
electroporated
control
Pix1-459
PixD80
pEGFP
0
10
20
30
40
50
60
B
GFP
Pax2
Hoechst
pEGFP
PixD80
Pix1-459

*Netrin-1 promotes association of β-Pix/Cool-1 with GTP-bound Rac1 and Cdc42 in commissural neurons*

β-Pix and Git-2 multimerization is required for the activation of Rac1 and Cdc42 GTPases downstream of growth factors, such as bFGF, and receptors, such as EGFR, to promote reorganization of F-actin during cell migration and dendritic spine morphogenesis (Park et al., 2003; Zhang et al., 2003; Zhang et al., 2005). These morphological changes result from activation of RhoGTPases and downstream effectors, including Pak1. Rac1, Cdc42, and Pak1 activation are required for netrin-1 induced filopodia formation and growth cone expansion in commissural neurons, although it remains unclear how RhoGTPase activation is regulated in this context (Shekarabi et al., 2005).

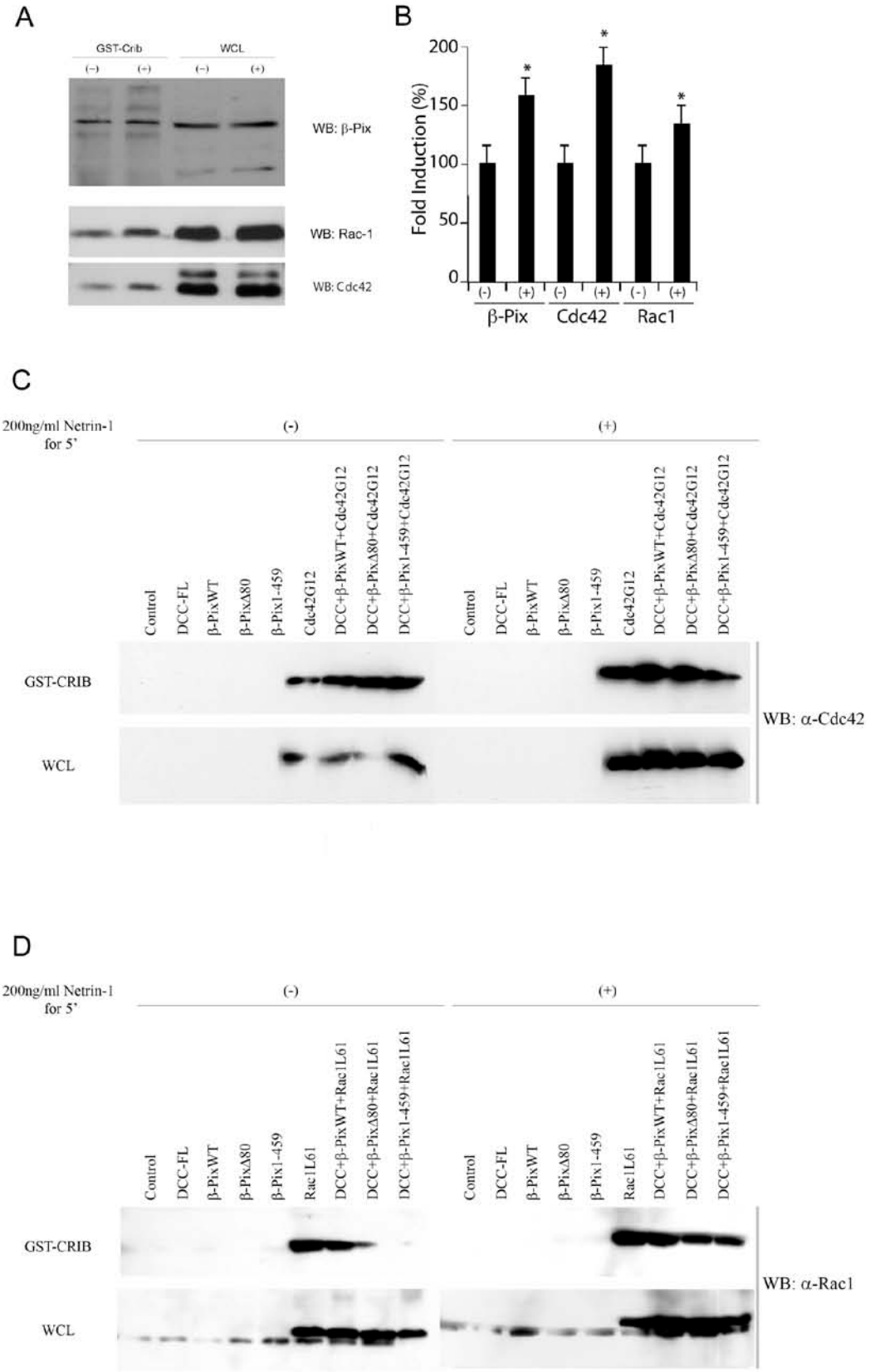
To investigate the role of β-Pix in Cdc42 and Rac1 activation in commissural neurons we analysed the capacity of β-Pix to associate with endogenously activated Cdc42 and Rac1. E13 rat commissural neurons cultured for 2 DIV were stimulated with 200 ng/ml of netrin-1 for 5 min. Cells were then lysed in a 0.5% NP-40 lysis buffer containing 20 mg of GST-CRIB protein. The CRIB domain in this chimeric protein is derived from Pak1 and selectively binds to GTP-loaded Cdc42 and Rac1. Following 5 minutes treatment with netrin-1, we observed a 1.2 fold increase in GTP-Rac1 and a 1.3 fold increase in GTP-Cdc42 binding to GST-CRIB (Figure. 3-7B). Similarly, the levels of β-Pix protein associated with activated Rac1 or Cdc42 showed a 1.2 fold increase. These results

suggest that β-Pix proteins associate with activated Rac1 and Cdc42 in commissural neurons and that netrin-1 induced activation of the RhoGTPases is associated with increased binding of β-Pix to GTP-Rac/Cdc42.

To investigate whether β-Pix could act as a GEF downstream of Netrin-1 to promote Rac1 and Cdc42 activity, we analysed the activity of these GTPases in 293T cells when expressing β-Pix mutants lacking GEF activity or the ability to associate with Pak. When we ectopically expressed DCC and a cDNA encoding WT β-Pix in addition to a wild-type Cdc42 construct (Cdc42G12) in 293T cells, an increase in the level of activated Cdc42 was detected following treatment with netrin-1 for 5 min, consistent with previous results (Shekarabi and Kennedy, 2002) (Figure 3-7C). Interestingly, the overexpression of a β-Pix mutant lacking the GEF domain abrogated this increased activation. Furthermore, a β-Pix mutant (PixΔ80) that is unable to associate with Pak1 but can still form multimers did not result in increased Cdc42 activity. These results provide evidence that activation of Rho GTPases by netrin-1 is required upstream of Pak1 activation.

**Figure 3-7. β-Pix associates with GTP-loaded Cdc42 and Rac1 in commissural neurons.** A-B) Commissural neurons 2DIV were treated with 200ng/ml netrin-1 for 5 minutes or left untreated. Cells were lysed in

0.5% NP-40, 50 mM Tris pH 7.4, 150 mM NaCl, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 g/ml aprotinin, 1 g/ml leupeptin and 20 mg of recombinant GST–CRIB. Following binding with glutathione-sepharose beads, pulled-down proteins were separated by SDS-PAGE on a 15% gel. Protein levels of GTP-bound Rac1, Cdc42 and of bound β-Pix were analysed by western blotting. Densitometry was performed using ImageJ software. C-D) 293T cells were transfected using Lipofectamine 2000 with the respective constructs as described in the materials and methods section. Transfected cells were treated with 200 mg/ml of Netrin-1 for 5 minutes after 2 days in vitro. GST-Crib pulldowns were performed on the purified lysates and associated proteins were separated by SDS-PAGE on a 15% gel. Protein levels of GTP-bound Rac1 and Cdc42 were analysed by western blotting.

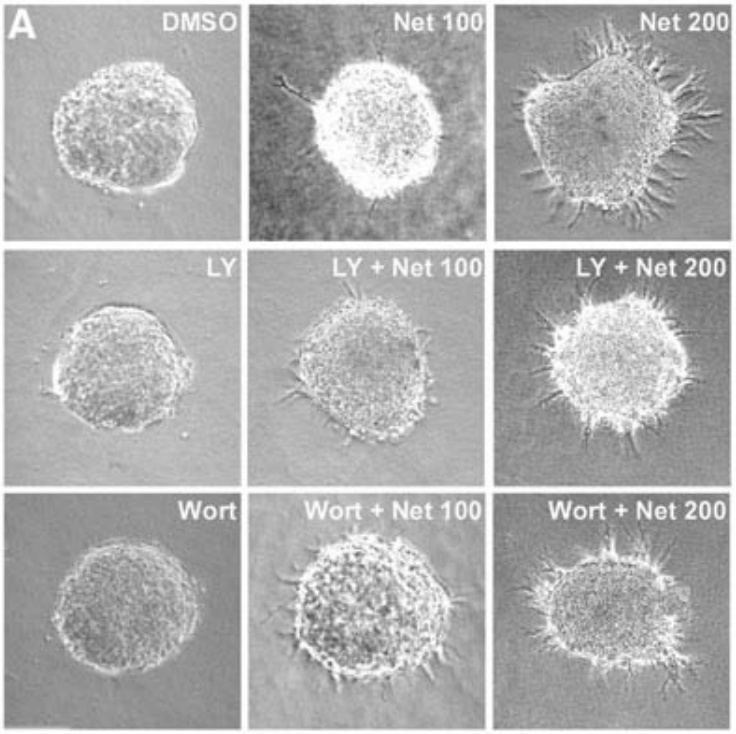

A
GST-Crib
WCL
(-) (+) (-) (+)
WB: β-Pix
WB: Rac-1
WB: Cdc42
B
Fold Induction (%)
200
150
100
50
0
(-) (+) (-) (+) (-) (+)
β-Pix Cdc42 Rac1
C
200ng/ml Netrin-1 for 5'
(-)
(+)
Control
DCC-FL
β-PixWT
β-PixΔ80
β-Pix1-459
Cdc42G12
DCC+β-PixWT+Cdc42G12
DCC+β-PixΔ80+Cdc42G12
DCC+β-Pix1-459+Cdc42G12
GST-CRIB
WCL
WB: α-Cdc42
D
200ng/ml Netrin-1 for 5'
(-)
(+)
Control
DCC-FL
β-PixWT
β-PixΔ80
β-Pix1-459
Rac1L61
DCC+β-PixWT+Rac1L61
DCC+β-PixΔ80+Rac1L61
DCC+β-Pix1-459+Rac1L61
GST-CRIB
WCL
WB: α-Rac1

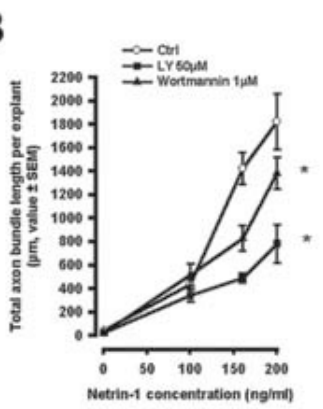
*PI3 kinase is required for activation of Cdc42 and Rac1 by netrin-1 in embryonic spinal commissural neurons*

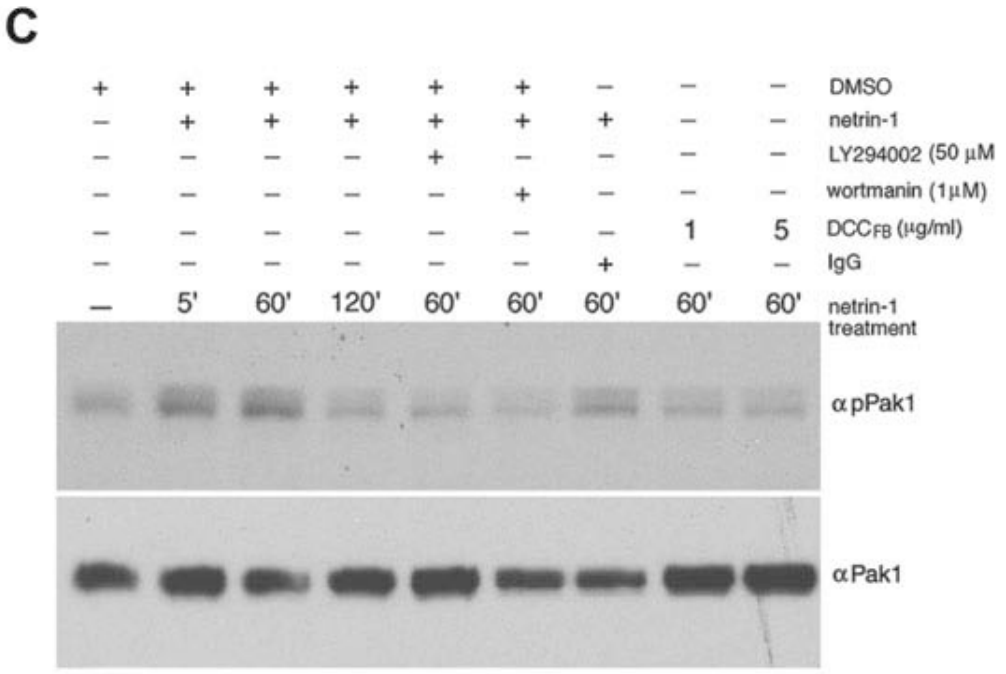
PI3 kinase (PI3K) plays multiple roles during neural development (reviewed by (Rodgers and Theibert, 2002). Importantly, PI3K regulates Rho GTPase activation (reviewed by (Fruman et al., 1998) and directly regulates GEFs such as βPix (Fukuda et al., 2002; Innocenti et al., 2003; Yoshii et al., 1999). Application of membrane permeable inhibitors of PI3K, 50 μM LY294002 or 100 nM Wortmannin, to cultured commissural neurons indicated that PI3K is required for netrin-1 to activate Rac1 and Cdc42 (data not shown). To investigate the functional consequence of inhibiting PI3K, axon outgrowth assays were performed using microdissected explants of E13 dorsal spinal cords cultured in a collagen gel. Application of either LY294002 or Wortmannin reduced, but did not completely block, axon outgrowth evoked by netrin-1 (Figure 3-8A and B). Importantly, cells in the explants are phase bright and appear healthy following treatment, suggesting that inhibition of PI3K is not triggering apoptosis at the time points examined. Furthermore, no increase in TUNEL staining of dissociated commissural neurons was observed following application of the PI3K inhibitors (not shown). Consistently with our laboratory's previous reports (Shekarabi et al., 2005), application of netrin-1 and either the DCC function blocking antibody, or the PI3K inhibitors LY294002 or Wortmannin, demonstrated that netrin-1 induced activation of Pak1 requires DCC and PI3K (Figure 3-8C).

**Figure 3-8**. **PI3 kinase inhibitors decrease axon outgrowth from netrin-1 treated embryonic dorsal spinal cord explants and block Pak1 activation.**

(A) Explants of dorsal E13 rat spinal cords were cultured in a collagen matrix with different concentrations of netrin-1 protein, with or without PI3 kinase inhibitors LY294002 (50 μM) and Wortmanin (100 nM) for 16 hours. (B) Quantification of the total length of axon outgrowth per explant. Both inhibitors significantly reduced netrin-1 induced axon outgrowth (* $p<0.05$). C) Netrin-1 activation of Pak1 requires DCC and PI3 kinase. Western blot analysis indicating that addition of 80 ng/ml netirn-1 protein to dissociated commissural neurons increases phospho-PAK1 immunoreactivity. Re-probing the same blot with anti-Pak1 confirms that comparable amounts of protein are present in each lane. The netrin-1 induced increase in phospho-PAK1 was blocked by 5 μg/ml anti-$DCC_{FB}$ or PI3 kinase inhibitors added to the cultures 1 hr before the addition of netrin-1.


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


B
Ctrl
LY 50µM
Wortmannin 1µM
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)
*
*


C
+ + + + + + – – – DMSO
– + + + + + + – – netrin-1
– – – – + – – – – LY294002 (50 µM
– – – – – + – – – wortmanin (1µM)
– – – – – – – 1 5 DCCFB (µg/ml)
– – – – – – + – – IgG
– 5' 60' 120' 60' 60' 60' 60' 60' netrin-1 treatment
αpPak1
αPak1

*β-Pix knockdown impairs Netrin-1 induced cytoskeletal remodelling in commissural neurons*

Our previous experiments provide evidence that the interaction of β-Pix with Pak1 and Git-2 is required for Netrin-1 signalling *in vitro* and *in vivo*. Additionally we demonstrated that netrin-1 treatment of commissural cultures results in an increased association of β-Pix with GTP-associated Rac1 and Cdc42. To directly test the hypothesis that β-Pix expression is required for Netrin-1 signaling, a cDNA was constructed encoding a micro RNA (miRNA) that targets β-Pix in embryonic rat commissural neurons.

MicroRNAs (miRNAs) are a growing class of small, noncoding RNAs (17-27 nucleotides) that regulate gene expression by targeting mRNAs for translational repression, degradation, or both. To facilitate delivery, we developed lentivirus vectors for the miRNAs. The lentivirus also included RFP as a marker to identify infected cells. As a control we used a miRNA scrambled sequence that is known not to be targeted by the RISC complex in cells (Dharmacon). Cultures of embryonic spinal commissural neurons were maintained as described for 1 DIV. Cells were infected overnight and fresh media added to the cultures after 18 hours. Commissural neurons were treated with netrin-1 for 5 min and then fixed with 4% paraformaldehyde followed by immunofluorescent staining of the actin cytoskeleton. Growth cone expansion, filopodia length and number were analysed as described in previous sections.

Preliminary results suggest that commissural neurons infected with lentivirus containing the β-Pix targeting miRNA leads to a decrease in netrin-1 induced filopodia formation, length and growth cone area expansion down to basal levels (Figure 3-9). Commissural neurons infected with lentivirus containing a scrambled control sequence that is not targeted by the RISC complex had filopodia number/length and growth cone measurements consistent with those not infected (Figure 3-9). Future experiments will include determining if βPix GEF activity is required for Rac1 and Cdc42 activation by netrin-1. Using the micro-RNA containing lentivirus will enable us to knockdown βPix and Git-2 in commissural neurons and assay GTP-bound Rac1 and Cdc42 using GST-PAK-CRIB pulldown assays as described previously (Shekarabi and Kennedy, 2002).

**Figure3-9. Netrin-1 mediated growth cone expansion and filopodia formation decreases with the knockdown of β-Pix.** Quantification of growth cone area, filopodia number (per 50μm of axon) and filopodia length in E13 SC commissural neuron cultures infected with a control miRNA (not targeted by RISC), or p26 miRNA that targets β-Pix mRNA sequence for degradation. n>10 in 3 separate experiments; error bars stand for SEM.

Filopodia number (μm)
0
10
20
30
40
50
60
(-)
(+)
(-)
(+)
control miRNA
p26 miRNA
Filopodia length (per 50μm)
0
1
2
3
4
5
6
7
8
9
(-)
(+)
(-)
(+)
control miRNA
p26 miRNA
GC Area (μm2)
-100
-50
0
50
100
150
200
250
300
350
400
(-)
(+)
(-)
(+)
control miRNA
p26 miRNA

## DISCUSSION

In recent years, the immediate signaling events occurring downstream of netrin-1 have rapidly been uncovered, but much of the signaling cascade leading to netrin-dependent cytoskeletal regulation remains poorly understood. The netrin-1 receptor DCC is constitutively bound to the adaptor protein Nck through its P2 domain in an SH3-proline rich domain interaction (Li et al., 2002a). Guirland *et al*, have provided evidence that netrin-dependent axon guidance signaling is localized to lipid rafts. More recently another study has demonstrated that the DCC receptor is localized to these rafts by palmitoylation of its transmembrane domain and upon ligand binding with this DCC receptor pool results in Erk activation and the promotion of axon outgrowth (Herincs et al., 2005). It is still unclear how the receptor is trafficked to these subcellular compartments and whether the signaling complexes shown to promote netrin-mediated axon guidance are also present at these sites.

Previous studies have provided evidence linking DCC to the F-actin cytoskeleton. Binding netrin-1 results in the recruitment and activation of the RhoGTPases Rac1 and Cdc42. The activation of these GTPases results in the recruitment of Pak1, a serine/threonine kinases, to a complex with the intracellular domain of DCC. N-WASP is also activated and recruited to DCC possibly through its interaction with Nck and Cdc42, although the exact mechanism still remains elusive. This scaffold can directly bind to actin and may possibly form a direct link between the

receptor and the actin cytoskeleton that promotes cell adhesion. Other laboratories have established that FAK and the src family member fyn are recruited to and can directly tyrosine phosphorylate DCC (Li et al., 2004; Ren et al., 2004) upon netrin-1 binding. It has been postulated that such an event is not only required for netrin-1 signaling in promoting axon outgrowth and turning may be the event that initiates activation of the RhoGTPases downstream of DCC.

Studies in C.elegans have shown that Unc-73 (trio), a Rho-GEF specific for Rac1, is required downstream of Unc-40 (DCC) in directed cell and growth cone migration (Levy-Strumpf and Culotti, 2007). Recent studies have provided evidence of roles for Trio and Dock180, two Rac1 specific guanine-nucleotide exchange factors, as regulators of Rac1 downstream of DCC during netrin-1 chemoattraction. However, in both reports most assays were performed using cell types other than commissural neurons making it unclear whether the same mechanisms contribute to netrin-1 mediated commissural guidance. Furthermore, Trio knockout mice exhibit a very modest phenotype compared to mice lacking netrin-1 or DCC. Specifically, the corpus callosum and ventral spinal commissure are both intact, two major axonal projects that require DCC and netrin-1 during neural development, providing evidence that while trio may contribute to signalling downstream of DCC, is not essentially for chemoattractant responses to netrin-1. Additionally, earlier findings have provided

evidence that Cdc42 activation appears to be upstream of Rac1 activity in embryonic spinal commissural neurons (Shekarabi and Kennedy, 2002).

Here we demonstrate that β-Pix isoforms are expressed by interneurons in the developing spinal cord and associate with the netrin-1 receptor, DCC, in commissural neurons. We show that β-Pix multimeric complexes and association with Pak are required for netrin-1 induced growth cone expansion, filopodia formation and maintenance. In ovo electroporation of β-Pix mutants into embryonic chick spinal cord deregulates commissural neuron axon extension toward the ventral midline. Furthermore, we demonstrate that application of netrin-1 increases the association of β-Pix with GTP-bound Cdc42 and Rac1. We propose a model in which a β-Pix/Git-2 complex constitutively associates with DCC in commissural neurons and that upon ligand binding src family members phosphorylate and activate β-Pix, resulting in the recruitment and activation of the RhoGTPases. Once active the RhoGTPases will bind and activate the downstream effector Pak1 resulting in actin polymerization and cytoskeletal rearrangement (refer to figure 4-2 for proposed model).

It would not be surprising that several GEFs were required downstream of DCC to promote axon extension in response of netrin-1, since their specificity could target various RhoGTPases depending on temporal and subcellular localization factors. Furthermore, different signaling cascades

may be targeted in different cellular and animal models. It is also possible that various factors may be needed during the activation of different events required for directed cell migration (as proposed in figure 4-2). For example, NFAT, a nuclear factor of activated T-cells is required for netrin-dependent axon outgrowth but not for axon turning (Graef et al., 2003). Furthermore it is plausible that proximal and long-distance signals downstream of netrin-1 may also differ (Baker et al., 2006). Immediate recruitment and activation of a protein complex that directly promotes actin polymerization may take place at initial stages of axon extension but the cytoskeletal turnover maintenance required to promote long distance targeting would likely involve other regulatory components. In summary, the data presented in this chapter identifies an additional Rac1 and Cdc42 GEF downstream of DCC signaling in commissural neurons. This GEF is important for netrin-1 mediated actin remodeling in both *in vitro* and *in vivo* settings. It remains to be established how β-Pix coordinates with other GEFs downstream of DCC to enable netrin-1 mediated chemoattraction.

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# CHAPTER 4:

# GENERAL DISCUSSION

## PREFACE

This final chapter begins with a discussion of the role of Netrin signaling throughout development. It remains unclear to what extent netrin expression in different tissues activates similar intracellular mechanisms. The possibility that netrin signaling may interact with other ECM associated proteins such as integrins is discussed. New intracellular models are proposed based on the identification of actin associated proteins as downstream effectors of netrin signaling in Chapter 2. The hypothesis that intracellular signaling nodes, such as specific GEFs, may be required for the activation of distinct actin remodeling events is proposed. The roles of identified Rac GEFs and how β-Pix may contribute to netrin mediated actin cytoskeleton signaling during chemoattraction is further discussed. Additionally, models of how intracellular signaling downstream of DCC functions to promote axon guidance in commissural neurons are outlined. The thesis concludes by highlighting the possible

contribution of netrin signaling beyond axon guidance in the embryonic spinal cord to development and disease progression.

## 4.1 Conclusion and Summary of Original Findings

The work presented in this thesis expands our understanding of the molecular mechanisms downstream of netrin-1/DCC signaling that regulate actin dynamics during commissural neuron chemoattraction. Although our analysis primarily focused on embryonic rat commissural neurons, the models proposed potentially provide insight into the mechanisms regulating other cellular events regulated by Netrin-DCC signaling, such as tissue morphogenesis of the mammary and lung epithelium, cell migration, neuronal process branching and tumorigenesis.

An exploratory study using mass spectrometry analysis of DCC-associated proteins isolated from netrin-1-treated E13 commissural neurons allowed us to identify 14-3-3 and Arp2/3 as additional links between DCC and actin. We demonstrated that both Arp2/3 and 14-3-3 are required for netrin mediated commissural growth cone expansion and filopodia formation. 14-3-3 association with DCC decreased following the addition of netrin-1 suggesting it functions to regulate the activation of DCC-induced actin polymerization. Arp2/3 association with a DCC complex increased upon netrin-1 treatment. Additionally, we showed that Arp2/3 activation by N-Wasp is required for netrin-1-mediated actin remodeling. These findings suggest that netrin-1-mediated actin remodeling is a tightly regulated mechanism with both positive and negative regulatory proteins participating.

In chapter 3 we identified β-Pix as a key regulator of Cdc42 and Rac1 downstream of DCC in the context of netrin-1 mediated commissural neuron chemoattraction and the underlying changes in cytoskeletal dynamics. β-Pix isoforms were identified as highly expressed proteins in the developing spinal cord. We further demonstrated that Cool-1/ β-Pix dimerization and association with p21-activated kinase (Pak-1) is required for netrin mediated filopodia formation, maintenance and growth cone expansion. Not surprisingly, a functional Cool-1/□β-Pix protein is shown to be required for axon extension toward the midline. These data provide compelling evidence that β-Pix is an important GEF downstream of DCC that regulates RhoGTPase and Pak-1 activation during the promotion of actin remodeling required for chemoattraction.

### 4.2 Netrins as guidance and adhesion molecules

Substantial evidence has accumulated indicating that netrin-1 functions as an adhesive ligand in the CNS in addition to its role as a diffusible chemotropic cue.

Only recently has a gradient of netrin protein been visualized, emanating from the floor plate and polarizing the neural epithelium directly in the path of extending commissural axons (Kennedy et al., 2006). These findings support the conclusion that netrin-1 functions as a long-range chemotropic axon guidance cue in the embryonic spinal cord. However, they do not

rule out the possibility that netrins guidance function may require, or be enhanced, by netrin protein being anchored to cell membranes or extracellular matrix. When netrin-1 protein was first purified from embryonic brain, the vast majority was found in high salt washes of cellular membranes, supporting the idea that the majority of netrin-1 protein is not freely soluble in the embryonic nervous system (Serafini et al., 1994). Further studies have demonstrated that the C domain of netrin-1 strong interacts with heparin, suggesting that it likely binds heparin sulfate proteoglycans in the extracellular matrix in vivo (Kappler et al., 2000; Manitt et al., 2001). Furthermore, netrin-1-heparin interaction has been demonstrated to be functionally important. The expression of the heparin sulfate synthesis enzyme, Ext1, by spinal commissural neurons is required for netrin-1 induced outgrowth and signaling events (Matsumoto et al., 2007).

Netrin's adhesive properties are further supported by analysis of paranodal junctions in netrin and dcc null mice. In the absence of either netrin or dcc oligodendroglial paranodes are able to establish initial contacts but with time become disorganized suggesting a role for netrin-1 in maintenance of the paranodal loops (Jarjour et al., 2008).

As initially proposed in the introductory chapter of this thesis it remains to be determined where netrin guidance in cell motility and axon targeting signals meet with netrin's signals in promotion of adhesion. It is very

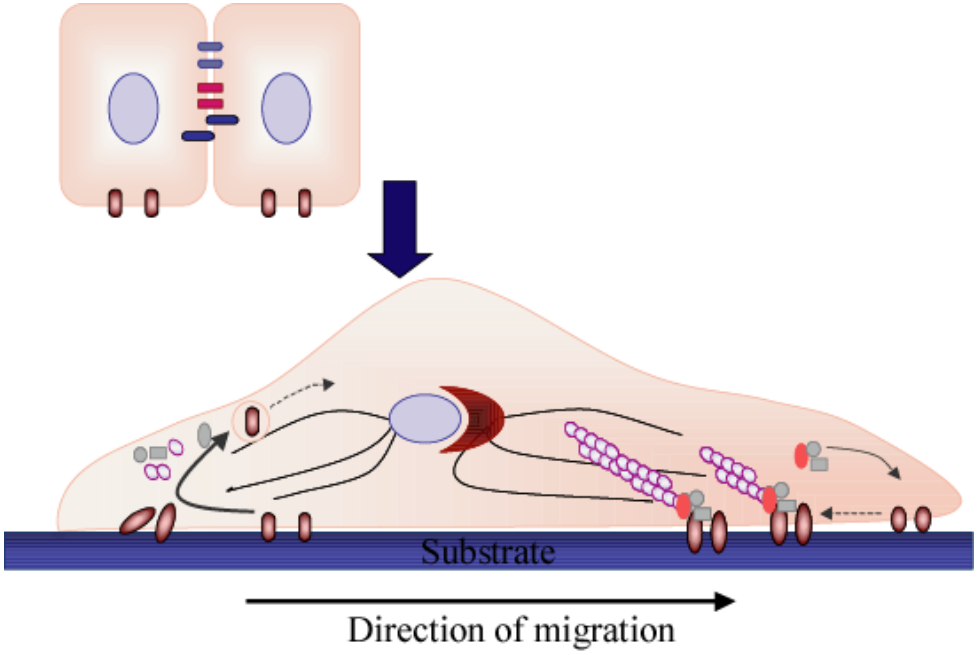
possible that these are not such distinguishable pathways as originally though. Recent evidence from Moore, et al. showed that spinal commissural neurons adhere to, and that their growth cones expand on, a substrate of netrin-1 using *in vitro* adhesion assays (Moore et al., 2008).

Additional evidence for such a proposal comes from signaling studies in other systems, in particular the dissection of intracellular signaling events taking place during cell migration have clearly demonstrated that cell-cell contact and cell-ECM adhesion are crucial events during cell migration in embryogenesis and tissue invasion (for a review refer to (Rozario and DeSimone, 2010).

## 4.3 Integrin regulation of actin dynamics – a signaling model for cell migration, adhesion and cell-ECM interaction

Adhesion foci with the substrate are fundamental structures not only for the propagation of migratory signals intracellularly but also to provide the anchoring and tension required to physically propel the cell body forward. These structures are complex molecular assemblies that bridge the extracellular matrix to the actin cytoskeleton via transmembrane receptors known as integrins. The initial response of a cell to a migration-promoting agent is the formation of membrane protrusions through the extension of the actin filaments towards a leading edge (Figure 4-1).

**Figure 4-1 EMT/cell migration dynamics.** Epithelial cells breakdown cell-cell adhesions and establish new substrate adhesions in the direction of cell migration. Actin regulatory complexes and membrane vesicles accumulate at the leading edge to form focal contacts, which then mature into focal adhesions. At the retracting end adhesions breakdown and receptors and protein complexes are internalized.



Large and broad membrane ruffles known as lamellipodia or spike-like membrane extensions known as filopodia are examples of some of these protrusions. Integrin and receptor tyrosine kinase receptor clustering at these edges allow for further actin polymerization and maturation of cell-matrix contacts required for cell motility (Friedl and Wolf, 2003; Ridley et al., 2003). Clustering of integrins signals the recruitment of scaffolds and adaptor proteins through their cytoplasmic tail. Such adaptor proteins form protein complexes through their SH2 and SH3 domains containing regulatory molecules such as PI3K or GEFs and GAPs, which regulate Rho GTPases and actin binding proteins that allow for the bridging of integrins to the actin cytoskeleton (Friedl and Wolf, 2003). Surface proteases are recruited to focal contacts and initiate cleavage of extracellular matrix components such as collagens, fibronectin and laminins, as well as pro-matrix metalloproteinases to generate active soluble MMPs necessary for the degradation of the surrounding extracellular matrix. The physical extension of the cell's cytoskeleton generates tension required for the contractibility of the cell body. As previously mentioned there are several types of cell-matrix adhesion foci. These include the well-characterized focal adhesion, the short-lived focal complexes, fibrillar adhesions and podosomes (Geiger et al., 2001). Initial contact with the substrate at the leading edge originates formation of focal contacts, which are small dot-like structures ~60nm thick and ~ 1 mm long (Chen and Singer, 1982; Wehrle-Haller and Imhof, 2002). These structures have a fast turnover and appear to be Rac dependent. Focal

contacts are predominantly enriched with $\alpha_V\beta_3$ integrins and apply stronger traction during migration than mature focal adhesions (Geiger and Bershadsky, 2001). Pak, a downstream effector of both Rac and Cdc42 appear to be a major regulator of focal complexes turnover. This serine kinase phosphorylates LIM kinase, which in turn phosphorylates and deactivates actin depolymerizing proteins ADF/cofilin (Edwards et al., 1999). Focal complexes can then mature into longer, denser structures known as focal adhesions via Rho activation. Rho activity leads to further polymerization and stabilization of actin stress fibers through its effectors ROCK and mDia. These structures are ~2-5□mm long, they are found at the cell periphery and are mainly constituted of FAK, Vinculin, Zyxin, Paxillin and $\alpha_V$ integrins (Zaidel-Bar et al., 2004).

Knockout studies of integrins have highlighted their importance in development. β□ chain knockouts show very early embryonic lethality, while $\alpha$ chain knockouts have demonstrated these receptors play an important role in the development of the nervous system (Anton et al., 1999; Fassler and Meyer, 1995). *In vitro* studies have not only demonstrated the specific affinities between integrin heterodimers and their extracellular matrix ligands but also mechanisms and biological responses downstream of particular integrin dimers. For example, while $\alpha5\beta1$ integrins are mainly found in small focal contacts, $\alpha V\beta3$ are only found in large focal adhesions at the ends of stress fibers (Raghavan et

al., 2003). Integrins are also required for proper signaling of certain growth factors, and have been shown to directly associate with some of them, examples are VEGF, EGF, Insulin receptor, PDGF (Giancotti and Ruoslahti, 1999). This cross-activation may lead to enhanced clustering of integrins at the cell surface and amplification of survival, migration, and growth signals. Importantly, upon ligand binding cytoskeletal proteins (Talin, tensin, $\alpha$-actinin,Vinculin, Paxillin) bridge integrins to the actin cytoskeleton and to downstream effectors of intra-cellular signaling pathways such as FAK, Src, and SHC and PKC. Activated Fak and src bind to several signaling molecules mediating integrin activation of the Erk and Ras pathways.

### 4.4 Integrins - Netrin signaling cross-talk

In neurons, β1 family integrin receptors were first identified as mediators of neurite outgrowth on ECM components using blocking antibodies *in vitro* (Reichardt and Tomaselli, 1991). It has since become clear that integrins also play important roles in neuroblast migration, synapse formation, glial cell development, and neural disease (Jones and Grooms, 1997; Milner and Campbell, 2002; Panicker et al., 2003).

An example of cross-talk between integrins with other cell surface receptors is the interaction between NGF and integrin signaling. NGF stimulates the accumulation of beta-1 integrins at the tips of growth cone filopodia of sympathetic neurons grown in the absence of an ECM substrate. Myosin inhibitors blocked this effect (Grabham and Goldberg, 1997). No mechanism has been proposed yet as to how this cross-talk may occur, however, the same authors have later demonstrated that NGF stimulates the movement of unliganded β1 integrin on the filopodia by an actomyosin-dependent mechanism of rapid forward transport. NGF further stimulated the coupling of cross-linked β1 integrin to the retrograde flow of actin (Grabham et al., 2000). Some of the proposed mechanisms on how NGF may be promoting this rapid movement of forward β1 integrin transport are through NGF activation of the myosin motors via second messengers or NGF increased efficiency of coupling of β1 integrin to the transport machineries. The increase in diffusion of β1 integrin, perhaps

caused by the release of a constraint in response to NGF, could make it more available to couple to the transport machineries.

A navigating axon can modify its actions in response to ECM, and also to diffusible factors, in an integrin-dependent manner. While increasing gradients of netrin-1 attracted Xenopus retinal neurons cultured on either glass, fibronectin, or poly-D-lysine, in contrast, growth cones of these same neurons turn away from a gradient of netrin-1 when cultured on laminin-1. Interestingly this switch was dependent on the cAMP levels being lowered by the presence of the laminin substrate (Hopker et al., 1999). It is not yet clear how the netrin-1-integrin intracellular cross-talk occurs but it is clear that many of the cytoskeleton regulating players activated downstream of netrin-1 have also been identified as key regulators in integrin signaling.

### 4.4.1 PI3K and β-Pix linking DCC to RhoGTPases

In studies using dominant-negative constructs, loss of Ras, Rac1, Cdc42, PI3K or ILK blocked neurite outgrowth (Sarner et al., 2000). Additionally these studies established the hierarchy of a signal transduction pathway capable of activating outgrowth (Sarner et al., 2000). Ras lies at the top of this pathway, signaling to PI3K and leading to the activation of Cdc42 and Rac1. A requirement for the small GTPase Rac1 in promoting integrin-dependent neurite outgrowth was also found in embryonic chicken motor

neurons when grown on laminin or fibronectin using blocking beta-1 antibody. Constitutively-active Rac1 resulted in actin filament accumulation in growth cones, while a decrease was seen in response to dominant-negative Rac1 (Kuhn et al., 1998). Similarly, as discussed throughout the previous chapters, netrin-1 signaled to promote adhesion, growth cone extension and turning is dependent on the activation of both of these Rho GTPases (reviewed in chapter 1 and chapter 3). It would not be surprising if similar hierarchical signaling mechanisms were activated to promote RhoGTPase dependent actin polymerization downstream of netrin-1 as they are downstream of integrins.

In fact, some evidence was presented in Chapter 3 supporting the hypothesis that effectors such as β-Pix and Pak, which activated in integrin signaling are also crucial for RhoGTPases' activity downstream of netrin-1. Although a requirement for Pak activity has not been tested using assays of commissural neuron adhesion to a netrin-1 substrate, it is likely that Pak is a key regulator of focal contact turnover in response to netrin-1, similar to its function downstream of Integrins. The results presented in chapter 3 demonstrate that Pak function is required for netrin-1 mediated axon chemoattraction. It is particularly interesting that Pak-1 complexes with β-Pix/Git proteins during the establishment of adhesive contacts in migrating cells and these complexes result in the regulation of Cdc42 and Rac1 proteins at these sites (Brown et al., 2005; Brown et al., 2002; Lamorte et al., 2003).

PI3K is a key regulator of RhoGTPase activity (reviewed by (Welch et al., 2003) and a direct regulator of β-Pix and Pak (Chan et al., 2008; Fukuda et al., 2002; Innocenti et al., 2003; Yoshii et al., 1999). In chapter 3 evidence is provided that PI3K activity is required for netrin-1 to activate Rac1 and Cdc42, and also required for netrin-1 to induced commissural axon outgrowth. Taking into consideration the potential role of PI3K as a positive regulator of β-Pix, we propose a model whereby a signaling cascade of DCC>PI3K>βPix/Pak/Cdc42/Rac is required for commissural axon chemoattraction to netrin-1 (refer to proposed models in section 4.5).

### 4.4.2 A role for 14-3-3 adaptor proteins downstream of Netrin

14-3-3 proteins participate in a plethora of cellular pathways, predominantly cell death and cell cycle regulation, raf signaling pathway, cell migration and disease progression (Aitken, 2006). Alpha4 integrins are used by leukocytes and neural crest derivatives for adhesion and migration during embryogenesis, immune responses and tumor invasion (Pinco et al., 2001; Rose et al., 2007). Recent work from Deakin et al. (Deakin et al., 2009) using FRET technology demonstrated that alpha4 integrin complexes with paxillin and 14-3-3zeta resulting in the localization of activated Cdc42 to lamellipodia and accelerated cell migration. In chapter 2 we demonstrate that 14-3-3 is also required for netrin induced filopodia formation and growth cone expansion. It remains to be elucidated

if this role involves a direct association between 14-3-3zeta and phosphorylated DCC, as is with the case of alpha4 integrins, and if such an association directly regulates Cdc42 localization to the edge of the neuronal growth cone. An alternative model was also proposed in chapter 2 where a SSH-14-3-3 complex functions further downstream of DCC to regulate cofilin, similar to functions proposed for SSH-14-3-3 in other cellular contexts (Gohla and Bokoch, 2002; Kligys et al., 2009; Ulvila et al., 2011).

### 4.4.3 Actin-nucleating activity downstream of Netrin-1

While it is unclear whether all guidance responses involve Arp2/3-mediated nucleation, Sema3A and netrin-1 likely utilize this pathway of actin assembly. In the mouse, DRG neurons respond to Sema3A as a repellent and exhibit a decrease in immunofluorescence staining intensity for Arp-2 and cortactin in a collapsing growth cone (Brown and Bridgman, 2009). Interestingly, in hippocampal neurons Arp2/3 was enriched in the central region of growth cones and also found at the cell periphery. Knocking down its expression appeared to have a more significant effect on axon guidance and microtubule stability than axon extension (Strasser et al., 2004). Studies from the Kennedy laboratory have provided evidence supporting a requirement for N-Wasp activation of Arp2/3 in netrin-1 mediated axon guidance (Shekarabi et al., 2005). In chapter 2, I have provided evidence that the Arp2/3 subunit p34 associates with a DCC

complex in commissural neurons and that the inhibition of N-Wasp activation of Arp2/3 impairs netrin-1 induced changes in the growth cone cytoskeleton, disrupting filopodia formation and growth cone expansion. Furthermore, dominant negative studies investigating the function of WASP, WAVE, and Arp2/3 in *Drosophila* and *C. elegans* have demonstrated that blocking their function results in aberrant axonal morphology and guidance defects for commissural and longitudinal axon projections (Norris et al., 2009; Shakir et al., 2008; Zallen et al., 2002). It remains to be determined if Arp2/3 only participates in specific events during axon guidance such as turning or adhesion or if it has a more general role as in actin nucleation. True loss-of-function experiments using p34 targeted siRNAs in mammalian neuronal cultures will be needed to dissect its function.

## 4.5 Models for netrin-1/DCC remodeling the cytoskeleton during axon outgrowth and turning

Netrin-1 binding to DCC recruits and activates the Rho GTPases Rac1 and Cdc42 (Shekarabi and Kennedy, 2002). Furthermore, Cdc42 and Rac1 function is required for netrin-dependent growth cone expansion and filopodia formation in commissural axons, conformational changes in growth cones that are required for growth cone chemotropism. The identity of the downstream effectors that are required for DCC activation of RhoGTPases remains unclear. Studies in C.elegans have shown that

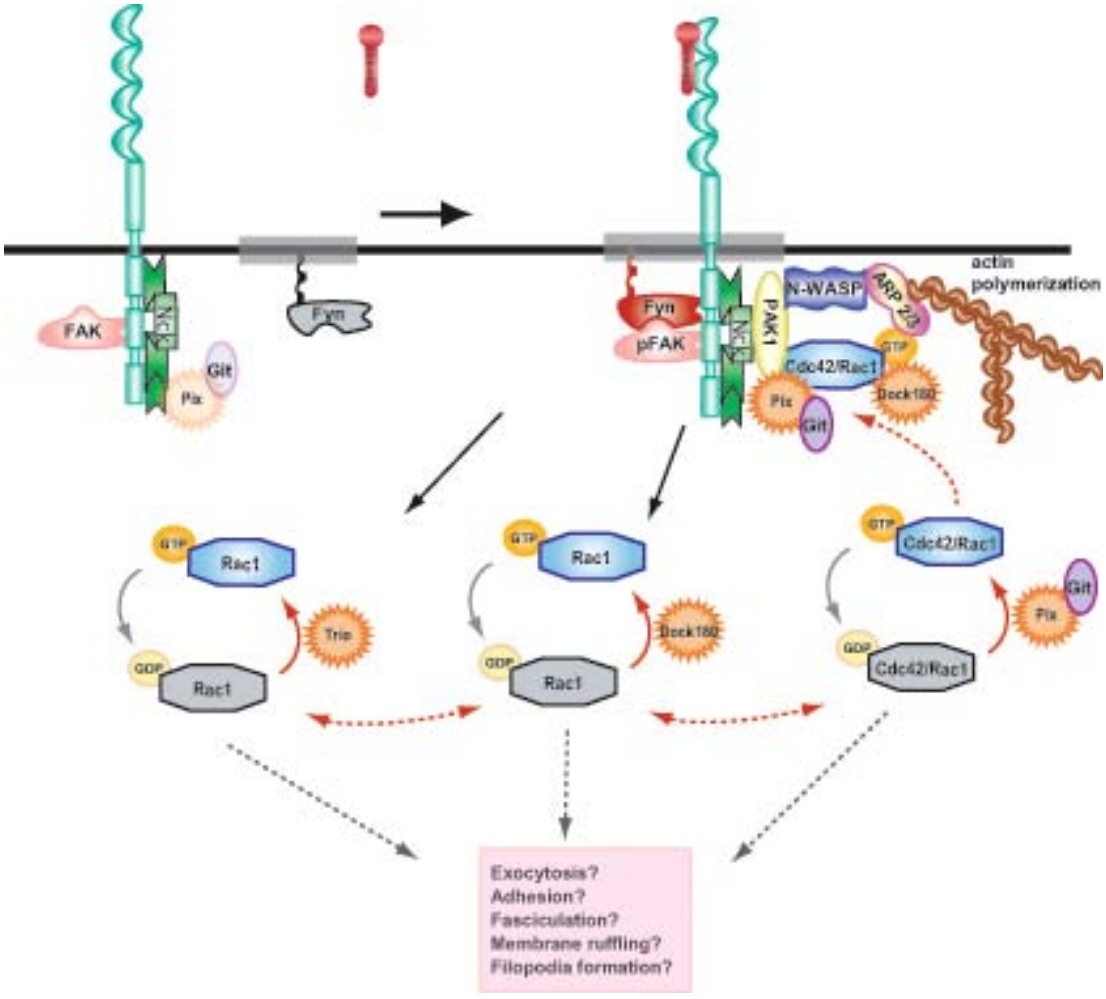
Unc-73 (trio), a Rho-GEF specific for Rac1, is required downstream of Unc-40 (DCC) in directed cell and growth cone migration (Levy-Strumpf and Culotti, 2007). Analyzing neurons from vertebrate species, recent studies have provided evidence for Trio and Dock180, two Rac1 specific guanine-nucleotide exchange factors, as regulators of Rac1 downstream of DCC during netrin-1 chemoattraction (Briancon-Marjollet et al., 2008; Li et al., 2008). However, as discussed in chapter 3, Trio knockout mice exhibit a very modest phenotype compared with the netrin-1 or DCC knockouts (Deiner and Sretavan, 1999). In particular, the corpus callosum and ventral spinal commissure are both intact in trio null mice, two major axonal projections that require DCC and netrin-1 during neural development. This finding alone provides strong evidence that while trio may contribute in some way to DCC signal transduction, is not essential to generate a chemoattractant axon guidance response to netrin-1. Furthermore, Dock180 knockout mice do not exhibit major defects in neural development (Briancon-Marjollet et al., 2008; Laurin et al., 2008). Such modest phenotypes might result from redundancy of function between different GEFs, or alternatively, may reflect specificity of GEF function in different cells types and biological contexts. Additionally, earlier findings provided evidence that activation of Cdc42 is required upstream of Rac1 activation in embryonic spinal commissural neurons (Shekarabi and Kennedy, 2002). This suggests that the response to netrin-1 in commissural neurons requires the activation of a GEF for Cdc42. In chapter 3 we demonstrated that blocking β-Pix, a Cdc42 and Rac1 specific

GEF, function resulted in reduced axon extension towards the floorplate *in vivo* and an inappropriate spreading of the axon bundles as they reached closer to the ventral midline. The phenotype detected was severe in axons expressing the β-Pix mutants. In light of these findings, future studies addressing the effect of miRNAs targeting β-Pix will provide important further insight into the requirement for β-Pix function in netrin-1 signaling. It is possible that β-Pix could be responsible for the activation of Cdc42 and/or Cdc42 and Rac1 while Trio and/or Dock180 may contribute to Rac1 activation further downstream. Alternatively, it remains possible that Rac and/or Cdc42 activation is required at different stages of chemoattraction. For example, trafficking of DCC containing cargo vesicles to the plasma membrane increases upon netrin-1 binding in a PKA dependent pathway (Bouchard et al., 2004). Rac and Cdc42 both influence vesicle recycling during cell migration (Harris and Tepass, 2010b; Ridley, 2006) and may influence DCC signaling by promoting receptor externalization that would then locally amplify the intracellular response to netrin-1. Cell migration is a complex process and it is possible that different GEFs will take part in RhoGTPase regulation in different ways. For example, Rac1 alone is implicated in reorganizing the cytoskeleton, directing vesicle trafficking, and influencing the establishment and maturation of adhesive contacts, all of which will likely be required for chemoattraction (see proposed model in figure 4-2).

A widely accepted paradigm is that a balance between the activation of multiple Rho GTPases determines how the cytoskeleton is reorganized in response to a specific signal. With this in mind, it is possible that activation of the same one Rho GTPase may result in varying responses, as a result of the activation state of other family members, and the specific cellular compartments in which they are localized. In chapter 3 we discussed the possibility that netrin-dependent axon guidance signaling is localized to lipid rafts. DCC is localized to such rafts by palmitoylation of its transmembrane domain and upon ligand binding with DCC pool results in Erk activation and the promotion of axon outgrowth (Herincs et al., 2005). It is still unclear how the receptor is trafficked to these subcellular compartments and whether the signaling complexes shown to promote netrin-mediated axon guidance are also present at these sites. We have observed from preliminary data the accumulation of DCC and β-Pix proteins (data not shown) in lipid raft-like membrane fractions isolated from commissural neurons. This accumulation increased with netrin-1 and Forskolin treatment. Additionally, PI3K, a key regulator of Rho GTPases and β-Pix, is required for netrin-1 mediated axon outgrowth (refer to chapter 3). Src family members, which bind and phosphorylate DCC upon netrin-1 binding, recruit and activate the PI3K pathway at lipid rafts (Arcaro et al., 2007). Furthermore, PAK dependent focal adhesion turnover is dependent on a PI3K/Pix/Cdc42/p38 pathway (Chan et al., 2008). Future studies will determine precisely how such complexes are localized with an axonal growth cone and contribute to the establishment of new sites of

adhesion, membrane extension, filopodia formation, receptor recycling, accumulation at the leading edge, and how all of these changes underlie growth cone chemoattraction in response to netrin-1.

**Figure 4-2. Proposed Models for Netrin-DCC signaling in commissural neuron chemoattraction.** β-Pix/Git complexes are constitutively bound to DCC. Once netrin-1 binds the receptor recruitment and activation of an array of proteins occurs, i.e. Fyn, Fak, Pak1 and the Rho GTPases Rac1 and Cdc42. β-Pix/Git complexes may coordinate the activation of Cdc42 and Rac1 with already previously identified GEFs Dock180 and Trio. All three of these GEFs could possibly target the GTPases downstream of netrin-1 depending on temporal and subcellular localization factors.



## 4.6 Future Directions and Implications

Netrin signaling continues to be elucidated in various systems. Piece by piece, new effectors are identified for the various functions that Netrin signaling impacts on during development, organogenesis, plasticity and even disease progression. Mass spectrometry results from chapter 2 have opened up new doors to identifying not just actin regulators downstream of Netrin but also potential new roles of DCC complexes in ribosomal protein translation, microtubule dynamics, or vesicle trafficking. Future studies are required to identify the precise function of many of these protein complexes in chemoattraction to netrin-1. True loss-of-function experiments will also be needed in mammalian systems to identify the contributions of Arp2/3 and N-Wasp in growth cone morphology and axon elongation. The identification of β-Pix as a possible Cdc42/Rac1 GEF downstream of netrin-1 in chapter 3 raises a number of new questions. Future studies are required to identify whether there is redundancy between the different β-Pix isoforms downstream of netrin-1. Experiments with FRET technology and live imaging may also help decipher the functional role each of the GEFs identified so far downstream of netrin-1. For example, it is possible that Trio and β-Pix may both be activated, but localized to different subcellular compartments in the growth cone during netrin-1 chemoattraction.

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## Appendix 1:

"A neuromodulatory role for sonic hedgehog signalling via smoothened: Regulation of commissural axon guidance by inhibiting DCC recruitment to the plasma membrane"

**DEVELOPMENT**

# A neuromodulatory role for sonic hedgehog signalling via smoothened: Regulation of commissural axon guidance by inhibiting DCC recruitment to the plasma membrane

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**SUMMARY**

Extending axons utilize intermediate targets to divide longer trajectories into more manageable steps, but how growth cones modify their response to guidance cues at different points along their trajectory is not fully understood. Activation of protein kinase A (PKA) recruits the netrin-1 receptor deleted in colorectal cancer (DCC) to the growth cone plasma membrane, enhancing embryonic spinal commissural axon chemoattraction to netrin-1. Here, we show that signalling by the transmembrane protein Smoothened (Smo), a downstream effector of Sonic Hedgehog (Shh), inhibits PKA, thereby antagonizing netrin-1 dependent axon extension by blocking recruitment of DCC to the plasma membrane. Immunohistochemical analyzes of embryonic spinal cord and functional studies of axon extension provide evidence that gradients of netrin-1 and Shh overlap only partially and do not act in parallel to simultaneously attract commissural neurons, but rather function in series. Netrin-1 is essential for long-range axon extension from the dorsal to ventral spinal cord, while Shh, in relatively closer proximity to the floor plate, contributes to silencing the response to netrin-1 and engages a PKA-independent mechanism of chemoattraction. These findings identify a novel neuromodulatory role for Shh signalling through Smo to regulate axon guidance in response to netrin-1.

## INTRODUCTION

An axon seeking its ultimate target in the developing nervous system must correctly interpret a multitude of cues present in a complex environment. One strategy used by extending axons is to utilize intermediate targets to break up long complex trajectories into smaller, more manageable steps. To reach the floor plate, an intermediate target located at the ventral midline of the embryonic spinal cord, commissural axons are initially directed by roof plate derived chemorepellents, BMP7 and GDF7, members of the Bone Morphogenic family of proteins (Augsburger et al., 1999;Butler and Dodd, 2003). Complementing roof plate derived repulsion, netrin-1 and Shh secreted by the floor plate attract commissural axons (Kennedy et al., 1994;Serafini et al., 1994;Charron et al., 2003). Upon reaching the floor plate, commissural axons lose their response to netrin-1 (Shirasaki et al., 1998;Zou et al., 2000) and redirect their growth longitudinally. How growth cones modify their response to guidance cues at different points along their trajectory is not well understood.

Vesicular trafficking regulates the receptors presented by the growth cone plasma membrane (Kamiguchi and Lemmon, 2000;Keleman et al., 2002;Williams et al., 2003;Bouchard et al., 2004;Bartoe et al., 2006;Bouchard et al., 2008) raising the possibility that this mechanism may modulate the response of axonal growth cones as they extend toward a target. Activation of PKA promotes the recruitment of DCC from an intracellular vesicular pool to the plasma membrane of embryonic rat spinal commissural neuron growth cones and this increase in cell surface DCC promotes axon chemoattraction to netrin-1 (Bouchard et al., 2004;Moore and Kennedy, 2006). Netrin-1 does not elevate cAMP or activate PKA in these cells (Bouchard et al., 2004;Moore and

Kennedy, 2006); however these previous studies suggest that extracellular cues that regulate PKA activity will alter the response of commissural axons to netrin-1 by regulating the amount of DCC inserted into the plasma membrane. We therefore sought to identify endogenous factors in the embryonic spinal cord that could act via this mechanism to regulate commissural axon extension *in vivo*. PKA and Shh often act antagonistically (Jiang and Struhl, 1995;Pan and Rubin, 1995;Li et al., 1995;Noveen et al., 1996;Epstein et al., 1996;Masai et al., 2005). Indeed, genetic and biochemical findings provide strong evidence that the Shh effector Smo signals through $G\alpha_i$ to reduce the intracellular concentration of cAMP and thereby inhibit PKA (DeCamp et al., 2000;Riobo et al., 2006;Ogden et al., 2008). Consistent with this signalling mechanism, Shh inhibits retinal ganglion cell axon extension during embryogenesis in chick by reducing the concentration of cAMP in these cells (Trousse et al., 2001). We therefore hypothesized that Shh secreted by the floor plate might regulate PKA in commissural neurons. Our findings indicate that the transmembrane protein Smo, a downstream effector of Shh signalling, inhibits PKA in embryonic spinal commissural neurons. Furthermore, we provide evidence that Smo inhibition of PKA reduces the amount of DCC presented by commissural growth cones, downregulating the response to netrin-1 as they approach the floor plate at the ventral midline of the embryonic spinal cord.

## MATERIALS and METHODS

### Reagents

The following primary antibodies were used in this study: polyclonal anti-trkB (provided by Dr. Louis Reichardt, UCSF, San Francisco, CA); polyclonal anti-TAG-1 (TG3) for western blot analysis (provided by Dr. Thomas Jessell, Columbia University, New York, NY); monoclonal anti-TAG-1 (4D7) for immunochemistry, and monoclonal anti-Shh clone 5E1 (Ericson et al., 1996) from the Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA); polyclonal anti-NCAM (AB5032) and anti-cAMP (AB306) from Chemicon (Temecula, CA); monoclonal anti–$DCC_{EX}$ (G92-13), and anti-$DCC_{IN}$ (G97-449) from PharMingen (Mississauga, Canada); anti-$DCC_{FB}$ (AF5), KT5720, SQ22536, TeTx, and SANT-1 from Calbiochem (LaJolla, CA); anti-phospho cAMP response element-binding protein (CREB) (Ser133, 1B6, P-CREB); anti-CREB (Cell Signaling Technology, Beverly, MA); rabbit polyclonal anti-netrin PN2 (Manitt et al., 2001;Kennedy et al., 2006); and monoclonal anti-β-tubulin from Research Diagnosics Inc, NJ. Forskolin (FSK) and poly-D-lysine (PDL) were obtained from Sigma-Aldrich, (Oakville, Canada); cyclopamine (CPA) from Toronto Research Chemicals (Toronto, Canada); Shh-N from R & D Systems (Minneapolis, MN); Minimum Essential Medium (SMEM) from BioWhittaker (Walkersville, MD); Neurobasal media and B27 supplement from Gibco Life Technologies (Burlington, Canada); and Glutamax, Heat Inactivated Fetal Bovine Serum (IFBS), and Penstrep from Bio Media (Boussens, France). Recombinant netrin-1 protein was purified from a HEK293T cell line secreting netrin-1 as previously described (Serafini et al., 1994).

**Commissural neuron culture**

Staged pregnant Sprague-Dawley rats were obtained from Charles River (St-Constant, Canada). The dorsal half of embryonic day (E) 13 rat spinal cords were isolated by microdissection, dissociated to produce a suspension of single cells, and cultured as described (Bouchard et al., 2004;Shekarabi et al., 2005). For analysis of growth cones, dissociated cells were plated and cultured for 2 days (~25,000 cells/well) in 24-well plates (Sarstedt, Montreal, Canada) on PDL coated (70-150 kD, 20 µg/ml) 12 mm round glass coverslips (No.0 Deckgläser, Carolina Biological, NC), as described (Bouchard et al., 2004). Two hundred nM KT5720, 1.6 nM TeTx, 2 µg/ml Shh-N or their respective vehicles were added to medium 15 min before the addition of netrin-1. Fifteen minutes later, the medium was supplemented with either 2.5 µM CPA, 300 nM SANT-1, 10 µM FSK, or vehicle.

For western blot analyses (Harlow and Lane, 1988), cells were plated and cultured as described above, for 6 days, at a density of ~250,000 cells per 35 mm PDL-coated tissue culture dish. Following treatments, cells were washed once with ice-cold PBS (pH 7.4) and lysed with Laemmli sample buffer or radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 20 mM Tris, pH 8.0, 1%, NP-40 (USB Corp., Cleveland, OH, USA), 0.5% Sodium deoxycholate, 0.1% SDS, 1 mM EDTA). Protein content was quantified using BCA (Pierce, Rockford, IL). Results were visualized using chemiluminescence (PerkinElmer Life and Analytical Sciences, Woodridge, Canada) and quantification was performed on scanned images of immunoblots (ScanJet 5300C, Hewlett Packard, Mississauga, Canada) using NIH Image software (United States National Institutes of Health, Bethesda, MD).

**Immunocytochemistry and immunohistochemistry**

Cultures were washed with ice-cold phosphate buffered saline (PBS, pH 7.4), fixed with ice-cold 4% paraformaldehyde (PFA) in PBS, pH 7.4, and blocked with 2% goat serum, 2% bovine serum albumine (BSA) in PBS, pH 7.4 for 2 hrs at room temperature. Cells were permeabilized using 0.1 % Tween20 in PBS (PBST) instead of PBS alone. Antibodies were used in blocking solution overnight at 4°C at the following dilutions: anti-$DCC_{IN}$ 1:500; anti-$DCC_{EX}$ 1:500; anti-TAG-1 1:500; anti-$trkB_{ECD}$ 1:500; anti-cAMP, 1:1000. The binding specificities of anti-$DCC_{IN}$ and anti-$DCC_{EX}$ have been characterized (Reale et al., 1994;Shibata et al., 1996;Meyerhardt et al., 1999). Cultures were subsequently washed with PBS (non-permeabilized cells) or PBST (permeabilized cells) and labelled with Alexa 546 or Alexa 488 secondary antibodies (Molecular Probes, Eugene, OR). Nuclei were stained with Hoechst 33258 (Sigma-Aldrich, Oakville, Canada). For netrin-1 immunohistochemistry using the PN2 antibody, E11 rat embryos were fixed in Carnoy's fixative, embedded in paraffin and 8 μm sections processed as described (Kennedy et al., 2006). E11 rat embryos were also fixed in ice cold 4% paraformaldehyde, 15 μm sections were cut with a cryostat and the distribution of Shh immunoreactivity was visualized using monoclonal antibody 5E1 (Ericson et al., 1996).

**Quantification of surface receptor density or cAMP immunoreactivity using fluorescence**

All micrographs used for quantification were taken using the same Axiovert microscope (Carl Zeiss Canada, Kirland, Canada), 100X objective lens, and exposure

time in order to facilitate comparison of measurements. Fluorescence was quantified using Northern Eclipse image analysis software (Empix Imaging Inc, Mississauga, Canada) by an observer blind to the experimental conditions. For image analysis of growth cones, both differential interference contrast (DIC) and fluorescent images were taken. Fluorescence intensity per $\mu m^2$ of the growth cone was quantified and expressed as mean ± SEM. Statistical significance was evaluated by a one-way analysis of variance with a Sheffe's *post hoc* test (Systat, Chicago, IL).

**Surface biotinylation**

E13 rat dorsal spinal cords were dissociated and commissural neurons were plated and cultured for 6 days at a density of ~2,000,000 cells per 100 mm PDL-coated tissue culture dish. On day 6, cells were treated with 1 mM SQ22536, 200 nM KT5720, 1.6 nM Tetanus Toxin, 2 µg/ml Shh-N or vehicles for 15 min, followed by addition of 50 ng/ml netrin-1 or vehicle to the culture media for 15 min. Neurons were then exposed for 15 min to either 2.5 µM CPA, 300 nM SANT-1, or 10 µM FSK. Cells were then washed with ice-cold PBS containing 0.1 mM calcium chloride and 1 mM magnesium chloride pH 7.4, to halt protein trafficking (Meyer-Franke et al., 1998). Surface biotinylation was performed by adding EZ-Link Sulfo-NHS-LC-biotin (Pierce, Rockford, IL), 5 ml per plate at 0.5 mg/ml in PBS at 4°C for 30 min (Lisanti et al., 1989), removed, and the reaction was quenched by the addition of 5 ml of 10 mM ice-cold glycine in PBS at 4°C for two 10 min periods. Subsequently, cells were washed twice with 5 ml ice-cold PBS and lysed with RIPA buffer. Biotinylated proteins were precipitated with streptavidin-agarose (Pierce, Rockford, IL) and analyzed by western blot.

**Embryonic spinal cord explant culture**

Dorsal spinal cord explants were dissected from E13 rat embryos (Serafini et al., 1994) and cultured for 16 hrs in three-dimensional collagen gels (Tessier-Lavigne et al., 1988) at 37$^{o}$C in Neurobasal, 10% IFBS, 2 mM Glutamine, 1 unit/ml penicillin, and 1 µg/ml streptomycin. Either 1 mM SQ22536, 200 nM KT5720, 1.6 nM TeTx, or 2 µg/ml Shh, 10 µg/ml anti-$DCC_{FB}$, or vehicle were added to medium 15 min before the addition of netrin-1. Following 15 min of treatment, the medium was supplemented either with 10 µM FSK, or 2.5 µM CPA, 300 nM SANT-1, or vehicle. All drugs were present throughout the experiment. Segments of E11 rat spinal cord were dissected as described (Placzek et al., 1990) embedded in collagen, and cultured for 36 hours in Neurobasal medium supplemented as descried above. For "spinal cord plus floor plate assays" E13 rat spinal cords were dissected in an open-book configuration and ~300 µm long segments isolated (Zou et al., 2000). Explants consisting of one half of the spinal cord with the floorplate attached were then embedded in collagen and cultured for 16 hrs at 37$^{o}$C in Neurobasal medium supplemented as described above. Explants were fixed in 4% PFA and immunostained for β-tubulin or TAG-1 as described (Kennedy et al., 1994).

Photomicrographs of embryonic spinal cord explants were taken with a Carl Zeiss Axiovert microscope, phase-contrast optics, a 20X objective lens, and a Magnafire CCD camera (Optronics, Goleta, CA), and were analysed using Northern Eclipse image analysis software (Empix Imaging, Mississauga, Canada). The total length of axon bundles, or the length and percentage of TAG-1 immunopositive axons that reach either the floorplate or β-tubulin post-crossing axons were measured and expressed as mean ±

SEM. Statistical significance of differences between means was evaluated by a one-way ANOVA with Sheffe post-hoc test (Systat, Chicago, Il).

## RESULTS

### Smo regulates cAMP concentration and PKA in commissural neurons

Activating PKA regulates DCC insertion into neuronal plasma membranes and this increased cell surface DCC promotes embryonic spinal commissural axon extension and chemoattraction in response to netrin-1 (Bouchard et al., 2004;Moore and Kennedy, 2006). We sought to identify cues that act via this mechanism to regulate commissural axon extension during embryogenesis. Studies in vertebrate and invertebrate organisms have provided evidence that PKA and Hedgehog family members antagonize each other's actions (Jiang and Struhl, 1995;Li et al., 1995;Noveen et al., 1996;Epstein et al., 1996;Trousse et al., 2001;Masai et al., 2005). We therefore tested the hypothesis that Shh, which is secreted by floor plate cells in the embryonic spinal cord (Ingham and McMahon, 2001), might attenuate commissural axon extension to netrin-1 by inhibiting PKA-dependent recruitment of DCC to the plasma membrane.

Using neuronal cultures highly enriched for embryonic rat spinal commissural neurons (Bouchard et al., 2004;Shekarabi et al., 2005), we determined if disrupting Shh signalling would influence PKA activity. These neuronal cultures are generated by dissociating microdissected E13 rat dorsal spinal cord (Moore and Kennedy, 2008). Over 90% of the cells obtained express Tag-1 and DCC, both markers of spinal commissural neurons (Dodd et al., 1988;Keino-Masu et al., 1996). In contrast, the motoneuron marker, choline acetyltransferase, ChAT, is not detected (Bouchard et al., 2004). As a positive control for PKA activation in these cells, western blot analysis indicated that forskolin (FSK, 10 μM), a direct activator of adenylate cyclase (Nairn et al., 1985), generated a robust increase in phosphorylation of the PKA substrate CREB, consistent with FSK

increasing the concentration of cAMP and activating PKA in these cells (Fig. 1A). Shh signals by binding to the transmembrane protein Patched (Ptc), relieving Ptc-dependent inhibition of the transmembrane protein Smo (Ingham and McMahon, 2001). We examined the effect of inactivating Shh signalling using two specific inhibitors of Smo: cyclopamine (CPA) (Incardona et al., 1998;Chen et al., 2002;Frank-Kamenetsky et al., 2002) and SANT-1 (Chen et al., 2002). Following 5 min application of 2.5 µM CPA or 300 nM SANT-1 to commissural neurons, we detected a significant increase in CREB phosphorylation, similar to that induced by FSK (Fig. 1A). The increased phosphorylation was blocked by SQ22536, a specific inhibitor of adenylate cyclase (Fabbri et al., 1991;Goldsmith and Abrams, 1991;Tamaoki et al., 1993), and KT5720, a specific inhibitor of PKA (Kase et al., 1987), indicating that the increase in CREB phosphorylation induced by CPA and SANT-1 requires active adenylate cyclase and PKA. In both cases, increased CREB phosphorylation was also blocked by an excess of Shh (2 µg/ml), consistent with the described competitive interaction between CPA and Shh (Frank-Kamenetsky et al., 2002). Five min application of Shh (2 µg/ml) alone to these cells did not alter CREB phosphorylation (Fig. 1A), suggesting that in the absence of Shh, sufficient Smo activity is present to inhibit adenylate cyclase in these cells under these conditions.

The western blot analysis illustrated in Figure 1A monitors global changes in cell homogenates and may not detect localized changes in PKA activation. To determine if Shh signalling might regulate the concentration of cAMP locally, we examined the relative levels of cAMP in commissural neuron growth cones immunocytochemically. As a positive control, FSK (10 µM) generated a robust increase in cAMP immunoreactivity

in growth cones (Fig. 1B, C). Consistent with the results of western blotting (Fig. 1A), CPA and SANT-1 produced significant increases in growth cone cAMP immunoreactivity, similar to that induced by FSK, whereas 2 µg/ml Shh alone did not (Fig. 1B, C). The increases in cAMP produced by CPA, SANT-1, or FSK, were all blocked by excess Shh (2µg/ml) (Fig. 1B, C). These findings are consistent with the conclusion that activation of Smo reduces the concentration of cAMP in growth cones, thereby negatively regulating PKA.

**Smo signalling regulates DCC presentation by growth cones**

We then determined if manipulating Shh signalling would regulate the amount of plasma membrane DCC in commissural neuron growth cones. To specifically visualize cell surface DCC, commissural neurons were labeled using a monoclonal antibody against an extracellular epitope of DCC (anti-$DCC_{EX}$) without permeabilizing the plasma membrane, as described (Bouchard et al., 2004). Commissural neurons cultured for 2 DIV were treated for 15 min with 2 µg/ml Shh, 2.5 µM CPA, 300 nM SANT-1, 50 ng/ml netrin-1, or 50 ng/ml netrin-1 in combination with 2 µg/ml Shh (Fig. 2A, B) and then immunostained with anti-$DCC_{EX}$.

We previously reported that application of netrin-1 alone to commissural neurons *in vitro* causes a modest, but significant increase in the level of cell surface DCC (Bouchard et al., 2004). Netrin-1 did not elevate the concentration of cAMP or activate PKA in these cells, nor did inhibiting PKA or exocytosis attenuate the relatively small increase in plasma membrane DCC induced by netrin-1 (Bouchard et al., 2004;Moore and Kennedy, 2006;Moore et al., 2008b). These findings suggested that netrin-1 is

required to stabilize DCC at the plasma membrane. Bouchard et al., (2004) also demonstrated that addition of netrin-1 and activation PKA generated a substantially larger increase in the amount of plasma membrane DCC than did application of netrin-1 alone. This PKA induced increase in cell surface DCC was also dependent on coincident addition of netrin-1, consistent with the hypothesis that netrin-1 retains DCC at the plasma membrane. Inhibiting adenylate cyclase, PKA, or exocytosis, but not protein synthesis, blocked the PKA induced increase in cell surface DCC. Bouchard et al., (2004). We therefore concluded that activating PKA mobilizes DCC to the plasma membrane from a pre-existing pool of intracellular vesicles.

Here, like the addition of FSK alone (Bouchard et al., 2004), application of Shh, CPA, or SANT-1, without netrin-1, evoked no change in DCC immunoreactivity on the surface of commissural neuron growth cones (Fig. 2A, B). Application of netrin-1 alone (15 min) evoked a modest increase in cell surface DCC immunoreactivity that was not blocked by Shh (Fig. 2A, B). Treatment with 50 ng/ml netrin-1 in combination with 2.5 μM CPA, 300 nM SANT-1, or 10 μM FSK for 15 min significantly increased cell surface DCC immunoreactivity (Fig. 2A, B), but did not alter cell surface immunoreactivity for TAG-1 (Fig. 2C).

To investigate the signal transduction mechanism regulating the increase in cell surface DCC caused by inhibiting Smo signalling, commissural neurons were exposed to different enzyme inhibitors 15 min before the addition of netrin-1, thus 30 min before the addition of CPA, SANT-1, or FSK to the media. Inhibition of adenylate cyclase using 1 mM SQ22536 blocked the increase in DCC surface immunoreactivity (Fig. 2A, B), indicating that the effect of CPA and SANT-1 required active adenylate cyclase. Pre-

treatment with 200 nM KT5720 to inhibit PKA also blocked the CPA or SANT-1 induced increase in DCC surface immunoreactivity, consistent with the increased cAMP produced by the adenylate cyclase acting via PKA (Fig. 2A, B). These findings indicate that PKA activation is required for the increase in cell surface DCC induced by inhibiting Smo signalling in the presence of netrin-1.

We then tested the hypothesis that recruitment from an intracellular store might contribute to the increase in plasma membrane DCC using tetanus toxin (TeTx), an inhibitor of exocytosis that acts by cleaving the v-SNAREs VAMP1 and VAMP2 (Schiavo et al., 1992). TeTx (1.6 nM) blocked the CPA and SANT-1-induced increase in cell surface DCC (Fig. 2A, B), consistent with this increase requiring exocytosis.

To test the specificity of action of CPA and SANT-1 on Smo signalling, Shh (2 µg/ml) was added to cultures pretreated with 2.5 µM CPA or 300 nM SANT-1 in the presence of 50 ng/ml recombinant netrin-1. Shh abolished the CPA and SANT-1-induced increase in DCC surface immunoreactivity, consistent with the described competitive interaction between Shh, CPA and SANT-1 (Chen et al., 2002;Frank-Kamenetsky et al., 2002), and with the effect of CPA and SANT-1 on DCC translocation resulting from the inhibition of signalling components downstream of Shh. These findings support the conclusion that inhibiting Smo elevates the intra-neuronal concentration of cAMP, activates PKA, and triggers translocation of DCC to the plasma membrane of neuronal growth cones.

**Inhibiting Smo produces a netrin-1-dependent increase in cell surface DCC via PKA-dependent vesicle recruitment**

The immunocytochemical detection of increased cell surface DCC described above could be the result of either a selective increase in the amount of plasma membrane DCC protein, or alternatively, clustering of plasma membrane DCC that is present diffusely before treatment. To differentiate between these possibilities, cell surface proteins were biotinylated and the relative amount of DCC on the neuronal surface quantified in each of the different conditions. We also used this method to determine if PKA induced DCC recruitment might be influenced by the maturation of these neurons in culture. Cell surface biotinylation was therefore examined in cultures maintained for 2 DIV (Fig. 3A, B) and also 6 DIV (Fig. 3C, D). Cells were then treated for 15 min with SQ22536, KT5720, tetanus toxin, or Shh. Netrin-1 (50 ng/ml) was then added to the culture media for 15 min. Following this, cultures were exposed to 10 μM FSK, 2.5 μM CPA or 300 nM SANT-1 for 15 min. Cell surface proteins were biotinylated, isolated using streptavidin-agarose beads, and examined by western blot analysis using an antibody against an intracellular epitope of DCC (anti-$DCC_{IN}$), anti-TAG-1, anti-NCAM, and anti-$trkB_{ECD}$. Analysis of biotinylated proteins indicated that netrin-1 in combination with either CPA or SANT-1 produced an approximately ten-fold increase in the amount of cell surface DCC compared to control (Fig. 3C, D). Pretreating commissural neurons at either 2 DIV or 6 DIV with SQ22536, KT5720, or TeTx prior to application of netrin-1 plus either CPA or SANT-1 significantly reduced the level of cell surface DCC, when compared to netrin-1 plus either CPA or SANT-1 (Fig. 3). Addition of 2 μg/ml Shh blocked the increase in cell surface DCC induced by CPA or SANT-1 in the presence of netrin-1. Amounts of plasma membrane TAG-1, trkB, or NCAM were not significantly altered by CPA or SANT-1 (Fig. 3). These findings support the conclusion that inhibiting

Smo activates PKA to recruit DCC to the neuronal plasma membrane. Furthermore, these results indicate that this response persists in the neurons during maturation of these cultures until at least 6 DIV.

**Inhibiting Smoothened signalling enhances netrin-1 induced commissural axon outgrowth**

Netrin-1 promotes the outgrowth of commissural axons from explants of embryonic dorsal spinal cord cultured in a three-dimensional collagen gel (Kennedy et al., 1994;Serafini et al., 1994). Using this axon outgrowth assay, we tested the hypothesis that inhibiting Smo signalling would activate PKA, recruit DCC to the plasma membrane of axonal growth cones, and promote commissural axon outgrowth in response to netrin-1. Explants of embryonic day 13 (E13) rat dorsal spinal cord were cultured in the presence of 2 µg/ml Shh alone, 2.5 µM CPA alone, 300 nM SANT-1 alone, 2.5 µM CPA plus 50 ng/ml netrin-1, or 300 nM SANT-1 plus 50 ng/ml netrin-1. At 50 ng/ml, netrin-1 evokes ~30% of maximal commissural axon outgrowth. We have previously demonstrated that this sub-maximal level of netrin-1 is ideal to best reveal the increase in outgrowth evoked by activating PKA in these cells (Bouchard et al., 2004). Following 16 hrs in culture, Shh, CPA, or SANT-1 alone did not affect axon outgrowth (Fig. 4A, B). In contrast, netrin-1 plus either CPA, SANT-1, or FSK (Fig. 4A, B) produced a dramatic increase in axon outgrowth compared with explants exposed to netrin-1 alone (Fig. 4A, B). In all cases, extending axons expressed TAG-1 (not shown), a marker for commissural axons (Dodd et al., 1988).

To determine if CPA and SANT-1 promoted axon outgrowth through adenylate cyclase and PKA, explants of dorsal spinal cord were exposed to different enzyme inhibitors 15 min before the addition of netrin-1, thus 30 min before the addition of CPA or SANT-1, and then cultured for 16 hrs. Application of either SQ22536 or KT5720 completely blocked the enhancement of axon outgrowth caused by inhibiting Smo, demonstrating that active adenylate cyclase and PKA are required (Fig. 4A, B). Treatment with 1.6 nM TeTx (16 hrs) (Fig. 4A, B) reduced the amount of axon outgrowth to the level found in the presence of netrin-1 alone, consistent with the hypothesis that increased axon outgrowth evoked by CPA and SANT-1 requires exocytosis. Importantly, TeTx does not non-specifically block axon outgrowth, as the neuronal v-SNAREs essential for axon outgrowth are insensitive to TeTx (Osen-Sand et al., 1996;Corset et al., 2000). Notably, the inhibitors KT5720, SQ22536, and TeTx, all reduced the netrin-1 dependent axon outgrowth produced by CPA and SANT-1 to the level evoked by 50 ng/ml netrin-1 alone, and not to the level of background found in the absence of netrin-1. This suggests that inhibiting Smo does not enhance DCC function or signal transduction, but rather that one pool of DCC protein is present on the surface of the cell in its baseline state, and that PKA activation induced by inhibiting Smo mobilizes additional DCC to the plasma membrane through a TeTx-sensitive mechanism.

**Shh inhibits DCC dependent axon extension to the ventral midline of the embryonic spinal cord**

Using explanted segments of embryonic spinal cord, we then tested the hypothesis that expression of Shh by floor plate cells in the embryonic spinal cord (Echelard et al.,

1993;Krauss et al., 1993) inhibits netrin-1 dependent commissural axon extension. Segments of E11 rat brachial spinal cords, ~3 somites long, were isolated and embedded in collagen (Fig. 5A, B) and the length of TAG-1 immunoreactive commissural axons quantified (Fig. 5C). Commissural axons in control explants followed their normal trajectory to the floor plate (Fig. 5A). Addition of exogenous Shh (2µg/ml) significantly reduced axon extension below the level of control, to an extent similar to that produced by the disruption of DCC with a monoclonal function-blocking antibody (10 µg/ml anti-$DCC_{FB}$). Inhibiting Smo with CPA or SANT-1 significantly increased the average axon length and the percentage of axons that reach the floor plate (Fig. 5A, C, D). Inhibiting PKA activation by pre-treating the explants with KT5720 blocked the facilitation of axon extension induced by CPA and SANT-1. Furthermore, inhibiting VAMP1/2 dependent exocytosis with TeTx, or application of 10 µg/ml anti-$DCC_{FB}$ for 15 min before the addition of CPA or SANT-1, also blocked the CPA and SANT-1 induced increase in axon extension (Fig 5A, C, D). Importantly, it has been demonstrated that similar manipulations of Smo signalling does not repattern explants of E11 rat spinal cord (Charron et al., 2003). These findings support the conclusion that Smo inhibits DCC dependent commissural axon extension to the ventral midline of the embryonic spinal cord by inhibiting PKA dependent recruitment of DCC to the neuronal plasma membrane. They also provide functional evidence that DCC is required for long-range guidance to the ventral midline, consistent with the phenotype found in *dcc* null mice (Fazeli et al., 1997). In contrast, following disruption of Shh signalling through Smo, commissural axons still reach the ventral midline, and in fact extend more rapidly. This finding is consistent with previous reports that following genetic deletion of Smo

(Charron et al., 2003) or the Shh receptor Boc (Okada et al., 2006) from commissural neurons, commissural axons still reach the floor plate and cross the ventral midline of the neural tube.

**Smo contributes to silencing the response of commissural axons to netrin-1 after crossing the floor plate**

Embryonic spinal commissural axons lose sensitivity to midline attractants, such as netrin-1, once they have crossed the floor plate (Shirasaki et al., 1998;Zou et al., 2000). To determine if Shh might contribute to silencing the response of commissural axons to netrin-1 as they approach and cross the midline we used an *in vitro* explant assay referred to as "spinal cord plus floor plate" (Zou et al., 2000). E13 rat spinal cords were microdissected as an "open-book" (Fig. 6A) and then further microdissected as illustrated to generate explants composed of one side of the cord with the floor plate attached. Culturing these spinal cord plus floor plate explants overnight allows commissural axons to cross the floor plate and extend into the surrounding collagen matrix. TAG-1 expression is rapidly downregulated after spinal commissural neurons cross the floor plate (Dodd et al., 1988). Axons extending into the collagen were therefore labelled with an antibody against β-tubulin (Fig. 6B), as previously described (Zou et al., 2000). Consistent with previous findings (Zou et al., 2000), application of 200 ng/ml netrin-1 to the explants did not increase axon extension into the collagen after they had crossed the floor plate (Fig. 6B, C, D). Nor did inhibiting Smo by application of either CPA or SANT-1 effect post-crossing axon extension into collagen (Fig. 6B, C, D). In contrast, application of netrin-1 and either CPA or SANT-1 significantly increased post-crossing

axon extension (Fig. 6B, C, D). Furthermore, the increase in post-crossing axon extension was blocked by pre-treatment with 10 µg/ml anti-$DCC_{FB}$ (Fig. 6B, C, D). These results provide evidence that Smo signalling contributes to silencing the DCC dependent response to netrin-1 as commissural axons cross the ventral midline.

**Distribution of Shh and netrin-1 in the embryonic spinal cord**

We then examined the distributions of Shh and netrin-1 proteins in the E11 rat spinal cord. Immunohistochemical analysis revealed netrin-1 protein in the floor plate, distributed throughout the ventral neuroeptihelium, and detectable at gradually decreasing levels extending into the dorsal spinal cord (Fig. 7A), consistent with previous findings (Kennedy et al., 2006). Immunoreactivity for Shh was also readily detectable in the floor plate, but in contrast to the distribution of netrin-1, relatively low levels of signal were detected in ventral neuroepithelium, and only in close proximity to the floor plate (Fig. 7A). Both Shh and netrin-1 are secreted proteins highly expressed by floor plate cells, however, the different distributions detected support the hypothesis that they contribute to commissural axon guidance at different points along the trajectory taken by these axons to the ventral midline (Fig. 7B).

## DISCUSSION

Shh functions as a secreted morphogen (McMahon et al., 2003) and as a guidance cue that directs axon extension. Initial studies demonstrated that Shh inhibited retinal ganglion cell axon extension *in vitro*, and that ectopic expression of Shh misdirected these axons *in vivo* (Trousse et al., 2001). A gradient of Shh, secreted by the floor plate was then shown to function as a chemoattractant for embryonic spinal commissural axons (Charron et al., 2003). Furthermore, genetic deletion of either the Shh receptor BOC (Okada et al., 2006) or the downstream signalling component Smo (Charron et al., 2003) from commissural neurons produced inappropriate lateral displacement of these axons in the ventral spinal cord. However, in spite of this deficit, in both cases the axons successfully reach the floor plate and cross the ventral midline.

Here we identify a novel neuromodulatory role for Smo, a transmembrane protein that signals downstream of Shh, regulating receptor recruitment to the plasma membrane of neuronal growth cones. We have previously reported that PKA activation promotes DCC translocation from a pre-existing vesicular pool to the neuronal plasma membrane and that this increased cell surface DCC promotes chemoattraction to netrin-1 (Bouchard et al., 2004;Moore and Kennedy, 2006). Here, we sought to identify endogenous cues in the embryonic spinal cord that act via this mechanism to regulate commissural axon extension *in vivo*. We report that Smo inhibits PKA in spinal commissural neurons, reduces the level of DCC presented by growth cones, and thereby attenuates the response to netrin-1. These findings support the hypothesis that Shh signalling through Smo contributes to silencing the response to netrin-1 as commissural axons cross the ventral midline of the embryonic spinal cord.

In the embryonic spinal cord (Zou et al., 2000) and metencephalon (Shirasaki et al., 1998) commissural axons lose sensitivity to netrin-1 after crossing the midline. Interestingly, the axons of retinal ganglion cells are initially attracted by netrin-1 as they exit the retina, but later in their trajectory are repelled by netrin-1 as they approach their ultimate target, the optic tectum (Shewan et al., 2002). Increasing intracellular cAMP in these axons as they approach the optic tectum causes them to switch from repulsion to attraction to netrin-1, indicating that the switch from attraction to repulsion is not necessarily permanent. Together with the findings reported by Shewan., et al. (2002), our demonstration that post-crossing commissural axons extend in response to netrin-1 following either inhibition of Smo signalling or PKA activation (Fig 6), and the finding that PKA induced recruitment of DCC persists in cultured commissural neurons up to 6 DIV (Fig. 3), provide evidence that in these cases, the capacity to respond to netrin-1 as a chemoattractant has not been lost after crossing the midline, but can be recovered by activating PKA.

The concentration of cAMP within a neuron plays a key role regulating the directionality of axon extension in response to a number of different extracellular guidance cues, including netrin-1, BDNF, NGF, and MAG. High levels of cAMP are associated with chemoattraction and low levels with repulsion (Song and Poo, 1999;Moore and Kennedy, 2006). Although Shh functions as an attractant for commissural neurons as they approach the floor plate, activation of Smo will reduce the concentration of intra-neuronal cAMP. As a consequence of our findings, we hypothesize that Shh will exert its described chemoattractive effect on growth cones (Charron et al., 2003) through an intracellular mechanism different from the cAMP sensitive mechanism

that underlies the response to the guidance cues listed above. Based on this, we predict that the chemoattractant response of embryonic spinal commissural neurons to Shh via Boc (Okada et al., 2006) will function independently of PKA activation.

Hedgehog (Hh) proteins activate Smo through a derepression mechanism (Fig. 7C). In the absence of Hh, Smo signalling is inhibited by Ptc. Signalling is initiated when Hh binds to Ptc, relieving inhibition of Smo (McMahon et al., 2003). Ptc inhibition of Smo is non-stoichiometric, with a large molar excess of Smo being inhibited by Ptc (Taipale et al., 2002). Our findings are consistent with a level of Smo activity in cultured embryonic commissural neurons that is sufficient to constitutively inhibit PKA. We conclude this based on finding that application of Shh alone to either commissural neurons grown in dispersed cell culture or to explants of embryonic dorsal spinal cord produced no measurable change in cAMP concentration, CREB phosphorylation or axon outgrowth. In contrast, inhibiting Smo in commissural neurons with CPA or SANT-1 increased cAMP, CREB phosphorylation, and potentiated netrin-1 induced axon outgrowth to an extent similar to that evoked by FSK. It is unlikely that sufficient levels of Shh to activate Smo are present in these dispersed cell cultures, as the floor plate, the endogenous source of Shh in the spinal cord, has been removed. In contrast to the lack of an effect of application of Shh alone to the neurons in culture (Fig. 1 and 2) or to dorsal spinal cord explants (Fig. 4 and Charron et al., 2003), addition of Shh alone to explanted segments of E11 spinal cord, which included the floor plate, inhibited commissural axon extension (Fig. 5). Conversely, inhibiting Smo increased the rate of commissural axon extension to the floor plate. The enhanced axon outgrowth required adenylate cyclase and PKA, and was comparable to the response to FSK. Interestingly, Ptc expression is

upregulated by Shh (Goodrich et al., 1996), and the absence of Shh may downregulate Ptc in cultures of dispersed neurons and dorsal spinal cord explants, thereby reducing Ptc repression of Smo and resulting in constitutively active Smo in the absence of floor plate. Our findings are consistent with commissural neurons in culture expressing Ptc at some level, as application of Shh overcomes inhibition of Smo, consistent with the competitive interaction between Shh, CPA, and SANT-1 (Chen et al., 2002;Frank-Kamenetsky et al., 2002).

Smo is a member of the Frizzled family of seven-transmembrane proteins that include G-protein coupled receptors. This led to speculation that Shh signalling might involve G protein mediated mechanisms (Alcedo et al., 1996;van den Heuvel and Ingham, 1996). In particular, the functional antagonism between Hh signalling and PKA has been suggested to result from Hh activating $G\alpha_i$, an adenylate cyclase inhibitor, reducing the intracellular concentration of cAMP and inhibiting PKA (Gilman, 1987). Evidence consistent with this mechanism has been obtained from a number of vertebrate and invertebrate systems (Hammerschmidt et al., 1996;Concordet et al., 1996;Ungar and Moon, 1996;Hammerschmidt and McMahon, 1998). Notably, stimulation of $G\alpha_I$ like activity by Shh has been documented in *Xenopus* melanophores (DeCamp et al., 2000), and constitutively active Smo has been reported to directly activate multiple mammalian $G\alpha_i$ family members (Riobo et al., 2006). Genetic and biochemical studies carried out in *Drosophila* have provided strong evidence that $G\alpha_i$ functions downstream of Smo, and that Hh application effectively reduces the intracellular concentration of cAMP (Ogden et al., 2008). Interestingly, PKA inhibition, as would be produced by $G\alpha_i$ activation, promotes activation of RhoA (Lang et al., 1996). Conversely, netrin-1 signalling through

DCC inactivates RhoA in commissural commissural axons and such RhoA inhibition potentiates chemoattraction to netrin-1 by recruiting DCC to the plasma membrane (Moore et al., 2008a). These findings suggest that RhoA may be a key point of convergence between netrin-1 and Shh signalling.

Shh and netrin-1 are both floor plate derived chemoattractants that collaborate to guide commissural neurons to the ventral midline of the embryonic spinal cord (Kennedy et al., 1994;Serafini et al., 1996;Charron et al., 2003). They have been proposed to function in parallel as overlapping approximately equidistant gradients that direct commissural neurons to the floor plate (Charron et al., 2003). This model of chemoattractant gradients operating in parallel predicts that inhibition of either attractant will reduce axon attraction to the floor plate by an amount proportional to the effectiveness of that particular cue. Our findings, both functional and immunohistochemical, provide evidence that Shh and netrin-1 are not distributed as equidistant overlapping gradients that function in parallel, but rather function in series; netrin-1 acting as a long-range guidance cue that directs axons from the dorsal to the ventral spinal cord, and Shh at relatively short-range closer to the floor plate (Fig. 7B). Consistent with this, as the earliest commissural axons approach the floor plate, netrin-1 protein extends well into the dorsal spinal cord, while Shh immunoreactivity was detected relatively close to the floor plate (Fig. 7A). The findings presented here, and a previous study (Bouchard et al., 2004) indicate that antibody-mediated disruption of DCC in embryonic spinal cord causes axons to stall along their trajectory to the floor plate (Fig. 5) mimicking the phenotype found in DCC or netrin-1 knockout mice (Serafini et al., 1996;Fazeli et al., 1997). In contrast, in the absence of DCC or netrin-1 function *in*

*vivo* (Serafini et al., 1996;Fazeli et al., 1997), Shh is not sufficient to rescue commissural axon extension to the floor plate (Serafini et al., 1996;Fazeli et al., 1997). Furthermore, when Smo signalling was disrupted with CPA or SANT-1 (Fig. 5) commissural neurons not only reached the floor plate, but inhibiting Smo facilitated axon extension to the floor plate. Together, these results argue against a model in which the two gradients operate in parallel, but support the conclusion that Shh signalling through Smo contributes to silencing the response to netrin-1 as commissural axons approach the midline. These findings identify a novel neuromodulatory role for Shh signalling through Smo, regulating receptor recruitment to the cell surface, and identify a mechanism utilized by axons to change their response to guidance cues as they approach an intermediate target such as the floor plate in the embryonic spinal cord.

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## FIGURE LEGENDS

**Figure 1: Inhibiting Smo increases growth cone cAMP concentration and activates PKA.**

Embryonic spinal commissural neurons were cultured for 2 DIV (B) or 6 DIV (A) before pre-treatment for 15 min with 1 mM SQ22536, 200 nM KT5720, or 2 µg/ml Shh. Neurons were then exposed to either 10 µM FSK, 2.5 µM CPA or 300 nM SANT-1 for 5 min. Panel (A) illustrates the results of western blot analyses of total cell extracts, probed with antibodies against phospho-CREB (P-CREB) and total CREB (~43 kDa). In panel (B) cells were fixed, permeabilized, and immunostained with anti-cAMP (Alexa 546-conjugated secondary antibody, magnification 100X, scale bar = 10 µm). The histogram in (C) illustrates quantification of cAMP immunofluorescence intensities (mean ± SEM; panel, A n=4; panel C, n=17-28 per condition, respectively, for A and B; * indicates $p < 0.01$ vs. control, and # $p < 0.01$ vs. CPA or SANT-1 alone).

**Figure 2: Distribution of DCC in growth cones is regulated by Shh and Smo.**

Commissural neurons were cultured for 2 DIV prior to treatment for 15 min with or without 50 ng/ml netrin-1. CPA (2.5 µM), SANT-1 (300 nM), or FSK (10 µM) was then added for 15 min in combination with KT5720 (200 nM), TeTx (1.6 nM), or Shh (2 µg/ml). In (A) cells were fixed without permeabilizing, and immunostained with anti-$DCC_{EX}$ or anti-TAG-1. Plasma membrane immunoreactivity associated with growth cones was imaged using Alexa 546 or Alexa 488-conjugated secondary antibodies. Corresponding DIC images of are shown next to the florescence images. (100X objective, scale bar = 10 µm). Panels (B) and (C) illustrate quantification of the fluorescence intensities of DCC and TAG-1, respectively (mean ± SEM; n=11-23 per condition; * indicates $p < 0.01$ vs ctrl, CPA, or SANT-1 alone; # indicates $p < 0.01$ vs netrin-1 plus CPA, netrin-1 plus SANT-1, or netrin-1 plus FSK).

**Figure 3: Inhibiting Smo signalling induces a netrin-1-dependent increase in cell surface DCC via a PKA dependent exocytotic mechanism.**

Commissural neurons were cultured for either 2 DIV (A, B) or 6 DIV (C, D) prior to treatment for 15 min with 50 ng/ml netrin-1. CPA (2.5 µM), SANT-1 (300 nM), or FSK (10 µM) was then added for 15 min in combination with 1 mM SQ22536, 200 nM KT5720, 1.6 nM TeTx, or 2 µg/ml Shh. (A, C) illustrate western blot analyses of biotinylated cell surface proteins isolated using streptavidin-agarose beads. Total cell lysates and isolated biotinylated proteins were analysed by western blot using antibodies against DCC (~180kDa), TAG-1 (~135 kDa), trkB (~145 kDa), or NCAM (~200 kDa). Panels (B and D) illustrate quantification of the optical density of biotinylated DCC shown in panels (A and C) (mean ± SEM, n = 4, * indicates $p < 0.01$ vs. 50 ng/ml netrin-1; # indicates $p < 0.01$ vs. 50 ng/ml netrin-1 plus 2.5 µM CPA or 50 ng/ml netrin-1 plus 300 nM SANT-1).

**Figure 4: Inhibiting Smo signalling enhances netrin-1 induced commissural axon outgrowth.**

(A) Commissural axon outgrowth was assayed for explants of dorsal E13 rat spinal cord embedded in collagen in the following conditions: control; 2 µg/ml Shh; 2.5 µM CPA; 300 nM SANT-1; 50 ng/ml netrin-1; 10 µM FSK plus 50 ng/ml netrin-1; 2.5 µM CPA plus 50 ng/ml netrin-1; 1 mM SQ 22536 plus 2.5 µM CPA plus 50 ng/ml netrin; 200 nM KT 5720 plus 2.5 µM CPA plus 50 ng/ml netrin-1; 1.6 nM TeTx plus 2.5 µM CPA plus 50 ng/ml netrin-1; 10 µg/ml anti-$DCC_{FB}$ plus 2.5 µM CPA plus 50 ng/ml netrin-1; 300 nM SANT-1 plus 50 ng/ml netrin-1; 1 mM SQ 22536 plus 300 nM SANT-1 plus 50 ng/ml netrin-1; 200 nM KT 5720 plus 300 nM SANT-1 plus 50 ng/ml netrin-1; 1.6 nM TeTx plus 300 nM SANT-1 plus 50 ng/ml netrin-1; and 10 µg/ml anti-$DCC_{FB}$ plus 300 nM SANT-1 plus 50 ng/ml netrin-1 (20X objective, phase contrast optics; scale bar = 100 µm). (B) Quantification of axon outgrowth illustrated in (A) (mean total axon bundle length per explant ± SEM for between 6-58 explants per condition; * indicates $p < 0.01$ vs control; # indicates $p < 0.01$ vs 50 ng/ml netrin-1 plus 2.5 µM CPA or 300 nM SANT-1).

**Figure 5: Smo signalling inhibits DCC dependent axon extension to the ventral midline of the embryonic spinal cord.**

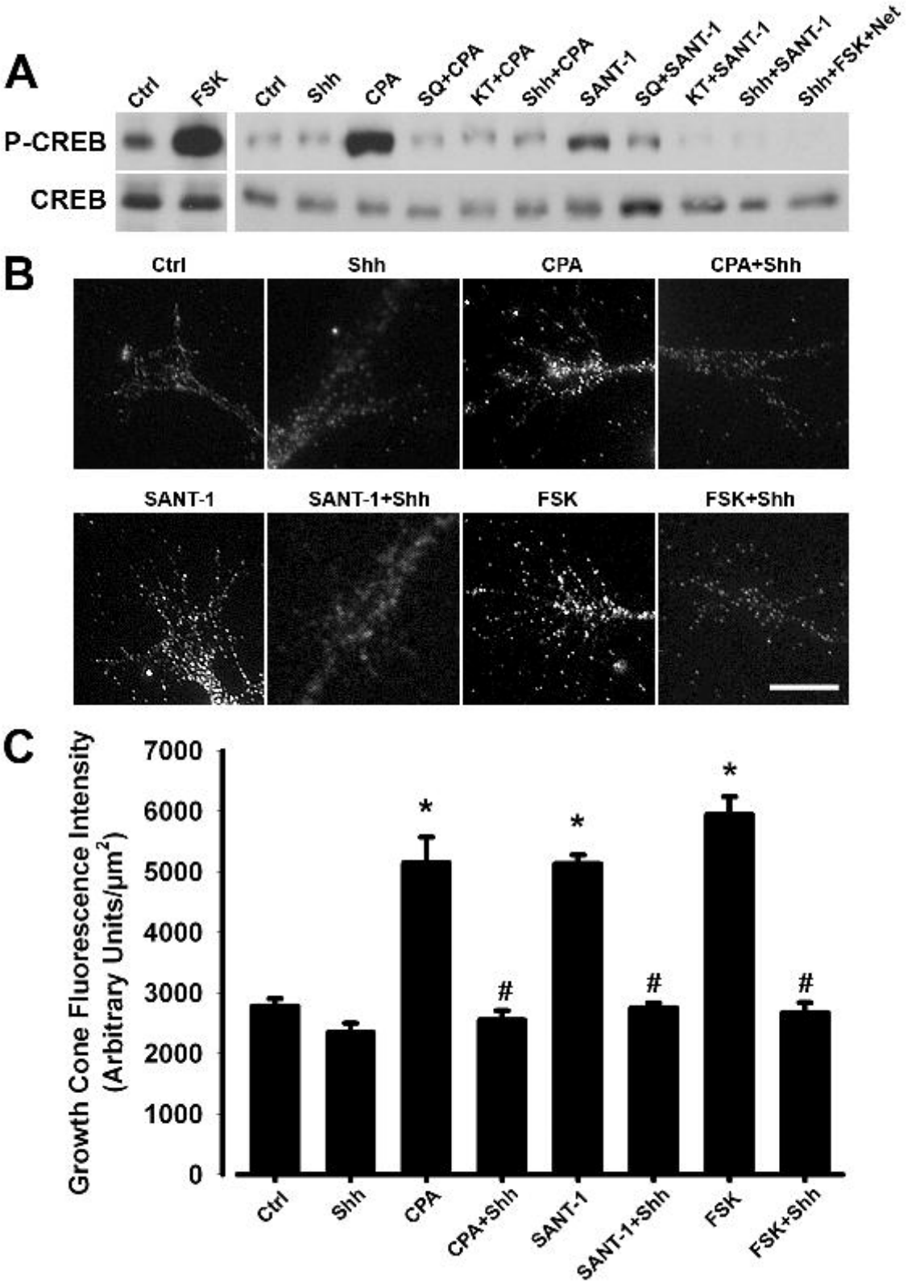
(A) Brachial segments of E11 rat dorsal spinal cords were embedded in collagen and cultured for 36 hrs in the following conditions: control; 2.5 µM CPA; 200 nM KT5720 plus 2.5 µM CPA; 1 nM TeTx plus 2.5 µM CPA; 10 µg/ml anti-$DCC_{FB}$ plus 2.5 µM CPA; 300 nM SANT-1; 200 nM KT5720 plus 300 nM SANT-1; 1 nM TeTx plus 300 nM SANT-1; 10 µg/ml anti-$DCC_{FB}$ plus 300 nM SANT-1 (TAG-1 immunoflorescence; B/W reversed image; 10X objective; scale bar = 100 µm). (B) Diagram of an E11 spinal cord explant illustrating commissural neuron cell bodies in the dorsal (D) spinal cord extending an axon ventrally (V) to the floor plate (FP; RP, roof plate; R, rostral; C, caudal). (C) Quantification of axon bundle length (mean ± SEM for 521-1186 axons per condition). (D) Quantification of the percentage of axons reaching the floorplate (mean ± SEM for 12-24 explants per condition; * indicates a p value < 0.01 vs. control; # indicates a p value <0.01 vs. 2.5 µM CPA or 300 nM SANT-1).

**Figure 6: Shh and Smo contribute to silencing the response of commissural axons to netrin-1 after crossing the floor plate.**

(A) Diagram depicting micro-dissection of spinal cord plus floor plate explants. E13 rat spinal cords were dissected as an "open-book". One side of the cord was removed and the remaining spinal cord plus floor plate explants were cultured in collagen overnight. (B) Immunofluorescence images of β-tubulin staining of spinal cord plus floor plate explants using a 20X objective to highlight axon outgrowth past the midline. The brightness of the images has been digitally inverted so that fluorescence is dark. (B) Representative pictures of control explants and explants treated with 100 ng/ml netrin-1, 600 nM SANT-1, 100 ng/ml netrin-1 plus 600 nM Sant-1, and 100 ng/ml netrin-1 plus 600 nM Sant-1 plus 10μg/ml DCC function blocking antibody. (C) and (D) illustrate quantification of axon outgrowth evoked by netrin-1 by axons that have crossed the floor plate (mean ± SEM for 30-60 explants per condition; * and # indicate respectively a p value $< 0.05$ vs. control or netrin-1 + CPA or SANT-1).

**Figure 7: Distributions of netrin-1 and Shh in E11 rat spinal cord suggest function in series directing commissural axons to floor plate.**

(A) Immunohistochemical distribution of Shh (left panel, PFA fixed tissue, cryostat section, monoclonal 5E1, immunoflorescence detection) and netrin-1 (right panel, Carnoy's fixative, paraffin section, anti-netrin PN2, BM purple detection) in the E11 rat spinal cord. (B) Model illustrating extending commissural axons first encountering a gradient of netrin protein, and subsequently a gradient of Shh closer to the floor plate. (C) Model illustrating the influence of Shh signalling on cell surface presentation of DCC. The dotted line indicates that extracellular Shh binding to Ptc leads to disinhibition of Smo. Active Smo inhibits adenylate cyclse (AC), inhibiting PKA dependent translocation of DCC to the plasma membrane. Conversely, in the absence of Shh, Ptc inhibits Smo, leading to AC activation, elevation of cAMP, and activation of PKA dependent translocation of DCC to the plasma membrane.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SANT-1
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Shh+FSK+Net
P-CREB
CREB
B
Ctrl
Shh
CPA
CPA+Shh
SANT-1
SANT-1+Shh
FSK
FSK+Shh
C
Growth Cone Fluorescence Intensity (Arbitrary Units/μm²)
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6000
5000
4000
3000
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1000
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CPA
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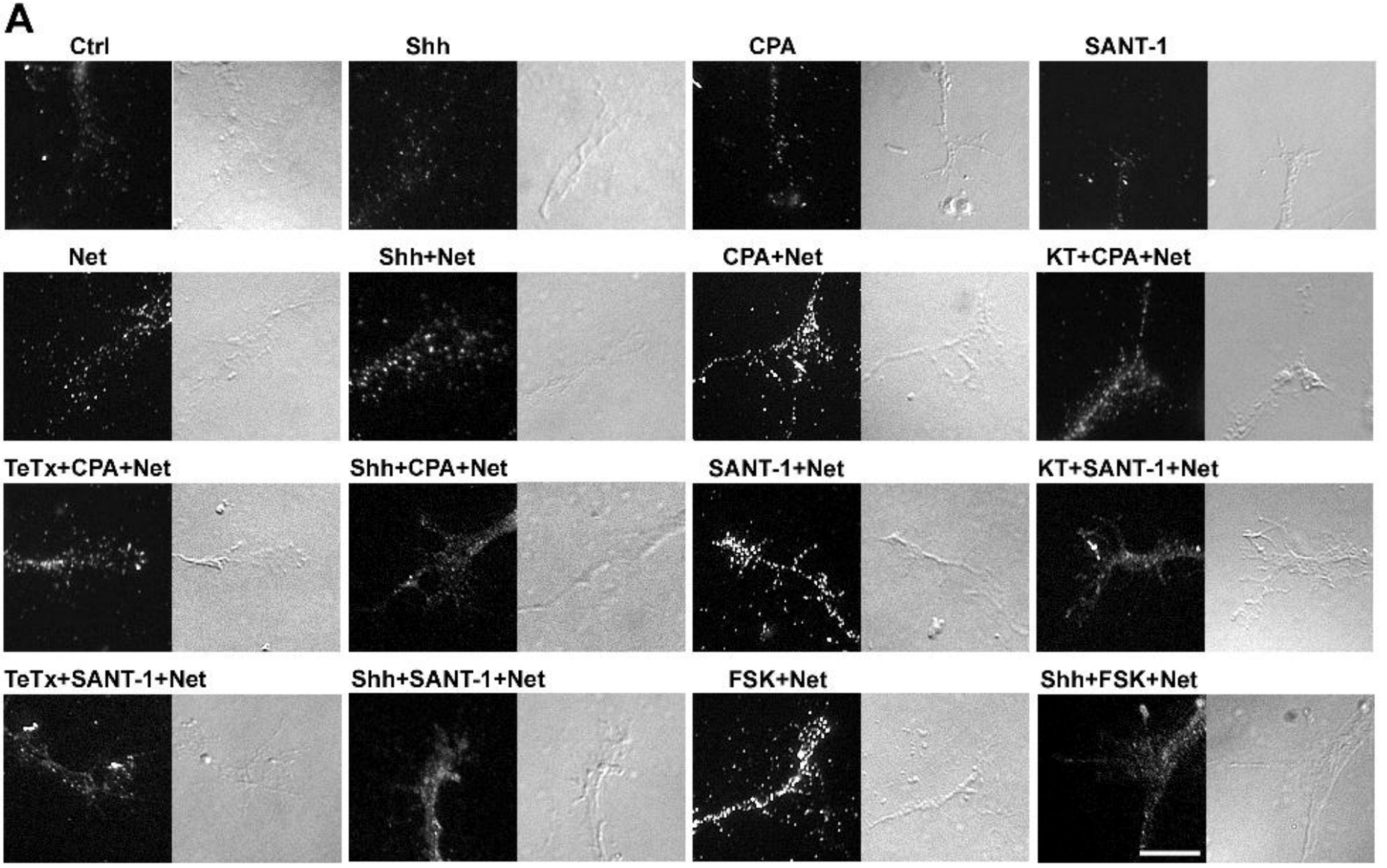
FSK
FSK+Shh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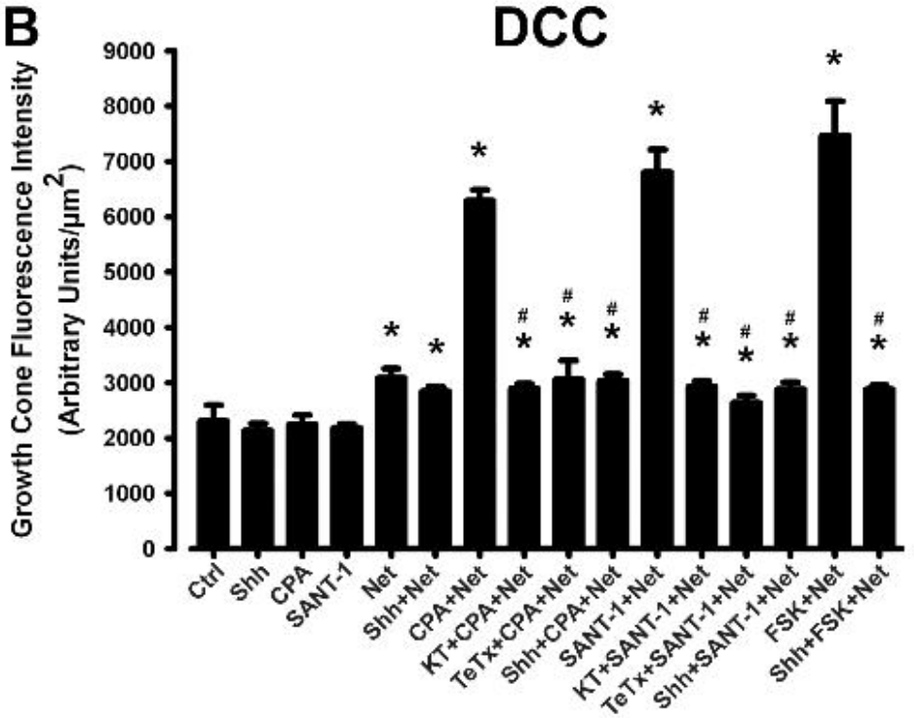
Shh+FSK+Net


B
DCC
Growth Cone Fluorescence Intensity (Arbitrary Units/μm²)
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
Ctrl
Shh
CPA
SANT-1
Net
Shh+Net
CPA+Net
KT+CPA+Net
TeTx+CPA+Net
Shh+CPA+Net
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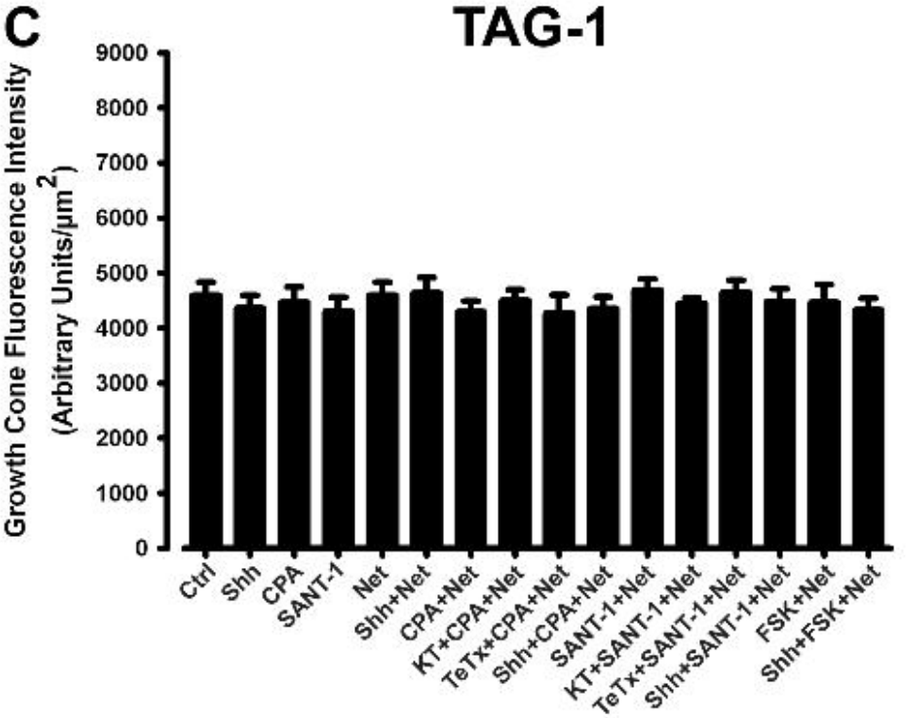
Shh+SANT-1+Net
FSK+Net
Shh+FSK+Net


C
TAG-1
Growth Cone Fluorescence Intensity (Arbitrary Units/μm²)
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6000
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4000
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2000
1000
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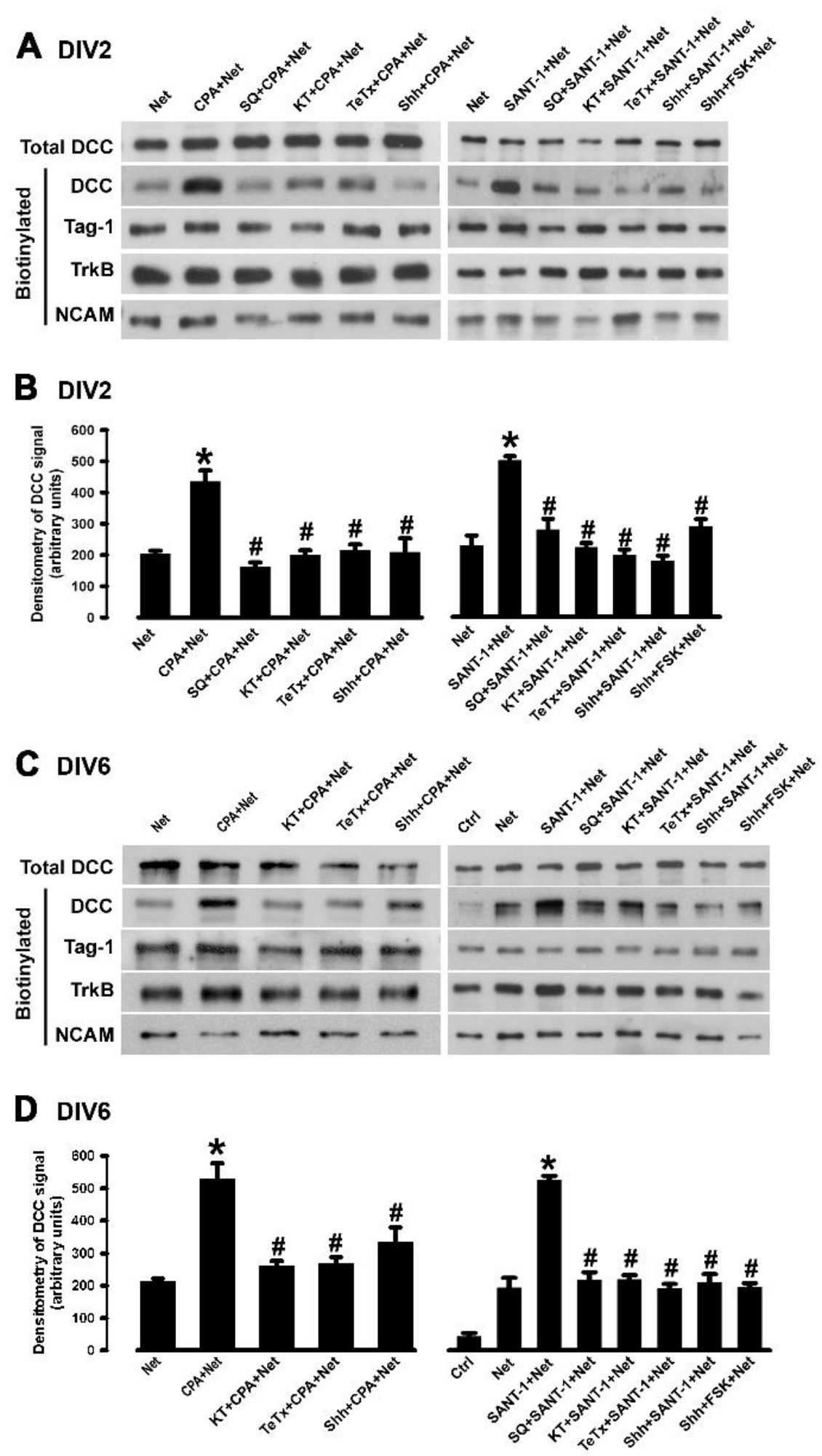
Ctrl
Shh
CPA
SANT-1
Net
Shh+Net
CPA+Net
KT+CPA+Net
TeTx+CPA+Net
Shh+CPA+Net
SANT-1+Net
KT+SANT-1+Net
TeTx+SANT-1+Net
Shh+SANT-1+Net
FSK+Net
Shh+FSK+Net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Net
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SQ+CPA+Net
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TeTx+CPA+Net
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KT+SANT-1+Net
TeTx+SANT-1+Net
Shh+SANT-1+Net
Shh+FSK+Net
Total DCC
Biotinylated
DCC
Tag-1
TrkB
NCAM
B DIV2
Densitometry of DCC signal (arbitrary units)
600
500
400
300
200
100
0
Net
CPA+Net
SQ+CPA+Net
KT+CPA+Net
TeTx+CPA+Net
Shh+CPA+Net
SANT-1+Net
SQ+SANT-1+Net
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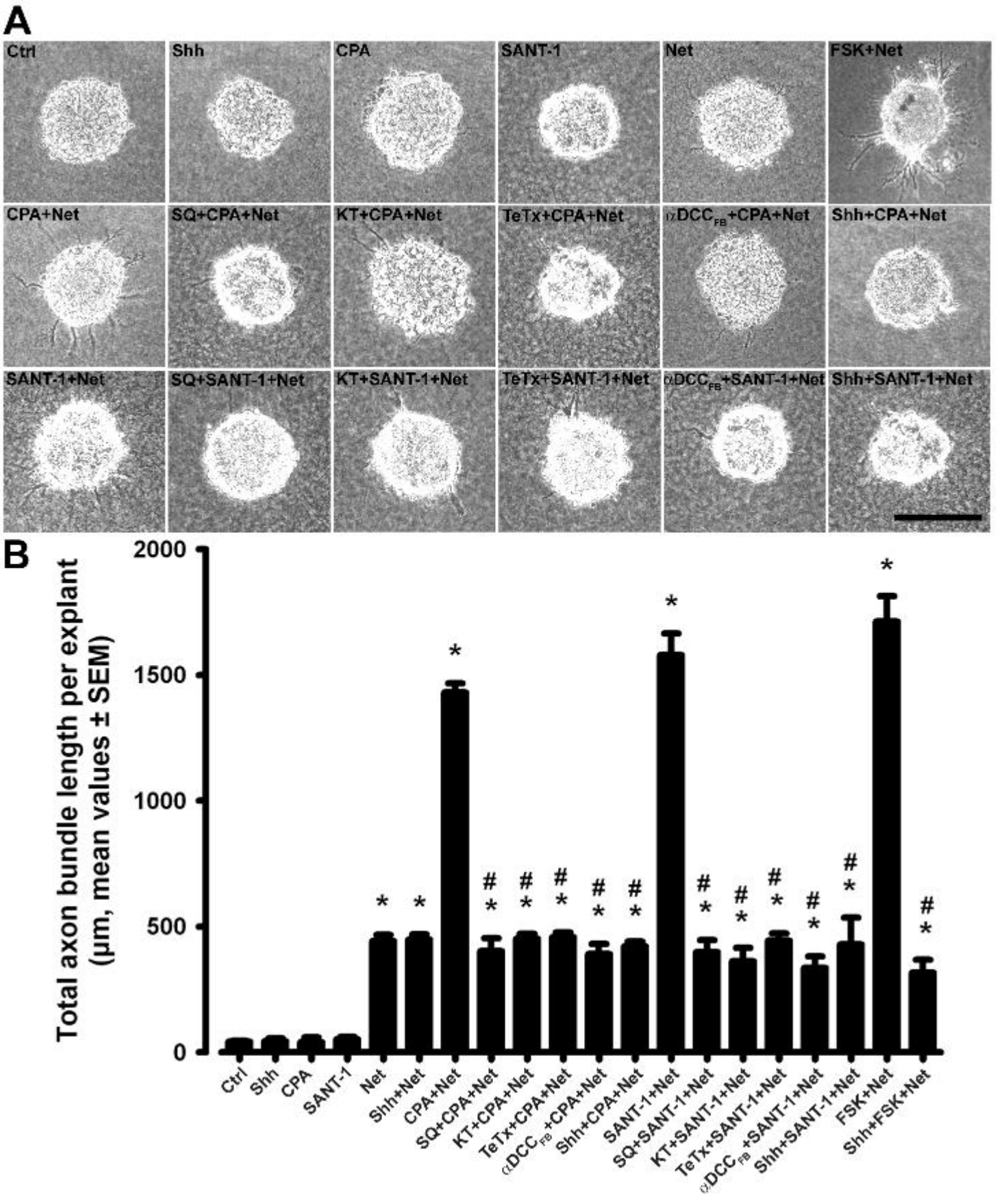
C DIV6
Ctrl
D DIV6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Ctrl
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CPA
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CPA+Net
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KT+CPA+Net
TeTx+CPA+Net
αDCC_FB+CPA+Net
Shh+CPA+Net
SANT-1+Net
SQ+SANT-1+Net
KT+SANT-1+Net
TeTx+SANT-1+Net
αDCC_FB+SANT-1+Net
Shh+SANT-1+Net
B
Total axon bundle length per explant
(µm, mean values ± SEM)
2000
1500
1000
500
0
Ctrl
Shh
CPA
SANT-1
Net
Shh+Net
CPA+Net
SQ+CPA+Net
KT+CPA+Net
TeTx+CPA+Net
αDCC_FB+CPA+Net
Shh+CPA+Net
SANT-1+Net
SQ+SANT-1+Net
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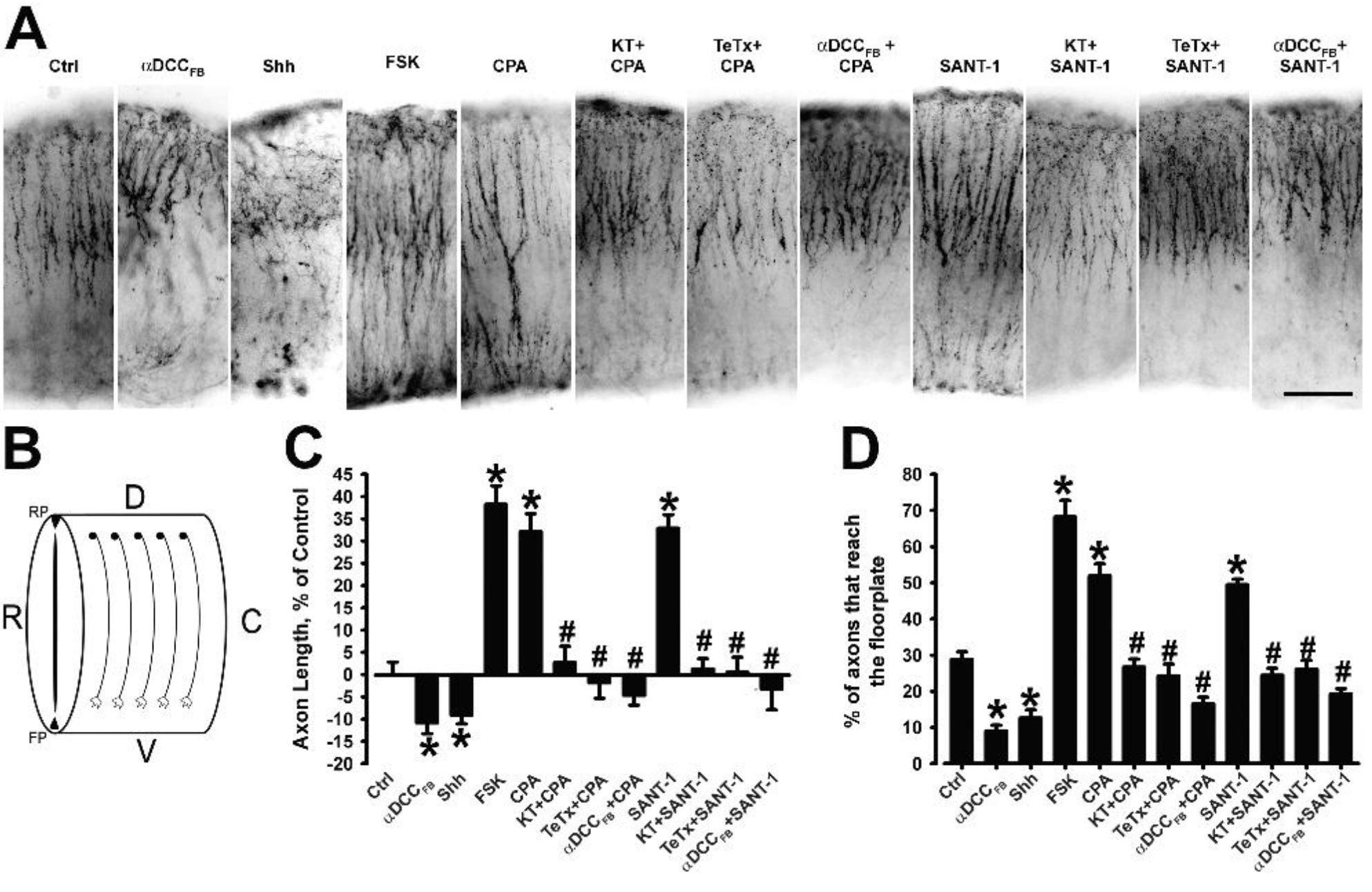
Shh+SANT-1+Net
FSK+Net
Shh+FSK+Net


A
Ctrl
αDCC$_{FB}$
Shh
FSK
CPA
KT+ CPA
TeTx+ CPA
αDCC$_{FB}$ + CPA
SANT-1
KT+ SANT-1
TeTx+ SANT-1
αDCC$_{FB}$+ SANT-1
B
D
RP
R
C
FP
V
C
Axon Length, % of Control
45
40
35
30
25
20
15
10
5
0
-5
-10
-15
-20
Ctrl
αDCC$_{FB}$
Shh
FSK
CPA
KT+CPA
TeTx+CPA
αDCC$_{FB}$ +CPA
SANT-1
KT+SANT-1
TeTx+SANT-1
αDCC$_{FB}$ +SANT-1
D
% of axons that reach the floorplate
80
70
60
50
40
30
20
10
0
Ctrl
αDCC$_{FB}$
Shh
FSK
CPA
KT+CPA
TeTx+CPA
αDCC$_{FB}$ +CPA
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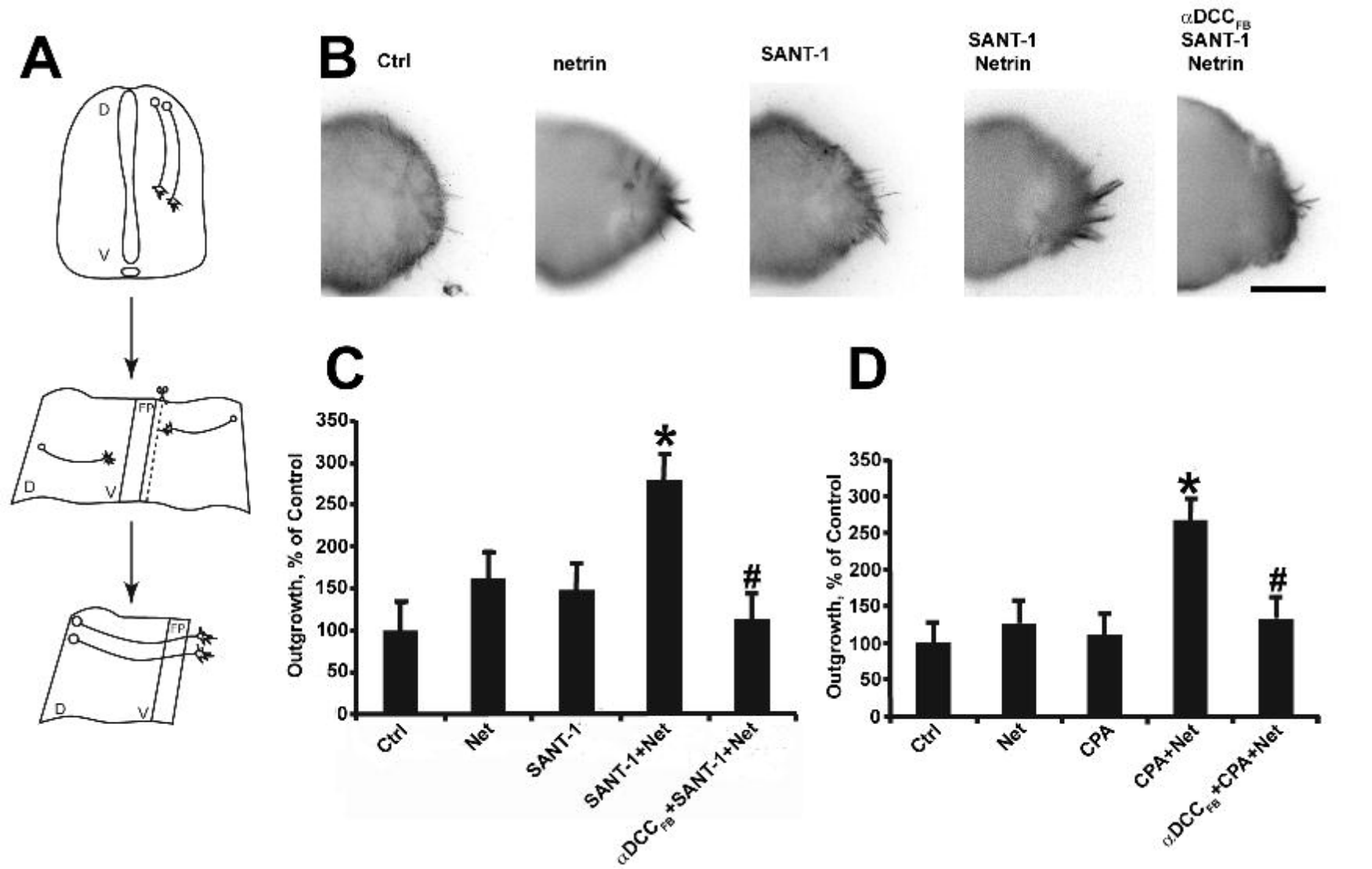
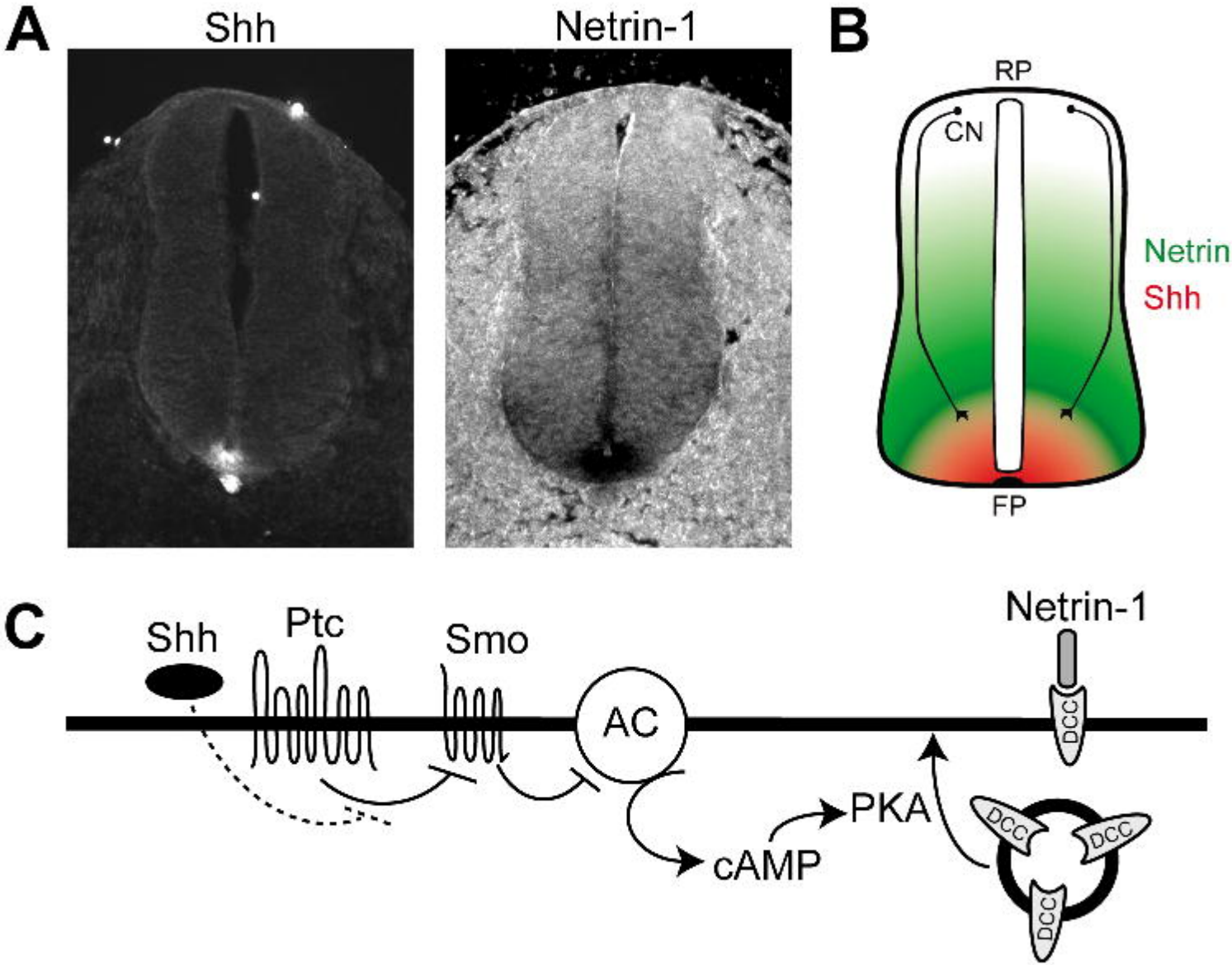
KT+SANT-1
TeTx+SANT-1
αDCC$_{FB}$ +SANT-1


A
D
V
FP
B
Ctrl
netrin
SANT-1
SANT-1
Netrin
αDCC FB
SANT-1
Netrin
C
Outgrowth, % of Control
350
300
250
200
150
100
50
0
*
#
Ctrl
Net
SANT-1
SANT-1+Net
αDCC FB +SANT-1+Net
D
Outgrowth, % of Control
350
300
250
200
150
100
50
0
*
#
Ctrl
Net
CPA
CPA+Net
αDCC FB +CPA+Net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Shh
Netrin-1
B
RP
CN
Netrin
Shh
FP
C
Shh
Ptc
Smo
AC
Netrin-1
DCC
PKA
cAMP
DCC
DCC
DCC

## Appendix 2:

"Rb/E2F regulates expression of neogenin during neuronal migration."

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# Rb/E2F Regulates Expression of Neogenin during Neuronal Migration▽

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**The Rb/E2F pathway has long been appreciated for its role in regulating cell cycle progression. Emerging evidence indicates that it also influences physiological events beyond regulation of the cell cycle. We have previously described a requirement for Rb/E2F mediating neuronal migration; however, the molecular mechanisms remain unknown, making this an ideal system to identify Rb/E2F-mediated atypical gene regulation *in vivo*. Here, we report that Rb regulates the expression of *neogenin*, a gene encoding a receptor involved in cell migration and axon guidance. Rb is capable of repressing E2F-mediated *neogenin* expression while E2F3 occupies a region containing E2F consensus sites on the *neogenin* promoter in native chromatin. Absence of Rb results in aberrant neuronal migration and adhesion in response to netrin-1, a known ligand for neogenin. Increased expression of neogenin through *ex vivo* electroporation results in impaired neuronal migration similar to that detected in forebrain-specific Rb deficiency. These findings show direct regulation of *neogenin* by the Rb/E2F pathway and demonstrate that regulation of *neogenin* expression is required for neural precursor migration. These studies identify a novel mechanism through which Rb regulates transcription of a gene beyond the classical E2F targets to regulate events distinct from cell cycle progression.**

The Rb pathway is best characterized for its role in regulating cell cycle progression through E2F-mediated transcriptional regulation of classical cell cycle machinery target genes. Recently, however, accumulating *in vivo* and *in vitro* evidence is emerging to suggest that Rb and E2F are capable of regulating expression of atypical target genes with functions other than cell cycle regulation in cell-type-specific manners (reviewed in reference 35). *In vivo*, several studies have emerged that implicate Rb and E2F interaction in novel processes beyond well-characterized roles in cell cycle regulation (10; for a review, see reference 6). In the nervous system, in particular, we have recently shown that an Rb-E2F3 interaction mediates migration of a subpopulation of GABAergic interneurons (34). In the same study, we also observed deregulation of a number of genes with known roles in neuronal migration in cell populations lacking Rb, suggesting a role for E2F3 in regulating transcription of novel targets (34). A second cell cycle-independent role for E2F3a in regulating Rb-mediated interneuron differentiation was also reported in the retina (9). Thus far, *in vivo* studies have failed to identify the mechanism through which these cell cycle-independent processes occur.

In parallel, *in vitro* several microarray studies examining changes in gene expression in response to various models of deregulated E2F expression have each identified groups of overlapping novel target genes with well-characterized roles in differentiation, development, and migration (5, 15, 25, 31, 39, 41, 60). More recently, chromatin immunoprecipitation (ChIP)-on-chip studies have identified putative E2F binding sites within the promoters of a number of genes unrelated to the cell cycle (3, 4, 7, 28, 46, 56, 57). Finally, by using an approach whereby novel genes induced by E2F1 are identified based on subtraction screening, genes with known roles in differentiation and migration were identified as being directly induced by E2F1 in a cell cycle-independent manner (26). Thus, these data provide evidence that our understanding of the significance of Rb/E2F function should be expanded to include transcriptional regulation of genes beyond the well-characterized subset of targets that regulate the cell cycle.

Our identification of a role for Rb/E2F3 in mediating neuronal migration represents an attractive model to identify novel cell cycle-independent E2F target genes in the context of an *in vivo* physiological function (16, 34). Given our previous observations revealing (i) deregulation of a number of genes in families of known chemotactic ligands and receptors implicated in neuronal migration in the absence of Rb; and (ii) the cell-autonomous requirement for Rb in neuronal migration, we hypothesized that Rb/E2F may modulate the transcription of novel target genes involved in neuronal migration. We focused our efforts on *neogenin*, a receptor for the netrin and repulsive guidance molecule (RGM) families of chemotropic ligands (reviewed in reference 14). Notably, neogenin is highly expressed by a subpopulation of interneurons migrating from the ventral forebrain and has been independently identified, in an *in vitro* overexpression system, as an E2F-regulated gene (26, 34). Here, we report that Rb directly regulates the expression of a nontraditional target, *neogenin*. Rb is capable of repressing E2F-mediated transcription of *neogenin* while E2F3 binds to a region containing a conserved E2F consensus site on

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the *neogenin* promoter in native chromatin. The absence of Rb results in aberrant neuronal migration and adhesion in response to the neogenin ligand, netrin-1. Finally, increased expression of neogenin through *ex vivo* electroporation results in impaired neural precursor migration similar to that observed in forebrain-specific Rb deficiency. From these findings, we show direct regulation of *neogenin* by the Rb/E2F pathway and demonstrate that correct regulation of *neogenin* expression is required for neural precursor cell migration. Through these studies we identify a novel mechanism through which Rb interacts with E2F to regulate transcription of genes beyond the classical E2F targets to influence biologically relevant events distinct from cell cycle progression.

## MATERIALS AND METHODS

**Mice.** Telencephalon-specific Rb-deficient mice were generated by crossing floxed Rb-F19 (33, 53) and Foxg1-cre mice (23), and mice were genotyped according to standard protocols with previously published primers (16, 17). For embryonic time points, the time of plug identification was counted as embryonic day 0.5 (E0.5). For all experiments littermate Rb conditional mutants ($Rb^{flox/flox}$ Foxg1-$cre^{+/-}$) and double heterozygous controls ($Rb^{flox/+}$ Foxg1-$cre^{+/-}$) were compared. Due to Rb autoregulation (49), Rb expression in heterozygous mice is similar to that of wild-type controls. All experiments were approved by the University of Ottawa's Animal Care ethics committee, adhering to the Guidelines of the Canadian Council on Animal Care.

**Western blotting.** Protein was isolated from neurospheres by treating cells with lysis buffer (10 mM Tris, 0.15 M NaCl, 1 mM EDTA, 0.4 mM sodium vanadate, and 0.5% Triton-X). Cells were incubated on ice for 20 min, followed by a 10-min centrifugation at maximum speed to remove debris. Western blotting was performed as previously described (16) with antibodies directed toward neogenin (H-175; Santa Cruz), Rb (Pharmingen), and beta-actin (Sigma). Immunoblotting was performed on three independent samples, and results were quantified using ImageJ software.

**Tissue preparation and *in situ* hybridization.** Tissue was dissected, fixed, cryoprotected, and sectioned as previously described (34). Nonradioactive *in situ* hybridization and digoxigenin probe labeling were performed according to previously described protocols (54). Neogenin, deleted in colorectal cancer (DCC), netrin, and RGMa, were generous gifts of Helen Cooper of the University of Queensland (20), Elke Stein of Yale University, and Silvia Arber of the University of Basel (40). All results shown are representative of those obtained with a minimum of three independent animals.

**Transcription factor binding sites.** The 5′ region of the mouse Neo1 locus containing the intergenic region, the untranslated region, and exon 1 was analyzed for putative E2F binding sites using the TRANSFAC Professional Library, version 10.2, through Mulan/MultiTF (http://rvista.dcode.org). All sites identified in Mulan were manually examined for their similarity to the consensus (TTTSSCGC) and nonconsensus (BKTSSCGS) E2F motifs.

**Chromatin immunoprecipitation.** Neurosphere cultures were prepared from CD1 embryos (Charles River) at embryonic day 14.5. Proliferating neurospheres were triturated, cross-linked with formaldehyde, lysed, sonicated, and centrifuged at 14,000 × *g* to remove cellular debris. Each immunoprecipitation was performed using 2 μg of antibody. Antibodies against E2F3 (sc-878), E2F1 (sc-193), and normal rabbit immunoglobulin G (sc-2027) were obtained from Santa Cruz Biotechnology. Immunocomplexes were captured using protein A/G9-Sepharose beads and washed extensively, and cross-links were reversed overnight, followed by treatment with RNase A at 37°C for 1 h and proteinase K at 65°C for 30 min. The purified DNA was examined by PCR using primers designed around the E2F consensus sites at bp −44 and bp −821 in the 5′ region of the neogenin gene. Immunoprecipitations were performed from three cultures obtained from three independent animals.

**DNA constructs.** The *neogenin* construct was PCR amplified from pSecTagA-Neogenin (rat) using the following primers: forward, GAACTGCAGACCATG GAAGAAAGA; reverse, CTTCTGCAGGTTGCCCTCTAGCTAG. The PCR fragment was then subcloned into pCIG2. The 5′ regulatory region of *neogenin* was PCR amplified from embryonic genomic DNA. Primers were designed as follows with flanking XhoI restriction sites inserted: full-length forward, AGAC TCGAGGAGGTGCAGAGGAGTCGC; full-length reverse, TGTCTCGAGG TTGAAAAACCAATTCCCG. To create 5′ truncations the following primers were used with full-length reverse primer: FTrunc247, AGACTCGAGAGCCG GGGGGTGG; FTrunc618, AGACTCGAGAAGCGATCCGCCTCCT. To create the 3′ truncation the following primer was used with full-length forward primer: RTrunc 247np, TGTCTCGAGCCACCCCCCGGCT. The construct consisting of bp 247 to 618 was made using the FTrunc247 and RTrunc 618bp (TGTCTCGAGGCGGATCGCTTCTCC) primers. PCR products were digested with XhoI and ligated into pGL4.24 (Promega). Sequence was confirmed by DNA sequencing (StemCore Laboratories, University of Ottawa).

**Luciferase reporter assays.** HEK293T cells were transfected using Lipofectamine (Invitrogen) as per the manufacturer's protocol. Briefly, cells were transfected with 500 ng of pGL4.24, pGL4.24-Neogenin, or pGL4.24-NeoTruncations, 10 ng of E2F3, 300 ng of Rb, and total transfected plasmid normalized with pcDNA3.1. Transfection efficiency was normalized using 10 ng of pRL *Renilla*-expressing vector. Cells were lysed at 24 h posttransfection and examined by spectrophotometer (LMaxII; Molecular Devices) for luciferase expression by a Dual-Glo luciferase kit (Promega).

**In vitro explant cultures.** *In vitro* explant cultures were performed as described previously (13, 29, 38, 43, 44) with some modifications. Briefly, brains were removed from E14.5 embryos in L-15 (Gibco) medium, and medial ganglionic eminence (MGE) was dissected as described previously (16); explants were subsequently divided into pieces approximately 200 μm in diameter by using sharpened tungsten needles. MGE explants were then transferred into collagen (PureCol, catalog number 5409; Inamed BioMaterials) inside culture dishes and allowed to solidify for 40 min prior to addition of Neurobasal medium (Gibco) supplemented with fetal bovine serum (FBS). Purified netrin-1 was added to the explant culture medium at a final concentration of 200 ng/ml.

Explants cultured alone were grown *in vitro* for 24 h. Images were captured with a Zeiss Axioscope microscope. For quantification of cell migration in collagen, the total number of cell bodies migrating from explants was counted. Two to four explants per embryo were measured, and values were averaged. Two-tailed *t* tests were performed to compare mean migration between genotypes or treatment groups. Differences were considered significant at a $P$ value of $<0.05$.

**Neural progenitor cultures.** Pregnant mice were euthanized at gestation day 14.5, embryos were removed, and the ganglionic eminences were isolated by microdissection. For determination of cell proliferation and cell death in single-cell preparations, ganglionic eminences were dissociated, and equal cell numbers were plated on poly-D-lysine- and laminin-1-coated dishes in duplicate. Cells were cultured in Neurobasal medium supplemented with 0.5 mM L-glutamine, 1% N-2, 2% B-27, 10 ng/ml fibroblast growth factor 2 (FGF-2), and 20 ng/ml epidermal growth factor (EGF) in either the presence or absence of netrin-1 at 200 ng/ml. Cells were treated with bromodeoxyuridine (BrdU) at a final concentration of 10 μg/ml for 45 min prior to fixation. After 24 h, cells were fixed for 15 min in 4% paraformaldehyde (PFA) and then treated sequentially with 2 N HCl and 0.01 M $NaB_4O_7$, followed by BrdU immunohistochemistry (anti-BrdU at 1:100; BD Biosciences, San Jose, CA) and Hoechst nuclear staining. The total cells and BrdU-positive cells were counted in three microscope fields per duplicate well. Rates of proliferation were obtained by calculating the proportion of BrdU-positive cells relative to the total cell number. Fold increase in proliferation in response to netrin-1 was calculated for each genotype by dividing the percentage of proliferating cells in the presence of netrin-1 by the percentage of proliferating cells in the absence of netrin-1. (Three separate embryos were analyzed in quadruplicate for both control and conditional mutant embryos). For Hoechst labeling, dead cells were identified by the characteristic condensation of chromatin. Fold increase in apoptotic nuclei was calculated in an analogous manner to cell proliferation. (Three separate embryos were analyzed in quadruplicate for both control and conditional mutant embryos).

**Substrate-bound adhesion assay.** To assess cell adhesion in the presence of netrin-1, a substrate-bound adhesion assay was performed as described previously (50). Briefly, 20 μl of 0.1% nitrocellulose (Hybond ECL; Amersham Biosciences) dissolved in methanol was dried on the bottom of a four-well plate. Plates were then incubated with either Hanks' balanced salt solution (HBSS) or 2 μg/ml netrin-1 in HBSS for 2 h at room temperature. Wells were then blocked for 1 h with 1% bovine serum albumin (BSA; Fisher Scientific) in HBSS and then again with 1% heparin (Sigma) in HBSS. A total of $2.5 \times 10^5$ cells from dissociated ganglionic eminences were plated in Neurobasal medium (Gibco) supplemented with 2% B-27 and 2 mM glutamine. Cells were cultured for 2 h at 37°C in 5% $CO_2$, gently washed once with phosphate-buffered saline (PBS), and fixed with 4% PFA in PBS overnight. Nuclei were labeled with 0.5 μg/ml Hoechst 33258 (Sigma) in PBS for 30 min. Experiments were performed on four wild-type and three mutant embryos. Paired, two-tailed *t* tests were performed to compare genotypes, with differences considered significant at a $P$ value of $<0.05$.

***Ex vivo* cortical electroporation.** Cortical electroporation and *ex vivo* slice culture were performed as described previously (16, 22, 42), with some modifications. Briefly, pregnant female mice (Charles River) were euthanized at E15

with a lethal injection of sodium pentobarbital. Embryos were removed and decapitated, and a 2 μg/μl solution of pCIG-Neogenin or empty pCIG vector (supplemented with 0.5% Brilliant Blue FCF for visualization) was injected, using a Picospritzer II (General Valve Corporation), into the lateral ventricles. Brains were subjected to 10 pulses at 70 V using an ElectroSquarePorator ECM830 (BTX/Genetronics San Diego, CA). Brains were then isolated and embedded in low-melting-point agarose. Agarose-embedded brains were sectioned coronally into 250-μm sections on a Leica VT1000S vibratome. Brain sections were collected and plated on poly-L-lysine–laminin-coated filter membrane inserts placed on top of the culture medium in each well of a six-well dish, as described previously (16, 42). Slices were then cultured for 72 h to assess the degree of migration. Two sections from five to six embryos from three litters were measured, and values were averaged. Migration was assessed by measuring the total area occupied by green fluorescent protein (GFP)-positive cells at both 24-h and 72-h time points. Degree of migration under each condition was assessed by subtracting the area occupied at 24 h from the area occupied at 72 h and then dividing this value by the initial area to obtain a percentage of migration over time. Degree of migration in neogenin-expressing samples relative to the control was obtained by dividing percent migration in neogenin by percent migration of the control. Two-tailed *t* tests were performed to compare migration between control and neogenin overexpression, with differences considered significant at a *P* value of $<0.05$.

## RESULTS

**Rb deficiency results in specific deregulation of *neogenin* expression *in vivo*.** Our previous studies have described a physiological requirement for Rb interacting through E2F to mediate nervous system development (34). Furthermore, we reported that Rb deficiency results in increased expression of mRNA encoding neogenin, a receptor involved in regulating axon guidance and cell migration during neural development (59; for a review, see reference 14).The physiological significance of Rb-mediated regulation of *neogenin* expression on nervous system development, however, is unknown. To assess the contribution of neogenin to Rb-mediated nervous system development, we first asked if deregulation is unique to *neogenin* or extends across the family of neogenin ligands and related receptors. To address this question in the telencephalon, sections from Foxg1-cre conditional Rb mutants were subjected to *in situ* hybridization to examine the expression profiles of neogenin, the closely related receptor deleted in colorectal cancer (DCC), and the known neogenin ligands repulsive guidance molecule (RGMa) and netrin-1 (Fig. 1A). Consistent with our previous microarray and *in situ* hybridization findings (34), we detected increased neogenin expression throughout the ventral and dorsal telencephalon in conditional Rb mutants. In the ganglionic eminence, a source of migrating interneurons, no difference was detected in the expression patterns of DCC, netrin-1, and RGMa between control and conditional Rb mutants ($n = 3$) (Fig. 1A). These expression patterns parallel results of other studies that have shown neogenin and netrin-1 overlapping protein expression in the ganglionic eminence (19, 52). Thus, of the members of the netrin signaling pathway, a significant change was detected in the expression only of neogenin within the ventrally derived population of neural precursor cells. To validate increased neogenin levels identified by *in situ* hybridization, we assessed expression at the protein level within the migrating cell population. Total protein was extracted from the population of ventrally derived neural progenitor cells from three separate embryos for each genotype, and a similar increase in neogenin protein was identified in conditional Rb mutants (Fig. 1B). Efficient excision of the Rb allele in the context of primary

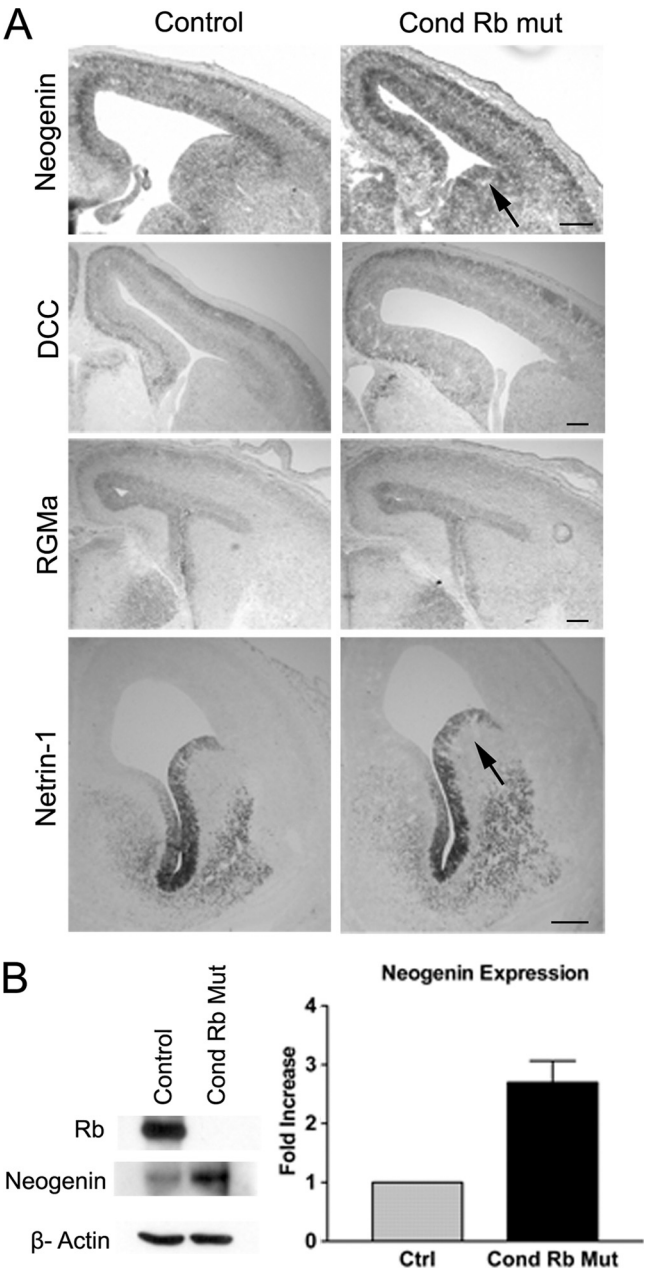


FIG. 1. Neogenin is upregulated in the absence of Rb in the developing forebrain. (A) In the absence of Rb (*Foxg1-Cre*/+; $Rb^{loxP/loxP}$) increased neogenin expression is detected in the subventricular zone, cortex, and striatum compared with results in controls (*Foxg1-Cre*/+; $Rb^{loxP/+}$). *In situ* hybridizations were performed on three or more independent samples for DCC, RGMa, netrin-1, and neogenin. Arrows indicate regions of overlapping expression between neogenin and netrin-1 in the ganglionic eminence. (B) Western blot analysis was performed on neural precursor cells isolated from E14.5 Rb conditional mutants and control embryos. Note efficient recombination of the floxed Rb allele, showing no detectable Rb. Rb mutants showed an upregulation of neogenin compared to levels in the control. Densitometric quantification of neogenin expression detected by Western blotting was performed using ImageJ analysis of three independent experiments. Significance was determined through a paired two-tailed *t* test for the control and conditional Rb mutant ($P < 0.05$).

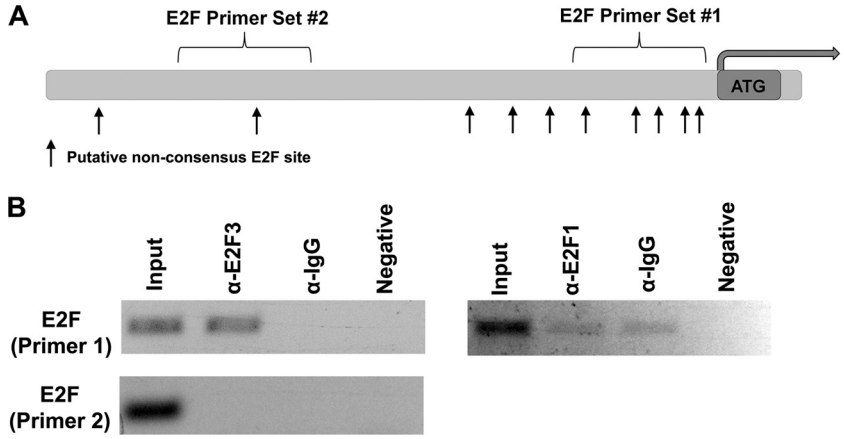


FIG. 2. E2F3 interacts with a region containing multiple putative E2F sites within the 5′ regulatory region of neogenin. (A) Putative E2F sites were identified in the 5′ region of the mouse (mm9) Neo1 gene using Mulan/rVista software and confirmed by manual sequence analysis (BKTSSCGS). (B) ChIP was performed on neurospheres isolated from the ganglionic eminence of E14.5 wild-type embryos. Immunoprecipitation was performed using an antibody specific to E2F3 or E2F1, followed by PCR amplification of the indicated regions in the 5′ regulatory region of the neogenin gene. ChIP was performed on three independent cultures per condition. An interaction was detected only with E2F3 at the region containing multiple clustered sites; the putative site at 852 bp showed no specific binding. Immunoprecipitations were performed on independent cultures from three animals.

ventral neural precursors was confirmed by protein levels (Fig. 1B). Together, these data support the hypothesis that Rb may play an important role in regulating expression of neogenin, a nontraditional E2F target gene.

**E2F3 binds the 5′ locus of neogenin *in vivo*.** Recent studies have reported that *neogenin* is among a novel class of atypical E2F target genes regulated in a cell cycle-independent manner (26). E2F1 was shown to be capable of directly inducing neogenin expression independent of growth stimulation; however, no classical E2F binding (TTTSSCGC) site was observed within the 5′ region (26). With the implementation of new bioinformatic techniques, a new broad E2F consensus binding site(s) (BKTSSCGS) has recently been characterized (45). We asked whether E2F could be mediating neogenin expression through one of these newly defined E2F sites contained in its 5′ regulatory region. Using Mulan/rVista software, the 5′ region of the mouse *neogenin* gene was examined for the presence of broad-spectrum BKTSSCGS E2F motifs (45). Consistent with previous reports, no classical E2F binding sites could be found in the mouse *neogenin* promoter (26). Closer examination revealed 10 broad E2F binding sites located within 1.23 kb upstream of the translational start site. The majority of these putative E2F sites are clustered within the first 600 bp upstream of the ATG (Fig. 2A).

Prior studies examined the regulation of neogenin expression by E2F1; however, as we have previously described unique roles for E2F3 in nervous system development (34, 36), we asked whether E2F3 interacted with the 5′ regulatory region of the *neogenin* gene in the context of native chromatin. To address this question, we performed chromatin immunoprecipitation (ChIP) in primary neural progenitor cultures to see if E2F3 associated with the regions containing atypical E2F sites. Chromatin was immunoprecipitated with antibodies to E2F3, followed by PCR with primers designed around the cluster of E2F sites immediately upstream of the translation start and the region containing only a single site 852 bp upstream (Fig. 2). ChIP with the primer set surrounding the cluster demonstrated enrichment of E2F3 binding, with no detectable E2F3 binding at the site at −852 bp (Fig. 2B). Consistent results were obtained from three independent primary cultures. Previous studies using a subtractive microarray analysis revealed that E2F1 could induce neogenin expression in rat embryonic fibroblasts; however, a direct interaction in the *neogenin* regulatory regions was not shown (26). We therefore asked if E2F1 might physically interact with the putative promoter region of *neogenin*. To this end we performed ChIP with an antibody directed to E2F1 and examined the region in which E2F3 binding was detected. No enrichment was found in this region (Fig. 2B), suggesting that E2F1 does not interact with the *neogenin* promoter in precursors from the ventral forebrain. These results demonstrate that, in the context of neural precursor cells, E2F3 specifically binds the *neogenin* promoter at the region (−600 to ATG) encompassing the multiple putative E2F consensus sites in an *in vivo* context, and this is consistent with the hypothesis that E2F3 is capable of modulating neogenin gene expression.

**Rb/E2F regulates transcriptional activity at the 5′ neogenin-regulatory region.** Given that we observe deregulated neogenin expression in the absence of Rb, we next asked whether Rb regulates this expression through its interaction with E2F. To address this question, we performed *in vitro* luciferase reporter assays in HEK293T cells. Ideally, these studies should be performed in primary systems; however, overexpression of E2F1 or E2F3 induces a rapid and robust apoptotic response in embryonic tissue and in primary neural precursor cells. As HEK293T cell lines express E1B preventing apoptosis (58), they withstand overexpression of "activating" E2F constructs without undergoing cell death. Thus, all reporter assays were performed in HEK293T cell lines. The *neogenin* promoter region was amplified from embryonic genomic DNA with primers designed to flank a 1.23-kb region containing the putative E2F binding sites (Fig. 3A). The fragment was then subcloned into a luciferase reporter vector (pGL4.24) which was subsequently transfected into HEK293 cells.

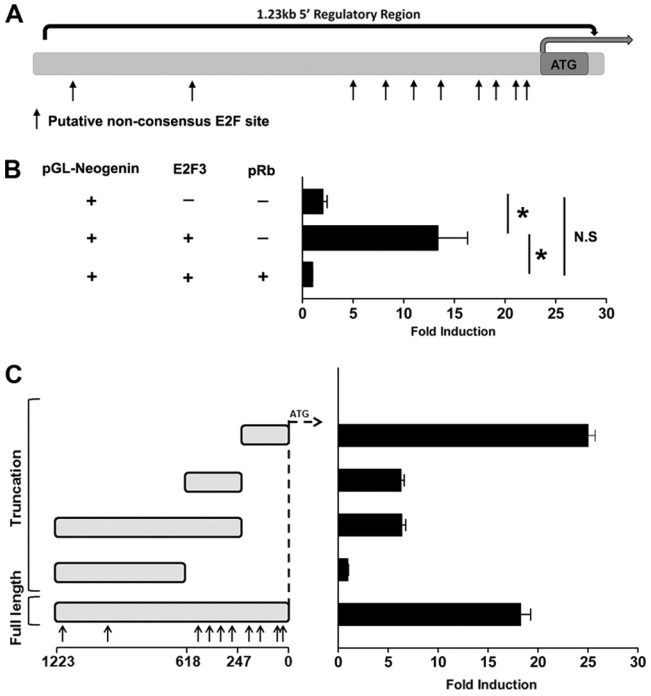


FIG. 3. The 5′ neogenin promoter is responsive to Rb/E2F regulation. (A) Schematic of the 5′ region of the neogenin gene. The 1.23-kb region isolated and cloned into the pGL4.24 vector contains 10 putative E2F binding sites identified using Dcode/Mulan software. (B) Dual-Glo luciferase (Promega) promoter assays in HEK293T cells utilizing the neogenin promoter reveal that the addition of E2F3 induces a 13-fold induction of neogenin promoter activity. When cells are stimulated with Rb and E2F3, the activation is eliminated, and luciferase levels return to that of promoter alone. (C) Luciferase analysis of truncations of the 5′ region of the neogenin gene. Activity is ablated upon removal of the initial 618 bp. The region between bp 247 and 618 results in slight E2F3 responsiveness; however, the first 247 bp recapitulates full-length promoter activity. *, $P < 0.05$; N.S., nonsignificant difference.

In the absence of exogenous E2F, the *neogenin* promoter displayed a minimal level of activation (Fig. 3B). Upon addition of E2F3, however, we observed a strong 13.3-fold increase in luciferase activity, demonstrating that E2F3 is capable of transcriptionally activating the *neogenin* promoter. We next asked if Rb is capable of repressing E2F-mediated activation of the *neogenin* promoter. Upon cotransfection of E2F3 and Rb expression plasmids, E2F3-mediated activation of the *neogenin* promoter was repressed back to basal levels (Fig. 3B). Together these results demonstrate that Rb acts to repress E2F3-mediated activation of the *neogenin* promoter through interaction in the 5′ regulatory region.

We next sought to determine the specific region in the neogenin promoter in which E2F3 was binding to activate transcription. Examination of the 5′-proximal promoter for both classical/nonclassical E2F binding motifs revealed 10 putative sites within the 1.23 kb examined. To define the essential regions required for E2F-mediated activation, we created multiple truncation constructs at roughly 250-bp intervals from both the 5′ and 3′ ends of the 1.23-kb promoter construct (Fig. 3C). While constructs lacking the regions upstream of bp −247 had no effect on E2F responsiveness, absence of the region from the ATG to bp −618 abolished the ability of E2F to activate transcription (Fig. 3C). This region was most enriched with eight putative E2F sites, and the results suggest that E2F3 is binding one or more of the several clustered sites in this region. To more precisely identify the sites, two further truncation constructs (bp 0 to 247 and bp 247 to 618) were made lacking each of the two clusters of E2F sites within the first 618 bp of the promoter (Fig. 3C). Introduction of the construct containing only the region encompassing bp 247 to 618 upstream of the promoter (Fig. 3C) resulted in slight activation in response to E2F (Fig. 3C). This result recapitulated the slight E2F responsiveness observed in the construct lacking the first 247 bp upstream of the ATG. To determine if the first 247 bp could confer E2F-mediated activation, this 247-bp fragment alone was ligated into the reporter construct, and full E2F responsiveness, equivalent to that of the full-length construct, was obtained. These results demonstrate that the cluster of E2F sites contained in the 247 bp upstream of the ATG are essential for neogenin promoter activation, consistent with the region identified by our E2F3 ChIP (Fig. 2B). These findings support the conclusion that Rb, acting through E2F3, directs the expression of neogenin, an atypical E2F target gene, that functions outside cell cycle progression.

**Rb deficiency results in aberrant neuronal migration in the presence of netrin-1.** We next sought to determine the functional consequences of deregulation of *neogenin* expression as a result of the ablation of the Rb gene. We hypothesized that if deregulated expression of *neogenin* contributes to the aberrant migration of ventrally derived neurons in the conditional Rb mutant, then neural precursor cells should elicit an aberrant response in the presence of neogenin ligand. During mammary gland development, netrin-1–neogenin interactions have been shown to be crucial for proper stabilization of the multipotent progenitor cell layer (19, 51). This interaction may play an analogous role during tangential migration in the developing forebrain. We therefore determined if netrin-1 is capable of influencing migration of MGE-derived cells under wild-type conditions. To effectively address this question, we employed a reductionist *in vitro* approach using primary neural precursor explants cultured in a collagen matrix. This approach created a defined extracellular environment containing netrin-1 alone and allowed us to determine the effect of the neogenin ligand, netrin, in the absence of other competing signals known to influence migration (32). Explants of ventral ganglionic eminence were microdissected from control and mutant E14.5 cerebral hemispheres and then cultured for 24 h supplemented with netrin-1, after which cell migration from the explant was quantified. We assessed the relative contribution of netrin-1, a ligand demonstrated to elicit neogenin-dependent chemoattractant responses in the developing nervous system. Explants were cultured in collagen, a matrix suitable for assessing chemoattractant responses (29, 38). Both control and conditional Rb mutant explants cultured in collagen alone exhibited modest numbers of cells migrating, with no appreciable difference in migration from either type of explant. In the presence of netrin-1, however, a clear difference was observed (Fig. 4A). While control explants exhibited a 4-fold increase in migration in the presence of netrin-1, there was no significant difference in the number of cells migrating from

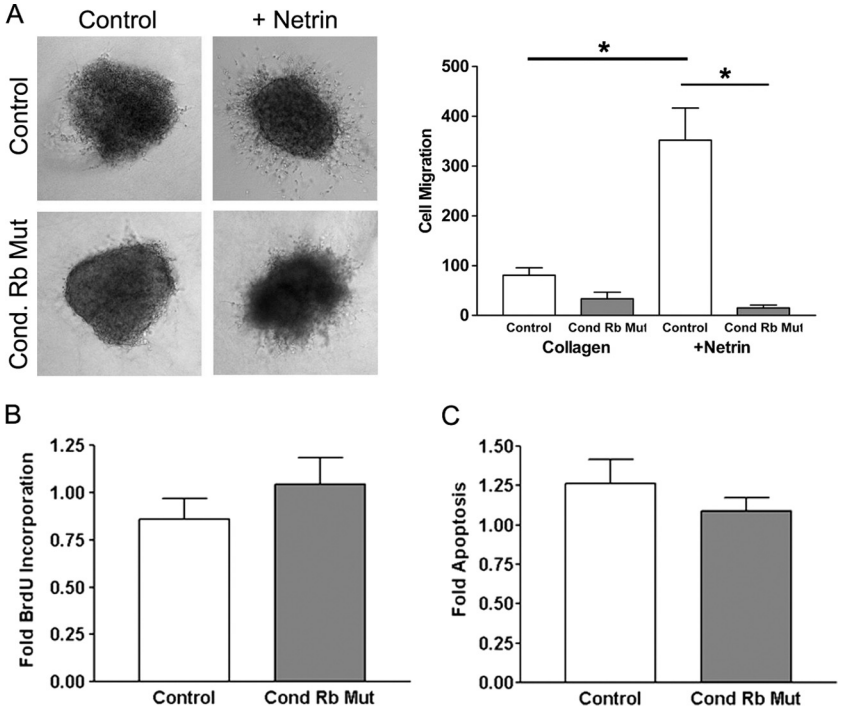


FIG. 4. Conditional Rb mutants display a defective migratory response to netrin-1. (A) Control (*Foxg1-Cre*/+; $Rb^{loxP/+}$) and conditional Rb mutant (*Foxg1-Cre*/+; $Rb^{loxP/loxP}$) MGE explants were cultured in collagen in the absence or presence of recombinant netrin-1. Migration was quantified by counting the individual cell bodies migrating from each explant. Bars in the graph at left represent the mean of the average number of cells migrating from an individual explant ± standard error of the mean. While control cells exhibit a nearly 4-fold increase in migration in the presence of netrin, no difference was observed in conditional Rb mutants between the presence and absence of netrin. Significance was determined through a paired two-tailed *t* test for explants of the same genotype and a two-tailed *t* test for explants of different genotypes. *, $P < 0.05$ ($n$ = 4 embryos per treatment, per genotype; two to three explants were examined per embryo). (B and C) Cells from the ganglionic eminence of control and conditional Rb mutants were dissected and cultured as single-cell preparations in the presence or absence of netrin-1. Quantification of the proportion of cells in S phase (BrdU) or dying (Hoechst) reveals no change upon addition of netrin-1 in either genotype. Three separate embryos were analyzed in quadruplicate for both the control and conditional Rb mutant.

conditional Rb mutant explants cultured in the presence or absence of netrin-1 (Fig. 4A).

Netrin-1 has been hypothesized to mediate cell proliferation and cell death (37; for a review, see reference 11). We therefore verified that the differences detected in migration were not due to either of these processes. To dissect the potential contribution of altered proliferation or cell death, netrin-1 treatment was performed under conditions that recapitulate those used in our *in vitro* explant culture in order to ensure that there are no changes in proliferation and cell death under those specific conditions. To examine proliferation, cultures were treated with bromodeoxyuridine (BrdU), and the proportion of cells in S phase of the cell cycle was counted. In three independent control and mutant cultures, netrin-1 treatment did not significantly impact the number of proliferating cells (Fig. 4B). Assessment of chromatin condensation revealed no significant change in cell death between control and mutant cultures upon addition of netrin-1 ($n$ = 3) (Fig. 4C). These results suggest that the increased number of cells migrating in response to netrin-1 is not a consequence of increased cell proliferation, nor can the absence of migration in the conditional Rb mutant be attributed to increased cell death. Thus, these data support a model whereby netrin-1 is capable of influencing migration of ventrally derived progenitors, an effect that is not observed in the conditional Rb mutants. These results suggest that ventrally derived progenitors from Rb mutants are inherently unable to elicit the appropriate migratory response to netrin-1 itself.

**Increased adhesion to substrate-bound netrin-1 in conditional Rb mutants.** A previous study demonstrated that netrin-1 and neogenin interact to mediate adhesion in the mammary gland (51). Given that we observe reduced migration in response to netrin-1 in the conditional Rb mutant, where *neogenin* expression is increased, we determined if the increased amount of neogenin present would increase adhesion of neural precursor cells. To address this, adhesion assays were performed which have been previously used to assess netrin-neogenin-mediated adhesion in fibroblasts (51) and adapted the assay for neural precursor cells (50). Using this assay, we examined the capacity of ventrally derived precursors from control and conditional Rb mutants to adhere specifically to immobilized netrin-1. The ventral telencephalon from E14.5 control and conditional Rb mutants was dissected and dissociated into single-cell suspensions. Cells were quantified, and then equal numbers were plated and allowed to adhere to culture dishes preadsorbed with nitrocellulose alone or with netrin-1 and nitrocellulose. After 2 h, cells were washed and fixed, and cell adhesion was quantified. Data were represented as the fold increase in adhesion upon netrin-1 treatment to eliminate the experimental variability observed from each in-

TABLE 1. Rb-deficient neural precursors show an increased propensity to adhere to substrate bound netrin-1

| Expt[a] | Embryo | Genotype | Cell adhesion (avg no. of cells/field)[b] | | Fold increase[c] |
|---|---|---|---|---|---|
| | | | Without netrin | With netrin | |
| CTL | MGA57 | *Foxg1-Cre*/+; $Rb^{loxP/+}$ | 382 | 925 | 2.421466 |
| CTL | MGA62 | *Foxg1-Cre*/+; $Rb^{loxP/+}$ | 235.5 | 513 | 2.178344 |
| CTL | MGA132 | *Foxg1-Cre*/+; $Rb^{loxP/+}$ | 121.5 | 285.5 | 2.349794 |
| CTL | MGA133 | *Foxg1-Cre*/+; $Rb^{loxP/+}$ | 127.75 | 291.4 | 2.281018 |
| $Rb^{-/-}$ | MGA134 | *Foxg1-Cre*/+; $Rb^{loxP}/Rb^{loxP}$ | 100 | 316.25 | 3.1625 |
| $Rb^{-/-}$ | MGA136 | *Foxg1-Cre*/+; $Rb^{loxP}/Rb^{loxP}$ | 113.5 | 504.75 | 4.447137 |
| $Rb^{-/-}$ | MGA59 | *Foxg1-Cre*/+; $Rb^{loxP}/Rb^{loxP}$ | 464 | 1635 | 3.523707 |

[a] CTL, control.
[b] Cells from the ganglionic eminence of control and conditional Rb mutants were dissected at E14.5. Cells were allowed to adhere to netrin-1 or noncoated wells for 2 h before they were fixed and stained. Cells were then imaged, and nuclei were counted. The total number of nuclei per field was averaged for each condition. In the absence of Rb, cells from the ganglionic eminence display more significant adherence to netrin-1 than cells from control littermates.
[c] Fold increase represents the increase in adhesion from noncoated to netrin-1-coated wells.

dependent assay. Independent experiments, however, produced highly consistent results (Table 1). In the presence of netrin-1, conditional Rb mutants displayed a 3.6-fold increase in adhesion, whereas control littermates displayed a significantly smaller 2.5-fold increase (Fig. 5). Our findings suggest that Rb-deficient neural precursor cells have increased adhesive properties, consistent with previous findings revealing a role for netrin-neogenin in mediating cellular adhesion (50, 51). Given the elevated levels of *neogenin* expression detected, increased adhesion in response to netrin-1 may be a key contributing factor to the migration defect present in Rb-deficient brains.

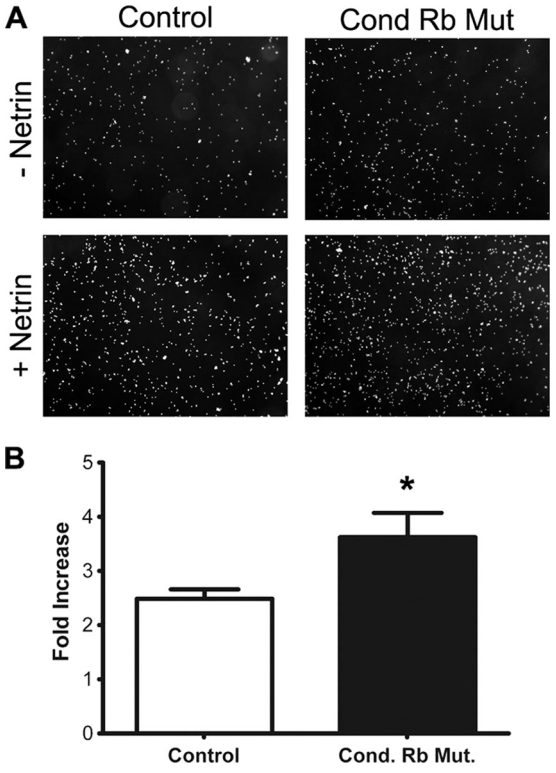


FIG. 5. Rb-deficient neural precursors show an increased propensity to adhere to substrate-bound netrin-1. (A) Cells from the ganglionic eminence of control (*Foxg1-Cre*/+; $Rb^{loxP/+}$) and conditional Rb mutants (*Foxg1-Cre*/+; $Rb^{loxP/loxP}$) were dissected at E14.5. Cells were allowed to adhere to netrin-1 or noncoated wells for 2 h and were then fixed and stained. Cells were then imaged, and nuclei were counted. In the absence of Rb, cells from the ganglionic eminence display more significant adherence to netrin-1 than cells from control littermates. (B) Fold increase represents the increase in adhesion from noncoated to netrin-1-coated wells. Error bars represent standard error of the mean ($n = 4$ for the controls and $n = 3$ for conditional Rb mutants). Significance was determined through a paired two-tailed $t$ test for control and conditional Rb mutant cultures. *, $P < 0.05$.

**Increased neogenin impedes neuronal migration.** While we have demonstrated that Rb is capable of regulating neogenin transcription through E2F in the developing nervous system, the consequence of increased neogenin expression remains unknown. We therefore asked if upregulation of neogenin as found in the Rb-deficient forebrain was sufficient to disrupt the migration of MGE-derived neurons. To determine whether increased neogenin expression could perturb neuronal migration, we performed *ex vivo* cortical electroporation of the full-length neogenin or a control internal ribosome entry site (IRES)-GFP vector into the ventral telencephalon of wild-type E15.5 embryonic brains (22). Following electroporation, brains were cultured as slices for 72 h to observe migration. Expression of the plasmid carrying GFP-positive cells was first observed at 24 h postelectroporation and subsequently at 72 h. At 24 h, brains electroporated with either control or neogenin-containing plasmids displayed GFP-positive cells lining the ventricular zone of the ventral forebrain, with no difference observed between controls and neogenin-electroporated cells (Fig. 6A, red). At 72 h, numerous GFP-positive cells from control slices were observed to have migrated considerably from their initial position within the ventricular zone. GFP-positive cells from neogenin slices, however, remained clustered within a similar band along the ventricular zone (Fig. 6A, green). Migration was quantified by measuring the total area occupied by GFP-positive cells at the endpoint, subtracting the initial area, and then dividing this value by the initial area to obtain the percent increase in migration [(total migration − initial migration)/total migration], and values were normalized to the percentage of the control. Upregulation of neogenin resulted in a 77% decrease in migration ($P < 0.05$) compared to that of control-electroporated embryos (Fig. 6B). We conclude that increased expression of neogenin by cells in the ganglionic eminence results in reduced migration of precursors away from the ventricular zone, paralleling the migration defect observed in the Rb-deficient forebrain.

Taken together, our results demonstrate a function for the

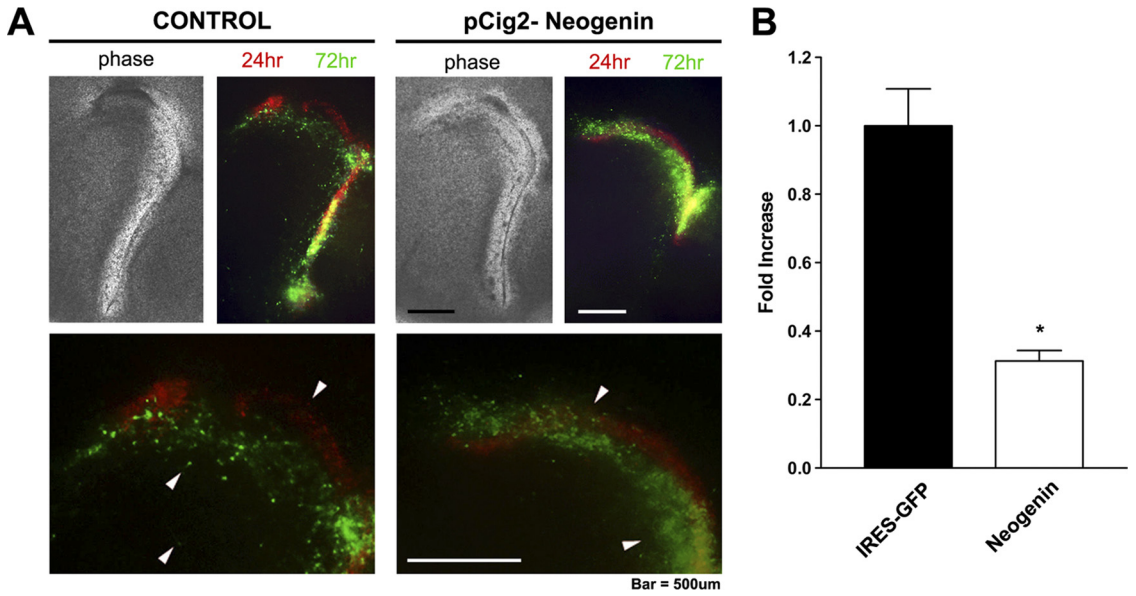


FIG. 6. Increased neogenin expression impairs migration of neuroblasts from the subventricular zone. (A) *Ex vivo* overexpression of control IRES-GFP- or neogenin-IRES-GFP-expressing plasmids in E15 embryos. Embryos were sectioned at 250 μm and plated on poly-L-lysine–laminin-coated inserts. Cells were visualized at 24 h postelectroporation (red) to determine their baseline vector expression and migration. Cells were imaged again at 72 h postelectroporation to assess the degree to which they migrated (green). Under both control and neogenin-overexpressing conditions cells expressing the plasmids initially line the ventricle (red, arrows). After migration (lower panels) the cells move away from the ventricular zone into the striatum (green, arrows) in the control; however, in the mutant they fail to shift position. (B) Quantification of the capacity of cells to migrate depicted in panel A. In order to quantify migration, fold increase was obtained by calculating (total migration − initial migration)/total migration and then normalizing values to wild-type migration levels. Error bars represent standard error of the mean ($n = 5$ for the control and $n = 6$ for the mutant, with two sections per embryo). Significance was determined through a paired two-tailed $t$ test of control versus neogenin-overexpressing slices. *, $P < 0.05$.

Rb pathway in regulating expression of a nontraditional E2F target gene, neogenin, during neuronal migration. Furthermore, we demonstrate that aberrant neogenin expression, similar to that found in conditional Rb mutants, leads to impaired migration. Overall, these finding support the conclusion that Rb/E2F regulation of *neogenin* expression, an atypical target, influences appropriate neural development in a manner beyond traditional regulation of the cell cycle.

## DISCUSSION

Here, we demonstrate the existence of an Rb/E2F-mediated molecular mechanism regulating expression of an atypical E2F target gene, *neogenin*. First, we have shown that *neogenin* expression is deregulated in the absence of Rb at the mRNA and protein levels in neural precursor cells. While neogenin has previously been shown to be an E2F-regulated target gene *in vitro*, here we complement previous findings by demonstrating that E2F3 is capable of activating neogenin expression, and we extend these findings by demonstrating the binding of E2F3 to the 5′ regulatory region of the neogenin promoter in neural precursor cells. It is possible that other E2Fs are contributing to regulation of neogenin expression in different biological contexts; however, given that E2F3 has previously been implicated in multiple aspects of nervous system development *in vivo* (9, 34, 36), these observations lend further support to the idea that E2F3 regulation is significant in this context. Finally, we demonstrate that E2F transcriptional regulation of neogenin is, in turn, strongly repressed by Rb activity. These results, along with our data regarding increased neogenin expression among migrating neurons in the absence of Rb, suggest a direct role for the regulation of neogenin by the Rb/E2F pathway in the developing forebrain.

Previously, we have shown that the Rb/E2F pathway mediates migration of a population of precursors from the ventral telencephalon during nervous system development (16, 34). Here, we provide mechanistic insight into this process, showing that in the absence of Rb, migrating ventral precursors exhibit a decreased response to the neogenin ligand, netrin-1. Consistent with decreased migration, we observe increased adhesion of ventrally derived Rb-deficient precursors to substrate-bound netrin-1. This suggests a mechanism in which increased neogenin expression results in augmented neuronal adhesion leading to the decreased migration. Our *ex vivo* manipulation of neogenin expression resulted in a defect in neuronal migration similar to that seen in the conditional Rb mutant (16). While we favor our model as a hypothesis to explain how Rb/E2F regulates migration, we note that neogenin is likely only one of many factors contributing to Rb-mediated neuronal migration. Indeed, neuronal migration is a complex phenomenon involving multiple genes and genetic pathways (24). Through our previous microarray analysis we identified several known genes that regulate migration in the central nervous system, and therefore dysfunction in their expression could also be contributing to several facets of the observed migration defect in the conditional Rb mutants (34). While the extent to which deregulated neogenin contributes to the migration defect in Rb mutants is unknown, our studies reveal that overexpression of

neogenin perturbs neuroblast migration in wild-type tissue (Fig. 6). Indeed, a rescue experiment in our conditional Rb mutants would be challenging as reducing neogenin expression to physiological levels without causing a complete knockdown would likely lead to variable results. As presented, our results provide strong evidence that, by regulating neogenin expression, Rb/E2F has an important physiological role beyond regulation of the cell cycle machinery, a phenomenon that has not yet been reported. It is probable that Rb is involved in the regulation of multiple genes, which through distinct mechanisms contribute to the regulation of neuronal migration.

The idea that the Rb/E2F pathway can regulate genes outside the prototypical cell cycle machinery in the context of nervous system development may also broaden its role in tumorigenesis. As Rb is the first identified tumor suppressor, intense interest has been focused on defining the molecular mechanisms through which it mediates tumor suppression. While early studies established the model that Rb-mediated tumor suppression is the result of its restraint of E2F transcription factors at the $G_1$/S transition, (reviewed in reference 55), more recent studies suggest that the role of Rb as a tumor suppressor is more complex than originally hypothesized. Indeed, roles for Rb in maintaining genome stability and promoting senescence have broadened the scope and complexity of Rb-mediated tumor suppression (reviewed in references 21 and 30). Further deregulation of the Rb pathway in cancer has been traditionally associated with sustained proliferation; however, Rb mutations are frequently found in metastatic cancers, including small-cell lung carcinoma and osteosarcoma, as well as invasive poor-prognosis glioblastomas (reviewed in reference 12).

Having demonstrated a novel role for the Rb/E2F pathway in mediating expression of a specific gene involved in neuronal migration, the data presented here raise the possibility that Rb activity could contribute to the regulation of other cellular processes involved in cancer beyond regulation of cell division. Recent studies employing conditional transgenic alleles to remove tumor suppressor genes specifically in adult neural precursor cells have shown the important nonoverlapping roles for Rb, PTEN, Nf1, and p53 (1, 27). The study of Jacques et al. correlated the ablation of Rb gene expression in the adult subventricular zone with the appearance of primitive neuroectodermal tumors (pNETS). These tumors display significant differentiation across all three neural lineages and ectopic infiltration of surrounding brain tissue. This lends itself to the hypothesis that Rb may also regulate the differentiation and localization of these tumor cells. Many families of genes which mediate neuronal migration, such as the netrin signaling axis (18, 48), have been implicated in multiple aspects of cancer and tumorigenesis. Ligands and receptors from these migration pathways are frequently found deregulated or are lost altogether in numerous cancers (reviewed in references 2 and 8). Our findings demonstrate a key role for Rb/E2F-regulated expression of neogenin. Contributing to neuronal migration gives rise to the possibility that Rb-mediated mechanisms may regulate expression of migration-related genes during steady-state events such as neurogenesis. The deregulation of these processes may contribute to facets of tumor progression that expand from the typical aberrant S-phase entry associated with Rb loss of function. Further exploration of this hypothesis in the context of tumorigenesis could lend new insight into our understanding of the mechanisms of Rb-mediated tumor suppression.

In conclusion, our results suggest that Rb/E2F is required for the regulation of neogenin during neuronal migration. Further, these results provide strong support to our overall hypothesis that Rb acts through E2F to mediate events distinct from cell cycle progression by regulating transcription of genes that are not classical E2F targets.

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