Cyclic adenosine monophosphate and rho guanine triphosphatase signaling in the guidance of axons to netrin-1

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It is hard to predict how science is going to turn out,
and if it is really good science it is impossible to predict.

- Lewis Thomas
'The Hazards of Science'

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ABSTRACT

The adult nervous system is a network of neurons connected to each other by thin processes, called axons. During development, axons are guided to their targets by patterned chemical cues. The leading tip of an axon, called the growth cone, pulls the axon to its target with cell surface receptors that sense these cues and trigger biochemical cascades which extend, retract or turn the axon. This thesis examines mechanisms involved in the guidance of axons to the cue netrin-1. I test the hypothesis that netrin-1 may function as an adhesive ligand. As well, I examine the role of two signaling pathways, the cyclic adenosine monophosphate (cAMP) and the rho guanine triphosphatase (Rho GTPase), in the guidance of axons to netrin-1.

I report that neurons efficiently attach to netrin-1 and that their growth cones display morphological changes consistent with its function as an adhesive ligand. Inhibition of the RhoA subfamily of Rho GTPases or elevation of cAMP levels increases the plasma membrane presentation of netrin-1's receptor DCC and promotes axon outgrowth and turning to netrin-1. This observation is consistent with the possibility that these two biochemical pathways are linked. However, an important difference exists in how these pathways are regulated by netrin-1; while netrin-1 inhibits RhoA activation, it does not affect cAMP levels.

Studies by others have reported that inhibiting RhoA activity or elevating cAMP concentration promotes axon regeneration following injury. These studies did not, however, examine the axon's guidance decisions past the injury site; while overcoming an inhibitory injury site likely involves ignoring or switching the response to inhibitory cues, guidance to their appropriate targets requires axons to sense and respond appropriately to the cues in their environment. Results presented here indicate that inhibiting RhoA or augmenting cAMP levels promote axonal attraction to the netrin-1 guidance cue.

RÉSUMÉ

Le système nerveux adulte comprend un réseau de neurones connectés les uns aux autres par des prolongements minces que l'on nomme des axones. Pendant le développement, les axones sont guidés vers leurs cibles par des signaux moléculaires. L'extrémité d'un axone, appelé cône de croissance, tire celui-ci vers sa cible grâce à des récepteurs de surface qui détectent les signaux moléculaires environnementaux et déclenchent des cascades biochimiques qui font s'allonger, se rétracter ou tourner l'axone. Cette thèse examine les mécanismes impliqués dans le guidage des axones vers le signal moléculaire nétrine-1. J'ai investigué l'hypothèse voulant que nétrine-1 puisse fonctionner comme ligand adhésif. De plus, j'ai examiné le rôle de deux cascades biochimiques : l'adénosine monophosphate cyclique (cAMP) et la rho guanine triphosphatase (Rho GTPase), dans le guidage de l'axone vers nétrine-1.

Le présent travail démontre que les neurones s'attachent efficacement à nétrine-1 et que leurs cônes de croissance axonaux affichent des changements morphologiques conformes à une fonction de ligand adhésif pour nétrine-1. L'inhibition de RhoA, une sous-famille de Rho GTPases, ou l'élévation des niveaux de cAMP augmente la présentation du récepteur de nétrine, DCC, à la surface de la cellule, promeut la croissance axonale et fait tourner l'axone vers nétrine-1. Ces observations suggèrent la possibilité que ces deux voies biochimiques soient inter-reliées. Cependant, une différence importante existe dans la façon dont ces voies biochimiques sont régulées par nétrine-1 : nétrine-1 diminue l'activation de RhoA, mais n'affecte pas les niveaux de cAMP. Des études précédentes avaient rapporté que le fait de diminuer l'activité de RhoA ou d'élever la concentration de cAMP promeut la régénération des axones après une lésion. Cependant, ces études n'ont pas examiné les décisions de l'axone, une fois passé le site de lésion : pour traverser un site de lésion, l'axone doit probablement négliger certains signaux inhibiteurs, mais son guidage vers ses cibles exige qu'il détecte et réponde de façon appropriée aux signaux environnementaux pertinents. Les résultats présentés ici indiquent que diminuer l'activité de RhoA ou élever la concentration de cAMP promeut l'attraction axonale à nétrine-1.

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LIST OF ABBREVIATIONS

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
DCC-fc: Recombinant protein of the extracellular domain of mouse DCC and the Fc portion of human IgG_1
DD: Death domain
DRG neurons: Dorsal root ganglion neurons
EB: Epidermoblasts
ECM: Extracellular matrix
EGF: Epidermal growth factor
ELISA: Enzyme-linked immunoSorbent assay
Ena/Vasp: Enabled/vasodilator-stimulated phosphoprotein
F-actin: Filamentous actin
FA: Focal adhesion
FAK: Focal adhesion kinase
FBS: Fetal bovine serum

FITC: Fluorescein-conjugated
FN3: Fibronectin type 3
FSK: Forskolin
GAP: GTPase activating protein
GDP: Guanine diphosphate
GDI: Guanine nucleotide dissociation inhibitor
GEF: Guanine nucleotide exchange factor
GFP: green fluorescent protein
CRIB: Cdc42/Rac interactive-binding
GST-RDB: Recombinant protein of glutathione-S-transferase and the Rho binding domain of rhotekin
GTP: Guanine triphosphate
GPI: glycosylphosphatidylinositol
HBSS: Hanks' balanced salt solution
HEK293T: Human embryonic kidney 293T
ICD: Intracellular domain
Ig: Immunoglobulin
IP3: Inositol triphosphate
LHRH neurons: Luteinizing hormone-releasing hormone neurons
LRR: Leucine rich repeats
MAG: Myelin associated glycoprotein
Mena: Mouse enabled
MN: Motoneuron
MOI: multiplicity of infection
MT: Microtubule
NCAM: Neural cell adhesion molecule
NCK: Non-catalytic region of tyrosine kinase adaptor protein
NG108-15: Neuroblastoma glioma 108-15
NGL: Netrin G ligand
NgR: Nogo receptor
N-WASP: Neuronal Wiskott-Aldrich syndrome protein
OMGP: Oligodendrocyte myelin glycoprotein
PAK: p21-activated kinase
PBS: Phosphate buffered saline
PC12 cells: Pheochromocytoma cells
PCOLCE: Type I C-proteinase enhancer proteins
PDL: Poly-D-lysine
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
SN: segmental nerve
sFRP: Secreted frizzled-related proteins
SRC: Rous sarcoma oncogene
TAG-1: transient axonal glycoprotein-1
TeTx: Tetanus toxin
TIMP: Tissue inhibitors of metalloproteinases
Tsp: Thrombospondin
UNC: Uncoordinated
X. laevis: Xenopus laevis

CONTRIBUTION OF AUTHORS

CHAPTER 1: ***Literature Review I - Axon Guidance during Development and Regeneration***

- **Simon W. Moore:** Drew all figures and co-wrote manuscript.

- **Timothy E. Kennedy:** Co-wrote manuscript.

CHAPTER 2: ***Literature review II - Netrins and their receptors***

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- **Marc Tessier-Lavigne:** Edited manuscript.

- **Timothy E. Kennedy**: Co-wrote Manuscript.

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- **Simon W. Moore:** Dissected spinal commissural neurons for dispersed (Figures 3.6) and explant culture (Figure 3.7). Dissected, cultured, immunofluorescently labeled and imaged E12 spinal cords (Figure 3.10). Drew figure 3.10B and edited manuscript.

- **Nicolas X. Tritsch:** Cultured explants (Figure 3.7) and edited manuscript.

- **Philippe P. Roux:** Performed some biotinylation experiments (Figure 3.8).

- **Masoud Shekarabi:** Developed dispersed rat spinal commissural neuron cultures.

- **Philip A. Barker:** Provided expertise during paper redaction.

- **Timothy E. Kennedy:** Dissected spinal commissural neurons for dispersed (Figures 3.1-3.6, 3.8) and explant culture (Figure 3.7, 3.9). Confocal imaging (Figure 3.1B), developed rational and co-wrote manuscript.

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CHAPTER 1

LITERATURE REVIEW I

Axon Guidance during Development and Regeneration

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PREFACE

This chapter is reprinted from the Textbook of Neural Repair and Rehabilitation, edited by Michael Selzer, Stephanie Clarke, Leonardo Cohen, Pamela Duncan and Fred Gage (Moore and Kennedy, 2006a)**.** General principles of axon guidance during development are reviewed. Of particular relevance to this thesis are descriptions of: (1) Rho GTPases and cAMP in axon guidance during development and regeneration, and (2) spinal commissural axon extension – a model system used throughout this thesis whose dissection and culture are described in Appendix I.

INTRODUCTION

During neural development, many neurons must extend an axon across a relatively large distance in order to reach their targets and make appropriate synaptic connections. Several models were contemplated during the 20th century to explain axon guidance. Late in the 19th century, Santiago Ramón y Cajal proposed a chemotropic model (Ramón y Cajal, 1892), speculating that axons reach their targets by sensing molecular cues. Later, based on observations of live neurons in cell culture, Ross Granville Harrison and Paul Weiss put forth a stereotropic model, proposing a form of mechanical guidance whereby axons respond to relatively non-specific physical constraints. This was inspired by finding that axons tend to follow mechanical discontinuities on a substrate, such as scratches on the bottom of a glass cell culture dish (Harrison, 1914;Weiss, 1934). Paul Weiss

elaborated on this model by proposing the resonance principle, which argues that a rough layout of neuronal connections established by stereotropism is subsequently refined by matching an axon's electrical activity with that of its target (Weiss, 1941). It was not until the early 1940s that Roger Sperry, a student of Paul Weiss, revived the hypothesis that chemical cues direct axon growth by demonstrating that axons regenerating along the frog optic nerve reconnect with their original targets in the tectum (reviewed in Sperry, 1963). Many subsequent studies, utilizing a variety of organisms and systems have established that, although activity may refine neuronal connections once they have been established, molecular cues are the major influence directing axons to their targets during development.

This chapter provides an overview of molecular mechanisms that guide axon extension during neural development. It begins by introducing the growth cone, a specialized motile structure at the tip of the axon responsible for sensing and responding to guidance cues. This is followed by a description of the trajectory of embryonic spinal commissural axons, which serves to illustrate fundamental characteristics of axon guidance. A brief overview of key axonal guidance cues is then presented, followed by a description of our growing understanding of the cellular and molecular mechanisms that transduce extracellular guidance cues into directed axon growth. The chapter concludes with a brief discussion of the possibility that cues now known to regulate axon guidance during development may subsequently influence axon regeneration in the adult CNS.

THE GROWTH CONE

The growth cone at the tip of an axon is a motile structure that is exquisitely sensitive to guidance cues in its environment. Santiago Ramón y Cajal, who gave the growth cone its name, described it as "a concentration of protoplasm of conical form, endowed with amoeboid movements" (Ramón y Cajal, 1890). While Ramón y Cajal hypothesized that the growth cone was a motile structure from his studies of fixed tissue, direct evidence of this was provided by Ross Granville Harrison in 1907 based on his examination of neurons extending axons into a three dimensional matrix in cell culture (Harrison, 1907). Notably, this first report provided an indication of just how motile

growth cones can be, with Harrison commenting that, "close observation reveals a continual change in form, especially as regards the origin and branching of the filaments. In fact the changes are so rapid that it is difficult to draw the details accurately." The first description of growth cones imaged within a living organism, a frog tadpole, was provided by Carl Caskey Speidel in 1933 (Speidel, 1933).

The peripheral domain of neuronal growth cones is made up of filopodia and lamellipodia, highly dynamic membrane protrusions at the motile leading edge of many cells (reviewed in Bentley and O'Connor, 1994). Filopodia are thin finger-like extensions that can reach out dozens of microns to probe the surrounding environment. Lamellipodia are flattened veils of ruffling membrane between the filopodia (Figure 1.1A). Disruption of these structures causes errors in axon guidance (Keshishian and Bentley, 1983;Bentley and Toroian-Raymond, 1986;Chien et al., 1993;Zheng et al., 1996). Conversely, contact of the tip of a single filopodium with an appropriate extracellular target is sufficient to cause a growth cone to turn (O'Connor et al., 1990;Chien et al., 1993), indicating that receptors for guidance cues are present, and perhaps enriched, at the tips of growth cone filopodia.

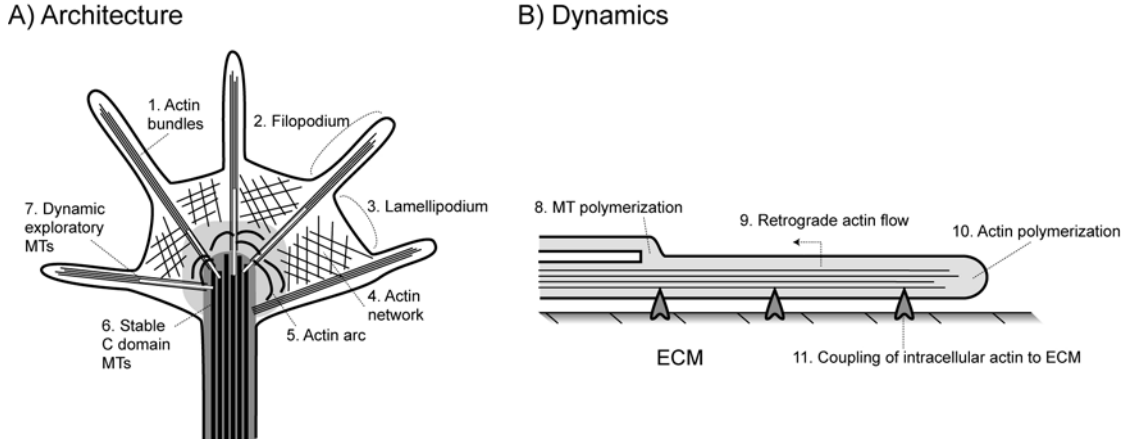

Figure 1.1 - Architecture and Dynamics of the Growth Cone Cytoskeleton: *The growth cone is composed of three domains: a central domain (dark shading), a peripheral domain (no shading) and transition zone (light shading). F-actin bundles (1) are located in filopodia (2), F-actin networks (4) in the peripheral domain and F-actin arcs (5) in the transition zone. F-actin polymerization (10) occurs at the leading edge of filopodia (2) and lamellipodia (3). F-actin networks and bundles move by retrograde flow towards the central domain of the growth*

cone (9). Dynamic unbundled MTs (7) polymerize into the peripheral domain (8) along filopodial F-actin bundles and are simultaneously cleared by depolymerization and coupling to retrograde F-actin flow (9). Protrusions are stabilized by bridging intracellular F-actin to the extracellular matrix (ECM) (11).

Growth cone morphology is a direct consequence of the organization of the two main components of its cytoskeleton, microtubules and filamentous actin (F-actin, Figure 1.1A). Both F-actin and microtubules are polarized polymers, both are tightly regulated, and both are required to be stable at some times and dynamic at others (Schaefer et al., 2002;reviewed in Dent and Gertler, 2003). Microtubules form a dense parallel array in the axon shaft and splay apart as they enter the growth cone (Letourneau, 1983;Forscher and Smith, 1988;Dailey and Bridgman, 1991;Tanaka and Kirschner, 1991). Although microtubules are the major cytoskeletal element of the axon shaft and the central domain of the growth cone (Figure 1.1A), they continuously probe into the growth cone periphery and will even extend into filopodia (Schaefer et al., 2002). In contrast, F-actin is concentrated in the peripheral domain of growth cones where it is arranged in two types of arrays: extended parallel bundles form the core of filopodia, while a meshwork underlies lamellipodia (Figure 1.1A). Like microtubules, actin filaments are also polarized, and grow through polymerization of their barbed end located near the membrane. A retrograde flow of F-actin travels back from the leading edge of growth cone filopodia and lamellipodia (Figure 1.1B, Forscher and Smith, 1988;Welnhofer et al., 1997;Mallavarapu and Mitchison, 1999). This retrograde flow can be slowed or stopped if a receptor that is linked intracellularly to F-actin becomes bound to an immobilized extracellular ligand, such as a component of the extracellular matrix (ECM). Reducing retrograde flow in this way will promote local extension due to the polymerization of F-actin that builds out the cytoskeleton and supports a leading edge of membrane (reviewed by Suter and Forscher, 2000). As growth cones probe their environment through fits of polymerization and depolymerization that extend and retract filopodia and lamellipodia, guidance in one direction or another is thought to occur through selective stabilization of

these F-actin based membrane protrusions on one side, coupled with the withdrawal and collapse of the trailing edge on the opposite side.

AXON GUIDANCE DURING DEVELOPMENT

An axon seeking its target faces enormous challenges in the embryo. Not only must it correctly interpret a multitude of cues present in a very rich environment, but the distance separating it from its final destination can be relatively large. Axons appear to use three main strategies to reach their goal: they extend early during development when distances are smaller, they utilize intermediate targets that break up long complex trajectories into smaller more manageable steps, and axons that extend later in development often fasciculate with and follow earlier pioneer axons. Factors that influence axon extension can be broadly divided into permissive and instructive cues. Permissive and non-permissive cues either promote or inhibit axon extension respectively, but without necessarily exerting a directional influence on axon growth. In contrast, an instructive cue directs axon extension, either attracting or repelling the growth cone. It has also been useful to describe axon guidance cues as having either short-range or long-range functions (Tessier-Lavigne and Goodman, 1996). Short-range refers to cues that remain close to or attached to the surface of the cell that synthesized them. These include membrane associated secreted proteins and transmembrane guidance cues. In contrast, a secreted long-range cue may be presented to a growth cone many cell diameters from the cell that produced it. In some cases, a gradient of an axon guidance cue may be established by graded expression of a short-range cue across a field of cells. Alternatively gradients may be generated by secretion of a long-range cue that polarizes the embryonic neural epithelium.

To illustrate the mechanisms employed by extending axons, we describe the trajectory followed by embryonic spinal sensory interneurons that pioneer the ventral commissure. This axonal projection can be broken into at least five distinct steps as illustrated in Figure 1.2A: (1) Initially, commissural axons are repelled ventrally along the lateral edge of the embryonic spinal cord by BMP7 and GDF7, members of the Bone Morphogenetic family of proteins secreted by the roof plate at the dorsal midline, an

example of long-range chemorepulsion (Augsburger et al., 1999;Butler and Dodd, 2003). (2) Complementary to this, netrin-1 and sonic hedgehog secreted by the floor plate attract commissural axons to the ventral midline, illustrating long-range chemoattraction (Kennedy et al., 1994;Serafini et al., 1994;Charron et al., 2003). (3) As the axons cross the floor plate, a contact mediated interaction between the cell adhesion molecules tag-1 (called axonin-1 in chick) on the growth cone and Nr-CAM on floor plate cells is required for commissural axons to traverse the midline (Stoeckli et al., 1997), a short-range permissive action of these cues.

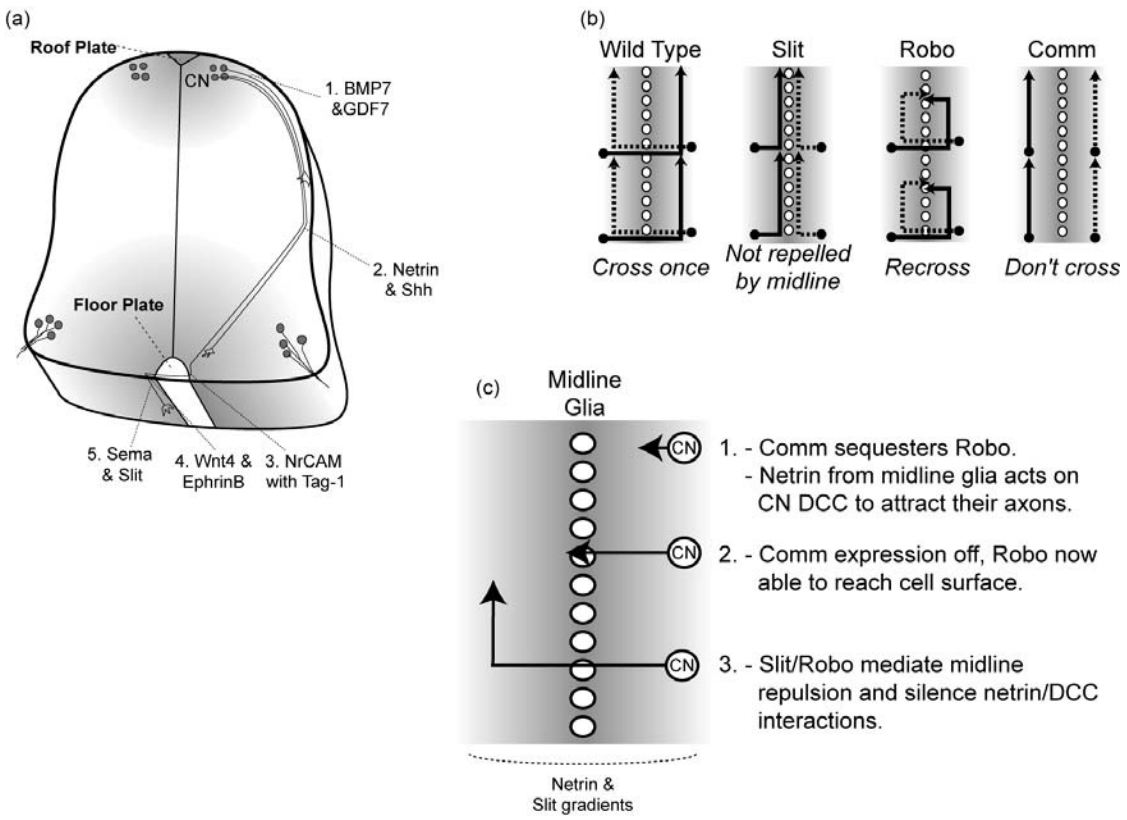

Figure 1.2 - Midline Guidance of Vertebrate and Drosophila Commissural Neuron Axons: *(A) The five stages of spinal commissural neuron (CN) axon guidance in vertebrates. (B) Schematic of the behavior of CN axons in Comm, Slit and Robo genetic mutants (adapted from Kidd et al., 1999). (C) Summary of the mechanisms regulating the response to guidance cues in Drosophila commissural neuron axon extension.*

(4) Once the axons have crossed to the contralateral side of the developing spinal cord, most extend longitudinally towards the head. Although the mechanisms regulating this turn are not well understood, expression of B-class ephrins and Wnt4 by the floor plate are implicated (Imondi and Kaprielian, 2001;Lyuksyutova et al., 2003). (5) As they extend longitudinally, commissural axons are directed by Semaphorin and Slit family members, secreted repellents that prevent them from re-crossing the midline and direct them out of the gray matter where they fasciculate into different longitudinal tracks (Zou et al., 2000;Long et al., 2004). These cues are described in more detail below, but the point to be made here is that commissural axons make their way along a complex trajectory by sequentially responding to guidance cues that are precisely positioned in the developing neural epithelium, first being directed circumferentially, then across the ventral midline, and finally longitudinally toward their ultimate synaptic targets.

AXON GUIDANCE CUES AND THEIR RECEPTORS

Although multiple families of axon guidance cues have been identified and their number continues to increase, the diversity of known cues still seems small in light of the immense complexity of the nervous system. The following provides an overview of several well-described families of axon guidance proteins, illustrating the range of molecules now known to direct axons to their targets.

Laminins

Multiple extracellular matrix (ECM) components influence axon extension during neural development (reviewed in Reichardt and Tomaselli, 1991). Among these, the laminin family is notable for several reasons. Many types of neurons, derived from either the CNS or PNS, readily extend axons on laminin, and laminin-1 is very commonly used as a permissive substrate that promotes axon outgrowth in cell culture. Laminins are a major component of basement membranes, a layer of ECM at the base of epithelia (Colognato and Yurchenco, 2000). Notably, the basal lamina secreted by Schwann cells in peripheral nerves promotes axon regeneration following injury (Ide et al., 1983). Depletion of laminin from preparations of peripheral nerve myelin substantially reduces

its capacity to promote axon growth, indicating that laminin is a key component of peripheral nerve basal lamina responsible for promoting regeneration. Interestingly, preparations of CNS myelin that are potent inhibitors of axon growth, actually promote axon growth following the addition of exogenous laminin-1, indicating that laminin-1 is a powerful stimulant of axon extension that can mask some of the growth inhibitory properties of CNS myelin (David et al., 1995).

Laminins are secreted as large cruciform heterotrimers made up of one α, one β and one γ subunit (Figure 1.3; reviewed in Beck et al., 1990;Engvall and Wewer, 1996). Ten different laminin chains and at least 12 different heterotrimers have been documented *in vivo* (reviewed in Erickson and Couchman, 2000). Multiple laminins are expressed early during embryonic development (Lentz et al., 1997). Among many important functions, they influence neural crest cell migration, Schwann cell migration, axon extension, and nerve-muscle synapse formation. Mutations of genes encoding specific laminins indicate that they make numerous essential contributions to the development of both the central and peripheral nervous systems (Colognato and Yurchenco, 2000). Although laminins are secreted proteins and axons will migrate up a gradient of a peptide fragment of laminin-1 (Adams et al., 2005), evidence for laminin gradients directing axon extension *in vivo* has not been obtained (McKenna and Raper, 1988;Matsuzawa et al., 1998;Dertinger et al., 2002).

Multiple proteins interact with laminins. Of particular importance as laminin receptors are the integrins, a large family of receptors for ECM proteins (Figure 1.3). Integrins are transmembrane hetero-dimers composed of combinations drawn from at least 16 α and 8 β subunits. They are linked intracellularly to F-actin and by acting as a transmembrane bridge between the ECM and the cytoskeleton, integrins function as key regulators of cell-ECM adhesion and of cell motility, including growth cone motility (Belkin and Stepp, 2000).

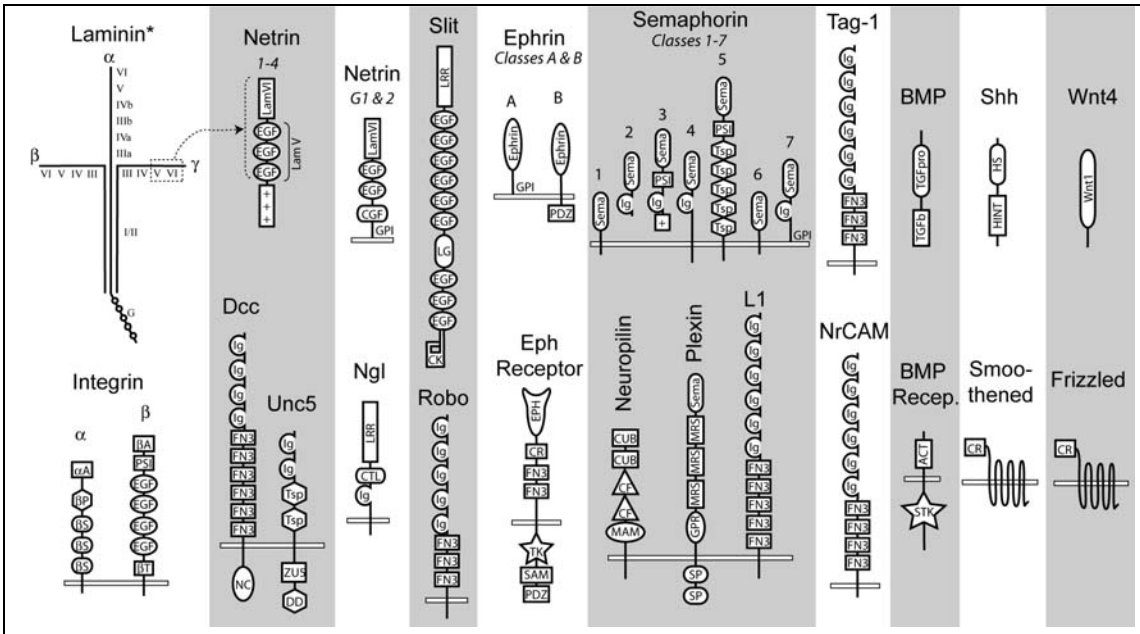

Figure 1.3 - Axon Guidance Cues and their Receptors: *For membrane proteins, domains above the plasma membrane are extracellular, while those below it are intracellular. *Due to its size, the domains of laminin are omitted. Domain abbreviations: ACT: Activin types I and II receptor; LG: Lamin G (also known as an ALPS spacer, Agrin, Laminin, Perlecan and Slit); βP: β propeller; βS: β sandwich; βT: β tail; CF: Coagulation Faction V, VIII homology; CGF: Cripto growth factor; CK: Cysteine knot; CR: Cysteine rich; CTL: Carboxy terminal leucine rich repeat; CUB: Complement binding; ; DD: Death Domain; EGF: Epidermal Growth Factor; EPH: Ephrin binding; Ephrin: Eph receptor binding; FN3: Fibronectin type III; GPI: Glycosyl Phosphatidyl Inositol anchor; Hint: Hedgehog/Intein; HS: Hedgehog signaling; Ig: Immunoglobulin; LRR: Leucine rich repeats; GPR: Glycine-proline rich region; LamVI: Laminin N-terminal domain VI; LamV: Laminin N-terminal domain V; MAM: Meprin, A5, Mu domain; MRS: Met related sequence; NC: Neogenin C-terminus; PSI: Plexins, Semaphorins and Integrins; SAM: Sterile alpha motif; SP: Sex-Plexin, STK: Serine/Threonin kinase; TGFb: Transforming growth factor beta like; TGFpro: TGF-beta propeptide; TK: Tyrosine kinase; Tsp: Thrombospondin type I; +: basic/positively charged. Domains were drawn based on NCBI conserved domain database.*

Netrins

Netrins, named for the Sanskrit word meaning 'one who guides', are a small family of axon proteins that direct axon outgrowth during embryogenesis. They are

bifunctional, attracting some axons and repelling others. Six netrins have been identified in vertebrates: netrins 1-4 and netrins G1 and G2 (reviewed in Manitt and Kennedy, 2002). All are ~75 kDa glycoproteins, with sequence homology to the amino terminus of laminins (Figure 1.3). Netrins 1-4 are secreted proteins, while netrins G1 and G2 contain a GPI (glycosylphosphatidylinositol) that attaches them to the plasma membrane. Although netrins 1-4 are secreted, their carboxyl terminal domain, a domain unrelated to laminins, contains many charged amino acids and netrin-1 binds heparin with high affinity (Kappler et al., 2000). Consistent with this, the majority of netrin-1 in the CNS is bound to cell surfaces and ECM (Serafini et al., 1994;Manitt et al., 2001;Manitt and Kennedy, 2002).

The functional assays used to identify netrins were based on the proposal, and subsequent demonstration, that the axons of embryonic spinal commissural neurons are attracted to the ventral midline of the neural tube by a cue secreted by the floor plate (Ramón y Cajal, 1899;Weber, 1934;Tessier-Lavigne et al., 1988;Placzek et al., 1990). Consistent with this, a source of netrin-1 attracts commissural axons, and netrin-1 is strongly expressed by floor plate cells at the ventral midline of the embryonic neural tube (Kennedy et al., 1994). Furthermore, netrin-1 is required for formation of the corpus callosum, hippocampal commissure and ventral spinal commissure, indicating that it is essential for the normal development of multiple axonal projections to the ventral midline of the developing CNS (Serafini et al., 1996).

Candidate netrin receptors were first identified genetically in *C. elegans*. *Unc-5* mutation caused defects in axon trajectories directed away from netrin expressing cells, while mutation of *unc-40* caused defects in axon extension toward these cells. Mutation of the *C. elegans* netrin homologue *unc-6* produced defects in both trajectories (Hedgecock et al., 1990;Ishii et al., 1992;Wadsworth et al., 1996). Both *unc-40* and *unc-5* encode transmembrane Ig superfamily members (Leung-Hagesteijn et al., 1992;Chan et al., 1996). DCC (deleted in colorectal cancer), the mammalian homologue of unc-40 (Keino-Masu et al., 1996), binds netrin-1, is expressed by spinal commissural neurons and is required for chemoattractant responses to netrin-1 (Fazeli et al., 1997). Members of the Unc-5 homologue family also bind netrin-1 and mediate the repellent response to

netrin-1. Four have been identified in mammals, Unc5h1 to 4, (Ackerman et al., 1997;Leonardo et al., 1997;Engelkamp, 2002). Many neurons express both an Unc5 homologue and DCC. The two classes of receptors interact, forming a netrin receptor complex, and neurons that express both can respond to netrin-1 as an attractant or a repellent (Hong et al., 1999). Interestingly, integrins have recently been shown to bind to the extreme carboxyl-terminus of netrin-1 (Yebra et al., 2003), however a role for this interaction in axon guidance has not been demonstrated.

Slits

A key challenge for axons that cross the midline during development is that once they have crossed to the contralateral side of the CNS, they must remain crossed and ignore the cues that directed them to the midline. An interesting group of mutations in *Drosophila melanogaster* led to the initial molecular insights into this process. The *Drosophila Slit* mutant phenotype was first identified over 20 years ago (Nusslein-Volhard et al., 1984) and then cloned in 1988, but its role in axon guidance was not appreciated until ten years later (Rothberg et al., 1988;Li et al., 1999;Brose et al., 1999;Kidd et al., 1999). The ventral nerve cord of a fly embryo is composed of symmetrical longitudinal projections connected by a series of commissures, making a ladder like structure (Figure 1.2B). In *Slit* loss of function mutants, the commissures disappear and the longitudinal projections merge (Kidd et al., 1999). *Slit* is expressed by specialized midline glia at the ventral midline of the *Drosophila* CNS and encodes a large secreted protein composed of leucine rich repeats (LRR), EGF repeats, and a laminin G domain (Rothberg et al., 1990). Slit is an essential midline repellent, that inhibits ipsilaterally projecting neurons from approaching the midline and prevents contralaterally projecting neurons from recrossing (Kidd et al., 1999;Simpson et al., 2000;Rajagopalan et al., 2000).

A complementary mutation, *Roundabout* (*Robo*), identified the Slit receptor (Seeger et al., 1993). In *Robo* mutant fly embryos, axons that would normally project ipisilaterally and contralaterally instead cross and recross the midline repeatedly (Figure 1.2B). Named after the circular roundabouts found at British intersections, loss of Robo

function generates a phenotype where the CNS collapses into a series of circles that are essentially repeated commissural crossings that go nowhere.

Robo is a single pass transmembrane protein that binds Slit. In mammals, three Robo homologues, Robo1, Robo2, and Rig-1, and three Slits, Slit1-3, have been identified (Taguchi et al., 1996;Holmes et al., 1998;Itoh et al., 1998;Brose et al., 1999;Yuan et al., 1999). Analogous to their role in *Drosophila*, Slits are expressed in the ventral embryonic spinal cord where they repel ipisilaterally projecting axons and prevent recrossing by contralaterally projecting axons (Long et al., 2004). Slits also regulate axon branching (Wang et al., 1999b) and are important guidance cues for axons in the dentate gyrus of the hippocampus, olfactory bulb, and retina (Nguyen Ba-Charvet et al., 1999;Li et al., 1999;Erskine et al., 2000;Long et al., 2004).

Semaphorins

Semaphorins, named after semaphore, a flag based method of signaling once used between ships and along railroads, constitute a large family of secreted and membrane associated proteins. The first evidence that Semaphorins might function as axonal chemorepellents was provided by the demonstration that collapsin-1, subsequently named Semaphorin 3A, could collapse sensory ganglion growth cones *in vitro* (Luo et al., 1993). The semaphorin family is divided into 8 subclasses. Four of these (classes 3-7) are found in vertebrates and play major roles as axon guidance cues during neural development (reviewed in Raper, 2000). Classes 1 and 2 are expressed in invertebrates. Interestingly, class 'V' is viral. All semaphorins share a characteristic 500 amino acid 'sema' extracellular domain, and may be secreted, transmembrane, or GPI-linked (Figure 1.3). Secreted class III semaphorins are well characterized for their role organizing the central projections of dorsal root ganglion sensory neurons into different laminae of the embryonic spinal cord (Messersmith et al., 1995). Although they are best understood for their role as repellents that affect axon steering, fasciculation, and branching (reviewed in Kolodkin and Ginty, 1997;de Wit and Verhaagen, 2003), like many axon guidance cues, they are bifunctional and also promote the growth of some axons (Wong et al., 1997;Song et al., 1998;Wong et al., 1999). These semaphorins signal via a receptor complex

composed of a neuropilin family member, the ligand binding component, and a plexin family member, which activates intracellular signaling (reviewed in Tamagnone and Comoglio, 2000). In addition, L1, a transmembrane Ig superfamily cell adhesion molecule, interacts with neuropilin-1 and is required for repellent responses to Sema3A (Castellani et al., 2000).

Ephrins

In the early 1940s, Roger Sperry's findings generated the chemospecificity hypothesis of axon guidance (reviewed in Sperry, 1963). Sperry's experiments took advantage of the capability of some lower vertebrates, such as frogs, to regenerate the precise array of axonal connections made between the retina and visual tectum. He demonstrated that if, following trans-section of the optic nerve, the eye was rotated 180° and reimplanted, the misaligned axons were able to find and reconnect with their original targets in the tectum. The rotated eye then generated a grossly misaligned visual signal that produced equivalently inappropriate motor responses, such as a frog jumping upward when aiming for a fly placed on the ground. The conclusion of such behavioral findings, confirmed by subsequent anatomical and physiological analyses, was that the regenerating axons found their targets by responding to precise distributions of chemical cues in the cellular environment, and not by responding to mechanical constraints, or based on activity directing the formation of appropriate connections.

It is now clear that graded expression of ephrins across the tectum, and complementary gradients of their receptors, the Eph tyrosine kinases, in the retina, play key roles directing the spatiotopic projection of the retina to the tectum. Eph receptors make up the largest family of receptor tyrosine kinases in the mammalian genome. Ephrins are either transmembrane (ephrinB1-B3) and bind EphB receptors (EphB1-B6), or GPI-linked (ephrinA1-A5) and bind EphA receptors (EphA1-A9; reviewed in Himanen and Nikolov, 2003). Graded expression of EphA receptors by retinal ganglion cells and ephrinAs in the tectum direct the topographic projection of retinal ganglion cell axons along the tectal anterior/posterior axis. Complementing this, graded expression of EphB receptors by retinal ganglion cells and ephrinBs in the tectum directs the formation of

lateral to medial projections into the tectum (reviewed in McLaughlin et al., 2003). Ephrins influence multiple CNS axonal projections including those of the vomeronasal axons, anterior commissure, corpus callosum and corticospinal tract (Drescher et al., 1995;Orioli et al., 1996;Yokoyama et al., 2001;Kullander et al., 2001;Coonan et al., 2001). Both classes of ephrins are membrane attached, and the interaction between ephrins and Eph receptors generates bi-directional signaling into both the "ligand" and the "receptor" expressing cells (reviewed in Kullander and Klein, 2002). Although ephrins have been intensively studied for their role as repellent axon guidance cues, it is now clear that they also influence adhesive interactions between cells, synaptic plasticity, cell migration, and vascular development (reviewed in Knoll and Drescher, 2002;Holmberg and Frisen, 2002). Reflecting this diversity of function, "Eph" is derived from their expression by an erythropoietin-producing human hepatocellular carcinoma cell line (Eph Nomenclature Committee, 1997), and "ephrin" from the contraction of 'Eph family receptor interacting protein', and ephoros, the ancient Greek word for overseer or controller.

Morphogens as guidance cues: Wnts, BMPs and Hedghogs

The Wingless(Wg)/Wnt, BMP (bone morphogenic protein), and Hedgehog families are all well-characterized morphogens: secreted proteins that direct target cells to adopt a particular fate. Surprisingly, members of each of these protein families have now been implicated as axon guidance cues during embryogenesis. In the embryonic spinal cord, the BMP family members BMP7 and GDF7, are secreted by roof plate cells and act as repellents that direct the initial outgrowth of commissural axons ventrally (Augsburger et al., 1999;Butler and Dodd, 2003). In contrast, Sonic hedgehog (Shh) secreted by the floor plate attracts commissural neurons to the ventral midline, and this chemoattractant response requires the Shh receptor, Smoothened (Charron et al., 2003). Wnt4 and its receptor Frizzled3 have been implicated in reorienting commissural axon growth longitudinally, once the axons have crossed the ventral midline (Lyuksyutova et al., 2003). Although known receptors for these proteins have been implicated as influencing

axon guidance in some cases, the signal transduction mechanisms underlying their effect on growth cone motility are not well understood.

REGULATION OF THE GROWTH CONE CYTOSKELETON

Growth cones integrate inputs from many guidance cues to produce directed motility. Although our understanding of the intracellular signaling mechanisms used by the receptors for guidance cues is currently incomplete, a point of convergence for guidance cue signal transduction is their influence on the organization of the growth cone cytoskeleton (Figure 1.4A). The Rho family of small GTPases are key regulators of the organization of F-actin in both neuronal and non-neuronal cells (reviewed in Hall, 1998;Dickson, 2001). They function as molecular switches that are inactive when bound to GDP and active when bound to GTP (Figure 1.4B). Cycling between GDP and GTP bound states is tightly regulated by multiple mechanisms. Guanine nucleotide exchange factors (GEFs) activate Rho GTPases by promoting the exchange of GDP for GTP. GTPase activating proteins (GAPs) inhibit Rho GTPases by promoting the hydrolysis of GTP to GDP. Guanine nucleotide dissociation inhibitors (GDIs) remove the GTPase from the membrane and prevent dissociation of GDP, thereby maintaining the GTPase in an inactive state. Downstream of the Rho GTPases, over 30 target effector proteins have been identified (reviewed in Bishop and Hall, 2000).

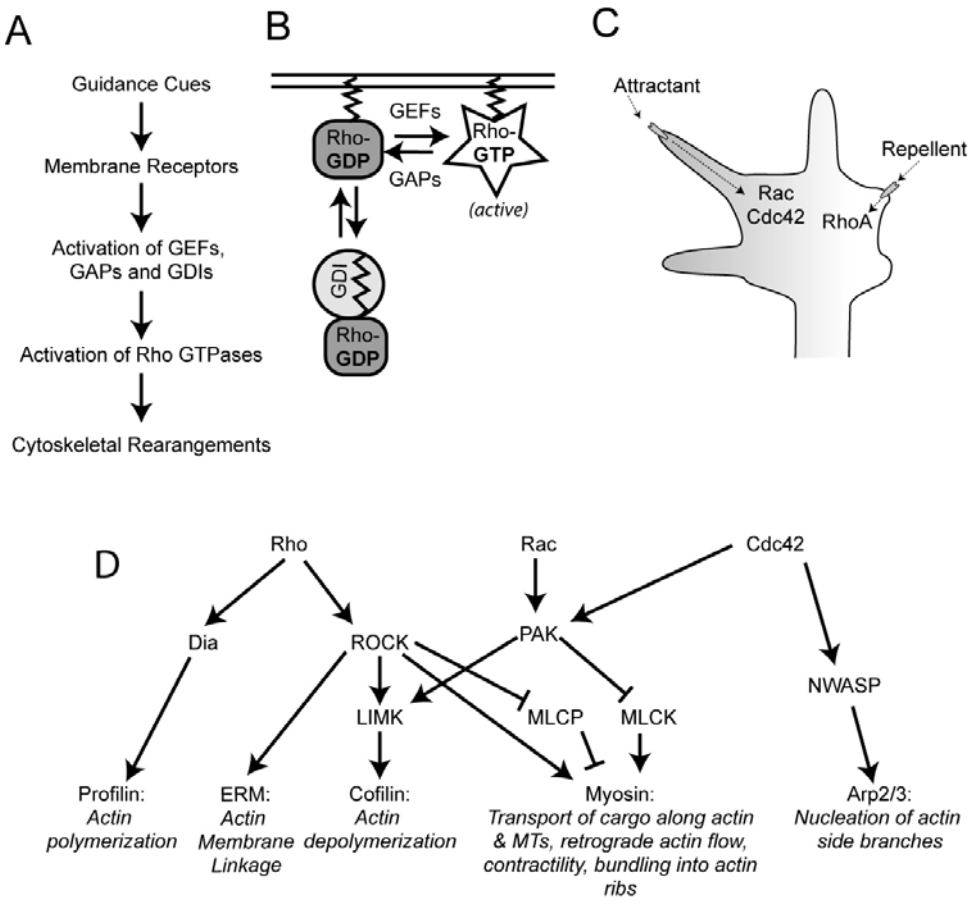

Figure 1.4 - Intracellular Signaling Mechanisms that Regulates F-Actin Architecture: *(A) Flow chart of how guidance cues affect the growth cone's cytoskeleton. (B) The cycling of Rho GTPases between inactive GDP-bound and active GTP-bound states is under the control of GEFs, GAPs and GDIs. (C) Activation of Rac and Cdc42 is correlated with a response to attractants, while activation of RhoA is correlated with a response to repellents. (D) Examples of how RhoA, Rac and Cdc42 activation can lead to remodeling of the actin cytoskeleton.*

In many cell types, the Rho family members Cdc42 and Rac1 regulate the formation of filopodia and lamellipodia, respectively. RhoA activation directs the formation of F-actin stress fibers and activates myosin contractility, potentially leading to increased retrograde flow of F-actin and process retraction. A current model suggests that attractant guidance cues will activate Cdc42 and Rac in growth cones, while repellents trigger growth cone collapse by activating RhoA (Figure 1.4C). Substantial experimental support for this model has now been obtained: netrins, ephrins, slits, and the semaphorins

all influence axon extension by signaling via the Rho GTPases and the mechanisms by which these cues regulate Rho GTPase activity in neurons are currently under intense scrutiny (reviewed in Govek et al., 2005).

MODULATING THE RESPONSE OF GROWTH CONES TO GUIDANCE CUES

As an axon extends along its trajectory, its growth cone has the capacity to rapidly change its response to local guidance cues. This is well illustrated by an interesting twist in the Robo/Slit story that first presented itself as the following paradox. If Slit prevents commissural axons from recrossing the midline, why are the axons not prevented from crossing as they approach the midline the first time? At least two mechanisms have now been identified that contribute to overcoming this challenge. In *Drosophila*, a protein named Commissureless (Comm), describing its loss of function phenotype (Figure 1.2B), regulates the vesicular traffic that carries newly synthesized Robo to the plasma membrane (Keleman et al., 2002). Before crossing the midline, commissural neurons express Comm, which targets newly synthesized Robo for degradation, and therefore the growth cone remains insensitive to Slit. In ipsilaterally projecting neurons and in commissural neurons after they have crossed the midline, Comm is not expressed. This allows newly synthesized Robo to travel unimpeded to the plasma membrane and the axons are repelled by midline derived Slit. Interestingly, a mammalian homologue of Comm has not been identified thus far. In contrast, a divergent member of the Robo family, Rig1, inhibits the ability of precrossing embryonic commissural axons to respond to Slits, although it does not appear to do this by regulating sorting analogous to Comm function in *Drosophila* (Marillat et al., 2004;Sabatier et al., 2004). In addition to turning on the response to Slit as they cross the midline, Robo binds to DCC inhibiting it, thereby silencing the response to the midline attractant netrin-1 (Stein and Tessier-Lavigne, 2001). These findings indicate that their encounter with the midline changes the commissural neurons, silencing their response to midline attractants, while activating their sensitivity to midline repellents, thereby allowing them to cross once and then preventing recrossing (Figure 1.2C).

Growth cones must rapidly respond to local guidance cues and this ability exhibits substantial autonomy from the neuronal cell body. Growth cones will even continue to migrate and respond to guidance cues hours after severing the axon, completely independent of any connection to the cell body (Shaw and Bray, 1977;Harris et al., 1987;Campbell and Holt, 2001;Brittis et al., 2002). Mechanisms that modify the response of growth cones to guidance cues include regulated presentation of receptors on the cell surface, receptor inactivation, degradation of receptors by proteolysis, and local protein synthesis in the growth cone (reviewed in Yu and Bargmann, 2001;Piper and Holt, 2004). In addition, the intracellular concentrations of the cyclic nucleotide cAMP and cGMP are key regulators of growth cone responsiveness (reviewed in Song and Poo, 1999). Decreasing the concentration of cAMP, or in some cases cGMP, in the neuron can convert attraction to repulsion (Ming et al., 1997;Hopker et al., 1999). Conversely, increasing the concentration of cAMP or cGMP can convert a repellent response to attraction. These findings suggest that extracellular cues that regulate the concentration of cAMP or cGMP in the growth cone may exert a profound influence on the response to guidance cues presented in parallel. An example of this is provided by Hopker et al (1999) who demonstrated that regulation of the concentration of cAMP in the growth cone by Laminin-1, changes the response of retinal ganglion cell axons to netrin-1 from attraction to repulsion as they exit the eye and enter the optic nerve.

A decrease in the intracellular concentration of cAMP also contributes importantly to the reduced capacity of neurons to regenerate during maturation (Cai et al., 2001). Increasing the intraneuronal concentration of cAMP, thereby activating protein kinase A (PKA), a major downstream effector of cAMP, enhances axon growth in the presence of myelin-associated inhibitors of axon extension (Cai et al., 2001), including promoting axon regeneration in the mature mammalian CNS following injury (Neumann et al., 2002;Qiu et al., 2002a). PKA induces increased expression of intracellular polyamines, which contribute to enhanced axon regeneration (Cai et al., 2002). Consistent with a requirement for changes in gene expression, the ability of PKA to promote regeneration also requires the activation of the transcription factor CREB (cAMP response element binding protein, Gao et al., 2004). PKA also recruits the netrin receptor DCC to the cell

surface enhancing axon outgrowth in response to netrin-1 (Bouchard et al., 2004). This suggests that PKA dependent regulation of the complement of receptors on the growth cone may influence the capacity of an axon to regenerate. PKA can also regulate the activity of the Rho GTPases, in particular, directly phosphorylating and inhibiting RhoA (Lang et al., 1996;Ellerbroek et al., 2003). As described below, inhibiting RhoA has a dramatic influence on the capacity of axons to regenerate in the CNS.

AXON GUIDANCE DURING REGENERATION

Inhibitors of Axon Regeneration

Although many neurons in the adult CNS have the capacity to regenerate a severed axon (David and Aguayo, 1981), maturation, and in particular myelination, in the mammalian CNS coincides with a dramatic decrease in the ability of injured axons to regenerate. CNS white matter contains multiple myelin-associated inhibitors of axon outgrowth and regeneration. Identified inhibitors include myelin associated glycoprotein (MAG), oligodendrocyte myelin glycoprotein (OMGP), and Nogo (McKerracher et al., 1994;Mukhopadhyay et al., 1994;GrandPre et al., 2000;Prinjha et al., 2000;Chen et al., 2000a;Wang et al., 2002;Kottis et al., 2002). MAG is a transmembrane immunoglobulin superfamily member expressed by myelinating glia in the PNS and CNS. OMgp is a GPI-linked membrane protein component of CNS myelin. Nogo is expressed by oligodendrocytes, but not Schwann cells, and is the protein recognized by IN-1, a monoclonal antibody that enhances axon outgrowth on substrates of myelin *in vitro* and promotes axon regeneration in the mammalian CNS (reviewed in Schwab and Bartholdi, 1996). Remarkably, these three structurally unrelated proteins all interact with the same cell surface receptor, NgR, a GPI-linked membrane protein widely expressed by neurons that was first identified as a receptor for Nogo (Figure 1.5, Fournier et al., 2001;Liu et al., 2002;Domeniconi et al., 2002;Wang et al., 2002). Although MAG, OMgp, and Nogo all influence axon extension, their functional role during development and in the intact adult CNS are not known. In addition to these three identified myelin-associated inhibitors, components of the glial scar that forms following injury, such as chondroitin sulphate

proteoglycans, are also potent inhibitors of axon regeneration (reviewed in Morgenstern et al., 2002).

RhoA Activity Inhibits Regeneration

Similar to their role downstream of axon guidance cues during neural development, the small Rho GTPases, and in particular RhoA, are thought to be a point of convergence for neuronal signal transduction mechanisms that inhibit axon regeneration in the adult. Inhibiting RhoA blocks growth cone collapse and promotes axon extension, both on myelin substrates *in vitro* and in the adult CNS following lesion (Jalink et al., 1994;Jin and Strittmatter, 1997;Lehmann et al., 1999;Winton et al., 2002;Dergham et al., 2002;Dubreuil et al., 2003;Fournier et al., 2003). Importantly, NgR, the receptor for MAG, Omgp and Nogo, forms a functional complex with the p75 neurotrophin receptor (p75NTR, Wang et al., 2002;Wong et al., 2002), a key upstream regulator of RhoA in neurons (Yamashita et al., 1999). Myelin associated inhibitors cause the intracellular domain of p75NTR to sequester a Rho-GDI (Yamashita and Tohyama, 2003) leading to the activation of RhoA. These findings support a model whereby the GPI membrane linked NgR acts as the ligand-binding component of a myelin associated-inhibitor receptor complex, leading to RhoA activation by p75NTR, growth cone collapse, and termination of axon regeneration (Figure 1.5).

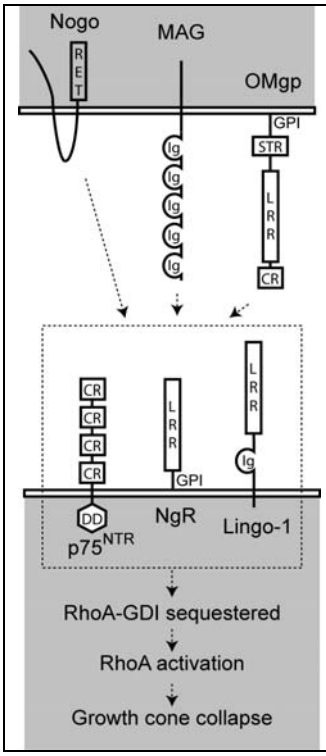

Figure 1.5 - Common Signaling Pathway of Nogo, MAG and OMgp: *Nogo, Mag and OMgp all lead to RhoA activation by acting through a receptor complex containing the GPI-linked Nogo Receptor (NgR), P75NTR and Lingo-1 (Mi et al., 2004). Intracellular space is shaded.*

Roles for Developmental Cues during Regeneration

Interestingly, several of the cues that guide axons during development are also expressed in the adult CNS, raising the possibility that they may also influence axon regeneration following injury. The semaphorins currently present the strongest case for an embryonic axon guidance cue subsequently influencing axon regeneration in the adult nervous system. Sema3A expression increases following lesion in the adult CNS, and this increased expression appears to contribute to restricting or blocking axon regeneration (Tanelian et al., 1997;Pasterkamp et al., 1998a;Pasterkamp et al., 1998b;Pasterkamp et al., 1999b;Williams-Hogarth et al., 2000). Notably, sema3A is strongly expressed by fibroblast like cells at the core of the scar that forms following lesion (Pasterkamp et al., 1999a). Additionally, sema4D, a transmembrane semaphorin shown to inhibit axon extension, is expressed by mature myelinating oligodendrocytes and strongly upregulated by oligodendrocytes at the edge of an adult spinal cord injury (Moreau-Fauvarque et al., 2003).

Netrin-1 is also expressed by mature oligodendrocytes in the adult CNS, making it a candidate myelin associated inhibitor of axon regeneration. If this is the case, neurons attempting to regenerate following injury should express Unc-5 homologues, receptors required for the repellent response to netrin-1. Both DCC and Unc5h2 expression persists in retinal ganglion cells following axotomy as their axons attempt to regenerate along the optic nerve or into a growth permissive peripheral nerve graft (Petrausch et al., 2000;Ellezam et al., 2001). Interestingly, studies carried out in lamprey, a primitive vertebrate with the ability to recover significant function following spinal cord transection (Cohen et al., 1988), have revealed a correlation between Unc-5 expression and poor axonal regeneration following lesion (Shifman and Selzer, 2000).

Although these axon guidance cues are expressed in the adult mammalian CNS, the functional significance of this expression remains unknown. An intriguing hypothesis is that proteins such as semaphorins and netrins function in the intact adult CNS as barriers that restrain axonal sprouting. During maturation of the spinal cord, neuronal expression of Unc5 homologues increases, while expression of DCC decreases, suggesting that Unc5 homologue repellent signaling may be the dominant response to

netrin in the adult spinal cord (Manitt et al., 2004) Notably, injection of antibodies that mask Nogo into the intact adult Cerebellum causes axonal sprouting of uninjured Purkinje cells (Buffo et al., 2000). These findings suggest that such cues may play an important role maintaining appropriate connections in the intact CNS by restraining inappropriate axonal sprouting. However, the price paid for this is that they subsequently inhibit the re-establishment of connections following injury.

CONCLUDING REMARKS

Overcoming the inhibition of axon growth characteristic of the adult CNS following injury is a major goal of contemporary neuroscience. Ultimately, functional recovery will require connecting regenerated axons to their appropriate targets. Although it is now clear that multiple guidance cues for axons in the embryo continue to be expressed in the adult intact CNS, the extent to which these cues will assist, block or misdirect regenerative growth, or might be manipulated to promote the regeneration of appropriate connections, remains to be determined.

CHAPTER 2

LITERATURE REVIEW II

Netrins and their Receptors

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PREFACE:

This chapter has been published online (http://www.eurekah.com/chapter/3230) and will appear in a book, edited by Dominique Bagnard, titled 'Axon Growth and Guidance' (Moore et al., 2007). As opposed to Literature Review I, this section focuses on netrins and their receptors in axon guidance. Of particular relevance to this thesis are overviews of: (1) netrin's structure and the evidence indicating that it is not freely soluble, but bound to surfaces *in vivo*, (2) signal transduction events that occur in response to netrin-1, and (3) intracellular events known to influence the guidance to netrin-1.

ABSTRACT

Netrins are a family of proteins that direct cell and axon migration during development. Three secreted netrins (netrin-1, -3 and -4) have been identified in mammals, in addition to two GPI-anchored membrane proteins, netrin-G1 and G2. Orthologues of netrin-1 play a highly conserved role as guidance cues at the midline of the developing CNS of vertebrates and some bilaterally symmetric invertebrates. In vertebrates, floor plate cells at the ventral midline of the embryonic neural tube secrete netrin-1, generating a circumferential gradient of netrin protein in the neuroepithelium. This protein gradient is bifunctional, attracting some axons to the midline and repelling others. Receptors for the secreted netrins include DCC (deleted in colorectal cancer) and the UNC5 homologues:

UNC5A, B, C and D in mammals. DCC mediates chemoattraction, while repulsion requires an UNC5 homologue and, in some cases, DCC. The netrin-G proteins bind NGLs (netrin G ligands), single pass transmembrane proteins unrelated to either DCC or the UNC5 homologues. Netrin function is not limited to the developing CNS midline. Various netrins direct cell and axon migration throughout the embryonic CNS, and in some cases continue to be expressed in the mature nervous system. Furthermore, although initially identified for their ability to guide axons, functional roles for netrins have now been identified outside the nervous system where they influence tissue morphogenesis by directing cell migration and regulating cell-cell and cell-matrix adhesion.

INTRODUCTION

The discovery of netrins can be traced back to insights provided by Santiago Ramón y Cajal at the end of the 19th century, when he proposed that axons may be guided by diffusible cues (Ramón y Cajal, 1999). Upon observing, in fixed sections, the projections of spinal commissural neuron axons towards the ventral midline of the embryonic spinal cord, he hypothesized that floor plate cells at the midline secreted a diffusible cue that established a chemotropic gradient in the neuroepithelium (Figure 2.1A). Direct evidence of chemotropic axon guidance began to accumulate in the 1980s through single cell turning assays and co-culture of explanted embryonic neural tissue (Tessier-Lavigne and Goodman, 1996). Notably, explants of embryonic rat spinal floor plate, when cultured at a distance from explants of dorsal spinal cord, evoked commissural axon outgrowth (Figure 2.1D, Tessier-Lavigne et al., 1988) and an ectopic floor plate co-cultured alongside an embryonic spinal cord attracted commissural axons, deflecting them away from their normal dorsal-ventral trajectory (Figure 2.1E, Placzek et al., 1990). These findings provided strong evidence for the existence of a chemotropic axon guidance factor(s) secreted by the floor plate.

In parallel, studies in the nematode *Caenorhabditis elegans* identified genes required for circumferential axon guidance (Brenner, 1974;Hedgecock et al., 1990). One of the genes identified, *unc-6*, encoded a secreted protein with sequence homology to laminins (Ishii et al., 1992). In 1994, using commissural axon outgrowth from explants of

embryonic rat dorsal spinal cord as a functional assay, two proteins were purified from homogenates of embryonic chick brain and discovered to be homologous to UNC-6 (Serafini et al., 1994). They were named netrin-1 and netrin-2 based on the Sanskrit word 'netr' meaning 'one who guides'. Netrin-1 is expressed by floor plate cells (Kennedy et al., 1994) and forms a gradient in the spinal neuroepithelium as commissural axons extend to the floor plate (Kennedy et al., 2006).

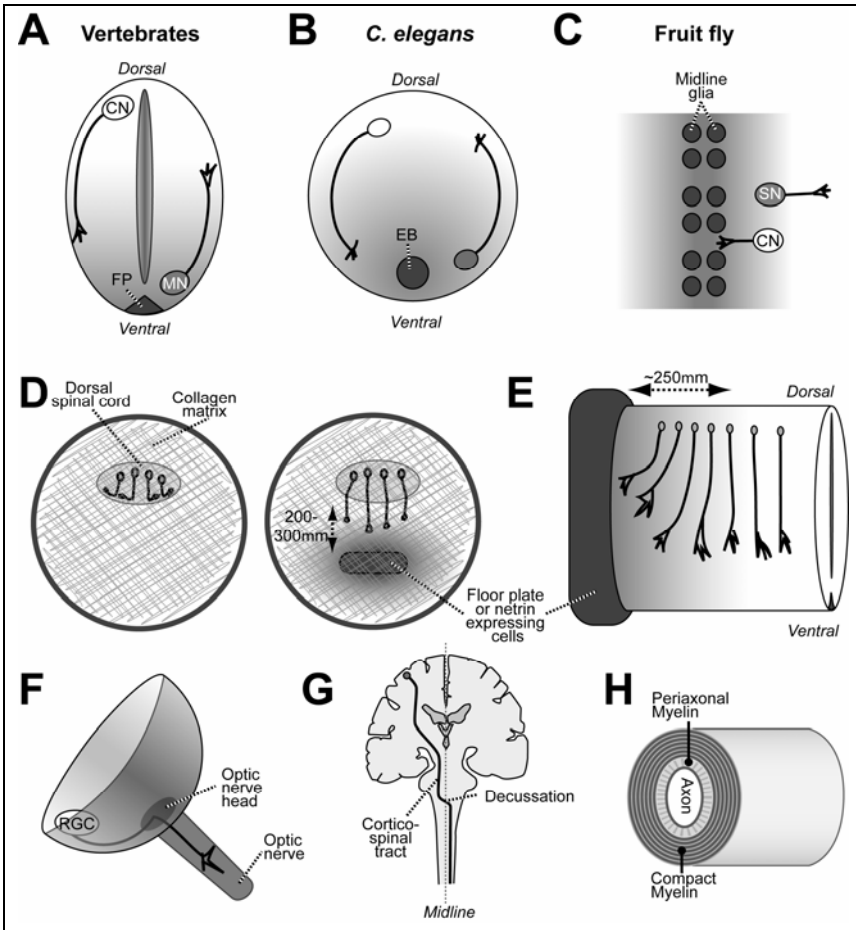
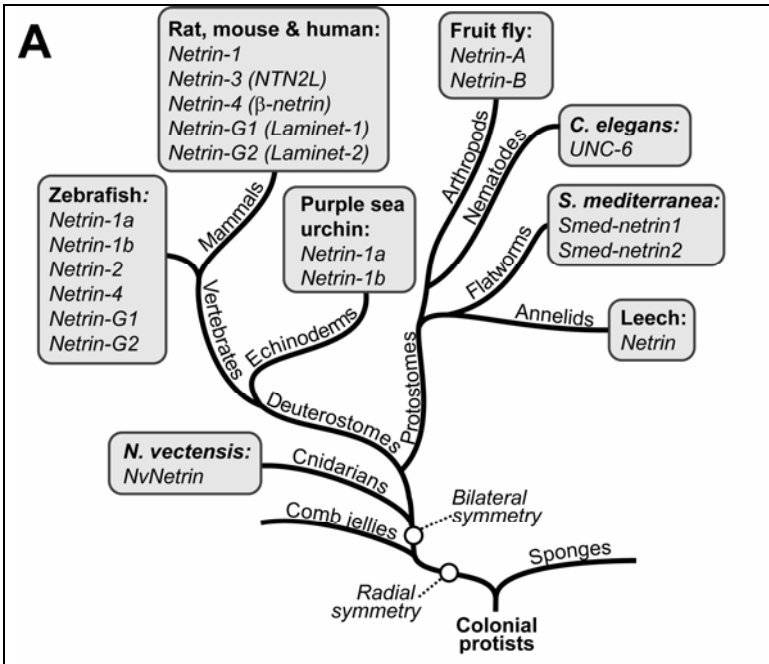


Figure 2.1 - Netrins are important midline axon guidance cues: *(A) Netrin-1 secreted by the floor plate (FP) attracts commissural neuron (CN) axons and repels motoneuron (MN) axons from the ventral midline. (B) During early neural development in C. elegans, axons are guided towards and away from a row of epidermoblasts (EB) expressing the netrin homologue UNC-6 at the ventral midline. (C) Netrin-A and -B emanating from midline glia guides commissural (CN) axons to and segmental nerve (SN) axons away from the D. melanogaster midline. (D) Embryonic spinal commissural axon outgrowth assay: An explant of dorsal embryonic rat spinal cord containing the commissural neuron cell bodies is embedded in a collagen matrix. In the absence of a source of netrin-1, such as the floor plate, the extending axons remain within the explant. In the presence of netrin-1, the axons emerge from the explant and grow into the collagen. (E) Embryonic spinal commissural axon turning assay: A segment of embryonic rat spinal cord is*

embedded into a collagen matrix and an explant of the floor plate is grafted onto one end. Neurons within ~250 μm of the ectopic floor plate turn away from their normal dorsal to ventral trajectory and grow toward the grafted floor plate. (F) Netrin-1, expressed at the optic nerve head, is required for retinal ganglion cell (RGC) axons to exit from the retina into optic nerve. (G) Netrin and its receptors DCC and UNC5C are required for the decussation of the corticospinal tract at the spinal medulla boundary. (H) In the mature mammalian CNS, netrin-1 is localized to periaxonal myelin suggesting a role regulating interactions between axonal and oligodendroglial membranes.

Engineering an aggregate of cells to express either netrin-1 or netrin-2, mimicked the commissural axon guidance activity of the floor plate (Figure 2.1D-E, Kennedy et al., 1994). Identification of the mouse ortholog of netrin-1, and generation of netrin-1 mutant mice, demonstrated that netrin-1 is essential for appropriate spinal commissural axon extension in the embryonic spinal cord (Serafini et al., 1996). In parallel, *C. elegans unc-6* was shown to be expressed at the ventral midline (Wadsworth et al., 1996) and to function as a long-range midline attractant guidance cue (Adler et al., 2006). Furthermore, two netrins, Netrin-A and Netrin-B, were implicated in midline attraction in *Drosophila* (Mitchell et al., 1996;Harris et al., 1996), although in this case netrin mediated attraction is apparently only essential at short-range close to the midline (Brankatschk and Dickson, 2006). Thus, a century after chemotropic mechanisms were proposed to direct axon guidance, netrins were identified as diffusible chemotropic cues that guide spinal commissural axon extension, with homologues implicated in long- and short-range guidance in worms and flies. Netrins are now known to function not only as attractants, but also as repellents, and to be essential for the development of numerous axonal tracts.



B

	DCC	UNC5	NGL
Mammals	Neogenin DCC	UNC5A *(UNC5h1)* UNC5B *(UNC5h2)* UNC5C *(UNC5h3, RCM)* UNC5D *(UNC5h4)*	NGL-1 NGL-2
Chicken	Neogenin cDCC	UNC5h2 cUNC5h3	
Zebrafish	zDCC zNeo	UNC5a UNC5b	
X. laevis	xDCC	xUNC5	
Fruit fly	Frazzled	UNC5	
C. elegans	UNC-40	UNC-5	
S. mediterranea	Smed-netR		

Figure 2.2 – Netrins and their receptors in various organisms: *(A) Evolutionary tree diagram highlighting the presence of netrin homologues in a wide variety of bilaterally symmetrical organisms. (B) Netrin 1-3 receptors (DCC and UNC5) and the netrin-G1 receptor (NGL1) in various organisms.*

NETRIN STRUCTURE

Netrins are highly conserved in the course of animal evolution. Illustrating this, a netrin homologue has recently been identified in the sea anemone *Nematostella vectensis*, an organism thought to exhibit some of the earliest hallmarks of bilateral symmetry (Figure 2.2A, Matus et al., 2006). Vertebrate species express the secreted netrins, netrins 1-4, and two related GPI-anchored membrane proteins, netrin-G1 and -G2 (Figure 2A). All netrins are composed of approximately 600 amino acids, and have a molecular mass of approximately 70 kilodaltons. They share two characteristic amino terminal domains, V and VI, that are homologous to domains V and VI found at the amino terminal ends of laminins (Figure 2.3A). Laminins are large secreted heterotrimers made up of α, β, and γ

subunits (Miner and Yurchenco, 2004). Domains V and VI of netrin-4 and netrin-Gs are most similar to β subunits of laminin, while those of netrins 1-3 are more similar to the γ subunits (Figure 2.3C, Yurchenco and Wadsworth, 2004).

Netrins 1, 3, 4, G1 and G2 are expressed in mammals, including rats, mice and humans, whereas orthologues of netrin-2 have thus far only been identified in chicken (Serafini et al., 1994) and zebrafish (Park et al., 2005). The amino acid sequences of netrins 1-3 are highly similar (Figure 2.3C) and, consistent with this, cellular sources of any of these proteins mimic the chemoattractant function of the floor plate (Kennedy et al., 1994;Serafini et al., 1994;Wang et al., 1999a). The sequences of netrin-4 and netrin-Gs are substantially divergent, notably exhibiting a higher degree of homology to laminins than to netrins 1-3 (Yin et al., 2000;Figure 2.3C, Koch et al., 2000;Nakashiba et al., 2000;Nakashiba et al., 2002). Orthologues of netrin-4 or the netrin-Gs have thus far only been found in vertebrates, while orthologues of netrins 1-3 have been identified in distantly related animals, including the nematode worm *C. elegans* (Ishii et al., 1992), the flatworm *Schmidea mediterranea* (Cebria and Newmark, 2005), the fruit fly *Drosophila melanogaster* (Mitchell et al., 1996;Harris et al., 1996), the leech *Hirudo medicinalis* (Gan et al., 1999) and the sea anemone *Nematostella vectensis* (Figure 2.2A, Matus et al., 2006).

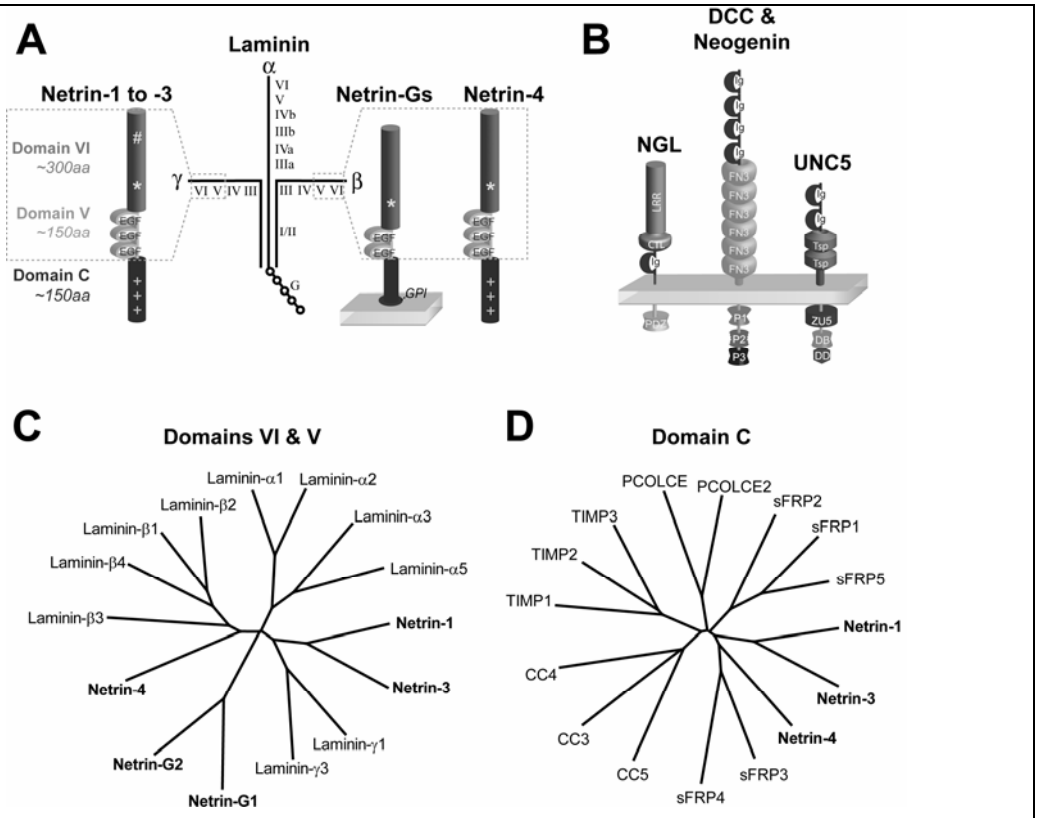


Figure 2.3 - Netrin and netrin receptor structure: *A) All netrins contain amino terminal domains V and VI related to corresponding amino terminal domains of laminins. Domain V is composed of cysteine-rich epidermal growth factor (EGF) repeats. Domain C in secreted netrins contains many positively charged, basic residues. B) DCC and UNC5 are receptors for netrin-1 to -3. Ngl1 is a receptor for netrin-G1. C) Tree illustrating a phylogenetic relationship based on sequence of the VI and V domains in human netrins and laminins. D) Phylogenetic tree based on human protein sequences related to the C domain of netrin-1 (see text for details).*

In laminins, domain VI, approximately 300 amino acids in length, is capable of binding heparin, cell surface receptors and ECM proteins (Colognato et al., 1997;Ettner et al., 1998) and is required for calcium-dependent multimerization between laminin molecules (Paulsson et al., 1988). Mutational studies carried out in *C. elegans* indicate that domain VI of netrin is critical for both axon attraction and repulsion (Lim and Wadsworth, 2002). The motif SXDXGXS/TW is present in domain VI of all netrins and mutation of these residues in the *C. elegans* netrin UNC-6 disrupts guidance functions

(Lim and Wadsworth, 2002;Yurchenco and Wadsworth, 2004). Interestingly, only the β subunits of laminin contain this motif. This is noteworthy because, as described above, netrins 1 through 3 are most homologous to the γ chain. Domain VI of netrins 1-3 also contains two cysteine residues not present in other netrins or laminins. One of these cysteines replaces a tryptophan that is strictly conserved among laminin subunits (Yurchenco and Wadsworth, 2004). Domain V of netrins contains three tandem arrays of cysteine-rich epidermal growth factor (EGF) repeats named V-1, V-2 and V-3, and is approximately 150 amino acids in size (Ishii et al., 1992). Mutation of domain V-3 in the *C. elegans* netrin UNC-6 disrupts attractant mechanisms, whereas repulsion is lost following mutation of either V-2 or V-3 domains (Wadsworth et al., 1996;Lim and Wadsworth, 2002).

Netrins 1-4 contain a conserved carboxyl terminal domain, domain C (Figure 2.3A), that has a predicted α-helical secondary structure and is homologous to domains found in the complement C3, 4 & 5 protein family (CC3, 4 & 5), secreted frizzled-related proteins (sFRP), type I C-proteinase enhancer proteins (PCOLCEs) and tissue inhibitors of metalloproteinases (TIMPs) (Figure 2.3D). Deletion of domain C from UNC-6 netrin in *C. elegans* does not appear to disrupt axon guidance, although increased axon branching has been detected (Wang and Wadsworth, 2002). Most netrin-1 protein in the vertebrate CNS is not freely soluble, but bound to cell surfaces or extracellular matrix (Manitt et al., 2001;Manitt and Kennedy, 2002). A notable feature of the netrin C domain is that it contains many basic amino acids. It has been hypothesized that these may bind to negatively charged sugars associated with proteoglycans on cell surfaces, such as heparin sulfate proteoglycans and chondroitin sulfate proteoglycans (Serafini et al., 1994;Kappler et al., 2000;Suzuki et al., 2006). Presentation of netrins closely associated with cell surfaces may be a common mode of action in the netrin family. Although the C domain is not conserved in the netrin-Gs, a C terminal GPI-link anchors them to cell surfaces.

FUNCTIONAL ROLES FOR NETRINS DURING NERVOUS SYSTEM DEVELOPMENT

During embryogenesis in *C. elegans* and *D. melanogaster*, secretion of the netrin UNC-6 and netrins A/B respectively, are essential for orienting cell and axon migration with respect to the ventral midline of the developing nervous system (Figure 2.1B,C; Hedgecock et al., 1990;Ishii et al., 1992;Hamelin et al., 1993;Harris et al., 1996;Keleman and Dickson, 2001). Similarly, netrin-1 expressed by the floor plate in mouse plays an essential role directing axon extension relative to the ventral midline of the embryonic spinal cord. Netrin-1 deficiency in mouse also disrupts the formation of major axon projections to the midline in brain, including the corpus callosum and hippocampal commissure (Serafini et al., 1996) indicating that numerous axon tracts require netrin-1 to cross from one side of the CNS to the other. Acting as a repellent, netrin-1 directs axon extension by subsets of motoneurons, including: trochlear motoneurons (Colamarino and Tessier-Lavigne, 1995), cranial motoneurons (Varela-Echavarria et al., 1997) and spinal accessory motoneurons (Dillon et al., 2005).

Away from the midline, netrin-1 expression at the optic nerve head is required for the axons of retinal ganglion cells to exit the retina and enter the optic nerve (Figure 2.1F, Deiner et al., 1997). Netrin-1 is also implicated in the guidance of dopaminergic axons within the ventral midbrain (Lin et al., 2005), in the thalamocortical projection (Braisted et al., 2000), as well as in the formation of axon projections within the hippocampus (Barallobre et al., 2000).

In contrast to netrin-1, the function of other netrin family members in vertebrates is relatively poorly understood. Netrin-3 can mimic the ability of netrin-1 to attract spinal commissural axons and repel trochlear motor neuron axons *in vitro* (Wang et al., 1999a), however, netrin-3 expression in the spinal cord begins after the initial commissural axons have pioneered the path to the floor plate. Netrin-3 is, however, expressed in dorsal root ganglia in the developing PNS, and by mesodermal cells that may influence axon guidance to peripheral targets (Puschel, 1999). Netrin-4 is widely expressed in the developing nervous system, including in the olfactory bulb, retina, dorsal root ganglia, as well as by cerebellar granule, hippocampal, and cortical neurons (Koch et al., 2000). In

the developing spinal cord, a relatively low level of netrin-4 is expressed adjacent to floor plate cells; however, like netrin-3, this begins after the first commissural axons have crossed the midline. Both netrin-G1 and -G2 are expressed primarily by neurons, with very limited expression outside the nervous system (Nakashiba et al., 2002;Yin et al., 2002). Netrin-G1 is expressed in the dorsal thalamus, olfactory bulb and inferior colliculus, while netrin-G2 is expressed in the cerebral cortex. *Netrin-G1* gene mutations in humans produce symptoms similar to Rett syndrome (Borg et al., 2005), characterized by normal early development followed by loss of purposeful use of the hands, distinctive hand movements, slowed brain and head growth, gait abnormalities, seizures, and mental retardation. Netrin G1-deficient mice have no obvious abnormalities in gross anatomy and neural circuitry, but exhibit altered synaptic responses and defects in sensorimotor gating behavior (Inaki et al., 2004). These findings led to the suggestion that the major role for netrin-G proteins may be in the maturation, refinement, and maintenance of synapses, rather than axonal outgrowth and guidance. Consistent with this, the netrin-G receptor NGL-2 influences the formation of glutamatergic synapses through an interaction with the post-synaptic scaffold protein PSD-95 (Kim et al., 2006).

NETRIN SIGNAL TRANSDUCTION

The signal transduction mechanisms regulated by netrins are currently the subject of intense scrutiny. The majority of the studies carried out have focused on the role of netrin-1 as a chemoattractant axon guidance cue and comparatively little is known regarding signal transduction by other netrins. The following provides an overview of signal transduction events implicated in the response to netrin-1 (for a detailed review see: Huber et al., 2003;Barallobre et al., 2005).

Netrin receptors in vertebrates include DCC (deleted in colorectal cancer), the DCC paralogue neogenin, and four UNC5 proteins, UNC5A-D (Figure 2.2B). Although DCC, neogenin, and the UNC5 proteins all bind netrin-1, the majority of studies of netrin signaling have focused on DCC. Attractant responses to netrin-1 require DCC. In contrast, repellent responses require expression of an UNC5 protein, with co-expression

of DCC in some cases. Interestingly, neogenin also interacts with a GPI-linked protein called Repulsive Guidance Molecule (Rajagopalan et al., 2004).

Netrin-1 mediated chemoattraction

Unc-40 encodes the *C. elegans* orthologue of DCC (Hedgecock et al., 1990;Chan et al., 1996). *C. elegans unc-40* mutants predominantly exhibit defects in ventrally-directed migration of cells and axons, in contrast to *unc-6* (*netrin)* mutants in which migrations both toward and away from the ventral midline are disrupted. Consistent with the *Unc-40* mutant phenotype in the nematode, application of DCC function blocking antibodies to explants of embryonic mouse spinal cord blocked netrin-1 induced commissural axon outgrowth (Keino-Masu et al., 1996). Furthermore, *dcc* gene knockout produced a phenotype very similar to that generated by loss of netrin-1 function, including loss of the spinal ventral commissure, corpus callosum and hippocampal commissure (Fazeli et al., 1997).

The extracellular domain of DCC is composed of six fibronectin type 3 (FN3) repeats and four immunoglobulin (Ig) repeats (Figure 2.3B). The DCC FN3 domains are implicated as netrin-1 binding sites, but exactly which FN3 domain binds netrin-1 remains controversial (Bennett et al., 1997;Geisbrecht et al., 2003;Kruger et al., 2004). The DCC intracellular domain has no known intrinsic catalytic activity, but contains several putative protein binding and phosphorylation sites. Based on particularly strong identity between human, chick and fly DCC family members, three regions of the intracellular domain of DCC, termed domains, P1, P2 and P3, have been identified (Figure 2.3B & 2.4A, Kolodziej et al., 1996). The P1 domain is a highly conserved 17 amino acid motif, the P2 domain is rich in proline residues, containing four PXXP putative SH3 domain-binding motifs (Figure 2.4A), and the P3 domain contains several highly conserved possible phosphorylation sites.

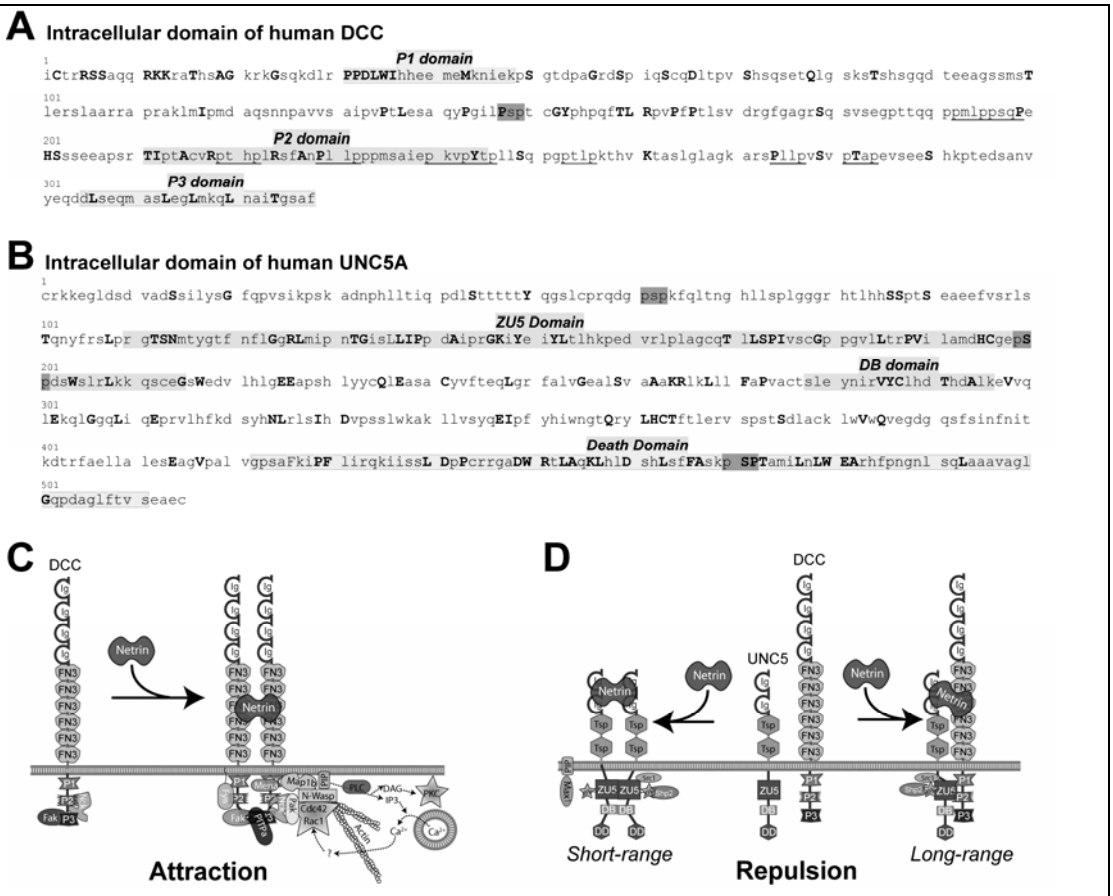


Figure 2.4 - Model of Netrin Signal Transduction: *Amino acid sequences of the intracellular domains of human DCC (A) and UNC5A (B). Amino acids conserved between C. elegans, X. laevis and humans are in bold capital letters. Assigned domains are lightly shaded, while WW class IV motifs (PSP) are more darkly shaded. Core SH3 PXXP motifs are underlined. Panel C and D summarize signaling events involved in attractive and repellent responses, respectively (see text for details).*

The ability of a cue to attract axon growth is thought to reflect its capacity to regulate membrane protrusions made by the growth cone. Rho GTPases are a family of intracellular proteins that coordinate cytoskeletal organization and adhesive interactions (Hall, 1998). In particular, the activation of the Rho GTPases Rac and Cdc42 has been shown to be essential for attractant responses to a number of guidance cues (Luo, 2000;Dickson, 2001), including netrin-1 (Causeret et al., 2004;Shekarabi et al., 2005). The exact sequence of events linking DCC to Rho GTPase activation, and their

downstream effectors, remains unclear. Multimerization of the DCC P3 domain following binding to netrin-1 is implicated as an initial event in mediating chemoattraction (Hong et al., 1999;Stein et al., 2001). The DCC intracellular domain associates with the adaptor protein Nck1 (Li et al., 2002), the tyrosine kinases Fak (Li et al., 2004) and Fyn (Meriane et al., 2004), the serine/threonine kinase Pak (Shekarabi et al., 2005), as well as the actin binding proteins Ena/Vasp (Lebrand et al., 2004) and N-WASP (Shekarabi et al., 2005). In addition to Rac and Cdc42 activation, application of netrin-1 leads to production of phosphoinositides by recruitment of phosphatidylinositol transfer protein-α (Xie et al., 2005), activation of phosphatidylinositol-3 kinase (Ming et al., 1999), and the breakdown of phosphoinositides by phospholipase C into IP3 and diacylglycerol (DAG, Ming et al., 1999) IP3 promotes intracellular calcium release from intracellular stores and DAG activates protein kinase C (Rhee, 2001). Supporting a role for IP3 production in netrin-1 mediated chemoattraction, elevating intracellular calcium is required for turning to netrin-1 (Hong et al., 2000). Notably, such calcium increases can contribute to Rac and Cdc42 activation (Jin et al., 2005). Figure 2.4C presents a speculative model of how these events may contribute to netrin-1 mediated axonal chemoattraction.

Netrin-1 mediated chemorepulsion

UNC5 netrin receptors were first implicated as mediators of repellent responses to the netrin UNC-6 from studies in *C. elegans* (Hedgecock et al., 1990;Leung-Hagesteijn et al., 1992). *Unc-5* mutants exhibit defects in dorsally-directed migrations, away from the ventral midline source of UNC-6 netrin, and misexpression of *unc-5* by neurons caused their axons to be redirected along a dorsal trajectory (Hamelin et al., 1993). As in *C. elegans,* a single UNC5 family member has been identified in *D. melanogaster* (Keleman and Dickson, 2001). Four have been found in mammals: UNC5A, B, C and D (Figure 2.2B, Ackerman et al., 1997;Leonardo et al., 1997;Przyborski et al., 1998;Engelkamp, 2002). UNC5s are composed of two extracellular Ig domains, that bind netrin, and two extracellular Tsp (thrombospondin) domains (Figure 2.3B, Geisbrecht et al., 2003). The UNC5 intracellular domain is made up of three conserved domains: a ZU5 domain, a DCC-binding (DB) domain and a death domain (DD, Figure 2.3B). The function of the

ZU5 domain is unknown, however it is homologous to a sequence in the scaffolding protein Zona Occludens-1 found at tight junctions (Itoh et al., 1997).

Studies in worms, flies and vertebrates suggest that long-range repulsion to netrin requires the cooperation of UNC5 and DCC, but that UNC5 without DCC is sufficient for short-range repulsion (Hong et al., 1999;Keleman and Dickson, 2001). Although the reason for this difference is not clear, it may be the case that DCC and UNC5 together form a more sensitive netrin receptor complex that is able to respond to lower concentrations of protein found at a greater distance from a source of netrin secretion. At long-range, direct association between the cytoplasmic domains of UNC5 and DCC appears to be essential (Hong et al., 1999;Merz et al., 2001). While mediating short-range responses to netrin independently of DCC, genetic studies in *C. elegans* have stressed the importance of an association between UNC5 cytoplasmic ZU5 and DD domains (Killeen et al., 2002). Several proteins have been proposed to interact with UNC5 family members in mediating a repellent response, including: the tyrosine kinase Src1, the tyrosine phosphatase Shp2 (Tong et al., 2001), the F-actin anti-capping protein Mena (Colavita and Culotti, 1998), the structural protein ankryn, and the adaptor protein Max1 (Huang et al., 2002). Repellent responses to netrin-1 are thought to involve tyrosine phosphorylation of UNC5's intracellular domains at multiple sites (Tong et al., 2001). Figure 2.4D outlines a speculative model of the intracellular events occurring during short and long-range repulsion.

Regulating the response to netrin-1

Growth cones respond rapidly to local guidance cues and exhibit substantial autonomy from the neuronal cell body. Growth cones react to netrin along a continuum that ranges from repulsion to unresponsiveness to attraction. The mechanisms that control this shift in netrin responsiveness are just beginning to be understood.

Many of the factors shown to regulate the response of growth cones to netrin can be correlated with changes in the expression of either UNC5 or DCC. At the transcriptional level, mis-expressing the homeobox transcription factor even-skipped in *D. melanogaster* resulted in disruption of *unc5* expression and motoneuron axon guidance

defects (Labrador et al., 2005). Local protein synthesis within the growth cone is required for chemoattraction of cultured *X. laevis* neurons to netrin (Campbell and Holt, 2001). The newly synthesized proteins have been suggested to influence either the recovery of growth cones from desensitization, or netrin signal transduction directly (Ming et al., 2002). Conversely, DCC function is negatively regulated by proteolysis, including both extracellular metaloproteinases implicated in shedding of the DCC ectodomain (Galko and Tessier-Lavigne, 2000), and ubiquitination of the DCC intracellular domain through an interaction with Siah-1, a RING domain containing protein that promotes DCC degradation via the ubiquitin-proteasome pathway (Hu et al., 1997;Kim et al., 2005). In mammals, the intracellular domains of UNC5 proteins are substrates for caspases (Tanikawa et al., 2003).

Intracellular concentrations of cyclic nucleotides are key regulators of growth cone responsiveness to several guidance cues. Manipulating the intracellular concentration of cAMP, thereby activating protein kinase A (PKA), regulates the response of growth cones to netrin-1. Initial experiments demonstrated that axons of cultured *X. laevis* spinal neurons attracted to a pipette puffing netrin-1, were instead repelled when PKA was inhibited (Ming et al., 1997). These studies led to the proposal that PKA controls the direction of growth cone turning by regulating intracellular signal transduction pathways downstream of netrin-1. PKA activation has been shown to selectively recruit DCC from an intracellular vesicular pool to the plasma membrane of commissural neuron growth cones, and the increased levels of DCC potentiate the outgrowth and turning response of these neurons to netrin-1 (Bouchard et al., 2004;Moore and Kennedy, 2006b). Interestingly, activation of protein kinase C (PKC) induces endocytosis of UNC5 homologues resulting in cultured cerebellar granule cell neurons switching from chemorepellent to chemoattractant responses to netrin-1 (Bartoe et al., 2006). These findings suggest that extracellular factors that regulate PKA and PKC will influence axon outgrowth by determining which receptors are presented by the growth cone.

Other potential netrin receptors

Other receptors, in addition to DCC and UNC5 proteins, have been suggested for netrins 1-3. The G-protein coupled adenosine receptor, A2B, was reported to bind netrin-1 and cooperate with DCC in spinal commissural axon guidance (Corset et al., 2000). However, subsequent studies provide evidence that argues against A2B binding to netrin-1, indicating that A2B is neither expressed by these neurons nor required for commissural axon guidance in response to netrin-1 (Stein et al., 2001). The α6β4 and α3β1 integrins bind netrin-1 and these interactions have been implicated in the development of the pancreas (Yebra et al., 2003). Given the homology of the N-terminus of netrin-1 to laminins, it might be predicted that netrins would bind integrins through N-terminal domains; but surprisingly α6β3 and α3β1 integrins interact with a highly charged sequence of basic amino acids at the C-terminus of netrin-1 that is not homologous to laminins. While these findings raise the exciting possibility that integrins may function as netrin receptors in other contexts, the significance of netrin-integrin interactions *in vivo* remains to be demonstrated. In contrast to the secreted netrins, netrin-G1 binds a transmembrane protein called the netrin-G ligand 1 (Ngl1, Figure 2.3B) and netrin-Gs do not appear to interact with DCC, neogenin, or the UNC5 proteins (Nakashiba et al., 2000;Lin et al., 2003). A receptor for netrin-4 has not so far been identified.

NETRIN IN THE ADULT NERVOUS SYSTEM

Netrins and netrin receptors are expressed in the adult vertebrate nervous system (Kennedy et al., 1994;Livesey and Hunt, 1997;Volenec et al., 1997;Volenec et al., 1998;Wang et al., 1999a;Koch et al., 2000;Nakashiba et al., 2000;Petrausch et al., 2000;Madison et al., 2000;Manitt et al., 2001;Ellezam et al., 2001;Nakashiba et al., 2002;Manitt et al., 2004). Netrin-1 is expressed by many types of neurons and by myelinating glia: oligodendrocytes in the CNS (Manitt et al., 2001) and Schwann cells in the PNS (Madison et al., 2000;Ellezam et al., 2001). Subcellular fractionation of CNS white matter indicated that netrin-1 is enriched in periaxonal myelin membranes (Figure 2.1H, Manitt et al., 2001) suggesting that it may normally mediate interactions between axonal and oligodendrocyte membranes. Expression by mature myelinating

oligodendrocytes raises the possibility that netrin-1 may influence axon regeneration. Notably, netrin-1, DCC and UNC5s influence the development of the corticospinal tract, which transmits information controlling voluntary limb movements, suggesting that netrin-1 might play an important role following spinal cord injury (Figure 2.1G, Finger et al., 2002;Harel and Strittmatter, 2006) During maturation of the mammalian spinal cord, DCC expression is downregulated, while UNC5 homologue expression increases (Manitt et al., 2004), indicating that UNC5 repellent signaling may be the dominant response to netrin in the adult spinal cord.

An examination of the consequences of spinal cord injury in the adult rat found that levels of netrin-1 mRNA and protein were substantially reduced at the site of injury itself, and this decreased expression persisted for at least 7 months (Manitt et al., 2006). Netrin-1 was not associated with the glial scar, but netrin-1 was expressed in an apparently normal distribution by neurons and oligodendrocytes adjacent to the lesion. The expression of DCC and UNC5 proteins was also reduced after injury. Although DCC expression remained low, UNC5 expression recovered and subsets of neurites adjacent to the lesion exhibited elevated UNC5 immunoreactivity. These findings are consistent with earlier studies carried out in the optic nerve, indicating that both DCC and UNC5B continue to be expressed by retinal ganglion cells following axotomy, albeit at reduced levels, as their axons attempt to extend along either the injured optic nerve itself or into a growth permissive peripheral nerve graft (Petrausch et al., 2000;Ellezam et al., 2001). While a role for netrin-1 in axon regeneration remains to be demonstrated directly, these findings suggest a role for netrin-1 as a component of CNS myelin that inhibits axon regeneration by neurons expressing UNC5 following injury.

Although the functional significance of netrin-1 expression in the adult CNS remains unknown, an intriguing hypothesis is that netrins may contribute to maintaining appropriate connections in the intact CNS by restraining inappropriate axonal sprouting. A consequence of this may be that netrins subsequently inhibit the re-establishment of connections following injury. In line with this hypothesis, studies carried out in lamprey, a primitive vertebrate with the ability to recover significant function following spinal cord transaction (Cohen et al., 1988), indicate a correlation between UNC5 expression and

poor axonal regeneration following lesion (Shifman and Selzer, 2000). Importantly, it may be possible to reverse such an inhibitory role for netrin in the adult mammalian CNS by manipulating cAMP levels within regenerating axons. As described above, increasing cAMP converts netrin-mediated repulsion to attraction, and encouraging findings indicate that increasing the concentration of cAMP in neurons promotes axon regeneration in the mature CNS following injury (Neumann et al., 2002;Qiu et al., 2002b).

CONCLUSION AND PERSPECTIVES

Since their discovery a little over a decade ago, significant insight has been gained into netrin function. Extending axons have been found to be directed by netrins in multiple contexts. Netrins also direct the migration of numerous cell types during development, including: inferior olivary (Bloch-Gallego et al., 1999), basilar pontine (Yee et al., 1999) and LHRH neurons (Schwarting et al., 2004), as well as, striatal neuronal precursors (Hamasaki et al., 2001), cerebellar granule cells (Alcantara et al., 2000), spinal accessory neurons (Dillon et al., 2005) and oligodendrocyte precursor cells (Jarjour et al., 2003;Tsai et al., 2003). An exciting new avenue of research has identified roles for netrins in the morphogenesis of a variety of tissues (Hinck, 2004;Baker et al., 2006). Netrins are now implicated in the development of the lung (Dalvin et al., 2003;Liu et al., 2004b), mammary gland (Srinivasan et al., 2003) and vascular networks (Lu et al., 2004;Park et al., 2004;Klagsbrun and Eichmann, 2005;Wilson et al., 2006). Although aspects of this work is in its initial stages, the studies described here identify roles for netrins in axon guidance, cell migration, tissue morphogenesis, and the maintenance of appropriate cell-cell interactions, supporting the conclusion that netrins influence development in a broad range of biological contexts.

RATIONAL AND OBJECTIVES

This thesis examines the RhoA subfamily of Rho GTPases and cyclic AMP in the guidance of axons to netrin-1. Although it was known that the RhoA and cAMP signaling pathways were linked in certain cells types (Lang et al., 1996;Schoenwaelder and Burridge, 1999), whether this applied to neurons and the biochemical mechanism underlying their function was not appreciated.

OBJECTIVE 1: HOW IS CYCLIC AMP SIGNLING INVOLVED IN AXON GUIDANCE TO NETRIN-1? (Chapters 3, 4 & 7)

At the onset of my studies, an emerging model indicated that cAMP was capable of switching the response of axons to a variety guidance cues, including netrin-1 (Ming et al., 1997). However, cAMP was also implicated in the signaling cascade to netrin-1 (Hopker et al., 1999;Corset et al., 2000). We therefore undertook experiments to determine how manipulationg cAMP affected the attraction of spinal commissural axons to netrin-1 and whether netrin-1 could induce cAMP production in this setting.

OBJECTIVE 2: IS NETRIN-1 AN ADHESIVE CUE? (Chapter 5)

Axon extension requires mechanical coupling of the cytoskeleton with the substrate (Suter and Forscher, 2000). Guidance cues are thought to steer the growth cone by locally affecting its traction. However, axon guidance cues are commonly described in terms of the biochemical cascades they engage and not the mechanical interactions they engage. Although it is possible that guidance cues indirectly regulate this traction by affecting interactions with other components in the extracellular space, it is equally possible that substrate-attached netrin-1 itself is used for traction. Colleen Manitt, a former PhD student in our lab, used fractionation experiments to demonstrate that most netrin-1 is physically associated with membranes *in vivo* (Manitt et al., 2001;Manitt and Kennedy, 2002). As such, an early goal was to explore whether netrin-1 employed mechanical aspects in its ability to guide axons.

OBJECTIVE 3: DO RHO GTPASES REGULATE NETRIN-1 MEDIATED ADHESION (Chapters 5 & 6)

At the start of my graduate studies, the importance of Rho GTPases in axon guidance were just beginning to be appreciated (Hall, 1998). Work done primarily in fibroblasts had demonstrated their role in actin and adhesive remodeling – two processes known to be important in the guidance of axons. Once we obtained evidence that netrin-1 was indeed an adhesive cue, we explored the possibility that Rho GTPases may be implicated in this process.

OBJECTIVE 4: IS SOLUBLE ADENYLYL CYCLASE INVOLVED IN THE GUIDANCE TO NETRIN-1? (Chapter 7)

Despite our extensive evidence that netrin-1 did induce cAMP production, a report emerged claiming that soluble adenylyl cyclase (sAC) was required for axonal responses to netrin-1 (Wu et al., 2006). Given this contradictory finding we explored this possibility in greater detail.

CHAPTER 3

Protein Kinase A Activation Promotes Plasma Membrane Insertion of DCC from an Intracellular Pool: A Novel Mechanism Regulating Commissural Axon Extension

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PREFACE

This chapter was published as a research article in the Journal of Neuroscience (Bouchard et al., 2004). A fundamental issue in axon guidance research is a clear understanding of the function of cyclic adenosine monophosphate (cAMP) and its downstream kinase, protein kinase A (PKA). Early studies suggested that local cAMP production may underlie a cue's ability to attract the growth of axons (Gundersen and Barrett, 1980). However, as presented in the Literature Review chapters, this simple model was later complicated by reports that global cAMP concentrations within the growth cone determine whether a particular cue is perceived as an attractant or a repellent (reviewed in Song and Poo, 1999). In this study we test these two models using dissociated spinal commissural neurons as a model system (the method is described in Appendix I of this thesis). These cultures offer the advantage of being a uniform population of neurons whose axons are attracted to netrin-1. Using this system, we uncovered a novel mechanism regulating axonal responses to netrin-1 – namely, that elevating cAMP levels results in a greater amount of netrin-1's receptor DCC on the plasma membrane. We also provide evidence, which will be elaborated in chapters 4 and 7, that netrin-1 itself does not induce cAMP production.

Acknowledgements

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ABSTRACT

Protein kinase A (PKA) exerts a profound influence on axon extension during development and regeneration; however, the molecular mechanisms underlying these effects of PKA are not understood. Here we show that DCC (deleted in colorectal cancer), a receptor for the axon guidance cue netrin-1, is distributed both at the plasma membrane and in a pre-existing intracellular vesicular pool in embryonic rat spinal commissural neurons. We hypothesized that the intracellular pool of DCC could be mobilized to the plasma membrane and enhance the response to netrin-1. Consistent with this, we show that application of netrin-1 causes a modest increase in cell surface DCC, without increasing the intracellular concentration of cAMP or activating PKA. Intriguingly, activation of PKA enhances the effect of netrin-1 on DCC mobilization and increases axon extension in response to netrin-1. PKA dependent mobilization of DCC to the plasma membrane is selective, as the distributions of TAG-1, NCAM, and trkB are not altered by PKA in these cells. Inhibiting adenylyl cyclase, protein kinase A, or exocytosis, blocks DCC translocation upon PKA activation. These findings indicate that netrin-1 increases the amount of cell surface DCC, that PKA potentates the mobilization of DCC to the neuronal plasma membrane from an intracellular vesicular store, and that translocation of DCC to the cell surface increases axon outgrowth in response to netrin-1.

INTRODUCTION

The direction taken by an extending axon depends on extracellular cues, the repertoire of receptors for these cues on the axonal growth cone, and the state of signal transduction mechanisms within the growth cone. Deleted in Colorectal Cancer (DCC) is a type I

transmembrane protein and a receptor for netrins (Keino-Masu et al., 1996;Stein et al., 2001). Netrins are a family of secreted axon guidance cues that attract some axons and repel others (reviewed by Yu and Bargmann, 2001). DCC is required for the attractant response to netrin-1 (Serafini et al., 1996;de la Torre et al., 1997) while both DCC and the UNC5 homolog family of netrin receptors mediate chemorepellent responses to netrin-1 (Ackerman et al., 1997;Leonardo et al., 1997;Hong et al., 1999;Keleman and Dickson, 2001;Merz et al., 2001).

PKA plays a key role regulating the response of axonal growth cones to netrin-1 (reviewed by Song and Poo, 1999). Inhibition of PKA in neurons dissociated from either the embryonic *Xenopus* spinal cord (Ming et al., 1997) or retina (Hopker et al., 1999) switches their response to netrin-1 from attraction to repulsion. Further investigation of growth cone turning using *Xenopus* CNS neurons suggests that manipulation of phosphatidylinositol 3-kinase, phospholipase Cγ (Hopker et al., 1999;Ming et al., 1999), MAP kinases (Forcet et al., 2002;Ming et al., 2002), protein synthesis (Campbell and Holt, 2001) and electrical activity (Ming et al., 2001), can all influence the response of a growth cone to netrin-1.

Activating PKA also promotes axon growth in the presence of myelin-associated inhibitors of axon extension (Cai et al., 2001), including promoting axonal regeneration in the adult mammalian CNS following injury (Neumann et al., 2002;Qiu et al., 2002a). Although PKA exerts profound effects on axon growth, the mechanisms underlying these actions during either neural development or axon regeneration are not known.

Here, we examined the role of PKA in the response of embryonic rat spinal commissural neurons to netrin-1. We report that DCC is normally present on both the neuronal surface and within an intracellular pool in these cells. We describe two mechanisms that regulate the amount of DCC on the neuronal plasma membrane. First, application of netrin-1 produced a modest increase in the amount of cell surface DCC. Netrin-1 did not increase the intracellular concentration of cAMP or activate PKA. Inhibiting PKA did not affect the netrin-1 induced increase in cell surface DCC or netrin-1 evoked axon outgrowth, providing evidence that PKA is not required for signaling downstream of netrin-1 in these cells. Activating PKA enhanced netrin-1 dependent

insertion of DCC into the plasma membrane and increased axon outgrowth evoked by netrin-1. Inhibition of adenylyl cyclase, protein kinase A, or exocytosis, but not protein synthesis, blocked the PKA-induced increase in cell surface DCC, consistent with DCC being mobilized from a pre-existing intracellular vesicular pool. Activated PKA did not alter the distribution of other membrane proteins, such as TAG-1, NCAM, or trkB, revealing a selective effect on the mobilization of DCC. These findings demonstrate that PKA activation potentiates the response to netrin-1 by translocating DCC to the plasma membrane.

MATERIALS and METHODS

Reagents

Polyclonal anti-trkB was provided by Dr. Louis Reichardt (UCSF). Polyclonal anti-TAG-1 (TG3) for western blot analysis was provided by Dr. Thomas Jessell (Columbia University). Monoclonal anti-TAG-1 (4D7), for immunocytochemistry, was obtained from the DSHB (University of Iowa). Polyclonal anti-NCAM (AB5032) and anti-cAMP (AB306) from Chemicon (Temecula, CA). Monoclonal DCC anti-DCC_{EX} G92-13, and anti-DCC_{IN} G97-449 were obtained from PharMingen (Mississauga, Canada) and anti-DCC_{FB} AF5, KT5720 and SQ22536, from Calbiochem (LaJolla, CA). Anti-phospho CREB (Ser133, 1B6, P-CREB) and anti-CREB were obtained from Cell Signaling Tech, MA. Cyclohexamide (CHX), forskolin (FSK), poly-D-lysine (PDL), tetanus toxin (TeTx), and 5'-n-ethyl-carboxamidoadenosine (NECA) were obtained from Sigma-Aldrich, (Oakville, Canada); SMEM from BioWhittaker (Walkersville, Maryland); Neurobasal media and B27 supplement from Invitrogen (Burlington, Canada); and Glutamax, IFBS and Penstrep from Bio Media (Boussens, France). Recombinant netrin-1 protein was purified from a HEK293T cell line secreting netrin-1 as described (Serafini et al., 1994;Shirasaki et al., 1996).

Commissural Neuron Culture

Staged pregnant Sprague-Dawley rats were obtained from Charles River (St-Constant, Canada). The dorsal half of embryonic day 13 rat spinal cords were isolated by

microdissection (Serafini et al., 1994) and dissociated to produce a suspension of single cells. In brief, dorsal spinal cords were incubated at 37^0C for 30 min in 0.0002% DNase (Sigma-Aldrich) in Ca^{2+}/Mg^{2+}-free Hanks' solution (Invitrogen). The tissue was then triturated to yield a suspension of single cells.

Dissociated cells were plated and cultured for either 2 days (~25,000 cells/well, growth cone analysis) or 6 days (~50,000 cells/well, neurite analysis) in 24-well plates (Sarstedt, Quebec). Cells were grown in the wells on PDL coated (70-150 kD, 20 μg/ml) 12 mm round glass coverslips (No.0 Deckgläser, Carolina Biological, NC). The first 24 hrs, cells were cultured in Neurobasal media containing: 10% IFBS, 2 mM Glutamine, 1 unit/ml penicillin, and 1 μg/ml streptomycin. The second day the medium was changed to serum-free Neurobasal supplemented with 1% B-27, 0.4 mM glutamine, 1 unit/ml penicillin, and 1 μg/ml streptomycin. Inhibitors (1 mM SQ22536, 200 nM KT5720, 1.6 nM TeTx, 100 μM CHX) 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 10 μM FSK or vehicle.

For western blot analysis (Harlow and Lane, 1999), cells were plated and cultured 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 RIPA buffer (Barker and Shooter, 1994). Protein content was quantified using BCA (Pierce, IL). Results were visualized using chemiluminescence (NEN Life Science Products, MA) and quantification performed on scanned images of immunoblots (ScanJet 5300C, Hewlett Packard, Canada) using NIH Image software (United States National Institutes of Health).

Immunocytochemistry

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 by using 0.1 % Tween20 in PBS (PBST) instead of PBS alone. Antibodies were used in blocking solution overnight at 4^0C at the following

dilutions: anti-DCC_{IN} 1:500; anti-DCC_{EX} 1:500; anti-TAG-1 1:500; anti-Tau 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 labeled with Cy2, Cy3, Alexa 488, or Alexa 546 secondary antibodies (Molecular Probes, Eugene, OR). Nuclei were stained with Hoechst 33258 (Sigma-Aldrich).

Quantification of surface receptor density or cAMP immunoreactivity

All micrographs used for quantification were taken using the same Carl Zeiss Axiovert microscope, 100X objective lens, and exposure time to allow comparison of measurements. Fluorescence was quantified using Northern Eclipse image analysis software (Empix Imaging Inc, Canada) by an observer blind to the experimental conditions. For image analysis of neurites or growth cones, both differential interference contrast (DIC) and fluorescent images were taken. Fluorescence intensity per μm^2 of the process was quantified and expressed as mean ± SEM. Statistical significance was evaluated by a one-way analysis of variance with a Sheffe post-hoc test (Systat, Chicago, IL).

Surface Biotinylation

E13 dorsal spinal cords were dissociated and commissural neurons 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 TeTx or vehicle for 15 min, followed by 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. 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 4^0C for 30 min (Lisanti et al., 1989), removed, and the reaction quenched by the addition of 5 ml of 10 mM ice-cold glycine in PBS at

4^0C 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 at 37^{o}C in Neurobasal, 10% IFBS, 2 mM Glutamine, 1 unit/ml penicillin, and 1 µg/ml streptomycin. Inhibitors, 1 mM SQ22536, 200 nM KT5720, 1.6 nM TeTx, or 1 to 100 µM NECA, 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 with 10 µM FSK. All drugs were present throughout the experiment.

Segments of E11 rat spinal cord were dissected as described (Tessier-Lavigne et al., 1988;Placzek et al., 1990), embedded in collagen, and cultured in Neurobasal containing: 2% B27, 2 mM GlutaMAX I, 100 units/ml penicillin, and 100 µg/ml streptomycin for 40 hours. TAG-1 immunoreactivity was visualized as described (Kennedy et al., 1994).

Photomicrographs were taken using a Carl Zeiss Axiovert microscope, phase-contrast optics, a 20X objective lens, Magnafire CCD camera (Optronics, CA), and analysed using Northern Eclipse image analysis software (Empix Imaging Inc, Canada). The total length of axon bundles or the length of TAG-1 immunopositive axons was 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).

RESULTS

Cell surface and intracellular pools of DCC

Little is known about the subcellular distribution of DCC protein. To investigate this, we developed a dissociated cell culture enriched in embryonic spinal commissural neurons. Greater than 90% of the cultured cells derived from E13 rat dorsal spinal cord were TAG-1 positive (Figure 3.1A), a marker for embryonic spinal commissural neurons

(Dodd et al., 1988). Immunolabeling with anti-DCC_{IN}, a monoclonal antibody raised against an intracellular epitope of DCC, showed that these neurons also express *dcc* (Figure 3.1B). Immunocytochemical and western blot analyses demonstrated that these cells do not express choline acetyl transferase, a marker for motoneurons (not shown). These findings indicate that these cultures are enriched with embryonic spinal commissural neurons. Confocal analysis of the distribution of DCC immunoreactivity revealed a punctate distribution of DCC protein in the cytoplasm of the cell bodies and proximal neurites (Figure 3.1B), consistent with a subset of DCC protein being associated with an intracellular vesicular pool.

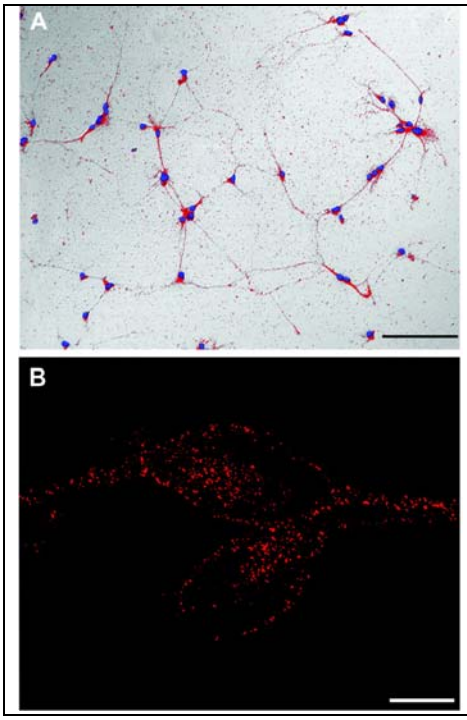

Figure 3.1 - Embryonic spinal commissural neurons in vitro. *A) Neurons derived from dissociated dorsal E13 rat spinal cord and cultured for 6 DIV. Cells were fixed, permeabilized, and immunostained for TAG-1 (red). Nuclei were stained with Hoechst 33258 dye (blue). Scale bar=100 µm. (B) Confocal microscopy revealed a punctate distribution of DCC immunoreactivity in the cytoplasm and outlining the surface of two commissural neurons cultured as in panel A. (anti-DCC_{IN}, Cy3 secondary, scale bar = 5 µm.).*

To specifically visualize cell surface DCC protein, dissociated commissural neurons were cultured for 6 days, fixed, and then processed with or without detergent (0.1% Tween 20). To verify that the cells were not permeabilized in the absence of detergent, they were labeled with either anti-DCC_{IN} or anti-DCC_{EX}, a monoclonal antibody raised against the extracellular domain of DCC, and with polyclonal anti-Tau, a microtubule-associated protein and intracellular marker (Figure 3.2). Anti-DCC_{IN} and anti-Tau produced a signal only in permeabilized cells (Figure 3.2A). Whereas, anti-

DCC_{EX} produced a signal in permeabilized and non-permeabilized cells (Figure 3.2C, D), the intensity of the signal in non-permeabilized cells was much weaker than in permeabilized cells, consistent with the presence of an intracellular pool of DCC protein. These findings suggested that much of the DCC protein expressed by commissural neurons is present intracellularly.

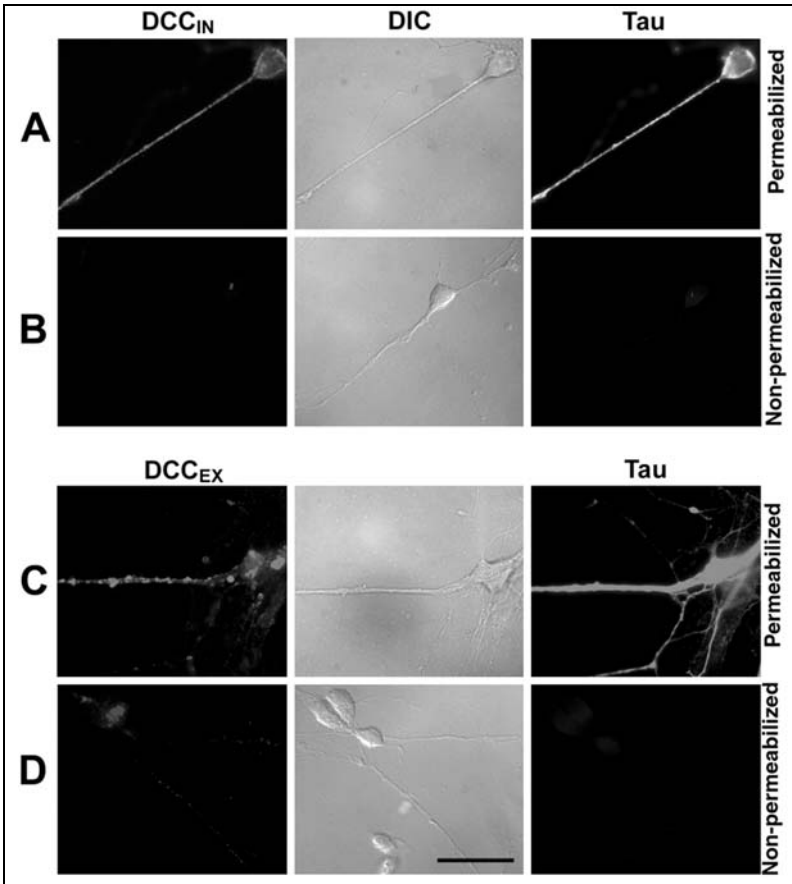

Figure 3.2 - Plasma membrane and intracellular DCC in commissural neurons. *Dissociated commissural neurons grown 6 DIV (A-D). Following fixation, cells in panel A and C were permeabilized with 0.1 % tween. Cells in panels B and D were not permeabilized. Cells were labeled with either anti-DCC_{IN} (A, B), anti-DCC_{EX} (C, D), or anti-Tau (A-D). Middle panels A-D, DIC optics. 100X objective. Scale bar = 25 µm.*

Netrin-1 increases the amount of DCC on the surface of commissural neurons

We then determined if netrin-1 alters the subcellular distribution of DCC, using cell surface biotinylation as an assay. Commissural neurons isolated from the E13 rat dorsal spinal cord were cultured for 6 days and then treated for 15 min with netrin-1 (50 ng/ml) or vehicle control. Cell surface proteins were then biotinylated, isolated using streptavidin-agarose beads, and examined by western blot analysis using anti-DCC_{IN}, anti-$trkB_{ECD}$, or anti-TAG-1. A single band was detected using anti-DCC_{IN} (Figure 3.3A)

at the expected molecular weight of full length DCC (~180 kDa). The same band was detected using anti-DCC_{EX} (not shown). Addition of netrin-1 increased the amount of cell surface DCC in comparison with control (Figure 3.3A). Under these conditions, the amount of either cell surface trkB or TAG-1 was not affected by the addition of netrin-1 (Figure 3.3A).

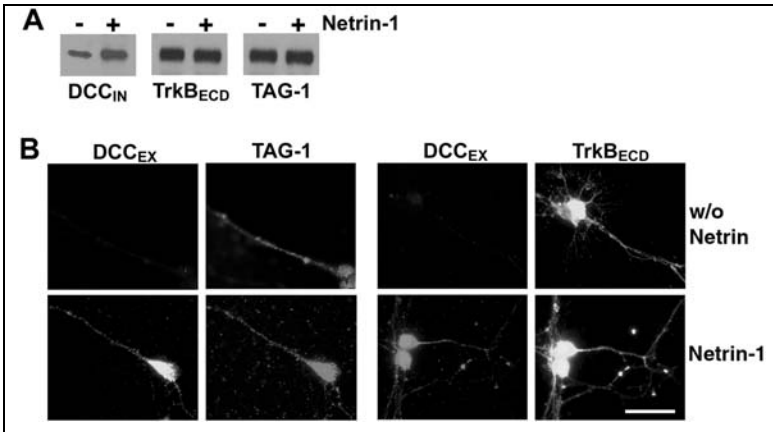

Figure 3.3 - Netrin-1 increases DCC immunoreactivity at the cell surface. *Dissociated commissural neurons were grown for 6 DIV prior to treatment for 15 min with 50 ng/ml netrin-1 or vehicle. (A) Cell surface proteins were biotinylated and then isolated using streptavidin-agarose beads. The biotinylated proteins were analysed by western blot with antibodies directed against DCC, trkB, or TAG-1. (B) Cells were fixed but not permeabilized, then immunostained with anti-DCC_{EX}, anti-TAG-1, or anti-$trkB_{ECD}$, all against extracellular epitopes. 100X objective. Scale bar = 25 µm.*

The netrin-1 induced increase in cell surface DCC protein was then evaluated immunocytochemically. Commissural neurons were treated for 15 min with either 50 ng/ml netrin-1 or vehicle, fixed without permeabilization, and then immunostained with anti-DCC_{EX} (Figure 3.3B). Netrin-1 caused a significant increase in cell surface DCC immunoreactivity but had no effect on cell surface levels of TAG-1 or trkB (Figure 3.3B, Table 3.1). Thus, netrin-1 selectively increased the amount of DCC on the neuronal surface.

PKA activation stimulates DCC translocation to the plasma membrane

PKA influences the response of neuronal growth cones to netrin-1 (reviewed by Song and Poo, 1999). We therefore determined if PKA activation might influence the distribution of DCC. Forskolin (FSK) activates the adenylyl cyclase, increases

intracellular cAMP, and activates PKA (reviewed by Nairn et al., 1985). Cells were treated with 10 µM FSK alone (Figure 3.4A), 50 ng/ml netrin-1 alone (Figure 3.3B), or 50 ng/ml netrin-1 in combination with 10 µM FSK (Figure 3.4C), and then immunostained with anti-DCC_{EX} to visualize DCC on the neuronal surface.

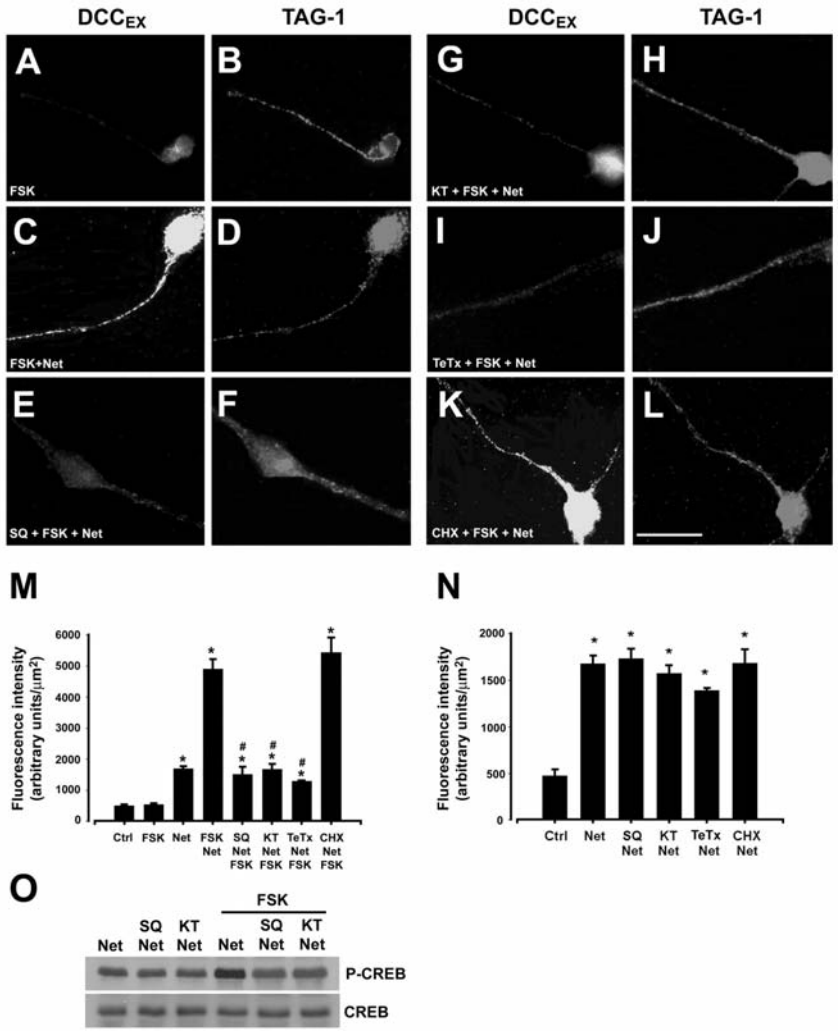

Figure 3.4 - DCC distribution is regulated by PKA and netrin-1*. Dissociated commissural neurons, 6 DIV (A-L). Cells in panels C-L were treated with 50 ng/ml netrin-1. Panels A and B were not. Cells were exposed for 15 min to (A-L) 10 µM Fsk; (E, F) 1 mM SQ 22536; (G, H) 200 nM KT 5720; (I, J) 1.6 nM TeTx; or (K, L) 100 µM CHX. Following treatment, cultures were fixed without permeabilization and immunostained with anti-DCC_{EX} (A, C, E, G, I, K) or anti-TAG-1 (B, D, F, H, J, L), both recognize extracellular epitopes. (A-L Cy2 or Cy3 conjugated secondary antibodies, 100X objective, scale bar=25 µm). Panel M illustrates quantification of DCC fluorescence intensity (mean ± SEM, n=6-14 per condition). * indicates $p < 0.01$ vs control or application of 10 µM FSK alone. # indicates $p < 0.01$ vs 50 ng/ml netrin-1 in combination with 10 µM FSK. Panel N illustrates the effect of 15 min application of netrin-1 alone on DCC*

*immuno-fluorescence intensity. * indicates $p < 0.05$ vs control. Panel O shows western blot analysis of total cell extracts for phospho-CREB (P-CREB) and total CREB (~ 43 kDa).*

FSK alone produced no change in DCC immunoreactivity (Figure 3.4A, M) whereas netrin-1 (15 min) produced a modest increase (Figure 3.4M, N). Substantially increased DCC immunoreactivity was detected in neurons treated with FSK and 50 ng/ml netrin-1 (Figure 3.4C, M). Netrin-1 plus FSK did not increase cell surface immunoreactivity for TAG-1 (Figure 3.4B, D, F, H, J, L, and Table 3.1) or trkB (Table 3.1).

	Ctrl	**Net**	**FSK+Net**	**SQ+ FSK+Net**	**KT+ FSK+Net**	**TeTx+ FSK+Net**	**CHX+ FSK+Net**
Neurites							
$DCC_{(EX)}$	461 ±80$^{\#}$	1658 ±102*$^{\#}$	4699 ±650*	1470 ±269*$^{\#}$	1677 ±201*$^{\#}$	1236 ±58*$^{\#}$	5408 ±612*
TAG-1	5456 ±443	5123 ±472	4681 ±886	5345 ±657	4993 ±457	5234 ±843	5033 ±702
$trkB_{(ECD)}$	6059 ±962	5879 ±934	6549 ±425	6166 ±420	5907 ±637	6143 ±762	5689 ±678
Growth Cones							
$DCC_{(EX)}$	239 ±44$^{\#}$	2034 ±213*$^{\#}$	6644 ±728*	1933 ±275*$^{\#}$	1433 ±346*$^{\#}$	1400 ±423*$^{\#}$	7623 ±710*
TAG-1	7354 ±266	7023 ±354	7493 ±455	8423 ±1003	8439 ±897	7799 ±910	8067 ±734

Table 3.1 - DCC, TAG-1, and trkB immunofluorescence intensity (f.i / μm^2) *Levels of DCC, TAG-1, and trkB present at the cell surface were compared by quantitative immunofluorescence with antibodies raised against extracellular epitopes of these proteins. Results are expressed as mean ± SEM of the neurite or growth cone surface fluorescence intensity (arbitrary units) (n of 6-14 per condition). Cells were treated with various inhibitors (1mM SQ22536, 200nM KT5720, 1.6nM TeTx, or 100µM CHX) or their respective vehicles for 15 min., following which, 50ng/ml netrin-1 or vehicle was added to culture media for 15 min. * $p < 0.01$ vs ctrl $^{\#}$ $p < 0.01$ vs 10µM FSK + 50ng/ml netrin-1*

To investigate the mechanism regulating the increase in cell surface DCC, cultures of dissociated commissural neurons were exposed to different enzyme inhibitors 15 min before the addition of netrin-1, thus 30 min before the addition of FSK to the media. To confirm that FSK was acting by increasing adenylyl cyclase activity, cells were treated

with 1 mM SQ22536 (Figure 3.4E,F), a specific inhibitor of adenylyl cyclase (Goldsmith and Abrams, 1991;Fabbri et al., 1991;Tamaoki et al., 1993). SQ22536 blocked the increase in DCC surface immunoreactivity (Figure 3.4E, M), consistent with the effect of FSK being due to adenylyl cyclase activation. To confirm that cAMP produced by the adenylyl cyclase was acting through PKA, commissural neurons were pretreated with 200 nM KT5720, a specific inhibitor of PKA (Kase et al., 1987). KT5720 blocked the increase in DCC surface immunoreactivity produced by FSK and netrin-1 (Figure 3.4G, M). FSK induced PKA activation was monitored by assaying phosphorylation of the PKA substrate CREB. Treatment of commissural neurons with FSK increased CREB phosphorylation, which was blocked by SQ22536 and KT5720 (Figure 3.4O). These findings indicate that PKA activation is essential for the increase in cell surface DCC induced by FSK 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 v-SNAREs (Schiavo et al., 1992). TeTx (1.6 nM) blocked the FSK-induced increase in surface DCC immunoreactivity (Figure 3.4I, M), consistent with the increase requiring exocytosis. The rapid increase in DCC protein on the neuronal surface, as early as 15 minutes following addition of FSK and netrin-1, suggests that it is unlikely to be accounted for by increased transcription or translation of DCC mRNA. Consistent with this, 15 min application of 100 μM cyclohexamide (CHX), a concentration sufficient to block protein synthesis (Twiss and Shooter, 1995), had no effect on the increase in cell surface DCC induced by FSK and netrin-1 (Figure 3.4 K, M). Insertion of locally translated protein into the plasma membrane has been detected in axonal growth cones (Brittis et al., 2002). Furthermore, Campbell and Holt (2001) provide evidence that protein synthesis is required for netrin-1 dependent growth cone turning. Our results indicate that a pre-existing intracellular pool of DCC protein is present in the neuron, that activation of PKA promotes the insertion of DCC into the plasma member, and that this recruitment of DCC occurs through a protein synthesis independent mechanism. Therefore we conclude that the PKA dependent increase in cell

surface DCC is not the protein synthesis sensitive step described by Campbell and Holt (2001).

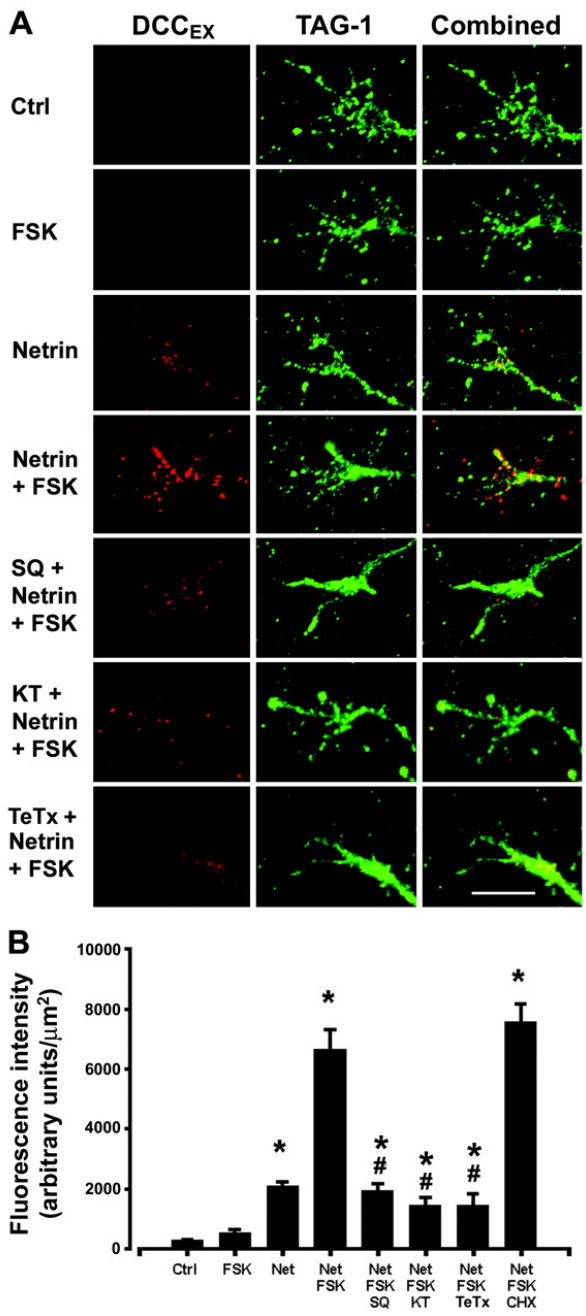

Figure 3.5 - Distribution of DCC in growth cones is regulated by PKA and netrin-1. *Dissociated commissural neurons were cultured for 2 DIV prior to treatment for 15 min with or without 50 ng/ml netrin-1. Ten μM FSK was then added for 15 min in combination with 1 mM SQ22536, 200 nM KT5720, or 1.6 nM TeTx. Cells were fixed without permeabilization, and immunostained with anti-DCC$_{EX}$ or anti-TAG-1. (Cy3 or Alexa 488-conjugated secondary antibodies, 100X objective, scale bar=10 μm). Panel B illustrates quantification of DCC fluorescence intensity. Values represent the mean ± SEM (n=6-8 per condition). * indicates p < 0.01 vs ctrl or 10 μM FSK alone. # indicates p <0.01 vs netrin-1 (50 ng/ml) plus FSK (10 μM).*

DCC insertion into the growth cone plasma membrane

We then examined the effect of PKA activation on the distribution of DCC protein on the surface of commissural neuron growth cones. Consistent with the findings presented above, treatment with FSK and netrin-1 increased DCC immunoreactivity on the surface of growth cones, netrin-1 alone produced a smaller increase, and FSK alone had no effect. FSK and netrin-1 had no effect on cell surface immunoreactivity for TAG-1 (Figure 3.5 and Table 3.1). Inhibition of adenylyl cyclase (1 mM SQ22536) or PKA (200 nM KT5720) blocked the increase in DCC surface immunoreactivity induced by FSK and netrin-1 (Figure 3.5), demonstrating that FSK produces this effect via the adenylyl cyclase and activation of PKA. In contrast, inhibition of protein synthesis using 100 μM CHX did not affect the increased DCC on the growth cone surface (Figure 3.5B). Application of 1.6 nM TeTx reduced the increase in cell surface DCC caused by FSK and netrin-1 to the level induced by netrin-1 alone (Figure 3.5). These findings support the conclusion that cAMP elevation potentiates the translocation of DCC to the plasma membrane of neuronal growth cones.

Netrin-1 does not activate PKA in commissural neurons and increases cell surface DCC by a PKA independent mechanism

Immunocytochemical evidence obtained using cultured *Xenopus* retinal neurons suggests that netrin-1 elevates the concentration of cAMP in neurons , raising the possibility that netrin-1 itself might promote DCC translocation by activating PKA. To test this hypothesis, cultures of embryonic rat spinal commissural neurons were treated with 50 ng/ml or 200 ng/ml netrin-1, or 10 μM FSK as a positive control, for either 5 or 15 min. Western blot analysis indicated that application of netrin-1 alone produced no detectable change in PKA-dependent phosphorylation of CREB, while the expected FSK induced increase in phospho-CREB was readily detectable (Figure 3.6A). Western blot analysis monitors global changes in CREB phosphorylation throughout the cell and may not detect localized changes in PKA activation. To determine if netrin-1 might regulate cAMP concentration locally, we examined the level of cAMP in the growth cones of commissural neurons immunocytochemically. Consistent with the results of western blot

analysis, 10 µM FSK added to the media increased cAMP immunoreactivity in growth cones, while 50 ng/ml or 200 ng/ml netrin-1 did not (Figure 3.6B).

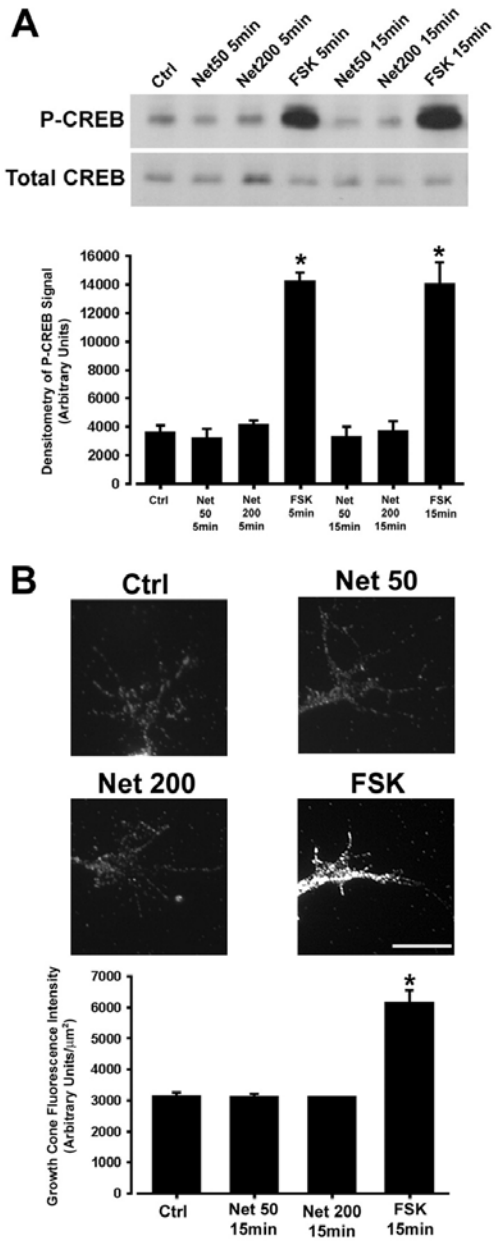

Figure 3.6 - Netrin-1 does not activate PKA in embryonic rat spinal commissural neurons. *Dissociated commissural neurons were cultured for 2 DIV (B) or 6 DIV (A) prior to treatment for 5 or 15 min with 50 ng/ml or 200 ng/ml netrin-1 or 10 µM FSK. Panel A shows western blot analysis of total cell extracts for phospho-CREB (P-CREB) and total CREB (~ 43 kDa). The histograms illustrate quantification of the optical density of CREB phosphorylation. In panel B, cells were fixed, permeabilized, and immunostained with anti-cAMP (Alexa 546-conjugated secondary antibody, 100X objective, scale bar = 10 µm). The histograms illustrate quantification of cAMP fluorescence intensities. Values represent the mean ± SEM (n=3 and 16 per condition, respectively for panels A and B). * indicates $p < 0.01$ vs ctrl.*

Furthermore, the increase in cell surface DCC triggered by addition of netrin-1 alone was not blocked by inhibition of either the adenylyl cyclase or PKA (Figure 3.4N). These findings indicate that netrin-1 does not increase the concentration of cAMP or activate

PKA in embryonic rat spinal commissural neurons and support the conclusion that the netrin-1 induced increase in DCC at the cell surface occurs through a PKA independent mechanism.

The adenosine A2b receptor activates the adenylyl cyclase (reviewed by Ralevic and Burnstock, 1998;Hopker et al., 1999). Increasing the concentration of intracellular cAMP by activating A2b has been used to modulate the response to netrin-1 (Shewan et al., 2002). Furthermore, evidence has been provided that A2b is a receptor for netrin-1 (Corset et al., 2000). We therefore tested the hypothesis that activating A2b might enhance the response to netrin-1 in commissural neurons. Consistent with evidence indicating that A2b is not expressed by embryonic rat commissural neurons (Stein et al., 2001), we found that the adenosine receptor agonist NECA does not effect netrin-1 induced commissural axon outgrowth (Figure 3.7), supporting the conclusion that A2b does not contribute to the response to netrin-1 in these cells.

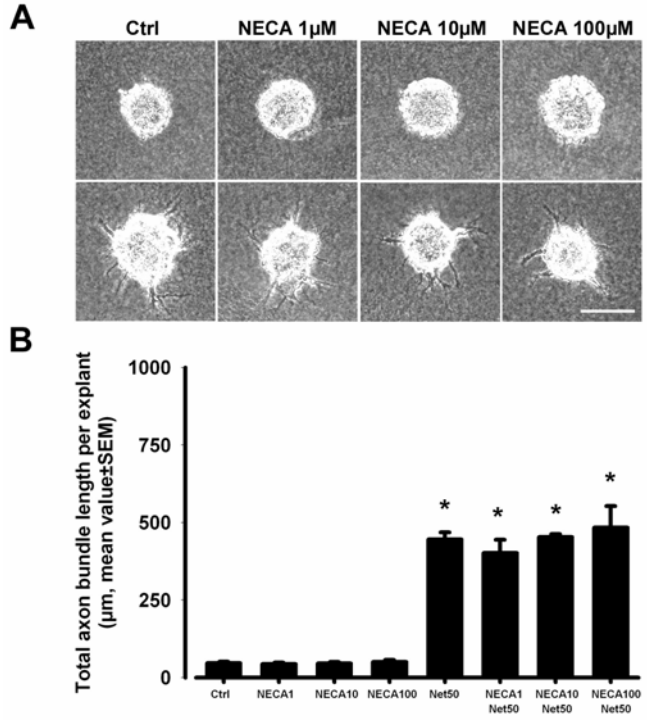

Figure 3.7 - Adenosine receptor agonist NECA does not effect embryonic rat spinal commissural axon outgrowth. *(A) Examples of E13 rat dorsal spinal cord axon outgrowth assayed in the following conditions: control, 1µM NECA, 10µM NECA, 100µM NECA, 50 ng/ml netrin-1, 50 ng/ml netrin-1 plus 1µM NECA, 50 ng/ml netrin-1 plus 10µM NECA, 50 ng/ml netrin-1 plus 100µM NECA. 20X objective. Scale bar=150 µm. (B) Quantification of axon outgrowth illustrated in panel A. * indicates p < 0.01 vs control. Results are expressed as mean total axon bundle length per explant ± SEM for between 4-5 explants per condition.*

PKA activation produces a netrin-1 dependent increase in cell surface DCC via a mechanism that requires exocytosis

The increased cell surface DCC detected immunocytochemically could be produced either by a selective increase in the amount of DCC protein on the cell surface, or by clustering DCC protein present more diffusely on the surface before treatment. To differentiate between these two possibilities, cell surface DCC protein was assessed directly by biotinylating cell surface proteins and quantifying the relative amount of DCC on the neuronal surface in different conditions. Dissociated commissural neurons were cultured for 6 days, and the cells treated for 15 min with SQ22536, KT5720, or TeTx. Netrin-1 (50 ng/ml) was added to the culture media for 15 min and cultures then exposed to 10 μM FSK for 15 min. Cell surface proteins were then biotinylated, isolated using streptavidin-agarose beads, and examined by western blot analysis using anti-DCC_{IN} and anti-$trkB_{ECD}$. A single ~180 kDa band was detected by anti-DCC_{IN} (Figure 3.8). Analysis of biotinylated proteins indicated that netrin-1 in combination with FSK produced a ten-fold increase in the amount of cell surface DCC compared to control (Figure 3.8). Pretreatment with SQ22536, KT5720, or TeTx prior to netrin-1 and FSK significantly reduced the level of cell surface DCC, when compared to netrin-1 plus FSK. Inhibition of protein synthesis with CHX did not affect the induced increase in cell surface DCC (not shown). Under the same conditions, the amount of biotinylated trkB, NCAM, or TAG-1 was not affected by cAMP elevation. Nor did we detect a change in non-biotinylated DCC protein, consistent with a relatively small amount of the total DCC being on the cell surface (Figure 3.8)

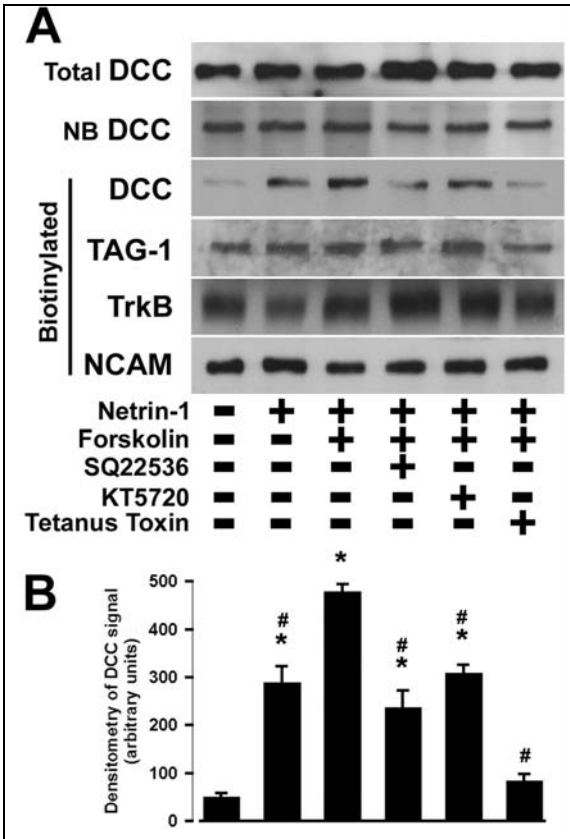

Figure 3.8 - Netrin-1 and FSK increase cell surface DCC. *Dissociated commissural neurons were cultured for 6 DIV prior to treatment for 15 min with or without 50 ng/ml netrin-1. Ten μM FSK was then added for 15 min in combination with 1 mM SQ22536, 200 nM KT5720, or 1.6 nM TeTx. Cell surface proteins were biotinylated and isolated. (A) Total, non-biotinylated, and biotinylated proteins were analysed by western blot with antibodies directed against either DCC (~180kDa), TAG-1 (~135kDa), trkB (~145kDa), or NCAM (~200kDa). (B) Quantification of the optical density of biotinylated DCC immunoreactivity. Values are mean ± SEM (n=4 per condition). * indicates a p value < 0.01 vs control. # indicates a p value <0.01 vs 50 ng/ml netrin-1 in combination with 10 μM FSK.*

PKA-dependent exocytosis promotes netrin-1 induced commissural axon outgrowth

Netrin-1 evokes commissural axon outgrowth from explants of embryonic dorsal spinal cord cultured in a three-dimensional collagen gel (Kennedy et al., 1994;Serafini et al., 1994). We tested the hypothesis that manipulation of PKA activation would cause DCC protein to be recruited to the surface of commissural axons and promote netrin-1 dependent axon outgrowth. Explants of E13 rat dorsal spinal cord were cultured in the presence of FSK (10 μM) alone or FSK (10 μM) and netrin-1 (50 ng/ml). At this concentration, netrin-1 alone evoked ~30% of maximal commissural axon outgrowth (not shown). Following 16 hrs of culture, FSK alone did not enhance axon outgrowth (Figure 3.9B, J). In contrast, FSK (10 μM) plus netrin-1 (50 ng/ml, Figure 3.9D, J) produced a dramatic increase in axon outgrowth compared with explants exposed to netrin-1 alone

(Figure 3.9C, J). In all cases, extending axons express TAG-1, a marker for commissural axons (not shown;(Dodd et al., 1988).

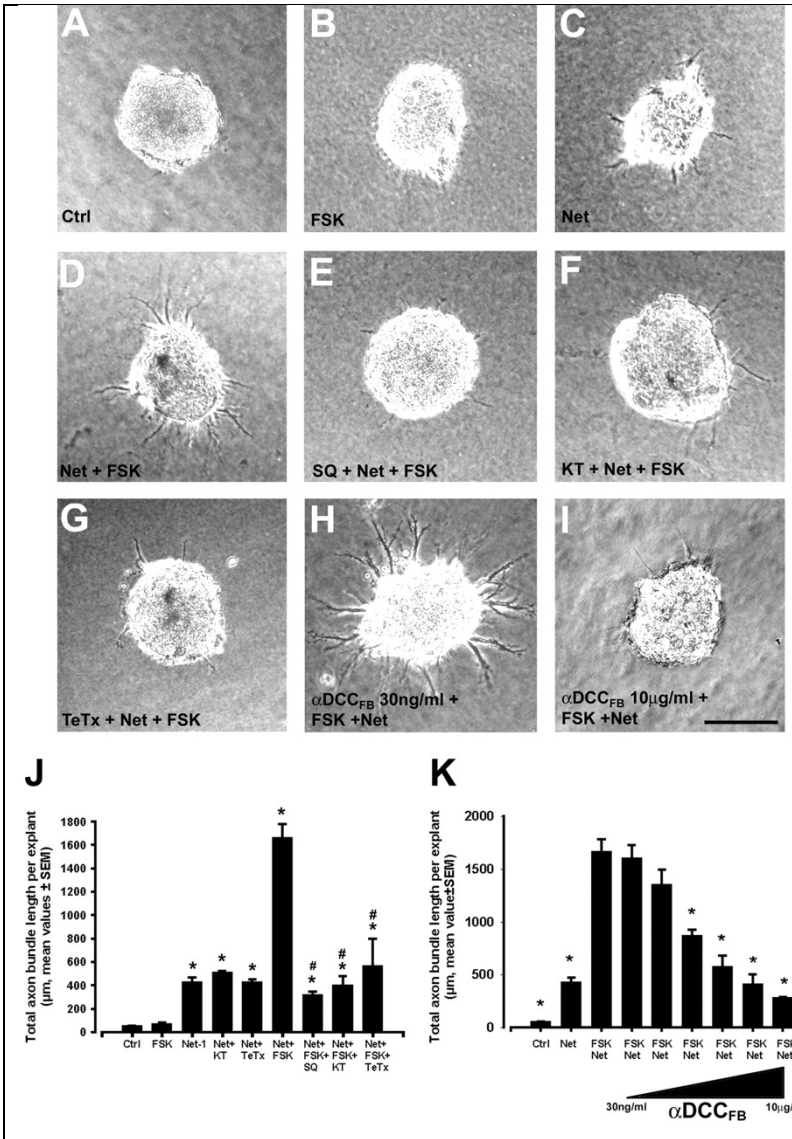

Figure 3.9 - PKA activation enhances netrin-1 dependent commissural axon outgrowth. *(A-I) E13 rat dorsal spinal cord axon outgrowth assays: (A) control, (B) 10μM FSK, (C) 50 ng/ml netrin-1, (D) 50 ng/ml netrin-1 plus 10μM FSK, (E) 50 ng/ml netrin-1, 10μM FSK, 1mM SQ 22536, (F) 50 ng/ml netrin-1, 10μM FSK, 200 nM KT 5720, (G) 50 ng/ml netrin-1, 10μM FSK, 1.6 nM tetanus toxin, (H, I) 50 ng/ml netrin-1, 10μM FSK and 30 ng/ml anti-DCC$_{FB}$ (H) and 50 ng/ml netrin-1, 10μM FSK and 10μg/ml anti-DCC$_{FB}$ (I). 20X objective. Scale bar=100 μm. (J) Quantification of axon outgrowth illustrated in panels A-I. * indicates p < 0.01 vs control. # indicates p <0.01 vs 50 ng/ml netrin-1 plus 10μM FSK. (K) Quantification of the effect of increasing concentrations of anti-DCC$_{FB}$ in the presence of netrin-1 and FSK. * indicates p < 0.01 vs 50 ng/ml netrin-1 plus 10μM FSK. J, K show mean total axon bundle length per explant ± SEM for between 4-26 explants per condition.*

To determine if FSK acts via the adenylyl 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 FSK, and then cultured for an additional 16 hrs. SQ22536 completely blocked the increase in axon outgrowth caused by FSK in the presence of netrin-1 (Figure 3.9E,J). KT5720 blocked the effect of FSK, demonstrating that PKA activation is required to produce the netrin-1 dependent increase in axon outgrowth evoked by FSK (Figure 3.9F,J). We then examined if the increase in cell surface DCC requires v-SNARE dependent exocytosis. Importantly, TeTx induced cleavage of v-SNAREs does not block axon extension because the neuronal v-SNAREs that are required for axon outgrowth are insensitive to TeTx (Osen-Sand et al., 1996;Martinez-Arca et al., 2001). Treatment with 1.6 nM TeTx (16 hrs) (Figure 3.9G,J) reduced axon outgrowth to the level found in the presence of netrin-1 alone, consistent with the increased outgrowth caused by netrin-1 and FSK requiring exocytosis. Notably, the inhibitors used reduced outgrowth to the level evoked by netrin-1 alone, suggesting that outgrowth evoked by netrin-1 alone does not require TeTx sensitive exocytosis.

Increased axon outgrowth evoked by FSK and netrin-1 requires DCC

The results described above suggest that FSK activates PKA, potentiating netrin-1 dependent outgrowth of commissural axons via a mechanism that requires exocytosis. To determine if the increased axon outgrowth caused by netrin-1 and FSK required DCC, dorsal spinal cord explants were exposed to increasing concentrations of DCC function blocking monoclonal antibody (anti-DCC_{FB}, from 30 ng/ml to 10 μg/ml) 15 min before the addition of netrin-1, thus 30 min before the addition of FSK to the media, and then cultured for 16 hrs. Anti-DCC_{FB} has been reported to block netrin-1 dependent commissural axon outgrowth *in vitro* at a concentration of 10 μg/ml (Keino-Masu et al., 1996). In the presence of FSK and netrin-1, anti-DCC_{FB} blocked axon outgrowth in a concentration-dependent manner (Figure 3.9H,I,K). The same concentrations of non-immune mouse IgG had no effect (not shown), indicating that the increased netrin-1 dependent axon outgrowth induced by FSK requires DCC

PKA modulates DCC dependent axon extension to the ventral midline of the embryonic spinal cord

Our findings predict that cell surface levels of DCC are co-regulated by netrin-1 and PKA. To determine if this contributes to commissural axon extension to the floor plate in the embryonic spinal cord, we utilized a semi-intact explant preparation. Segments of E11 rat brachial spinal cords, ~3 somites long, were isolated and embedded in collagen (Figure 3.10A,B) and the length of extending TAG-1 immunoreactive commissural axons quantified. Commissural axons in control explants, cultured for 40 hrs, followed their normal trajectory to the floor plate (Figure 3.10A). Consistent with the results of assaying axon outgrowth into collagen (Figure 3.9), activating PKA with FSK significantly increased the length of commissural axons extending within explanted spinal cords (Figure 3.10A,C,D). Furthermore, inhibiting PKA with KT5720 significantly reduced axon extension to the floor plate suggesting that endogenous PKA activity normally facilitates axon growth in the embryonic spinal cord. In contrast, inhibiting PKA with KT does not reduce axon outgrowth into collagen below the level evoked by netrin-1 alone (Figure 3.9), suggesting that the neuroepithelium may contain a PKA agonist that is not present when the axons grow into collagen. Anti-DCC_{FB} (10 µg/ml) significantly reduced axon extension to the floor plate. Furthermore, application of KT5720 or 10 µg/ml anti-DCC_{FB} to the explants 15 min before the addition of FSK, significantly reduced the FSK induced increase in axon extension (Figure 3.10A,C,D). These findings indicate that the enhancement of commissural axon extension in the embryonic spinal cord caused by activating PKA requires cell surface DCC.

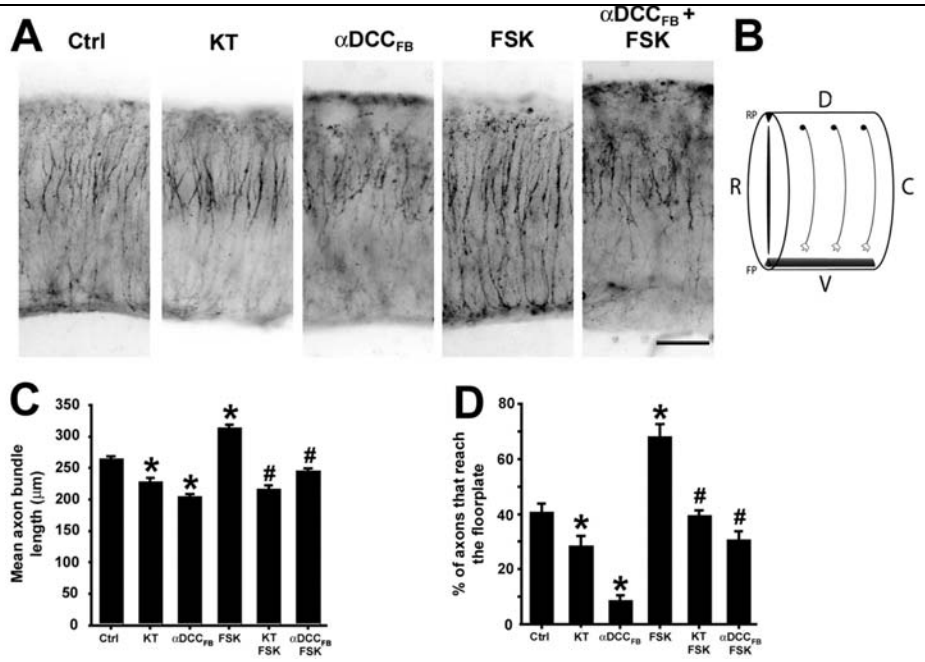

Figure 3.10 - PKA regulates axon extension to the ventral midline of the embryonic spinal cord in a DCC dependent manner. *(A) Brachial segments of E11 rat dorsal spinal cords were embedded in collagen and cultured for 40 hrs in the following conditions: control; 200 nM KT5720; 10 μg/ml anti-DCC$_{FB}$; 10 μM FSK; 10 μg/ml anti-DCC$_{FB}$ + 10 μM FSK. 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 183-548 axons per condition). (D) Quantification of the percentage of axons reaching the floorplate (mean ± SEM for 6-10 explants per condition; * indicates p < 0.01 vs control; # indicates p <0.01 vs 10μM FSK).*

DISCUSSION

The findings reported here indicate that post-translational recruitment of DCC to the cell surface from an intracellular pool regulates the response of axons to netrin-1. Application of netrin-1 alone produced a modest increase in the amount of DCC on the neuronal surface. Activation of PKA coincident with addition of netrin-1 potentiated the insertion of DCC into the plasma membrane and increased axon outgrowth. Blocking DCC function significantly reduced the effect of PKA activation in each assay used.

Furthermore, inhibiting PKA in explanted intact embryonic spinal cord assays reduced axon extension (Figure 3.10), providing evidence that the embryonic spinal neuroepithelium may contain an endogenous PKA agonist. These results identify a novel modulatory role for PKA in the growth cone, regulating the presentation of DCC and thereby enhancing the extension of commissural axons in response to netrin-1.

Netrin-1 does not activate PKA in commissural neurons nor is PKA activation required for commissural axon outgrowth evoked by netrin-1

Previous studies carried out using either *Xenopus* retinal neurons or spinal neurons grown in dispersed cell culture indicate that the intracellular level of cAMP plays a key role in determining if a growth cone responds to netrin-1 as an attractant or a repellent (Ming et al., 1997;Hopker et al., 1999;Nishiyama et al., 2003). Low levels of intracellular cAMP correlate with a repellent response, while high levels of cAMP, and presumably activation of PKA, correlate with an attractant response. Netrin-1 itself has been reported to increase the concentration of intracellular cAMP in *Xenopus* retinal ganglion cell growth cones *in vitro* (Hopker et al., 1999). cAMP immunoflorescence in RGC growth cones supports this conclusion, but additional biochemical data was not provided. On the basis of these studies, models of netrin-1 signal transduction place activation of PKA directly downstream of DCC (Song and Poo, 1999;Nishiyama et al., 2003).

In contrast, our findings provide direct evidence that netrin-1 does not elevate intracellular cAMP or activate PKA in embryonic rat spinal commissural neurons. Furthermore, they indicate that activating PKA is not required for netrin-1 evoked axon outgrowth. This conclusion is based on the finding that application of netrin-1 while inhibiting the adenylyl cyclase (SQ22536, Figure 3.9) or PKA (KT5720, Figure 3.9) did not reduce axon outgrowth below the level produced by netrin-1 alone. The finding that PKA inhibition does not affect netrin-1 evoked commissural axon outgrowth (Figure 3.9) also appears to be at odds with (Ming et al., 1997) and (Nishiyama et al., 2003) which report that reduced levels of cAMP in cultured *Xenopus* spinal neurons causes growth cones to be repelled by netrin-1. Further analysis will be required to determine if these differences are due to the species, cell types, or methodologies used. However, based on

our findings, we conclude that current models do not provide a widely generalizable description of the neuronal response to netrin-1.

We conclude that a major effect of activating PKA on netrin-1 induced commissural axon outgrowth is to potentiate translocation of DCC to the plasma membrane. We do not rule out a role for PKA exerting other effects on axon extension; however, our conclusion is supported by the finding that blocking DCC function dramatically reduced both the FSK induced enhancement of netrin-1 dependent axon outgrowth into collagen (Figure 3.9), and the FSK induced enhancement of axon extension toward the floor plate in the explanted embryonic spinal cord (Figure 3.10). These results indicate that a major component of the effect of FSK on netrin-1 evoked axon outgrowth requires DCC.

Interestingly, different mechanisms may underlie the increase in cell surface DCC caused by netrin-1 alone, and the recruitment of DCC triggered by netrin-1 and activation of PKA. Notably, the increase induced by netrin-1 alone was not blocked by inhibiting PKA or blocking TeTx sensitive exocytosis. In contrast, the increase in cell surface DCC triggered by netrin-1 and PKA activation was blocked by TeTx. Similarly, application of TeTx or inhibiting PKA did not affect commissural axon outgrowth in response to netrin-1, but reduced the PKA induced increase in axon outgrowth to the level produced by netrin-1 alone (Figure 3.9). These findings suggest that a TeTx sensitive v-SNARE protein is required for PKA dependent translocation of DCC, but is not required for the increase in cell surface DCC caused by netrin-1 alone.

Specificity of DCC translocation

There are multiple examples of the elevation of intracellular cAMP causing the translocation of proteins from an intracellular vesicular store to the plasma membrane. These include transporters (Yao et al., 1996), ion pumps (Schwartz and Al Awqati, 1986), ion channels (Barres et al., 1989), and trophic factor receptors (Meyer-Franke et al., 1998). Here, both immunoflorescent and surface biotinylation analyses indicate a surprising level of specificity in the PKA dependent increase in DCC at the cell surface. We hypothesize two mechanisms that may account for this specificity. First, commissural

neurons may contain vesicles that specifically traffic DCC to the cell surface. A second, but not necessarily mutually exclusive mechanism is suggested by the finding that increased cell surface DCC requires the presence of netrin-1. Activation of PKA alone produced no detectable increase in plasma membrane DCC (Figure 3.4, 5.6), while PKA activation plus netrin-1 increased the amount of cell surface DCC and increased axon outgrowth. This suggests that netrin-1 is required to hold DCC at the plasma membrane. In this case, the vesicle bringing DCC to the cell surface may or may not exhibit specificity for DCC, but the presence of netrin-1 selects DCC and stabilizes it on the cell surface. Similarly the PKA independent increase in cell surface DCC produced by netrin-1 alone may be due to netrin-1 dependent stabilization of DCC on the cell surface and not netrin-1 induced DCC translocation.

Interestingly, this selection and cell surface capture model predicts that DCC would accumulate at the cell surface in regions of the cell in contact with extracellular netrin-1, a prediction that we are currently testing. We have previously reported that netrin-1 and DCC direct the organization of F-actin, causing Rac1 and Cdc42 dependent cell spreading and filopodia formation (Shekarabi and Kennedy, 2002). Together, these findings suggest that DCC will accumulate at the cell surface in regions corresponding to high concentrations of extracellular netrin-1, locally triggering filopodia formation and the extension of a leading edge, thereby directing axon outgrowth. Interestingly, local redistribution of receptors for guidance cues to the leading edge has been observed in directionally migrating lymphocytes and *Dictyostelium* (reviewed by Manes et al., 2003), suggesting that this may be a general mechanism used by directionally migrating cells.

Recruitment of receptors to the cell surface: a post-translation mechanism regulating axon extension

Our findings support a model in which a post-translational mechanism plays a key role regulating the presentation of DCC. DCC is a member of a large family of Type 1 transmembrane proteins containing IgG repeats and fibronectin type III repeats that includes Roundabout (Robo) and L1 (reviewed by Brummendorf and Lemmon, 2001). Selective trafficking of such adhesion molecule-like receptors may be a widespread

mechanism regulating the response of growth cones to extracellular cues that influence motility. For example, endo-exocytic recycling regulates the distribution of L1 in growth cones (Kamiguchi and Lemmon, 2000). Furthermore, presentation of Robo on the cell surface determines if an axon will cross the ventral midline of the embryonic CNS (Keleman et al., 2002).

Increasing the amount of UNC5 homologue expressed by cultured embryonic *Xenopus* spinal neurons causes axons that would normally be attracted to netrin-1 to be repelled (Hong et al., 1999). This study and genetic manipulation of UNC5 expression (Hamelin et al., 1993) indicate that changing the complement of netrin receptors expressed by a neuron can alter its response to netrin. Recently, (Keleman and Dickson, 2001;Williams et al., 2003) provided evidence that PKC activation triggers the internalization of ectopically expressed UNC5H1 from neuronal growth cones, and that this reduces the probability that growth cones will collapse in response to netrin-1. The mechanism underlying the ability of a neuron to switch its response to netrin-1 from attraction to repulsion remains unclear. We have not ruled out that PKA regulated alterations in intracellular signal transduction may contribute to this; however a straight-forward alternative is that growth cones change their response to netrin-1 based on the selective presentation of different classes of netrin receptors on the plasma membrane.

The role identified for PKA regulating cell surface presentation of receptors for axon guidance cues may extend beyond embryonic neural development. A decrease in the steady state level of cAMP inside a neuron during maturation contributes to a decrease in the capacity of axons to regenerate in the adult mammalian CNS (Cai et al., 2001). Furthermore, PKA activation promotes regeneration of sensory axons in the CNS (Neumann et al., 2002;Qiu et al., 2002a). The mechanisms underlying this change in neuronal response are not known, but the findings presented here raise the possibility that modulation of PKA activity may influence the ability of an axon to regenerate by regulating the complement of receptors presented by the growth cone.

CHAPTER 4

Protein Kinase A Regulates the Sensitivity of Spinal Commissural Axon Turning to Netrin-1, but does not Switch between Chemoattraction and Chemorepulsion

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PREFACE

This chapter has been published as a brief communication in the Journal of Neuroscience (Moore and Kennedy, 2006b). In the previous chapter, we demonstrated that: (1) Protein kinase A (PKA) promotes trafficking of the netrin-1 receptor DCC to the plasma membrane, (2) that this increases the ability of spinal commissural neurons to extend toward a source of netrin-1, and (3) that netrin-1 does not induce cAMP elevation. In this chapter we extend these observations by examining how the cAMP pathway affects turning to netrin-1. We also perform a more thorough examination of the possibility that netrin-1 might induce cAMP production. Consistent with chapter 3, we found that netrin-1 is incapable of triggering cAMP production. Moreover, we found that contrary to the behaviour of *Xenopus* spinal neurons, reducing cAMP signaling does not induce rat spinal commissural axons to respond to netrin-1 as a repellent; rather, we observe only a reduction in the sensitivity of axons to netrin-1.

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ABSTRACT

Bifunctional axon guidance cues have been grouped into two classes depending on whether changes in intracellular cAMP or cGMP switch the growth cone's response between attraction and repulsion. According to this model, axons respond to netrin-1, a group I guidance cue, as a chemoattractant when cAMP levels are high in the growth cone, but switch and are repelled when the intra-neuronal concentration of cAMP is low. The model is complicated by the proposal that cAMP dependent kinase, PKA, functions as a downstream effector for several guidance cues, including netrin-1, suggesting a close inter-relationship between guidance cue signal transduction and mechanisms regulating the switch between attraction and repulsion. Here, we examine possible interactions between netrin-1 mediated axon guidance and cAMP signaling in embryonic rat spinal commissural neurons. We report that netrin-1 does not alter the concentration of cAMP or PKA activity in these neurons, across a wide range of netrin-1 concentrations and time points following application, leading us to conclude that netrin-1 does not regulate PKA in these cells. In contrast to the cyclic nucleotide switch model, we report that despite inhibiting PKA, embryonic spinal commissural axons were always attracted to netrin-1 and never repelled. Instead, manipulating PKA regulated the sensitivity of chemoattraction to netrin-1: PKA inhibition reduced, and PKA activation increased, the distance over which axons turn toward a source of netrin-1. These findings indicate that the mechanisms underlying cyclic nucleotide regulated switching are separable from the signal transduction mechanisms required for chemoattraction to netrin-1.

INTRODUCTION

During neural development, axons are directed to their targets by extracellular cues. Bifunctional guidance cues have been classified into one of two groups based on whether the growth cone's response is converted between attraction and repulsion by changes in intracellular cAMP or cGMP (Song and Poo, 1999;Song and Poo, 2001). According to this model, netrin-1 is a group I guidance cue: growth cones containing high levels of cAMP are attracted to netrin-1, whereas those with low cAMP levels are

repelled (Ming et al., 1997). Netrin-1 has also been reported to signal by elevating intracellular cAMP (Hopker et al., 1999;Corset et al., 2000). Together, these findings have led to a model in which cAMP and PKA are downstream effectors of netrin-1 signaling, while also regulating the directionality of the axonal response to netrin-1 (Song and Poo, 1999;Hopker et al., 1999;Shewan et al., 2002;Nishiyama et al., 2003)

We tested this model of netrin-1 chemotropic function using embryonic rat spinal commissural neurons, cells that extend an axon to the floor plate at the ventral midline of the developing spinal cord. These axons are initially repelled by BMP7 and GDF7 secreted by the roof plate at the dorsal midline (Augsburger et al., 1999;Butler and Dodd, 2003) and then attracted by netrin-1 and sonic hedgehog secreted by the floor plate (Kennedy et al., 1994;Serafini et al., 1994;Charron et al., 2003). We have previously reported that activating PKA recruits DCC to the cell surface from an intracellular pool, enhancing axon extension in response to netrin-1 (Bouchard et al., 2004). This study provided evidence that inhibiting PKA slowed commissural axon extension to the floor plate, but did not examine a role for PKA in commissural axon chemotropic turning to netrin-1. Here, we provide evidence that application of netrin-1 does not affect cAMP signaling in embryonic rat spinal commissural neurons across a wide range of netrin-1 concentrations and time points. Furthermore, we demonstrate that PKA regulates the sensitivity of embryonic rat spinal commissural axon turning towards a source of netrin-1. Activating PKA increased the distance over which axons turned toward a source of netrin-1, while PKA inhibition reduced this distance. However, in contrast to the cyclic nucleotide switch model, inhibiting PKA did not cause these axons to be repelled by netrin-1. We conclude that mechanisms underlying chemoattraction to netrin-1 are independent of mechanisms required for cyclic nucleotide-dependent switching. Our findings indicate that PKA regulates the sensitivity of spinal commissural axon chemoattraction to netrin-1 and are consistent with our previous report that PKA mobilizes DCC from an intracellular vesicular pool to the growth cone plasma membrane.

EXPERIMENTAL PROCEDURES

Reagents

Monoclonal anti-Tag-1 (4D7) was obtained from the Developmental Studies Hybridoma Bank (University of Iowa) and KT5720 from Calbiochem (LaJolla, CA). Rabbit antibodies against CREB and phosphorylated CREB (Ser133, 1B6) were obtained from Cell Signaling Tech (Beverly, MA). Forskolin (Fsk), DNase and poly-D-lysine (PDL, 70-150 kD) were obtained from Sigma-Aldrich, (Missisauga, Canada). Neurobasal, heat inactivated FBS (iFBS), B-27 supplement, GlutaMAX-1, Penicillin-Streptomycin and Ca^{2+}/Mg^{2+}-free HBSS were purchased from Invitrogen Canada (Burlington, ON). 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).

Spinal Commissural Neuron Cultures

Staged pregnant Sprague-Dawley rats were obtained from Charles River Canada (St-Constant, QC). The dorsal half of embryonic day 13 (E13, vaginal plug = E0) rat spinal cords were isolated by microdissection and dissociated, as previously described (Placzek et al., 1990;Bouchard et al., 2004). In brief, dorsal spinal cords were incubated at 37^0C for 30 min in 0.0002% DNase Ca^{2+}/Mg^{2+}-free HBSS. The tissue was then triturated with flamed glass pipettes to yield a suspension of single cells. For both cAMP ELISAs and phospho-CREB western blots, two million dissociated cells were plated and cultured in 35 mm tissue culture dishes (Corning Inc, Corning, NY) previously coated with 20 µg/ml PDL for 2 hrs at room temperature. For the first 12 hrs, cells were cultured in Neurobasal supplemented with 10% iFBS, 2 mM GlutaMAX-1, 100 units/ml penicillin, and 100 µg/ml streptomycin. The medium was then changed to Neurobasal supplemented with 2% B-27, 2 mM GlutaMAX-1, 100 units/ml penicillin, and 100 µg/ml streptomycin for an additional 28 hrs prior to cell lysis**.** More than 90% of the cells in these cultures express TAG-1 and DCC, markers of commissural neurons in the dorsal spinal cord. Furthermore, choline acetyl transferase, expressed by motoneurons, was not detected in these cultures (Bouchard et al., 2004).

cAMP ELISA

Following treatments, commissural neuron cultures were lysed and the cAMP levels measured using a low pH cAMP ELISA (R&D Systems, Minneapolis, MN) as per the manufacturer instructions for acetylated lysates. Absorbance in each well was measured on a Model 680 microplate reader (Bio-Rad, Hercules, CA). Concentrations of each condition were normalized across experiments by determining percent changes relative to the average value of controls, in the absence of netrin-1.

Phospho-CREB Analysis

Following treatments, cells were lysed in RIPA/Laemmli buffer (60 mM tris pH 6.8, 5% glycerol, 2.5% SDS, 1.25% BME, 7.25% DTT, 1% NP-40, 0.5% deoxycholate and 150 mM NaCl). The relative amounts of unphosphorylated and phosphoryalted CREB in lysates were assessed by western blot analysis (Harlow and Lane, 1999). Films were exposed using chemiluminescence (PerkinElmer BioSignal, Montreal, QC) and scanned on a ScanJet 5300C (Hewlett-Packard, Mississauga, ON). Intensities of each band were measured using Photoshop 7.0 (Adobe, San Jose, CA). Intensities across experiments were compared as percent change in intensity relative to controls, without netrin-1.

Commissural Axon Turning Assays

Segments of E11 rat spinal cord (vaginal plug = E0) were dissected and aggregates of netrin expressing HEK 293-EBNA cells were prepared, as previously described (Placzek et al., 1990;Kennedy et al., 1994;Shirasaki et al., 1996). Cell aggregates were immobilized in collagen alongside dissected E11 spinal cords. The explanted tissue was cultured for 40 hrs in supplemented Neurobasal (10% iFBS, 2 mM GlutaMAX-1, 100 units/ml penicillin, 100 µg/ml streptomycin), and then fixed with 4% paraformaldehyde (PFA). The trajectories of commissural axons were fluorescently labeled with Tag-1 (4D7) antibodies followed by an Alexa 546 coupled secondary against mouse IgM. Digital images were acquired using a Magnafire CCD camera (Optronics, Goleta, CA) on an Axiovert microscope (Carl Zeiss Canada, Toronto, ON). Images were

printed and the deflection distances determined by an observer blind to the experimental conditions. Distances were compared across experiments as the percent distance in each condition relative to the average value of controls. Statistical significance was evaluated by a one-way ANOVA with Sheffe post-hoc test (Systat 9, Point Richmond, CA).

E13 Spinal Cord Dorsal Explant Cultures

Dorsal spinal cord explants were dissected from E13 rat embryos, as previously described (Tessier-Lavigne et al., 1988). Explants were embedded and cultured for 14 hrs in Neurobasal supplemented with 10% iFBS, 2 mM Glutamine, 100 unit/ml penicillin, and 100 μg/ml streptomycin. Digital images of Tag-1 positive commissural axons were acquired as described above.

RESULTS

Netrin-1 does not regulate cAMP concentration or PKA activity in embryonic rat spinal commissural neurons

Elevation of cAMP results in PKA-dependent phosphorylation of CREB (pCREB) on Ser133 (Gonzalez and Montminy, 1989). Based on cAMP immunocytochemistry and pCREB analysis, we previously reported that netrin-1 does not affect cAMP-PKA signaling following netrin-1 application (Bouchard et al., 2004). We have now extended these findings, carrying out cAMP ELISAs and pCREB western blot analyses across a wide range of time points and netrin-1 concentrations. Application of 200 ng/ml netrin-1 generates maximal commissural axon outgrowth from explants of E13 dorsal spinal cord, while 50 ng/ml generates approximately half-maximal outgrowth. Although these concentrations of netrin-1 elicited substantial commissural axon outgrowth (Figure 4.1A), in no case did we find that they altered the concentration of cAMP or activation of PKA. Specifically, no significant difference in cAMP (Figure 4.1B) or pCREB (Figure 4.1C) was observed after 15 min of 50, 100 or 200 ng/ml netrin-1 stimulation. Furthermore, 200 ng/ml netrin-1 also failed to increase cAMP (Figure 4.1D) or pCREB (Figure 4.1E) after 5, 15 or 30 min. In contrast, large increases in

intracellular cAMP and pCREB were detected within 5 min of application of the adenylyl cyclase agonist forskolin (10 M Fsk, Figures 1B-E, Metzger and Lindner, 1981).

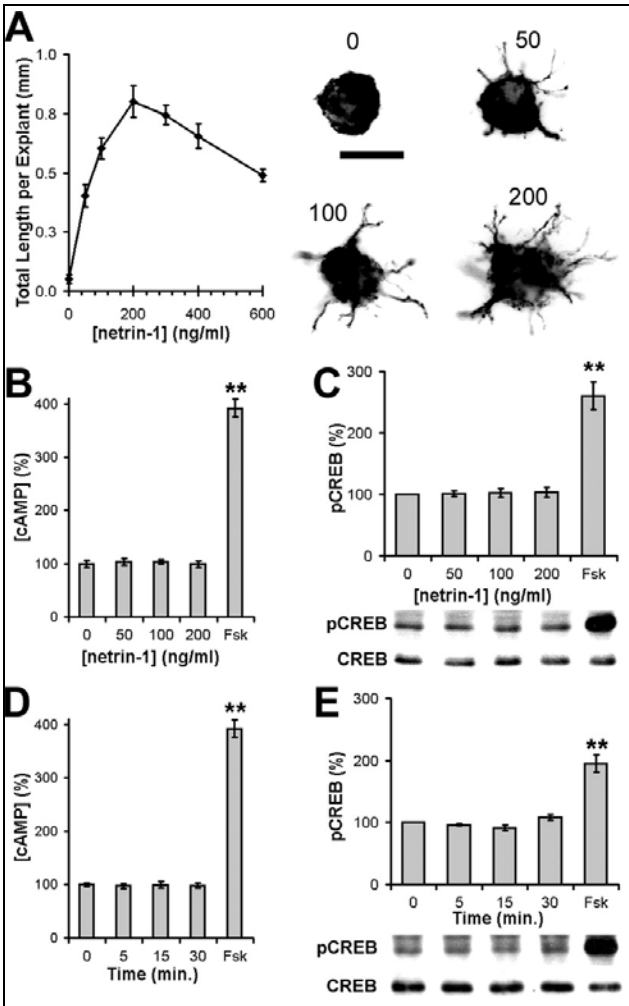

Figure 4.1 - Netrin-1 does not alter cAMP levels or PKA activity in rat spinal commissural neurons. *Netrin-1 induces the outgrowth of Tag-1 positive commissural neurons from dorsal spinal cord explants when present at concentrations between 50 and 200 ng/ml after 14 hrs in culture (A; Serafini et al., 1994). Numbers above each explant indicate the concentration of netrin in ng/ml. In lysates of commissural neurons cultured for 40 hrs, concentrations of cAMP and PKA activities were measured using cAMP ELISAs (B,D) and pCREB Western blots (C,E), respectively. The same concentrations of netrin-1 that induced extensive outgrowth, failed to increase cAMP levels (B) or PKA activity (C) after 15 min. Furthermore, 200 ng/ml netrin-1 failed to increase cAMP levels (D) or PKA activity (E) after 5, 15 or 30 min. However, 5 min of forskolin (Fsk) increased cAMP levels (n=4, ** p< 0.01, B, D) and PKA activity (n=3, ** p< 0.01, C, E). In histograms B & C, values are expressed as % of 0 ng/ml netrin-1 +/- SEM, and in D and E expressed as % of time 0 +/- SEM. Scale bar = 100 μm.*

Inhibiting PKA reduces commissural axon sensitivity to netrin-1, but does not switch attraction to repulsion

An aggregate of netrin-1 expressing cells placed against the cut edge of an E11 spinal cord explant causes embryonic spinal commissural axons to deviate from their dorsal-ventral trajectory and turn toward the source of netrin-1 (Figure 4.2A,D; Kennedy

et al., 1994). Similarly placing an explant of roof plate or BMP expressing cells causes these axons to be repelled (Figure 4.2F, Augsburger et al., 1999;Butler and Dodd, 2003). Given that commissural axons have the capacity to be either attracted or repelled, we used this turning assay to test the hypothesis that inhibiting PKA will switch the response of spinal commissural neurons to netrin-1 from attraction to repulsion.

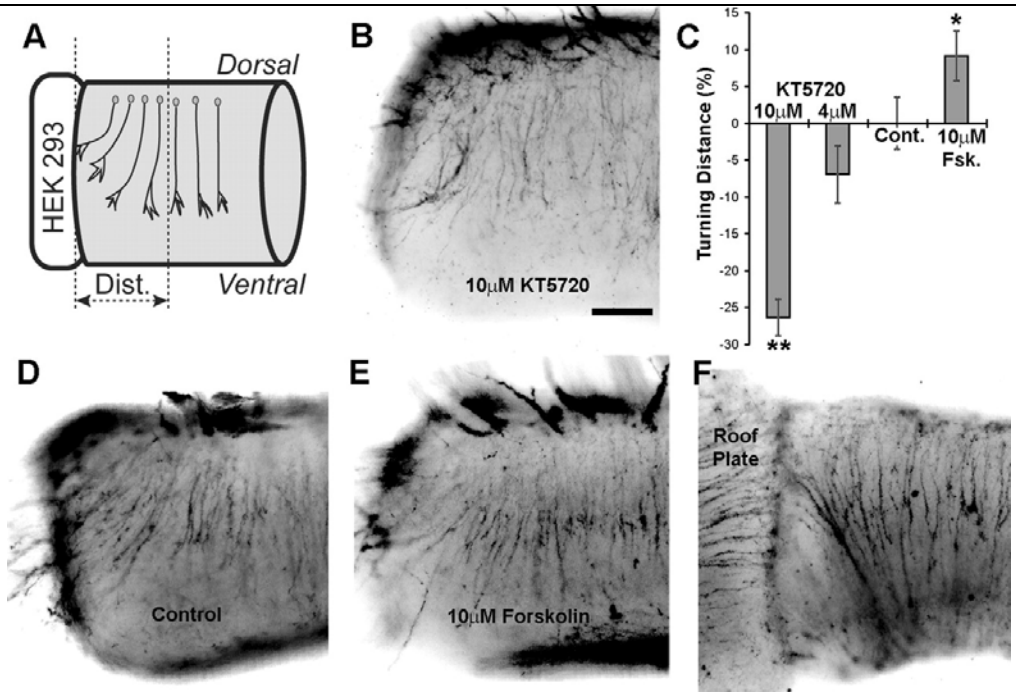

Figure 4.2 - cAMP and PKA regulate the range over which axons turn towards netrin-1. *Aggregates of netrin-1 expressing HEK 293-EBNA cells were cultured alongside explants of E11 rat spinal cord, as shown in panel A. Explants were cultured for 40 hrs in the absence of drugs (D) or in the presence of 4 μM KT5720, 10 μM KT5720 (B) or 10 μM forskolin (E). Commissural axons were fluorescently labeled with Tag-1 (4D7). Deflection distances were measured, as shown in panel A, and plotted in C as the percent distance relative to the absence of drugs. 10 μM KT5720 resulted in a 26.3% reduction (n=27, **p<0.01) of the turning distance, whereas 10 μM forskolin increased the turning distance by 9.2% (n=60, *p<0.025). Repellent turning was never observed to a netrin-1 source, despite commissural axon's ability to be repelled by roof plate, as previously described (F ; Augsburger et al., 1999). Scale bar = 100 μm.*

We found that application of 4 μM or 10 μM KT5720, an inhibitor of PKA (Kase et al., 1987), generated a dramatic reduction in pCREB following 12 hrs in culture (Figure 4.3). This effect is consistent with the reported 3.3 μM IC50 of KT5720 for PKA at physiological ATP concentrations (Davies et al., 2000). Although KT5720 may influence targets other than PKA, we conclude that KT5720 inhibits PKA at the concentrations used. However, despite inhibiting PKA, commissural axons continued to be attracted to netrin-1. Although repellent turning was never observed, 10 μM KT5720 reduced by 26% the distance over which rat commissural neuron axons turned toward the ectopic source of netrin-1 (n = 27, $p<0.01$, Figure 4.2B-D).

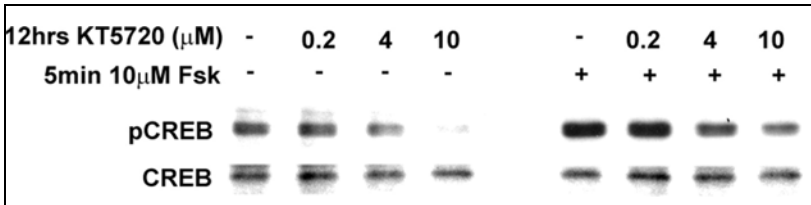

Figure 4.3 - KT5720 inhibits PKA activity in rat spinal commissural neurons. *KT5720 is an ATP analog that competitively inhibits PKA (Kase et al., 1987). Following 24 hrs in culture, E13 spinal commissural neurons were then cultured for an additional 12 hrs with KT5720. As indicated, some cultures were then lysed immediately, while others were stimulated with 10 μM forskolin for 5 min prior to lysis.*

Increasing cAMP Concentration and Activating PKA Increases the Sensitivity of Commissural Axon Turning to Netrin-1

We have previously reported that activating PKA recruits DCC from an intracellular vesicular pool to the plasma membrane of embryonic rat spinal commissural neurons, and that this increases axon extension in response to netrin-1 (Bouchard et al., 2004). These findings led us to hypothesize that activating PKA will enhance the sensitivity of commissural neurons to a gradient of netrin-1, increasing the distance over which chemoattractive turning occurs. In contrast to this prediction, cAMP and PKA have been reported not to alter the sensitivity of cultured *Xenopus* spinal neurons as they turn in response to netrin-1 (Ming et al., 1997). We therefore tested our hypothesis using the embryonic rat spinal commissural axon turning assay described above. Elevation of cAMP by application of 10 μM forskolin (Figure 4.1B-E) significantly increased the

range over which axons turn toward a source of netrin-1 (n = 60, $p<0.025$, Figure 4.2C-E). This is consistent with our previous report that PKA activation recruits DCC to the growth cone plasma membrane (Bouchard et al., 2004).

DISCUSSION

Here we examined the interaction of netrin-1 and cAMP-PKA signaling in embryonic rat spinal commissural neurons. Application of netrin-1 evoked no significant change in either the concentration of cAMP or the activity of PKA across a wide range of time points and netrin concentrations. Furthermore, despite inhibiting PKA, commissural axons did not switch their response to netrin-1. Rather, inhibiting PKA reduced, and activating PKA increased, the distance over which commissural axons turn toward a source of netrin-1. We conclude that activating PKA is not required for spinal commissural axon chemoattraction to netrin-1, and that mechanisms underlying netrin-1 chemoattraction are separable from mechanisms required for cyclic nucleotide regulated switching. Consistent with PKA recruiting DCC to the growth cone plasma membrane from an intracellular vesicular pool (Bouchard et al., 2004), our findings indicate that cAMP and PKA regulate the sensitivity of spinal commissural axon chemoattractive turning toward netrin-1 (Figure 4.4).

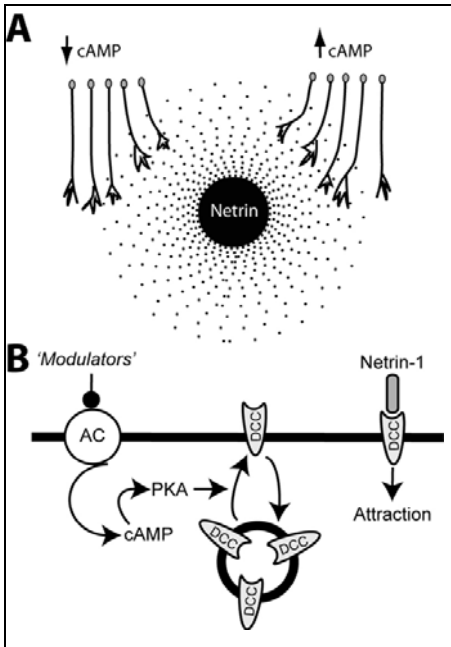

Figure 4.4 - Model: Activating PKA recruits DCC to the growth cone plasma membrane, increasing the sensitivity of commissural axon turning toward a source of netrin-1. *(A) Increasing the concentration of intracellular of cAMP increases the range over which commissural axons turn to a source of netrin-1. (B) Activating adenylyl cyclase (AC) increases cytosolic cAMP concentration, activating PKA, and triggering the recruitment of DCC to the growth cone plasma membrane, thereby increasing the sensitivity of the growth cone to netrin-1. Netrin-1 binding to DCC directs cytoskeletal rearrangements underlying chemoattraction, independent of downstream activation of PKA.*

The absence of a cAMP mediated switch between attraction and repulsion contrasts with findings obtained using cultured *Xenopus* retinal ganglion cells (RGCs) or *Xenopus* spinal neurons, whose axons are either attracted or repelled by netrin-1 depending on the intra-neuronal concentration of cAMP (Song et al., 1997;Ming et al., 1997;Song et al., 1998;Hopker et al., 1999). Attraction to a source of Netrin-1 requires DCC, whereas members of the Unc5 homologue netrin receptor family (Unc5A-D) are required for the repellent response (reviewed in Huber et al., 2003). It is not clear which netrin-1 receptors are expressed by the *Xenopus* spinal neurons assayed *in vitro* and the heterogeneous mixture of cells in these cultures confounds biochemical analyses. RGCs have been shown to express both DCC and multiple Unc5 homologues in zebrafish, rats, and mice (Deiner et al., 1997;Petrausch et al., 2000;Ellezam et al., 2001). *Xenopus* RGCs express DCC (de la Torre et al., 1997;Shewan et al., 2002), and although the single identified *Xenopus* Unc5 homologue is not expressed by RGCs (Anderson and Holt, 2002), it seems likely that they express a different family member. Two reports suggest that the relative amounts of DCC and Unc5 homologues expressed dictates whether an axon responds to netrin-1 as an attractant or repellent (Hamelin et al., 1993;Hong et al., 1999). In *C. elegans,* ectopic expression of Unc5 caused axons to be repelled rather than attracted to a source of the netrin Unc6 (Hamelin et al., 1993). Similarly, growth cones of cultured *Xenopus* spinal neurons were repelled by netrin-1 when they were engineered to over-express Unc5 (Hong et al., 1999). Interestingly, recent findings indicate that the cell surface presentation of DCC and Unc5 homologue netrin receptors is regulated by PKA and PKC, respectively (Williams et al., 2003;Bouchard et al., 2004). These, and our current findings, support an emerging model in which kinase activity regulates the amounts of DCC and Unc5 homologues inserted into the growth cone plasma membrane, that in turn determines if the axonal response to netrin-1 promotes or inhibits outgrowth. Embryonic rat spinal commissural neurons, however, do not express Unc5 homologues as they are extending to the floor plate (Leonardo et al., 1997). The responses observed therefore reflect DCC function in the absence of Unc5 homologues. Thus, the difference in the response to netrin-1 found between embryonic rat spinal commissural neurons and

Xenopus spinal and retinal neurons may be due to differences in the netrin receptors expressed by these cells.

PKA is often included as a downstream component of netrin-1 chemoattractive signaling (Song and Poo, 1999;Nishiyama et al., 2003), however little direct evidence supports this conclusion. A modest increase in cAMP immunoreactivity was reported in *Xenopus* RGC growth cones following exposure to netrin-1 (Hopker et al., 1999), but biochemical analyses to support this finding have not been carried out. The adenosine 2b receptor (A2b), a G-protein coupled receptor that generates an increase in cAMP when bound to adenosine (Ralevic and Burnstock, 1998), has been proposed to function as a receptor for netrin-1 in embryonic rat commissural neurons (Corset et al., 2000). In contrast, subsequent studies demonstrated that embryonic rat commissural neurons do not express A2b as they extend axons to the floor plate, and pharmacological manipulations of adenosine receptor activity did not affect axon outgrowth or turning in response to netrin-1 (Stein et al., 2001;Bouchard et al., 2004). It has been shown that manipulating A2b activity in *Xenopus* RGCs alters the concentration of cytosolic cAMP and switches between attractant and repellent response to netrin-1 (Shewan et al., 2002), however, these experiments did not provide evidence that netrin-1 itself signals through A2b. Our finding that netrin-1 does not regulate the cytosolic cAMP concentration in commissural neurons reinforces the conclusion that A2b is not a receptor for netrin-1 in these cells.

Although PKA is not activated downstream of netrin-1 and DCC in commissural neurons, we do not rule out that other cues encountered by a migrating commissural growth cone may influence attraction to netrin-1 by regulating PKA. For example, laminin-1 has been reported to switch netrin-1 from an attractant to a repellent of RGC axon outgrowth by reducing cAMP (Hopker et al., 1999). Furthermore, inhibiting PKA reduced spinal commissural axon extension to the floor plate (Bouchard et al., 2004), consistent with the action of an as yet unidentified endogenous activator of PKA in the neuroepithelium of the embryonic rat spinal cord.

Our findings indicate that the capacity of cAMP-PKA signaling to switch between chemoattraction and chemorepulsion is not ubiquitous to all cells that respond to netrin-1. We conclude that netrin-1 does not activate cAMP-PKA signaling in embryonic rat spinal

commissural neurons, that PKA activation is not required for chemoattraction to netrin-1, and that mechanisms underlying chemoattraction to netrin-1 are independent of mechanisms required for cyclic nucleotide dependent switching.

CHAPTER 5

Deleted in Colorectal Cancer Binding Netrin-1 Mediates Cell Substrate Adhesion and Recruits Cdc42, Rac1, Pak1, and N-WASP into an Intracellular Signaling Complex That Promotes Growth Cone Expansion

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PREFACE

This chapter was published as a research article in the Journal of Neuroscience (Shekarabi et al., 2005). As presented in the literature reviews, Rho GTPases are a family of intracellular proteins known to coordinate the remodeling of the actin cytoskelton and adhesive contacts (Hall, 1998). A previous report from our lab demonstrated that the Rho GTPases, Rac1 and Cdc42, are activated by netrin-1 in DCC-transfected human embryonic kidney 293T (HEK293T) and neuroblastoma glioma 108-15 (NG108-15) cell lines (Shekarabi and Kennedy, 2002). In this paper, we extend these finding to spinal commissural neurons. Aside from further implicating Rho GTPase signaling in axon attraction to netrin-1, we propose that netrin-1 functions as an adhesive cue: We delineate a physical link between extracellular netrin-1 and the cytoskeleton of the growth cone through the association of several intracellular molecules to the intracellular domain of the netrin-1 receptor DCC. Moreover, we report that dissociated spinal commissural neurons adhere to, and that their growth cones expand on, a substrate of netrin-1.

Acknowledgements:

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ABSTRACT

Extracellular cues direct axon extension by regulating growth cone morphology. The netrin-1 receptor deleted in colorectal cancer (DCC) is required for commissural axon extension to the floor plate in the embryonic spinal cord. Here we demonstrate that challenging embryonic rat spinal commissural neurons with netrin-1, either in solution or as a substrate, causes DCC-dependent increases in growth cone surface area and filopodia number, which we term growth cone expansion. We provide evidence that DCC influences growth cone morphology by at least two mechanisms. First, DCC mediates an adhesive interaction with substrate-bound netrin-1. Second, netrin-1 binding to DCC recruits an intracellular signaling complex that directs the organization of actin. We show that netrin-1-induced growth cone expansion requires Cdc42 (cell division cycle 42), Rac1 (Ras-related C3 botulinum toxin substrate 1), Pak1 (p21-activated kinase), and N-WASP (neuronal Wiskott-Aldrich syndrome protein) and that the application of netrin-1 rapidly activates Cdc42, Rac1, and Pak1. Furthermore, netrin-1 recruits Cdc42, Rac1, Pak1, and N-WASP into a complex with the intracellular domain of DCC and Nck1. These findings suggest that DCC influences growth cone morphology by acting both as a transmembrane bridge that links extracellular netrin-1 to the actin cytoskeleton and as the core of a protein complex that directs the organization of actin.

INTRODUCTION

Axon guidance is achieved by integrating the response to cues regulating adhesion and to cues that direct the reorganization of the growth cone cytoskeleton. Netrins are a family of

secreted proteins that guide migrating cells and axons, including spinal commissural axons, during neural development (Kennedy, 2000). Receptors for netrin-1 in the vertebrate CNS include deleted in colorectal cancer (DCC), neogenin, and the UNC-5 homologs (Dickson, 2002). DCC is a type I transmembrane Ig superfamily member that is expressed by embryonic spinal commissural neurons and is required for their axons to be attracted toward a source of netrin-1 (Keino-Masu et al., 1996;Fazeli et al., 1997).

Lamellipodia and filopodia form at the leading edge of a growth cone by continuous remodeling of the actin cytoskeleton (Bentley and O'Connor, 1994;Tanaka and Sabry, 1995). DCC is enriched in filopodia, and in response to netrin-1 it exerts a powerful influence on the organization of actin (Shekarabi and Kennedy, 2002). Intracellularly, the organization of the actin cytoskeleton is regulated by Rho-GTPases that act as molecular switches, cycling between active and inactive forms (Hall, 1998). Based on studies initially performed in fibroblasts, RhoA has been implicated in stress fiber formation, Ras-related C3 botulinum toxin substrate 1 (Rac1) has been implicated in lamellipodia formation, and cell division cycle 42 (Cdc42) has been implicated in filopodia formation (Ridley, 2001). These GTPases also play key roles in regulating growth cone morphology and axon outgrowth (Mueller, 1999;Dickson, 2001).

We have reported previously that netrin-1, through DCC, activates Cdc42 and Rac1, causing filopodia formation and cell spreading in human embryonic kidney 293T (HEK293T) and neuroblastoma glioma 108-15 (NG108-15) cell lines (Shekarabi and Kennedy, 2002). Consistent with this, genetic analysis in *Caenorhabditis elegans* indicates that *ced-10*, a Rac-like GTPase, is required for axons to respond to the netrin homolog UNC-6 (Gitai et al., 2003). In addition, we have identified a role for the Src homology 2 (SH2) and SH3 domain-containing adaptor protein Nck1, demonstrating that it binds the intracellular domain (ICD) of DCC and is required for DCC-induced outgrowth of neurite-like processes from neuroblastoma 1E-115 (N1E-115) cells (Li et al., 2002).

The majority of netrin-1 in the embryonic CNS is associated with either cell membranes or the extracellular matrix (Serafini et al., 1994;Manitt et al., 2001;Manitt and Kennedy, 2002), indicating that, although netrin-1 is a secreted protein, most is not freely

diffusible *in vivo*. Here we show that netrin-1, added in solution or as a substrate, causes embryonic rat commissural neuron growth cone expansion and that this response requires DCC. We provide evidence for an adhesive interaction between substrate-bound netrin-1 and cell-surface DCC, suggesting that DCC-mediated adherence contributes to netrin-1-induced changes in growth cone morphology. In addition, we show that netrin-1 recruits Cdc42, Rac1, neuronal Wiskott-Aldrich syndrome protein (N-WASP), and the serine/threonine kinase p21-activated kinase 1 (Pak1) into a complex with the DCC ICD; that netrin-1 activates Cdc42, Rac1, and Pak1 in commissural neurons; and that Cdc42, Rac1, Pak1, and N-WASP are required for netrin-1-induced growth cone expansion. These findings provide evidence that DCC functions as a transmembrane bridge between netrin-1 and the cytoskeleton and identify a signal transduction complex recruited to the DCC ICD that directs the organization of actin in the growth cone.

MATERIALS AND METHODS

Reagents and cell culture

The following antibodies were used: affinity-purified rabbit polyclonal netrin antibody PN3 (25 μg/ml) (Manitt et al., 2001), purified nonimmune rabbit IgG (25 μg/ml; Invitrogen, San Diego, CA), function-blocking DCC mouse monoclonal anti-DCC_{FB} (AF5; Calbiochem, La Jolla, CA), mouse monoclonal anti-DCC_{IN} (G97-449; PharMingen, Mississauga, Ontario, Canada), rabbit polyclonal anti-Cdc42 (SC-87; Santa Cruz Biotechnology, Santa Cruz, CA), mouse monoclonal anti-Rac1 (Transduction Laboratories, Lexington, KY), rabbit polyclonal anti-Pak1 (New England Biolabs, Beverly, MA), anti-phospho-specific (Ser^{198} and Ser^{203}) Pak1 (provided by M. Greenberg, Harvard University, Cambridge, MA) (Shamah et al., 2001), goat polyclonal anti-N-WASP D15 and mouse monoclonal anti-glutathione *S*-transferase (anti-GST; Santa Cruz Biotechnology), and anti-Flag epitope tag (Sigma-Aldrich, Oakville, Ontario, Canada). Filamentous actin was visualized using fluorescein-conjugated (FITC) phalloidin (Sigma-Aldrich). Recombinant netrin-1 protein was purified from a 293-Epstein-Barr virus nuclear antigen (EBNA) cell line secreting netrin-1, as described previously (Serafini et al., 1994;Shirasaki et al., 1996).

Dominant-negative (N17) and constitutively active (V12) forms of Cdc42- and Rac1-expressing adenoviruses were provided by Dr. J. Bamburg (Colorado State University, Fort Collins, CO). Expression constructs encoding GST fusion proteins of wild-type Cdc42 and Rac1 were provided by G. Bokoch (The Scripps Research Institute, La Jolla, CA) (Bagrodia et al., 1995). The Pak peptide and control peptide (Kiosses et al., 2002) were provided by M. A. Schwartz (The Scripps Research Institute). An adenovirus expressing a Flag-tagged dominant-negative mutant form of N-WASP was constructed as described previously (He et al., 1998). The N-WASP mutant [Δcofilin N-WASP (Δcof N-WASP)] contains a 4 aa deletion in its C-terminal domain that compromises its ability to bind the Arp2/3 complex and therefore does not promote actin polymerization. Recombinant protein was visualized by Flag epitope tag immunoreactivity, and endogenous N-WASP was detected by the use of anti-N-WASP.

Embryonic rat spinal commissural neurons were cultured as described previously (Bouchard et al., 2004). Briefly, dorsal halves of the spinal cord of embryonic day 13 (E13; E0, vaginal plug) rat embryos were microdissected (see Figure 5.1A), 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) plus 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 plastic was coated with 20 μg/ml poly-D-lysine (PK; Sigma, St. Louis, MO) at 37°C for 2 h. 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. For biochemical analysis of proteins, the dissociated neurons were cultured at 4 x 10^6 cells per 60 mm plate. For immunostaining, the neurons were plated on 13 mm glass coverslips (Carolina Biological Supply, Burlington, NC) at 7 x 10^3 cells per coverslip. At 36 h after plating, the cultures were washed, changed to B-27-free Neurobasal, and incubated for another 6 h before stimulation with 80 ng/ml purified netrin-1 protein. Then the cells were 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 a one-way ANOVA with Scheffé's *post hoc* test (Systat, Chicago, IL).

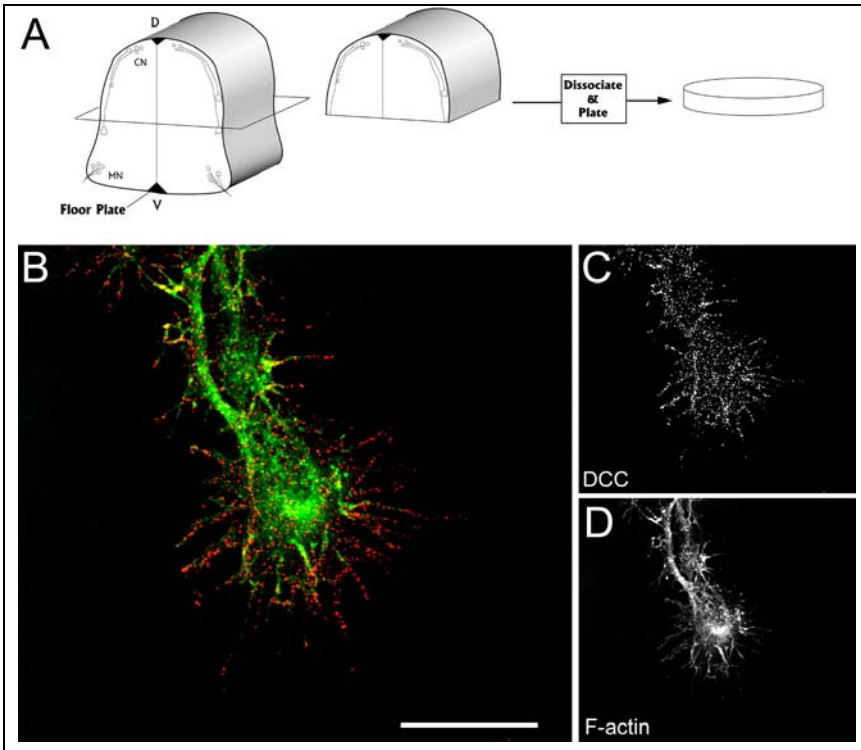

Figure 5.1 - Distribution of DCC in cultured embryonic rat commissural neuron growth cones. *A, The dorsal halves of E13 rat spinal cords were microdissected, dissociated, and plated (D, dorsal; V, ventral; CN, commissural neurons; MN, motoneurons). B, The morphology of commissural neuron growth cones grown for 2 DIV in the presence of netrin-1 and then immunolabeled for DCC (red) and for F-actin with FITC-coupled phalloidin (green). C, DCC staining alone. D, F-actin staining alone. Scale bar, 10 µm.*

Immunofluorescence

Photomicrographs were taken with an Axiovert microscope (Zeiss, Oberkochen, Germany) and a Magnafire CCD camera (Optronics, Goleta, CA) and analyzed with Northern Eclipse image analysis software (Empix Imaging, Mississauga, Ontario, Canada) by an observer blind to the experimental conditions. Values are expressed as the mean ± SEM. Statistical significance was evaluated by a one-way ANOVA with a Scheffé's *post hoc* test (Systat).

Cell substrate adhesion assay

To assay cell substrate adhesion, we dried 20 μl of 0.1% nitrocellulose (Hybond ECL; Amersham Biosciences, Piscataway, NJ) dissolved in methanol (histological grade; Fisher Scientific, Houston, TX) at the bottom of a four-well plate, followed by incubation with either HBSS or 2 μg/ml netrin-1 in HBSS for 2 h at room temperature. All substrates then were blocked for 1 h with 1% BSA (Fisher Scientific) in HBSS and then again with 1% heparin (Sigma) in HBSS. Substrates were incubated with one of the following (in μg/ml): 25 anti-netrin PN3 (Manitt et al., 2001), 5 DCC-Fc (R & D Systems, Minneapolis, MN), or 25 nonimmune rabbit IgG (Invitrogen) for 1 h. All substrates were washed once and kept in HBSS before 2.5 x 10^5 cells from dissociated dorsal spinal cords were plated in Neurobasal supplemented with 2% B-27 and 2 mM glutamine. Cells were cultured for 2 h at 37°C, 5% CO_2, gently washed three times with PBS, and fixed with 4% PFA in PBS. For cell counting, the nuclei were labeled with 0.5 μg/ml Hoechst 33258 (Sigma) in PBS for 30 min.

GTPγS loading assay

GTPγS loading assays were performed as described previously (Knaus et al., 1992). Neurons cultured in 60 mm plates were treated with purified 80 ng/ml netrin-1 protein for 3 min and lysed in ice-cold lysis buffer [LB; containing 150 mM NaCl, 25 mM HEPES, pH 7.5, 25 mM NaF, 1 mM EDTA, 1 mM sodium orthovanadate plus 1% NP-40 and 0.25% sodium deoxycholate] with 10 mM $MgCl_2$, 5% glycerol, and protease inhibitors (containing 2 μg/ml leupeptin, 2 μg/ml aprotinin, 1 μg/ml pepstatin plus 2 mM PMSF). Lysates were incubated with 100 μM GTPγS in the presence of 10 mM EDTA for 12 min at 31°C. GTP-bound Cdc42 and Rac1 were pulled down with 20 μl of glutathione-coupled Sepharose 4B beads (Amersham Biosciences) that had been loaded with 10 μg of bacterially expressed GST-Pak1-Cdc42/Rac interactive-binding (CRIB) domain fusion protein (amino acids 56-272) (Sander et al., 1999). Components of the protein complex were resolved by SDS-PAGE and Western blot analysis, using anti-Rac1 or anti-Cdc42. Signals were detected using ECL (PerkinElmer, Wellesley, MA). Densitometry and quantification were performed using NIH Image software.

GST-Cdc42 and GST-Rac1 pull-down assays

GST-Cdc42 and GST-Rac1 fusion proteins were expressed in bacteria and isolated as described previously (Sander et al., 1999). Cultured commissural neurons were treated with 80 ng/ml netrin-1 protein for the times indicated. Cell lysates were incubated with GTPγS, 10 mM EDTA, and either GST-Cdc42 or GST-Rac1 fusion proteins at 31°C for 12 min. The protein complex was isolated using glutathione-coupled Sepharose 4B beads as described above. The pellet was washed with LB, and the bound proteins were resolved by SDS-PAGE.

Coimmunoprecipitation

For coimmunoprecipitation (coIP) analyses the commissural neurons were plated in 60 mm PK-coated cell culture plates at 5 x 10^6 cells per plate as described above. Cells were then treated with 100 ng/ml netrin-1 for either 5 or 30 min, washed with ice-cold PBS, and then lysed in ice-cold LB with protease inhibitors for 15 min on ice. Cell lysate was collected and centrifuged at 15,000 x *g* for 10 min at 4°C; the supernatant was used as the input for coIP. Protein G-Sepharose beads (Sigma) were blocked for 1 h at 4°C in LB plus 10% BSA, washed four times, and resuspended in LB. Immunoprecipitations were performed using 1 µg of anti-DCC_{IN}, 2 µg of anti-Pak1, or 12 µl of goat polyclonal anti-N-WASP (D15) on a rocking platform at 4°C. Blocked beads were added after 1 h of incubation with the primary antibody and allowed to incubate for 1 h. Beads and associated proteins were then pelleted and washed three times in LB. Proteins were eluted from the beads using 25 µl of PAGE loading buffer and characterized by Western blot analysis.

RESULTS

Netrin-1 causes DCC-dependent commissural neuron growth cone expansion

Commissural neurons express *dcc* as they extend an axon toward the floor plate at the ventral midline of the embryonic spinal cord (Keino-Masu et al., 1996). To investigate the morphological and biochemical response of these neurons to netrin-1, we

microdissected and dissociated dorsal halves of E13 embryonic rat spinal cords and then cultured these cells (Figure 5.1A). More than 90% of the cells in these cultures express TAG-1 (transient axonal glycoprotein-1) and DCC, both markers of embryonic spinal commissural neurons *in vivo* (Dodd et al., 1988;Keino-Masu et al., 1996;Bouchard et al., 2004). DCC immunoreactivity was detected throughout the growth cones of commissural neurons grown in vitro, including along filopodia (Figure 5.1B-D).

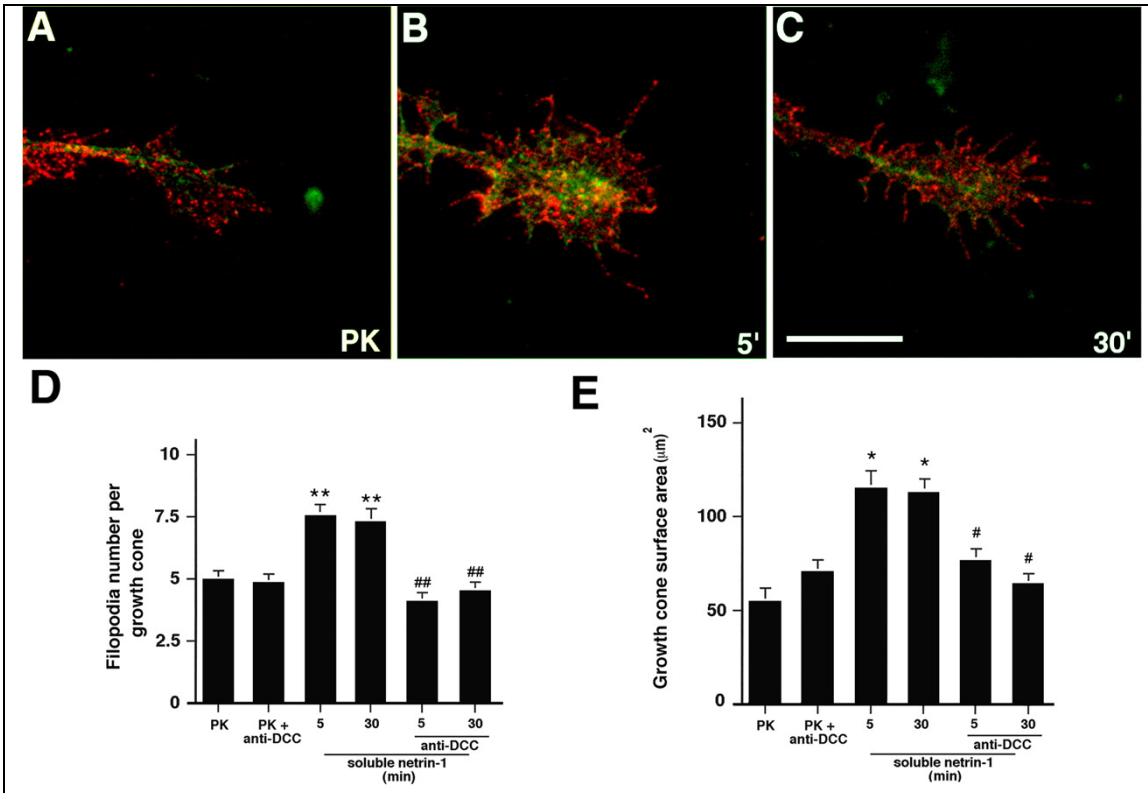

Figure 5.2 - Netrin-1 causes commissural neuron growth cone expansion. A-C, Representative examples of commissural neuron growth cone morphologies grown on a PK substrate *(A) or for 5 min (B) and 30 min (C) after the addition of 80 ng/ml netrin-1 protein to the culture media. DCC immunoreactivity is shown in red. FITC-phalloidin staining of F-actin is green (100x objective). Scale bar, 10 µm. D, E, Quantification of the increase in growth cone filopodia number (D) and surface area (E) after application of 80 ng/ml netrin-1 for 5 or 30 min. Netrin-1 produced a significant increase in mean filopodia number per growth cone and growth cone surface area ($^{*}p < 0.05$; $^{**}p < 0.01$). Anti-DCC_{FB} (5 µg/ml) blocked netrin-1-induced growth cone expansion ($^{\#}p < 0.05$; $^{\#\#}p < 0.01$), whereas the application of anti-DCC_{FB} alone had no effect (n = 25 per condition; error bars indicate SEM).*

Growth cone turning involves actin-dependent membrane extension on one side of the growth cone, which is coordinated with membrane withdrawal on the other side (Mueller, 1999). Growth cone collapse has been used widely as an assay to study mechanisms underlying the action of repellent guidance cues (Castellani and Rougon, 2002). Netrin-1 is an attractant for embryonic spinal commissural neurons; therefore, we assessed the possibility that it might exert the opposite effect. The addition of netrin-1 (80 ng/ml) to spinal commissural neurons *in vitro* induced a rapid increase in the number of filopodia and growth cone surface area (Figure 5.2), an effect that we describe as growth cone expansion.

Substrate-bound netrin-1 promotes growth cone expansion and adhesion

We have reported previously that the majority of netrin-1 protein is not freely soluble *in vivo* but is bound to cell surfaces or the extracellular matrix (Manitt et al., 2001;Manitt and Kennedy, 2002). This suggested that netrin-1 might influence growth cone morphology by acting as an extracellular anchor that promotes cell adhesion. Consistent with a functional role for substrate-bound netrin-1, we detected growth cone expansion when the cells were plated on coverslips that had been precoated with a 5 µg/ml solution of netrin-1 (Figure 5.3A-E). The addition of a function-blocking antibody against DCC (DCC_{FB}) abolished the effect of soluble or substrate-bound netrin-1, indicating that DCC is required for netrin-1-induced commissural neuron growth cone expansion (Figures 5.2D,E, 5.3C-E). Interestingly, the addition of netrin-1, either soluble or substrate-bound, did not affect the rate of axon outgrowth from these cells significantly, indicating that netrin-1 can influence growth cone morphology without affecting the rate of axon extension (PK, 40.15 ± 3.99 µm; PK plus netrin, 44.48 ± 2.9 µm; PK plus s-netrin, 42.37 ± 3.85 µm; PK plus s-netrin plus anti-netrin, 39.98 ± 3.5 µm; "plus netrin" indicates the addition of soluble netrin-1, and "plus s-netrin" indicates netrin-1 substrate). These results are consistent with previous findings indicating that netrin-1 can induce growth cone turning without altering the rate of axon growth (Ming et al., 1997).

To determine whether a netrin-1 substrate promotes adhesion, we plated cells derived from dissociated E13 rat embryonic spinal cord on substrates of either netrin-1 or BSA and counted the number of adherent cells. A substrate of netrin-1 resulted in a more than sevenfold increase in the number of adherent cells when compared with control BSA substrates (Figure 5.3F-K). Adhesion was blocked by preincubating the netrin-1 substrate for 1 h with 25 μg/ml anti-netrin-1 (Figure 5.3I). Control IgG (25 μg/ml) had no effect on the number of adherent cells (Figure 5.3K). Preincubating the netrin-1 substrate with a DCC-Fc recombinant protein chimera encoding the extracellular domain of DCC fused to an antibody Fc domain also blocked adhesion to netrin-1, consistent with DCC mediating an adhesive interaction with substrate-bound netrin-1 (Figure 5.3J).

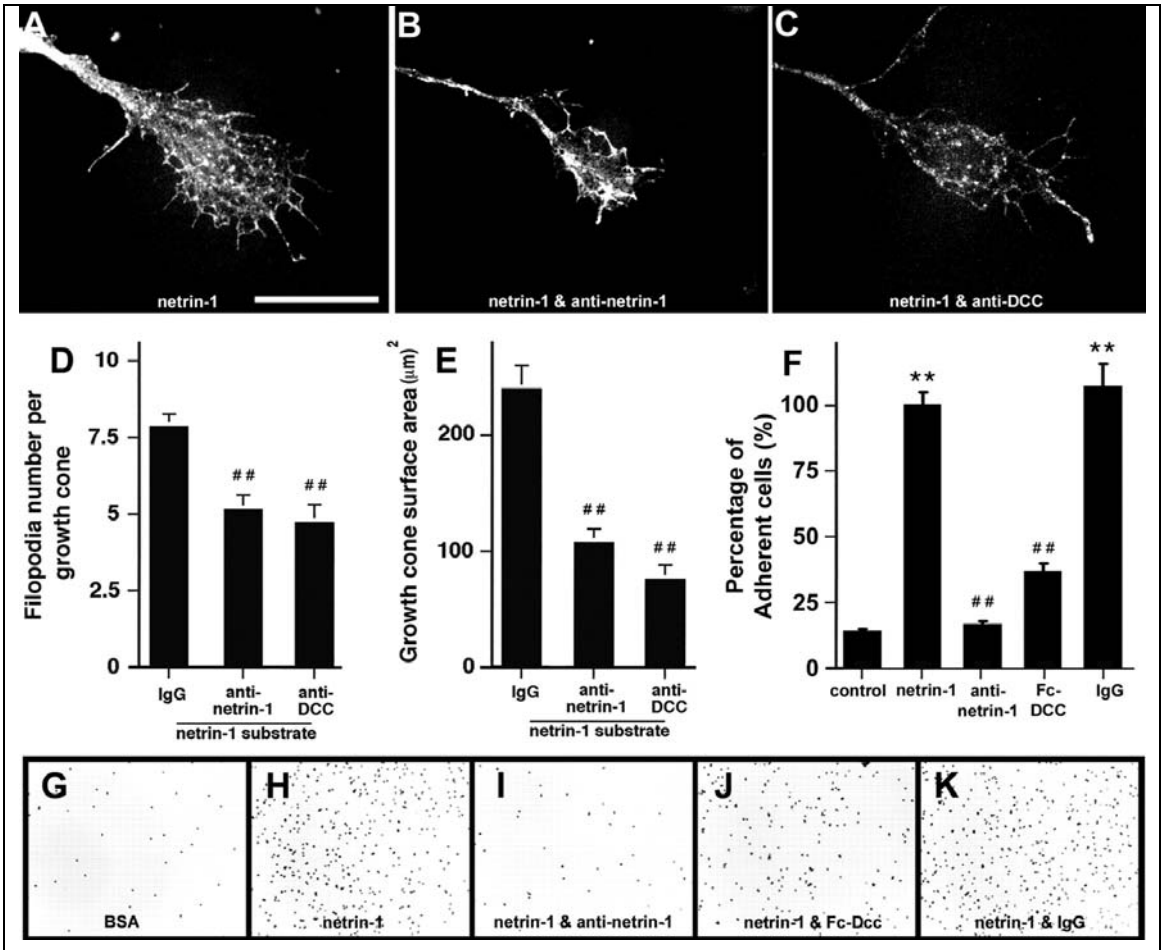

Figure 5.3 - Substrate-bound netrin-1 induces growth cone expansion: evidence for an adhesive interaction between DCC and netrin-1. *A-C, Commissural neuron growth cones cultured on a substrate of netrin-1 in the absence (A) or in the presence (B) of 25 μg/ml anti-*

netrin-1 or in the presence (C) of 5 μg/ml anti-DCC. Shown is the quantification of the number of filopodia per growth cone (D) and growth cone surface area (E) for the conditions shown in A-C. F, Quantification of cell substrate adhesion of dissociated E13 rat dorsal spinal cord cells. Representative examples of the assay are shown in G-K (10x objective lens; Hoechst-stained nuclei; grayscale inverted). A netrin-1 substrate generates a more than sevenfold increase in the number of adherent cells compared with a control BSA substrate. Preincubation of the substrates with anti-netrin-1 (I; 25 μg/ml PN3) or DCC-Fc (5 μg/ml) significantly reduced the number of adherent cells compared with netrin-1. Control IgGs (K) did not affect adherence to netrin-1 (ANOVA; $^{**}p < 0.005$ compared with control; $^{\#\#}p < 0.005$ compared with netrin-1 or IgG control; mean ± SEM).

Netrin-1-induced growth cone expansion requires Rac1 and Cdc42

We have reported previously that netrin-1 causes cell spreading and filopodia formation in cell lines transfected to express DCC (Shekarabi and Kennedy, 2002). These studies demonstrated that DCC independently activates Cdc42 and Rac1 in HEK293T cells and NG108-15 neuroblastoma glioma cells. To assess the role of Cdc42 and Rac1 in the morphological changes induced by netrin-1 in the growth cones of embryonic rat commissural neurons, we infected cells [20 multiplicity of infection (MOI)] with adenoviruses encoding either dominant-negative (N17Cdc42, N17Rac1) or constitutively active (V12Cdc42, V12Rac1) forms of Cdc42 and Rac1. Adenoviral vectors encoding green fluorescent protein (GFP) served as controls. Recombinant Cdc42, Rac1, and GFP were myc epitope tagged. At 48 h after infection, the neurons were treated with 80 ng/ml netrin-1 for 30 min. Cells expressing recombinant protein were identified immunocytochemically by the myc epitope tag. The distribution of F-actin was visualized using FITC-coupled phalloidin. Expression of dominant-negative Cdc42 (N17Cdc42), but not GFP alone, significantly reduced the effect of netrin-1 on the number of growth cone filopodia and growth cone surface area (Figure 5.4A,B). Expression of dominant-negative Rac1 (N17Rac1) blocked the netrin-1-induced increase in growth cone surface area (Figure 5.4B) and significantly reduced the netrin-1-induced increase in the number of filopodia (Figure 5.4A). Expression of either constitutively active Cdc42 (V12Cdc42) or constitutively active Rac1 (V12Rac1) was sufficient to increase significantly both the

growth cone surface area and the number of filopodia in these neurons (Figure 5. 4A,B). Interestingly, the morphological changes induced by constitutively active Cdc42 and Rac1 were significantly less than those induced by the addition of netrin-1 to control GFP-expressing cells, suggesting that activation of either Cdc42 or Rac1 alone is not sufficient to recapitulate the effect of netrin-1 on the growth cone.

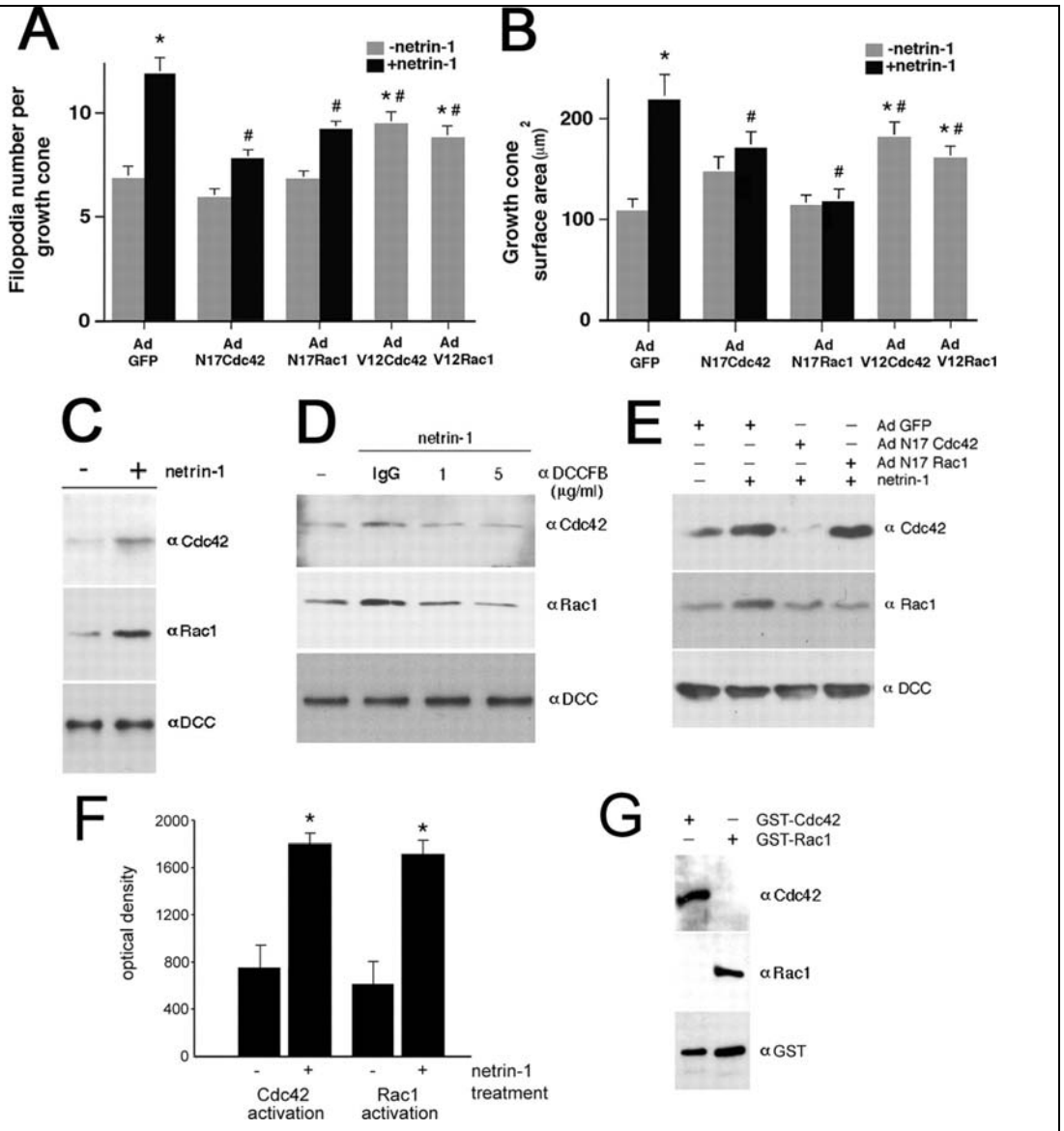

Figure 5.4 - Netrin-1-induced commissural growth cone expansion requires DCC and activated Cdc42 and Rac1. *A, B, Commissural neurons were infected with adenovirus expressing dominant-negative Cdc42 or Rac1 (N17Cdc42, N17Rac1), constitutively active Cdc42 or Rac1 (V12Cdc42, V12Rac1), or GFP. At 48 h after infection, 80 ng/ml netrin-1 was*

added, and 30 min later, the cells were fixed and immunostained. Netrin-1 significantly increased filopodia number and growth cone surface area in cells expressing GFP alone (mean ± SEM; $^{*}p < 0.05$, significant increase compared with control cells expressing GFP in the absence of netrin-1). Expression of either N17Cdc42 or N17Rac1 significantly decreased the number of filopodia per growth cone and growth cone surface area (n = 25; $^{\#}p < 0.05$, significant decrease compared with control cells expressing GFP in the presence of netrin-1). In the absence of added netrin-1, the expression of V12Cdc42 or V12Rac1 significantly increased both filopodia number and growth cone surface area (n = 15), but to an extent significantly less than netrin-1 applied to adenoviral (Ad) GFP controls. C-E, Dissociated embryonic spinal commissural neurons were treated with 80 ng/ml netrin-1 or carrier for 5 min and then lysed and incubated with GTPγS at 31°C for 12 min. GTPγS-bound Cdc42 and Rac1 were isolated by using GST-Pak1-CRIB fusion protein and assayed by Western blotting with antibodies against Rac1 or Cdc42. Total cell lysates were probed with anti-DCC to confirm that equal amounts of total protein were loaded per lane. D, The addition of anti-DCC_{FB} (1 or 5 μg/ml) blocked the activation of Cdc42 and Rac1 by netrin-1. E, Expression of N17Cdc42 blocked the netrin-1-dependent activation of Rac1. In contrast, expression of N17Rac1 did not block netrin-1-induced activation of Cdc42. F, Quantification of netrin-1-induced activation of endogenous Cdc42 and Rac1 in commissural neuron homogenates (after a 3 min application of 80 ng/ml netrin-1; n = 4; mean ± SEM; $^{*}p < 0.05$). G, Western blot analysis of recombinant GST-Cdc42 and GST-Rac1 (~100 ng) illustrates specificity of the antibodies against Cdc42 and Rac1. Anti-GST immunoreactivity confirms that similar amounts of recombinant protein were loaded in each lane.

Netrin-1 activates Cdc42 and Rac1 in embryonic rat spinal commissural neurons

We then determined whether netrin-1 activates Cdc42 and Rac1 in these neurons. A technical challenge encountered during investigation of the activation of Cdc42 and Rac1 was the relatively small number of commissural neurons obtained by microdissection and the limited amount of endogenous Cdc42 and Rac1. These limitations were overcome via the adaptation of a GTPγS loading assay. Rho-GTPases are activated by guanine nucleotide exchange factors (GEFs) that catalyze the exchange of GDP for GTP. GTPγS binds irreversibly to Rho-GTPases, trapping them in the bound state. When we incubated lysates of commissural neurons with GTPγS and isolated

GTPγS-bound endogenous Cdc42 and Rac1 with the GST-Pak-CRIB fusion protein, it was possible to visualize and quantify the activation of endogenous Rho-GTPases with antibodies specific for Cdc42 and Rac1 (Figure 5.4G). An analysis of the time course of Cdc42 and Rac1 binding to GTPγS indicated that binding was saturated after 30 min of incubation (data not shown). Subsequently, GST-Pak-CRIB binding to GTPγS was assayed after 12 min of incubation in commissural neuron homogenates, a nonsaturated time point. We detected Cdc42 and Rac1 activation within 3 min of netrin-1 application to the intact cells (Figure 5.4C-F). Adding anti-DCC_{FB} (1 or 5 μg/ml) with netrin-1 blocked the activation of both Cdc42 and Rac1, indicating that netrin-1-induced activation of these GTPases requires DCC (Figure 5.4D). Adenoviral-mediated expression of N17Cdc42 blocked the activation of Rac1 (Figure 5.4E), whereas N17Rac1 expression did not block the activation of Cdc42 (Figure 5.4E), suggesting that Cdc42 activation is upstream of Rac1 in embryonic rat spinal commissural neurons. Furthermore, because this assay measures the accumulation of GTPγS bound to Cdc42 or Rac1, the increase in GTPγS binding implicates netrin-1 in the activation of a GEF in commissural neurons.

Netrin-1 activates Pak1 and recruits Cdc42, Rac1, and Pak1 to the DCC ICD

The serine/threonine kinase Pak1 is an effector of Cdc42 and Rac1 and a key component of a well established signal transduction pathway that promotes actin polymerization (Bokoch, 2003). Activated Cdc42 and Rac1 bind directly to Pak1, regulating its activity (Bagrodia and Cerione, 1999). Pak1 activation can be assessed by using antibodies that recognize phospho-Ser^{198} and phospho-Ser^{203} of Pak1 (Sells et al., 2000;Shamah et al., 2001).

To determine whether netrin-1 promotes an interaction between Pak1 and activated Cdc42 or Rac1, we again used GTPγS loading. Purified GST-Cdc42 or GST-Rac1 fusion proteins were added to lysates of dissociated commissural neurons in the presence of GTPγS and interacting proteins identified by Western blotting. The results obtained indicate that the addition of netrin-1 to commissural neurons promotes the association of activated Cdc42 and Rac1 with phospho-Pak1 and DCC (Figure 5.5A).

Using coIP of endogenous DCC and Pak1, we then tested the hypothesis that Pak1 might be recruited to a complex with DCC. Increased amounts of Pak1 were determined to coimmunoprecipitate with DCC from homogenates of commissural neurons exposed to netrin-1 (Figure 5.5B). Furthermore, increased amounts of DCC were found to coimmunoprecipitate with anti-Pak1 from homogenates of commissural neurons after the application of netrin-1 (Figure 5.5C), indicating that netrin-1 promotes the formation of a complex that includes Pak1 and DCC.

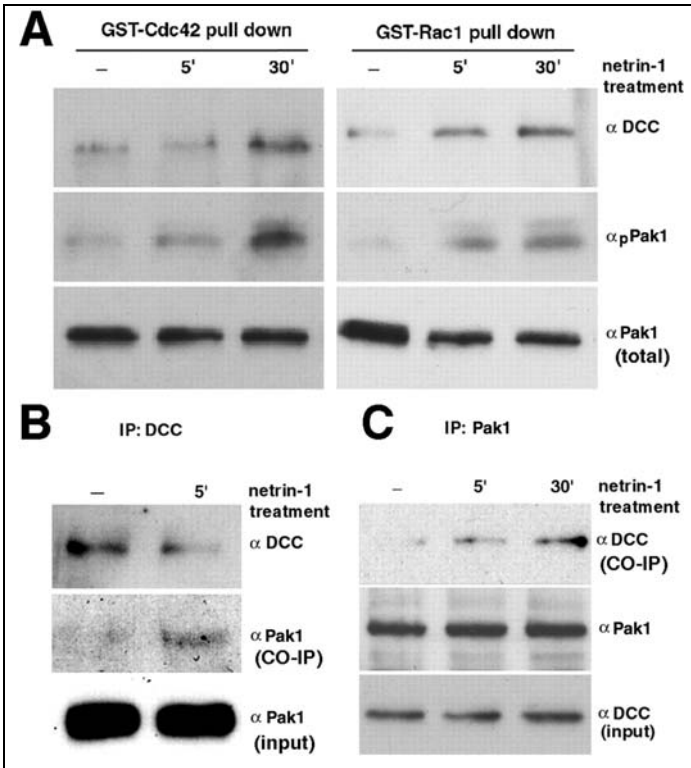

Figure 5.5 - Netrin-1 promotes Cdc42, Rac1, Pak1, and DCC complex formation*. A, After treatment with netrin-1 (5 or 30 min) commissural neuron cell lysates were incubated with recombinant GST-Cdc42 or GST-Rac1 and GTPγS. Phospho-Pak1 protein associated with the GST fusion proteins was isolated and analyzed by Western blot. Pak1 immunoreactivity in the whole-cell lysate (total) is shown in the last row. B, DCC was immunoprecipitated (1 μg of anti-DCC_{IN}) from commissural neuron lysates and then analyzed by Western blot with the use of anti-Pak1. Treatment of the cells with 80 ng/ml netrin-1 for 5 min increased the amount of Pak1 protein found to coimmunoprecipitate with DCC. The coIP results are shown above Pak1 immunoreactivity in corresponding whole-cell lysates. C, Increased amounts of DCC were detected in a coIP with anti-Pak1 after treatment with 80 ng/ml netrin-1 for 5 min. The coIP results are shown above the Western blots showing immunoreactivity for Pak1 and DCC in corresponding cell lysates.*

We then determined whether Pak1 might be activated by netrin-1 in commissural neurons. Immunostaining commissural neuron growth cones revealed a significant increase in phospho-Pak1 (pPak1) within 5 min of the addition of netrin-1 (Figure 5.6A-E). Western blot analysis of the relative levels of phospho-Pak1 in commissural neuron lysates indicated that netrin-1 activated Pak1 within 5 min of application (Figure 5.6H,I). Phospho-Pak1 levels remained elevated for at least 1 h. Coincident application of netrin-1 and the DCC function-blocking antibody indicated that netrin-1-induced activation of Pak1 requires DCC.

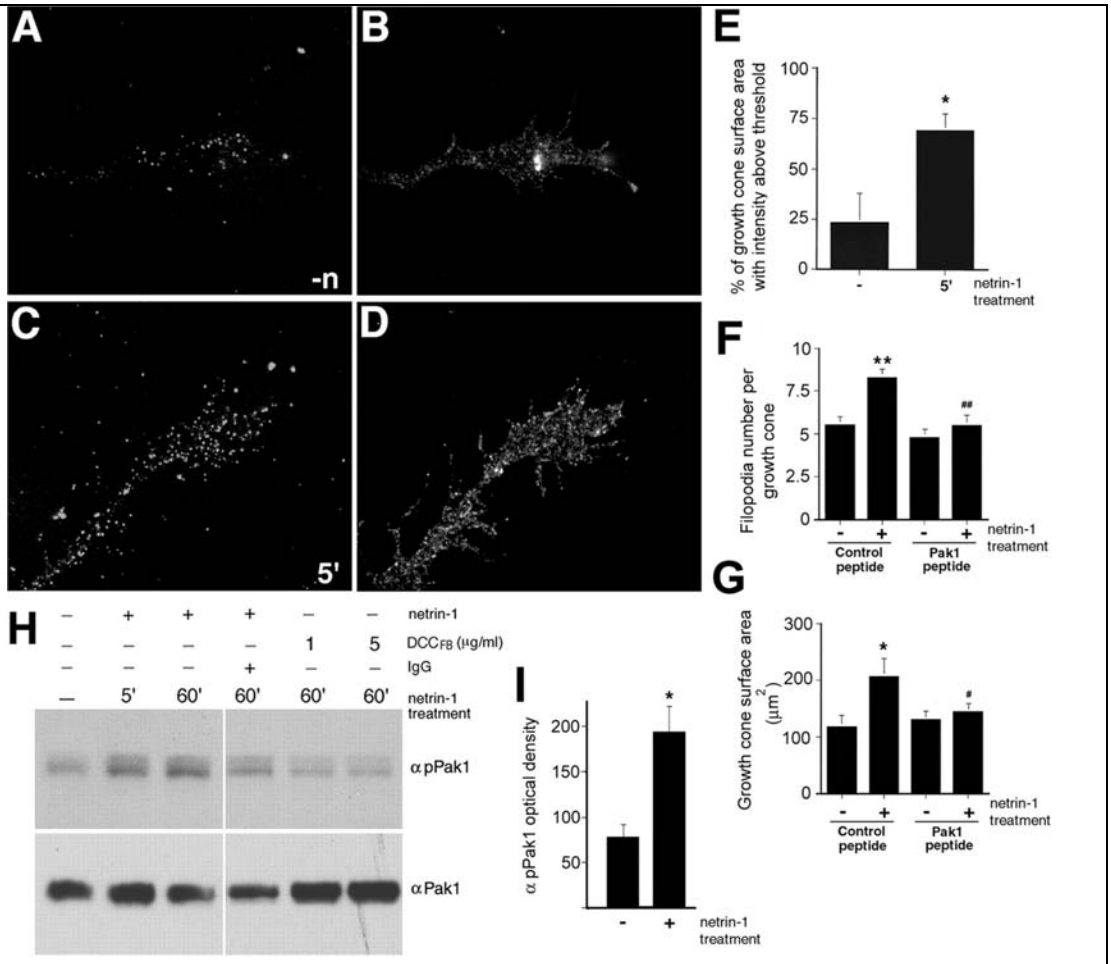

Figure 5.6 - DCC-dependent activation of Pak1 by netrin-1*. Commissural neurons double-immunolabeled for phospho-Pak1 (A, C) and DCC (B, D) are shown. Increased phospho-Pak1 immunoreactivity was detected in growth cones after 5 min of exposure to netrin-1 (-n in A indicates without added netrin-1). E, Quantification of phospho-Pak1 immunoreactivity (n =*

28; mean ± SEM; $^{*}p < 0.05$). F, G, The addition of control peptide did not affect the netrin-1-induced increase in growth cone surface area or filopodia number (mean ± SEM; $^{*}p < 0.05$; $^{**}p < 0.005$). Adding the Pakpeptide 40 min before the addition of netrin-1 blocked the netrin-1-induced increase in filopodia number and growth cone surface area (n = 25; mean ± SEM; $^{\#}p < 0.05$; $^{\#\#}p < 0.005$). H, The addition of 80 ng/ml netrin-1 to commissural neurons increased phospho-Pak1 as assayed by Western blot, consistent with the change in immunofluorescence shown in E. The same blot was reprobed with anti-Pak1, confirming that comparable amounts of protein are present in each lane. The netrin-1-induced increase in phospho-Pak1 was blocked by anti-DCC_{FB} added to the cultures 1 h before the addition of netrin-1. I, Quantification of increased phospho-Pak1 as detected by Western blot analysis (after a 5 min application of 80 ng/ml netrin-1; n = 3; mean ± SEM; $^{*}p < 0.05$).

Recruitment of Pak1 is required for netrin-1-induced growth cone expansion

Nck1, an adaptor protein composed of one SH2 and three SH3 domains, binds directly to Pak1 through its second SH3 domain (Li et al., 2001). We have reported that the intracellular domain of DCC binds directly to the first and third SH3 domains of Nck1 (Li et al., 2002). Furthermore, expression of dominant-negative Nck1 inhibited the DCC-induced extension of neurite-like processes from N1E-115 neuroblastoma cells and blocked DCC-dependent activation of Rac1 by netrin-1 in fibroblasts. These findings suggest that the intracellular domain of DCC may form a complex with Nck1 and Pak1.

To determine whether Pak1 binding Nck1 contributes to netrin-1-induced growth cone expansion, we used a cell-permeable peptide that is a competitive inhibitor of Pak1 binding Nck1. This peptide (Pak peptide) consists of 13 aa corresponding to the first proline-rich domain of Pak1, fused to the polybasic sequence of the human immunodeficiency virus Tat protein, which facilitates entry into cells. The proline-rich domain binds to the second SH3 domain of Nck1 (Hing et al., 1999), inhibiting the interaction between Pak1 and Nck1 (Kiosses et al., 2002). A control peptide mutated at two prolines does not affect Nck1/Pak1 binding. The addition of the Pak peptide (20 μg/ml) to cultures of embryonic rat spinal commissural neurons 45 min before the addition of netrin-1 (80 ng/ml) blocked the netrin-1-induced increase in growth cone surface area and filopodia number, whereas the application of the control peptide did not

(Figure 5.6F,G). Together, these results provide evidence that netrin-1 causes a DCC-dependent activation of Cdc42, Rac1, and Pak1 in spinal commissural neurons and recruits Cdc42, Rac1, and Pak1 to a complex with DCC and Nck1.

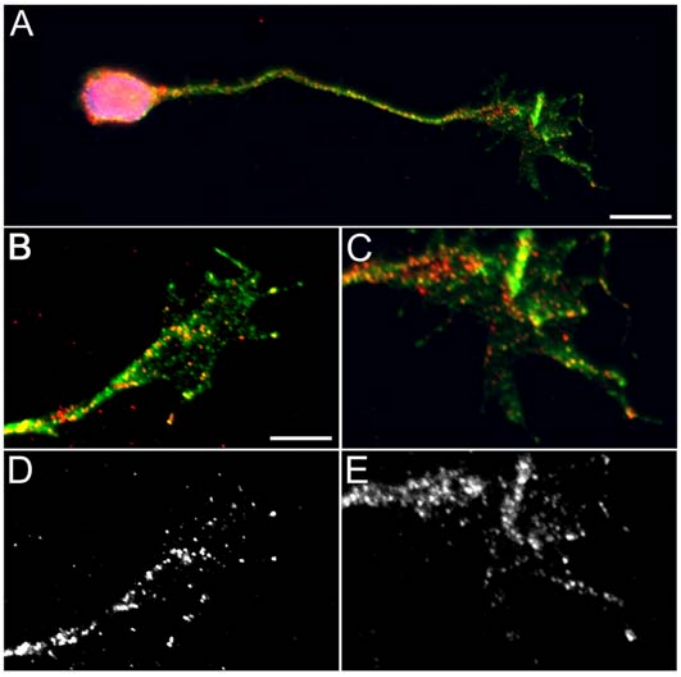

Figure 5.7 - Distribution of N-WASP and DCC in commissural neuron growth cones. *Commissural neurons cultured on a control PK substrate (B, D) or 30 min after the addition of 80 ng/ml netrin-1 (A, C, E) were labeled with anti-DCC (green) and anti-N-WASP (red, A-C; white, D, E). N-WASP immunoreactivity was detected readily along the axon, throughout the growth cone, and along filopodia; C and E present an enlargement of the growth cone shown in A, illustrating the punctate distribution of N-WASP immunoreactivity. Scale bars: A, 10 µm; (in B), B-E, 5 µm.*

N-WASP is required for netrin-1-induced growth cone expansion

N-WASP binds directly to Cdc42 and to Nck1 (Millard et al., 2004) and functions as a downstream effector of active Cdc42 that regulates actin polymerization (Mullins, 2000). Immunocytochemical analyses detected N-WASP in commissural neuron growth cones, including filopodia, both when they were cultured on a substrate of PK alone (Figure 5.7B,D) and 30 min after the addition of 80 ng/ml netrin-1 (Figure 5.7A,C,E). To determine whether netrin-1 promotes an interaction between Cdc42 and N-WASP or Pak1, we isolated proteins binding to GST-Cdc42 in commissural neuron lysates. After treatment of commissural neurons with 80 ng/ml netrin-1 for 5 or 30 min, increased binding of N-WASP and Pak1 was detected (Figure 5.8C). Furthermore, increased

amounts of DCC were detected to coimmunoprecipitate with N-WASP from commissural neuron lysates after the application of netrin-1 (Figure 5.8D).

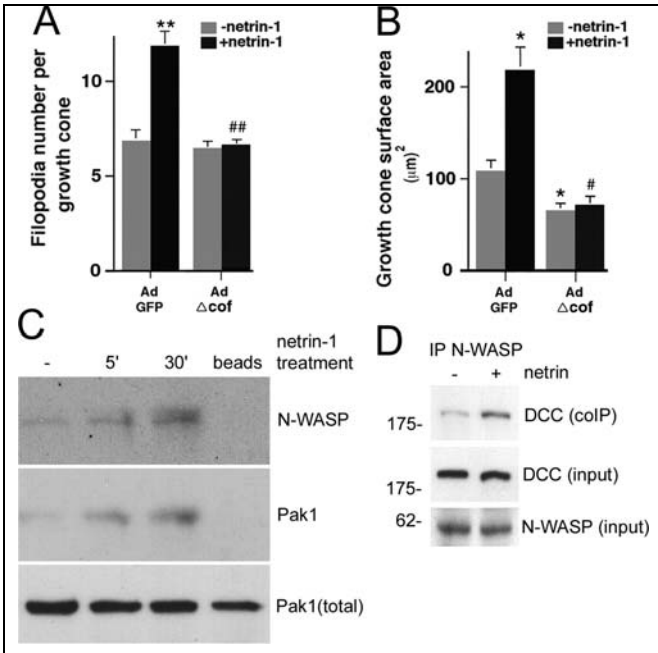

Figure 5.8 - N-WASP is recruited to a complex with DCC and required for netrin-1-induced growth cone expansion. *A, B, After the infection with adenoviral vectors encoding either GFP or Δcof N-WASP, growth cone morphology was quantified. The addition of netrin-1 (80 ng/ml, 30 min) significantly increased the number of filopodia and growth cone surface area ($^{*}p < 0.05$; $^{**}p < 0.005$). Expression of Δcof N-WASP blocked the increase in both the number of filopodia (A) and surface area (B) of commissural growth cones treated with netrin-1 ($^{\#}p < 0.05$; $^{\#\#}p < 0.005$). Expression of Δcof N-WASP significantly reduced the growth cone surface area below the level found in the presence of netrin-1 or control (n = 15; mean ± SEM). C, After treatment with netrin-1 (5 or 30 min) commissural neuron cell lysates were incubated with recombinant GST-Cdc42. Netrin-1 promotes the association of Pak1 and N-WASP with GST-Cdc42. Pak1 immunoreactivity in corresponding whole-cell lysates is shown in the bottom row. D, Increased amounts of DCC were detected in coIPs from commissural neuron lysates by the use of anti-N-WASP. The levels of DCC and N-WASP present in the whole-cell lysates are shown below the blot of the coIP.*

To investigate a functional role for N-WASP in the response of commissural growth cones to netrin-1, we generated an adenovirus expressing a Flag-tagged dominant-negative mutant form of N-WASP, Δcof N-WASP. This mutation contains a 4 aa deletion in the N-WASP C-terminal domain, rendering the protein incapable of binding the Arp2/3 complex and unable to promote actin polymerization. It therefore functions as a dominant negative, blocking Cdc42-induced filopodia formation and neurite extension (Banzai et

al., 2000;Hufner et al., 2002). Cultured commissural neurons were infected with Δcof N-WASP or GFP adenoviruses (20 MOI). At 48 h after infection, cells were exposed to 80 ng/ml netrin-1 for 30 min and then fixed and stained with an antibody against the Flag tag to identify the cells expressing Δcof N-WASP. Quantification of growth cone morphology indicates that interfering with N-WASP function blocks both the netrin-1-dependent increase in filopodia number and growth cone surface area (Figure 5.8A,B). These results indicate that netrin-1 recruits N-WASP into a complex with DCC and that N-WASP is required for the growth cone response to netrin-1.

DISCUSSION

Our findings indicate that netrin-1 binding to DCC profoundly affects embryonic rat spinal commissural neuron growth cone morphology, approximately doubling growth cone surface area and filopodia number. These findings are consistent with reports that the application of netrin-1 increases growth cone complexity *in vitro* (de la Torre et al., 1997;Lebrand et al., 2004) and with observations of increased growth cone complexity *in vivo* at points along axon trajectories where guidance decisions are made (Mason and Wang, 1997). We provide evidence that netrin-1 activates Cdc42, Rac1, and Pak1 in embryonic spinal commissural neurons and that it recruits Cdc42, Rac1, Pak1, and N-WASP into a complex with the DCC ICD. Disruption of Pak1 recruitment or the binding of N-WASP to downstream effectors blocks netrin-1-induced growth cone expansion. We also demonstrate that DCC promotes adhesion to substrate-bound netrin-1. These findings suggest that DCC functions as a transmembrane bridge linking immobilized extracellular netrin-1 to the cytoskeleton and that mechanisms of substrate-cytoskeletal coupling (Suter and Forscher, 2000) may contribute to netrin-1-dependent axon guidance.

Netrin-1 activates Cdc42 and Rac1 in commissural neurons

Here, netrin-1-induced activation of Cdc42 and Rac1 was assayed with a GTPγS loading assay. Because GTPγS is not hydrolyzed to GDP, the increases that were detected suggest that a GEF is activated by netrin-1. Our findings do not rule out the possibility that netrin-1 may influence other regulatory mechanisms such as GTPase-activating

proteins or guanine nucleotide dissociation inhibitors. The observed activation of Cdc42 in the presence of dominant-negative Rac1 suggests the action of a mechanism that does not require active Rac1. In contrast, dominant-negative Cdc42 blocks Rac1 activation, suggesting that Rac1 is activated either by a GEF having shared specificity for Cdc42 and therefore sequestered by dominant-negative Cdc42 (Feig, 1999) or by a mechanism activated downstream of Cdc42. The latter possibility is consistent with reports that Cdc42 can act upstream to activate Rac1 (Kozma et al., 1995;Nobes and Hall, 1995).

A complex of DCC, Nck1, Pak1, and N-WASP regulates embryonic spinal commissural neuron growth cone morphology

The serine/threonine kinase Pak1 is an important downstream effector of Cdc42 and Rac1 (Bagrodia and Cerione, 1999). The DCC ICD binds the adaptor Nck1 (Li et al., 2002). We show that disrupting Nck1 binding to Pak1 blocks netrin-1-induced growth cone expansion. Pak1 activation plays an essential role in the cytoskeletal changes underlying neurite outgrowth in PC12 (pheochromocytoma) cells (Manser et al., 1998), and Pak1 is recruited rapidly to the leading edge of leukocytes as they respond to extracellular chemoattractants (Dharmawardhane et al., 1999). Pak1 also may exert an influence on motility by regulating neuronal myosins (Lin et al., 1996).

Nck1 and Nck2 are closely related adaptor proteins, both of which are expressed in the early embryonic spinal cord (Bladt et al., 2003). Mice lacking Nck1 and Nck2 exhibit a severe phenotype, including failure of the embryonic neural tube to close dorsally and embryonic lethality at approximately E9.5, which has prevented the use of these animals to identify roles for Nck1 and Nck2 during axon guidance. Dock, the *Drosophila* ortholog of Nck, is expressed widely by neurons in the fly CNS and enriched in growth cones (Desai et al., 1999). Pak binds Dock, and loss of Dock function generates defects in longitudinal and commissural axon guidance (Hing et al., 1999;Desai et al., 1999). In mammalian cells Nck also associates with activated focal adhesion kinase (FAK) (Schlaepfer et al., 1997), which binds the DCC ICD and is required for axonal chemoattraction to netrin-1 (Ren et al., 2004;Li et al., 2004;Liu et al., 2004a). These reports also indicate that Fyn, a Src family tyrosine kinase, is activated downstream of

DCC and FAK in response to netrin-1. Src family members regulate Rho family GTPase activity (Hoffman and Cerione, 2002), and therefore are possible candidates to activate Cdc42 and N-WASP in response to netrin-1. Supporting this, DCC ICD phosphorylation by Fyn is required for netrin-1-dependent activation of Rac1 (Meriane et al., 2004).

Filopodia and lamellipodia formation is initiated downstream of Cdc42 and Rac1 by members of the WASP family of proteins (Zigmond, 2000). We detect N-WASP, a broadly expressed WASP family member, in the growth cones of embryonic rat spinal commissural neurons. Furthermore, the addition of netrin-1 recruits N-WASP into a complex with the DCC ICD, and disrupting N-WASP function blocks netrin-1-induced growth cone expansion. The N-terminal domain of N-WASP binds directly to F-actin, potentially providing a link between DCC and the cytoskeleton. The C terminus of N-WASP, a domain conserved in all WASP family members, binds to and activates Arp2/3, a protein complex that catalyzes the formation of actin filaments (Mullins, 2000). A recent model proposes that filopodia are formed by Arp2/3 nucleating a population of barbed ends, generating a dendritic array of F-actin typical of lamellipodia, which then align to form the bundles of F-actin at the core of a filopodium (Svitkina et al., 2003;Vignjevic et al., 2003). Arp2/3 is typically absent from established filopodia (Svitkina et al., 2003), and extension of actin filaments at the tip of a filopodium is promoted by anti-capping proteins, such as the Enabled/vasodilator-stimulated phosphoprotein (Ena/VASP) family (Krause et al., 2003). This model is consistent with our detection of N-WASP in growth cone lamellipodia and proximally along filopodia, where the nucleation of new actin filaments is expected to occur, but rarely at filopodia tips (Figure 5.7), where anti-capping proteins regulate extension (Lanier et al., 1999).

The Ena/VASP homolog UNC-34 is required for axon chemoattraction to netrin in *C. elegans* (Gitai et al., 2003), and recent findings indicate that netrin-1-induced filopodia formation requires Ena/VASP function (Lebrand et al., 2004). The model described above suggests that members of the WASP and Ena/VASP families act sequentially and in a spatially segregated manner; however, their actions may be related more closely. Genetic analysis in *C. elegans* indicates that these two protein families play substantially overlapping roles during neural development (Withee et al., 2004). Furthermore, the

neuronal scaffold protein Tuba binds to both N-WASP and Ena/VASP proteins (Salazar et al., 2003), and WASP itself binds to VASP (Castellano et al., 2001). It is possible that DCC may activate Ena/VASP activity at the tips of filopodia to promote elongation and also initiate filopodia formation by activating N-WASP more proximally in the growth cone. Alternatively, N-WASP and Ena/VASP may interact more closely downstream of DCC. Additional investigation is required to unravel the specific roles of these two families of proteins in axonal growth cones.

Evidence that DCC and substrate-bound netrin-1 form an adhesive receptor-ligand pair

Cell adhesion molecules (CAMs) regulate growth cone motility by acting as a transmembrane bridge that links an immobilized extracellular cue to the cytoskeleton (Suter and Forscher, 2000). Although netrins often are described as diffusible axon guidance cues, the majority of netrin-1 protein is not freely soluble *in vivo* but is bound to either cell membranes or the extracellular matrix (Serafini et al., 1994;Manitt et al., 2001;Manitt and Kennedy, 2002). Our findings indicate that substrate-bound netrin-1 both influences growth cone morphology and promotes adherence of DCC-expressing cells, suggesting that DCC and immobilized netrin-1 may form an adhesive receptor-ligand pair.

Recent reports implicate netrin-1 in the regulation of cell-cell interactions, including evidence that netrin-1 regulates epithelial morphogenesis in the mammary gland, pancreas, and lung at least in part by influencing cell-cell adhesion (Manitt et al., 2001;Yebra et al., 2003;Srinivasan et al., 2003;Slorach and Werb, 2003;Hebrok and Reichardt, 2004;Liu et al., 2004b). In the developing mammary epithelium, netrin-1 and the DCC homolog neogenin mediate an adhesive interaction between cell layers (Srinivasan et al., 2003). It is currently not clear whether neogenin mediates adhesion to netrin-1 directly or induces a netrin-1-independent adhesive mechanism. Binding of α6β4or α3β1 integrins to the C terminus of netrin-1 regulates the adhesion and migration of embryonic pancreatic epithelial cells (Yebra et al., 2003). Preliminary findings that use peptide inhibitors of this binding suggest that these integrins are not required for the DCC-

dependent adhesion to netrin-1 that is described here (data not shown). Our findings suggest that netrin-1 contributes directly to cell adhesion, because the cells must interact with the netrin-1 substrate, and that DCC, an IgG superfamily CAM-like transmembrane protein, either directly mediates an adhesive interaction with substrate-bound netrin-1 or is required to engage an additional netrin-1-dependent adhesive mechanism.

Figure 5.9 illustrates the intracellular molecular complex recruited to the DCC ICD in response to netrin-1. We have reported previously that Nck1 constitutively binds to the DCC ICD (Li et al., 2002). With netrin-1 binding, Nck1 serves as a scaffold for the recruitment of Pak1, Cdc42, Rac1, and N-WASP. FAK, bound constitutively to the DCC ICD, activates Fyn in response to netrin-1 (Meriane et al., 2004;Ren et al., 2004;Li et al., 2004;Liu et al., 2004a), which we speculate may be upstream of Cdc42 activation. We hypothesize that DCC promotes filopodia formation and membrane extension via two complementary mechanisms: DCC functions as a transmembrane bridge linking netrin-1 to the actin cytoskeleton and as the core of a protein complex that directs the organization of F-actin, leading to filopodia formation and membrane extension in response to netrin-1.

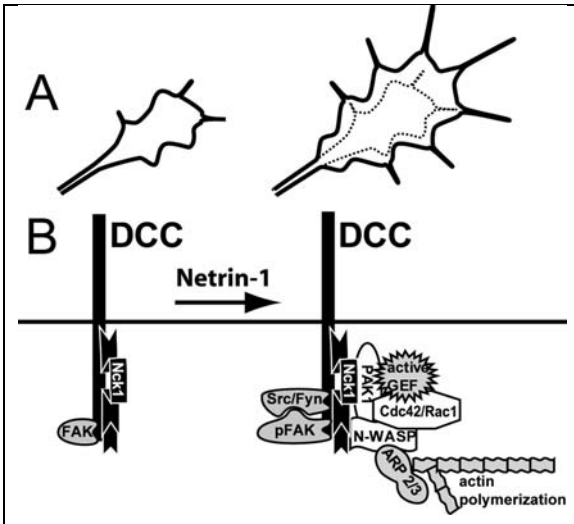

Figure 5.9 - The DCC ICD recruits a complex of signaling proteins to the plasma membrane. *A, The addition of a uniform concentration of netrin-1 induces growth cone expansion, namely an increase in surface area and in the number of filopodia. B, Model of the molecular mechanisms that act downstream of netrin-1 and DCC. Nck1 binds DCC constitutively by its first and third SH3 domains. FAK, bound to the DCC ICD, recruits and activates the tyrosine kinases Src or Fyn in response to netrin-1. Netrin induces the activation of an as-yet-unidentified GEF, leading to the activation of Cdc42, Rac1, and Pak1. We speculate that the activation of a member of the Src family may regulate the activation of Cdc42 by regulating a GEF. Activated Cdc42 activates N-WASP, which promotes the nucleation of F-actin via the Arp2/3 complex.*

CHAPTER 6

Rho Inhibition Enhances Axon Chemoattraction to Netrin-1

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PREFACE

A manuscript of this chapter is currently being revised for resubmission to the journal Development. This section explores the role of Rho subfamily of Rho GTPases in the guidance of axons to netrin-1. As discussed in the Literature Review I, activation of the Rho subfamily has been implicated in the repulsion of axons and in the inability of CNS axons to regenerate in the adult following injury. Here we explore its function in the response to netrin-1 in an attractive axon guidance setting. In contrast to what we observed for Rac and Cdc42 Rho GTPases in Chapter 4, here we report that Rho is inhibited by netrin-1 stimulation. We also extend our findings from the previous chapter that netrin-1 acts as an adhesive cue by demonstrating that inhibition of Rho promote cellular adhesion and growth cone expansion onto a substrate of netrin-1.

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ABSTRACT

Extracellular guidance cues direct axon extension by regulating cytoskeletal organization and remodeling adhesive contacts made by growth cones. The Rac and Cdc42 subfamilies

of Rho GTPases play well-recognized roles in axon extension and chemoattraction, while the Rho subfamily mediates growth cone repulsion or collapse. Here we investigated the hypothesis that Rho might influence axon chemoattraction. We show that netrin-1, through its receptor DCC, inhibits RhoA in embryonic rat spinal commissural neurons, demonstrating regulation of RhoA activity by an axonal chemoattractant. We then examined the consequences of inhibiting Rho on chemoattraction to netrin-1. Based on the well-established roles for Rho regulating cytoskeletal and adhesive remodeling, we anticipated that inhibiting Rho would disrupt the capacity of axons to turn in response to netrin-1. In contrast, when Rho signaling was inhibited spinal commissural neuron axon chemoattraction was not only intact, but occurred over a greater distance. The increased responsiveness of spinal commissural neuron axons was accompanied by increased plasma membrane DCC in neuronal growth cones. Additionally, using an adhesion assay, we detected enhanced DCC mediated adherence to substrate bound netrin-1. We conclude that netrin-1 inhibition of RhoA promotes axonal chemoattraction by increasing plasma membrane DCC in growth cones and by altering mechanisms that regulate cytoskeletal organization and adhesion. Notably, inhibiting Rho signaling after CNS injury enhances regeneration by making axons insensitive to growth inhibitors associated with myelin and the glial scar. Our results, in contrast, indicate that axons retain the capacity to respond to a chemoattractant guidance cue, netrin-1, in spite of disruption of Rho signaling.

INTRODUCTION

During development, axons are directed to their targets along defined pathways by extracellular cues (reviewed in Huber et al., 2003;Moore and Kennedy, 2006a). Netrins are a family of secreted axon guidance proteins with homology to laminins (Serafini et al., 1994;Yurchenco and Wadsworth, 2004). In the developing spinal cord, netrin-1 is secreted by the floor plate and guides the axons of spinal commissural neurons (SCNs) to the ventral midline (Kennedy et al., 1994;Kennedy et al., 2006). Axon chemoattraction to netrin-1 requires the transmembrane receptor DCC (Keino-Masu et al., 1996), association of N-WASP, Pak1 and Fak with the intracellular domain of DCC (Ren et al., 2004;Li et al., 2004;Liu et al., 2004a;Shekarabi et al., 2005), elevation of cytosolic calcium levels

(Hong et al., 2000), activation of phospholipase C (Ming et al., 1999) and activation of the Rho GTPases Rac and Cdc42 (Shekarabi and Kennedy, 2002;Shekarabi et al., 2005).

Growth cone turning is thought to involve the asymmetric formation of adhesive contacts that stabilize protrusions, leading to membrane extension on one side, coordinated with retraction of the trailing edge (reviewed in Dickson, 2002;Huber et al., 2003). Rho GTPases are a family of intracellular proteins that cycle between an inactive GDP-bound state and an active GTP-bound state (reviewed in Etienne-Manneville and Hall, 2002). The Rac and Cdc42 sub-families are implicated in directing cytoskeletal rearrangements within growth cones in response to chemoattractant guidance cues (reviewed in Govek et al., 2005), including netrin-1 (Shekarabi and Kennedy, 2002;Yuan et al., 2003;Shekarabi et al., 2005). The third sub-family, Rho, has three mammalian members (RhoA, B and C) and is implicated in generating repellent responses and growth cone collapse (Wahl et al., 2000;Hu et al., 2001;Driessens et al., 2001;Jain et al., 2004). Rho GTPases also regulate the formation of adhesive structures in growth cones called point contacts (Renaudin et al., 1999;Woo and Gomez, 2006). Rac activity promotes the formation of point contacts, while stabilization of point contacts requires inhibiting Rac and activating RhoA (Woo and Gomez, 2006).

Although the Rho subfamily of Rho GTPases have been implicated in promoting cell migration (Worthylake et al., 2001;Worthylake and Burridge, 2003), little attention has been paid to their potential role in axon chemoattraction. Here we report that netrin-1, through its receptor DCC, inhibits RhoA in embryonic rat SCNs. We then demonstrate that inhibiting Rho signaling enhances the response of SCN axons to netrin-1, including increased axon outgrowth from explants and turning toward a source of netrin-1 over a greater distance. Investigating this enhanced sensitivity to netrin-1, we show that Rho inhibition recruits increased levels of DCC to the neuronal plasma membrane, and using an adhesion assay, we demonstrate enhanced DCC dependent adherence of cells to substrate bound netrin-1. These findings support the conclusion that netrin-1 inhibition of RhoA promotes axonal chemoattraction by increasing plasma membrane DCC in growth cones, and by altering intracellular mechanisms that regulate cytoskeletal organization and adhesion.

MATERIALS AND METHODS:

Reagents

The following antibodies and reagents were used: mouse IgM anti-Tag1 (4D7) for embryonic spinal cord immunohistochemistry (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA); rabbit anti-Tag1 (TG3) for western blot analysis (provided by Dr. Thomas Jessell, Columbia University, New York, NY); rabbit anti-integrin β1 (AB1952, Chemicon, Temecula, CA); mouse IgG anti-RhoA (26C4) and goat anti-DCC (A-20, Santa Cruz Biotechnology, Santa Cruz, CA); mouse IgG anti-DCC (AF5) and Y-27632 (Calbiochem, LaJolla, CA); DCC-fc, a recombinant protein composed of the extracellular domain of mouse DCC and the Fc portion of human IgG_1 (R & D Systems, Minneapolis, MN); mouse IgG anti-ROCKII (BD Biosciences, Missisauga, Canada); rabbit anti-PRK2 (Cell Signaling, Danvers, MA); DNase, poly-D-lysine (PDL, 70-150 kD) and Hoechst 33258 (Sigma-Aldrich, Missisauga, Canada); Neurobasal, iFBS, B-27 supplement, GlutaMAX-1, Penicillin-Streptomycin, Ca^{2+}/Mg^{2+}-free HBSS, Alexa 546 coupled phalloidin and Jasplakinolide were purchased from Invitrogen Canada (Invitrogen Canada, Burlington, ON). 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). C3-07, a fusion peptide of C3-transferase and proline-rich sequences (Winton et al., 2002), was provided by Lisa McKerracher (Bioaxone, Montreal, QC).

Explant cultures

Staged pregnant Sprague-Dawley rats were obtained from Charles River Canada (St-Constant, QC). embryonic day 11 (E11) rat spinal cord (vaginal plug = E0) and E13 dorsal spinal cord explants were dissected as described (Placzek et al., 1990;Serafini et al., 1994). For turning assays, aggregates of netrin-expressing HEK 293-EBNA cells were prepared and immobilized alongside E11 spinal cords, as illustrated in Figure 6.4A (Kennedy et al., 1994;Shirasaki et al., 1996). Turning assays were cultured for 40 hours and E13 dorsal spinal cord explants for 14 hours in Neurobasal/iFBS (Neurobasal

supplemented with 10% iFBS, 2 mM GlutaMAX-1, 100 unit/ml penicillin, and 100 µg/ml streptomycin). A Magnafire CCD camera (Optronics, Goleta, CA) and an Axiovert 100 microscope (Carl Zeiss Canada, Toronto, ON) were used to capture digital images of Tag1 positive SCN axons. For quantification of turning assays, images were printed and the deflection distances determined by an observer blind to the experimental condition. Outgrowth from dorsal explants was measured using Northern Eclipse image analysis software (Empix Imaging, Mississauga, Canada).

RhoA Activation and Cell Surface Biotinylation Assays

RhoA activation and biotinylation assays were performed on SCNs obtained by microdissection and dissociation of the dorsal halves of E13 rat spinal cords, as described (Bouchard et al., 2004). Neurons were plated in 6-well tissue culture dishes previously coated for 2 hours at RT with 2 ml of 10 µg/ml PDL. For the first 12 hours, the media consisted of Neurobasal/iFBS. The medium was then changed to Neurobasal/B-27 (Neurobasal supplemented with 2% B-27, 2 mM GlutaMAX-1, 100 units/ml penicillin, and 100 µg/ml streptomycin)**.**

For G-LISA assays, two million dissociated SCNs were plated as described above. After a total of 40 hours in culture, the relative amounts of active, GTP-bound RhoA in each condition was measured as per the manufacturer's instructions (BK124, Cytoskelton, Denver, CO). The purification of GST-RBD and RhoA pulldown assays were performed as described (Ren and Schwartz, 2000), except that the lysis buffer for SCNs was 50 mM Tris (pH 7.3), 1% NP-40, 200 mM NaCl, 10 mM DTT, 2 µg/ml aprotinin, 5 µg/ml leupeptin and 1 mM PMSF. Ten million cells were plated per condition for RhoA-GTP pulldown assays. Western blots were visualized using chemiluminescence (PerkinElmer BioSignal, Montreal, QC) and films scanned (ScanJet 5300C, Hewlett-Packard, Mississauga, ON). Band intensities were measured using Photoshop 7.0 (Adobe, San Jose, CA).

Biotinylation of extracellular protein was carried out as described (Bouchard et al., 2004). Briefly, after 40 hours in culture two million SCNs were pretreated for 1 hour with, either: 10 µg/ml C3-07 or 10 µM Y-27632; and then, in some cases, stimulated for 5

minutes with 50 ng/ml netrin-1. Cells were washed with Ca/Mg PBS (0.1 mM $CaCl_2$, 1 mM $MgCl_2$ in PBS) and labeled for 30 minutes at 4˚C with 2.5 mg of EZ-Link Sulfo-NHS-biotin (Pierce, Rockford, IL) dissolved in 2.5 ml of Ca/Mg PBS. The reaction was quenched with 10 mM Glycine in PBS and the cells lysed in RIPA buffer (10 mM phosphate, pH 7.5, 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% deoxycholate, 2 μg/ml aprotinin, 5 μg/ml leupeptin, 1 mM EDTA and 1 mM PMSF). Labeled proteins were bound to steptavidin-agarose beads (Pierce) for 2 hours at 4˚C, then washed several times and analyzed by western blot.

Immunostaining

12 mm round cover glasses (no 0 Assistent, Carolina Biological, Windsor, ON) were coated with 400 μl of 10 μg/ml PDL for 2 hours at RT for all conditions except those examining growth cone area. For these experiments, 1 μg/ml PDL was applied for 5 minutes followed by either: a 3 hour incubation at RT with HBSS +/- 2 μg/ml netrin-1 or, for a circular substrate of netrin-1, a 2 μl drop of 100 μg/ml netrin-1. Dissociated SCNs were plated and cultured for 24 hours in Neurobasal/iFBS then pretreated for 1 hour with, either: 10 μg/ml C3-07 or 10 μM Y-27632, and then stimulated for 5 minutes with 50 ng/ml netrin-1. The cells were then fixed for 30 sec at 37˚C in 4% PFA, 0.1% Glutaraldehyde, 250 mM sucrose in PBS pH 7.5. For plasma membrane DCC labeling, cells were blocked for 1 hour in 3% BSA in PBS, then incubated with 250 ng/ml mouse anti-DCC (AF5, raised against the extracellular domain of DCC) in 1% BSA in PBS overnight at 4˚C. The coverslips were then washed several times with PBS. For all immunofluorescent labeling, cells were permeabilized for 5 minutes at RT in 0.15% triton X-100 PBS and then blocked for 1 hour at RT with 0.1% triton X-100, 3% BSA. Primary and secondary antibodies were diluted in PBS with 0.1% triton X-100 and 1% BSA and incubated for 1 hour at RT. The following dilutions were used: 1 μg/ml mouse anti-RhoA, 400 ng/ml goat anti-DCC, 1 μg/ml donkey anti-mouse Alexa 546, 1 μg/ml donkey anti-goat Alexa 488, 0.8 U/ml Alexa 546 coupled phalloidin and 500 ng/ml Hoechst 33258. Coverslips were mounted with SlowFade (Invitrogen) and immobilized with nail polish before imaging.

Adhesion Assays

Adhesion assays were performed as described (Shekarabi et al., 2005). Briefly, 20 µl of 0.1% nitrocellulose (Hybond ECL; Amersham Biosciences, Piscataway, NJ) dissolved in methanol (Fisher Scientific, Houston, TX) was dried on the bottom of NUNC four-well plates (VWR International, Mississauga, ON). Substrates were incubated with HBSS +/- 2 µg/ml netrin-1 for 2 hours at RT, blocked for 1 hour at RT in 1% BSA (Fisher Scientific) in HBSS and then 1% heparin (Sigma) in HBSS. As indicated, some substrates were incubated with 25 µg/ml anti-netrin PN3 (Manitt et al., 2001) or 5 µg/ml DCC-fc for 1 hour. SCNs, $2.5x10^5$ per well, were cultured for 2 hours in Neurobasal/B-27 medium in the presence of 10µg/ml C3-07, 10µM Y-27632 and/or 100nM Jasplakinolide. Unbound cells were removed by washing with three changes of PBS and the remaining cells fixed with 500 µl 4% PFA in PBS. Nuclei were labeled with 500 ng/ml Hoechst 33258 in PBS for 30 minutes and counted using Northern Eclipse software.

RT-PCR analysis

Total RNA was extracted from 10 million E14 rat SCNs cultured for 2DIV on a PDL coated (12ml of 10µg/ml PDL for 2hrs at RT) 10cm dish using TRIzol® (Invitrogen Life Technologies, Burlington, Ontario). RT-PCR was performed with 0.5 µg of total RNA per reaction using the QIAGEN® OneStep RT-PCR Kit (Qiagen, Mississauga, Ontario). The following primer pairs were annealed at 60^oC: RhoA 5'AAAGTCGGGGTGCCTCA3' and 3'GAGGGCGTTAGAGCAGTGTC5; RhoB 5'ATGTGCTTCTCGGTAGACAG3' and 3'AGAAAAGGACGCTCAGGAAC5'; RhoC 5'GCCTACAGGTCCGGAAGAAT3' and 3'GCACCAACCTAGTTCCCAGA5'; ROCKI 5'GTAATCGGCAGAGGTGCATT3' and 3'TCCAGACTTATCCAGCAGCA5'; ROCKII 5'CTAACAGTCCGTGGGTGGTT3' and 3'AGACCACCAATCACATTCTCG5'; PRK1 5'TGTGTGAGAAGCGGATTTTG3' and 3'ACGGCTCGAGTGTAGGATGT5'; PRK2 5'TTTGCATGTTTCCAAACCAA3' and 3'GACTCTCCGACGAGCATTTC5'.

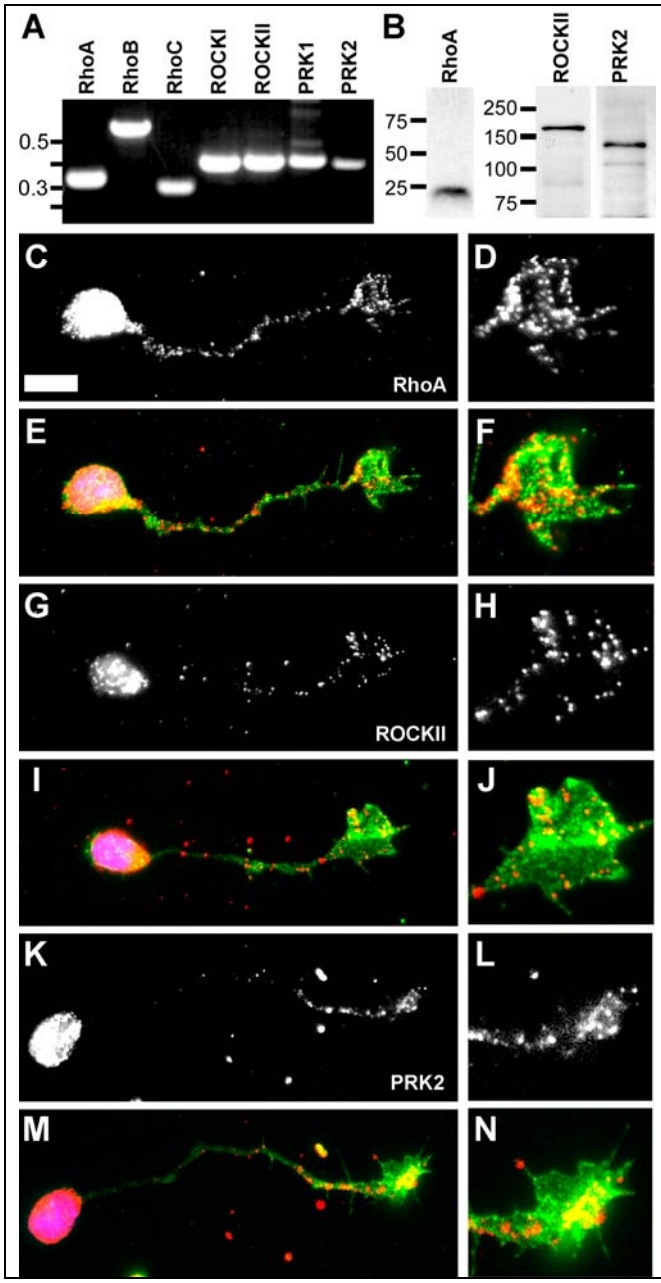

Figure 6.1 - Dorsal spinal cord neurons express Rho, ROCK and PRK family members. *(A) RT-PCR amplification indicates that RhoA, B and C, as well as, ROCKI, ROCKII, PRK1 and PRK2 are expressed by SCNs. (B) Western blot analysis of SCN lysate using RhoA, ROCKII and PRK2 antibodies. Immunofluorescent localization of RhoA (C,D), ROCKII (G,H) and PRK2 (K,L). In panels E, F, I, J, M and N, DCC is labeled in green and nuclei in blue; RhoA (E,F), ROCKII (I,J) and PRK2 (M,N) are labeled in red. Scale bar = 20 μm for panels C, E, G, I, K and M and 10μm for panels D, F, H, J, L and N.*

RESULTS:

Netrin-1 inactivates RhoA in SCNs

Although RhoA is activated in response to repellent axon guidance cues (Wahl et al., 2000;Hu et al., 2001), there have been no reports describing how RhoA might be regulated by axonal chemoattractants. Here we examine how RhoA signaling contributes to the response of embryonic rat SCNs to netrin-1. RT-PCR and western blot analysis

indicates that SCN cultures express RhoA (Figure 6.1A,B) and RhoA immunoreactivity was detected throughout the growth cones of SCNs, consistent with a potential capacity to influence chemoattraction (Figure 6.1C-F). RhoA activation was then examined in SCNs at several time points following application of netrin-1. A significant reduction in total GTP-bound RhoA was detected within 15 min after adding 200 ng/ml netrin-1, using both an ELISA-based (G-LISA) assay and a Rhotekin pulldown assay (Ren and Schwartz, 2000). Specifically, we observed a 13% reduction using the G-LISA assay (Figure 6.2A) and a 27% reduction using Rhotekin pulldown (Figure 6.2B). Inhibition of RhoA by netrin-1 after 15 minutes was blocked by application of a DCC receptor-body (2 µg/ml DCC-fc) or by function blocking antibodies against DCC (5 µg/ml DCC-fb, Figure 6.2A). We conclude that netrin-1 inhibits RhoA in SCNs through a DCC dependent mechanism.

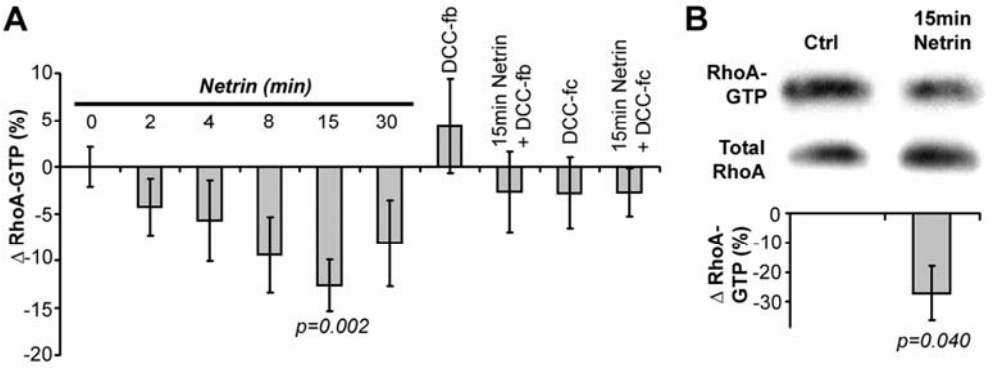

Figure 6.2 – Netrin-1 Inhibits RhoA in spinal commissural neurons. *G-LISA (A) and GST-RBD pulldown (B) assays were used to evaluate the relative amounts of RhoA-GTP in SCNs. GST-RBD pulldown assays measured a 27% (p=0.040) average reduction across three separate experiments. G-LISA assays measured an average reduction of 13% after 15 minutes of 200 ng/ml netrin-1 (n=23, p=0.002). This was blocked with either 5 µg/ml of DCC function blocking antibodies (DCC-fb) or 2 µg/ml of DCC's extracellular domain (DCC-fc). Tukey post-hoc tests of means. Error bars = SEM.*

Rho, ROCK and PRK family members are expressed in embryonic spinal commissural neurons

As described below, we utilized two pharmacological reagents: C3-07 and Y-27632, to investigate the functional role of Rho signaling in the response to netrin-1. C3-07 is a membrane permeable analog of C3-exozymes (Winton et al., 2002), a family of bacterial enzymes that inactivate all Rho family members (RhoA, B and C) through ADP-ribosylation (reviewed in Aktories et al., 2004). Y-27632 is a cell permeable ATP analog that selectively inhibits ROCK and PRK family of kinases (Uehata et al., 1997;Davies et al., 2000), both of which are downstream effectors of Rho signaling (reviewed in Karnoub et al., 2004). RT-PCR analysis indicated that SCNs express all Rho, ROCK and PRK family members (Figure 6.1A), and immunoreactivity for RhoA, ROCKII, and PRK2 protein were detected in whole cell homogenates of 2 DIV SCNs by western blot analyses (Figure 6.1B). The distribution of these proteins was then examined in the growth cones of embryonic SCNs in dispersed cell culture. Compared to the distribution of RhoA, which was detected throughout the growth cones of SCNs (Figure 6.1C-F), ROCKII was distributed along the periphery (Figure 6.1G-J) and PRK2 in the central region (Figure 6.1K-N). Interestingly, a similar distribution of these proteins has been reported in migrating cells, with ROCKII enriched at the leading and trailing edges and PRK2 localized more centrally in the soma (reviewed in Wheeler and Ridley, 2004). These findings indicate that all the known targets of C3-07 (RhoA, B and C) and Y-27632 (ROCKI & II, PRK1 & 2) are expressed by SCNs.

Rho inhibition increases DCC-dependent outgrowth to netrin-1

We then examined the effect of inhibiting Rho signaling on netrin-1 dependent SCN axon outgrowth. In the absence of netrin-1, few SCN axons emerge from an explant of E13 rat dorsal spinal neuroepithelium when cultured for 14 hours (Figure 6.3A). Outgrowth was not significantly increased in the presence of C3-07 ($n=5$, $p=0.624$) (Figure 6.3D), while Y-27632 produced only a modest increase in the mean axon outgrowth per explant from 0.012 mm to 0.149 mm ($n=5$, $p=0.001$) (Figure 6.3G). In control conditions, without drugs inhibiting Rho signaling, plotting the amount of axon outgrowth versus the concentration of netrin-1 generates a bell-shaped curve that reaches a maximum at approximately 200 ng/ml netrin-1 (Serafini et al., 1994;Moore and

Kennedy, 2006b). The consequences of Rho inhibition on SCN axon outgrowth were tested at three different netrin-1 concentrations: sub-maximal at 50 ng/ml, optimal at 200 ng/ml, and super-saturating at 600 ng/ml. At each concentration, C3-07 and Y-27632 dramatically increased outgrowth to netrin-1 (Figure 6.3B,E,H,J). These results provide evidence that across a wide spectrum of netrin-1 concentrations, Rho signaling remains active in SCNs and acts to restrain axon extension. Application of DCC function blocking antibodies, DCC-fb (Figure 6.3C), significantly reduced outgrowth in the presence of C3-07 (Figure 6.3F) or Y-27632 (Figure 6.3I) at all concentrations of netrin-1 tested (Figure 6.3K), indicating that DCC is required for the increased outgrowth to netrin-1 induced by inhibiting Rho signaling.

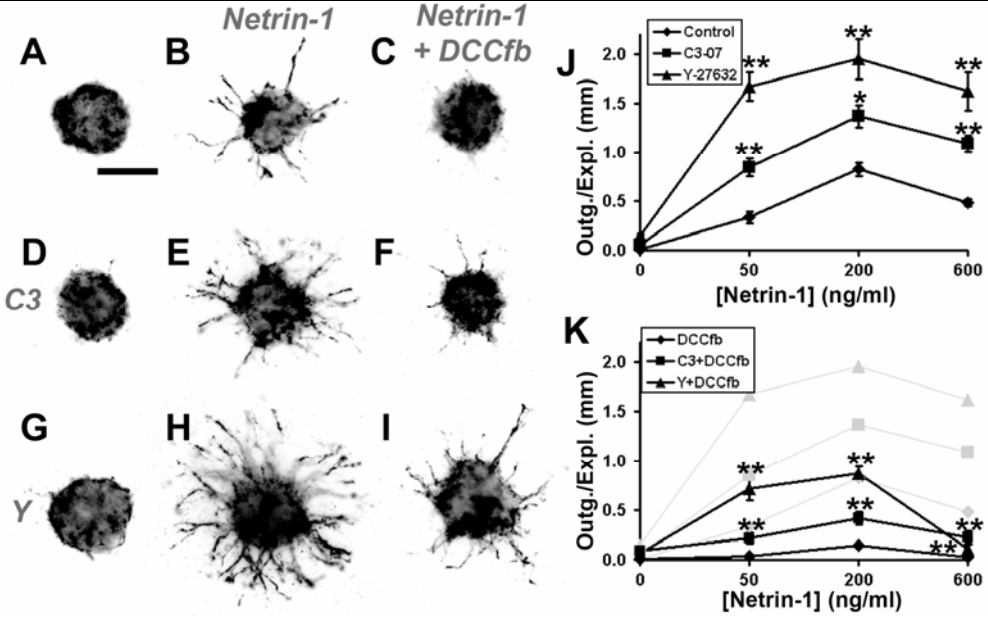

Figure 6.3 - Inhibiting Rho signaling increases DCC-dependent spinal commissural neuron axon outgrowth evoked by netrin-1. *E13 rat dorsal spinal cord explants were cultured for 14 hours with various amounts of netrin-1 in the presence of 10 µg/ml C3-07 or 10 µM Y-27632. SCN axons were labeled using the 4D7 monoclonal antibody against Tag1. C3-07 increased total outgrowth per explant by 155% (n=5, p=0.006) at 50 ng/ml netrin-1, 66% (n=4, p=0.041) at 200 ng/ml netrin-1 and 126% (n=4, p=0.003) at 600 ng/ml netrin-1 (J). Y-27632 increased outgrowth by 404% (n=5, p<0.001) at 50 ng/ml netrin-1, 137% (n=5, p<0.001) at 200 ng/ml netrin-1 and 236% (n=5, p=0.000) at 600 ng/ml netrin-1 (J). Outgrowth in the presence of C3-07 or Y-27632 was significantly reduced at each netrin-1*

*concentration (n=5, p<0.01) in the presence of 5 μg/ml DCC-fb (K). Specifically, total outgrowth with C3-07 was reduced by 74% (n=5, p=0.001) at 50 ng/ml netrin-1, by 70% (n=5, p<0.001) at 200 ng/ml netrin-1 and 79% (n=5, p<0.001) at 600 ng/ml. Total outgrowth with Y-27632 was reduced by 57% (n=5, p<0.001) at 50 ng/ml netrin-1, 55% (n=5, p<0.001) at 200 ng/ml netrin-1 and 93% (n=5, p<0.001) at 600 ng/ml netrin-1. *p<0.05, **p<0.01. Tukey Post-hoc tests of means. Error bars = s.e.m. Scale bar = 100 μm.*

Rho Inhibition promotes axon turning to netrin-1

Inhibiting Rho signaling with either Y-27632 or C3 exoenzyme hinders monocyte migration during transendothelial migration by disrupting cytoskeletal reorganization and interfering with adhesive mechanisms (Worthylake et al., 2001;Worthylake and Burridge, 2003). As such, the increased SCN axon outgrowth to netrin-1 evoked by Rho inhibition could reflect a severe deregulation of the mechanisms that normally direct axon extension. We hypothesized that such a disruption would interfere with the ability of SCN axons to turn in response to a gradient of netrin-1. To determine if inhibiting Rho signaling might enhance axon outgrowth, while disrupting the capacity of an axon to turn, we utilized an explanted embryonic spinal cord turning assay. In this assay, an aggregate of netrin-1 expressing cells is cultured immediately adjacent to the cut edge of a segment of intact E11 spinal cord and the two are immobilized in a three-dimensional collagen gel (Figure 6.4A). Typically, this source of netrin-1 attracts extending SCN axons over a distance of approximately 250 μm (Kennedy et al., 1994). In contrast to our expectations, neither C3-07 nor Y-27632 hindered the ability of SCN axons to turn toward the source of ectopic netrin-1. In fact, the inhibitors increased the average distance over which these axons turned by approximately 16% (Figure 6.4B,C,E,G). The increased axon turning was sensitive to the DCC monoclonal function blocking antibody (Figure 6.4B,D,F,H), indicating that the enhanced chemoattraction requires DCC.

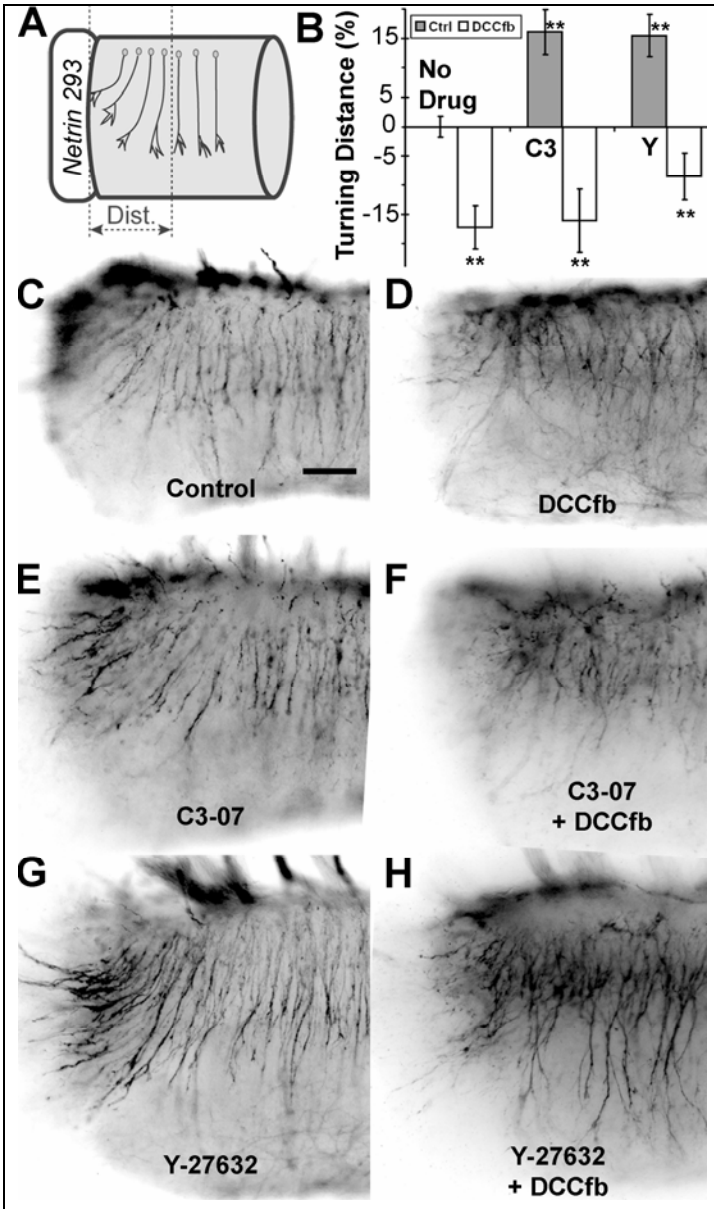

Figure 6.4 - Inhibiting Rho signaling promotes spinal commissural neuron axon turning to netrin-1. *Aggregates of netrin-1 expressing HEK 293-EBNA cells were cultured alongside explants of E11 rat spinal cord, as shown in panel A. Explants were cultured for 40 hours in the absence of rho inhibitors (C,D), in the presence of 10 μg/ml C3-07 (E,F) or 10 μM Y-27632 (G,H). SCN axons were fluorescently labeled with Tag1 (4D7). Deflection distances were measured as shown in panel A and graphed in B as the percent distance relative to the absence of drugs. Both C3-07 (E, n=30, p=0.005) and Y-27632 (G, n=30, p=0.007) resulted in a 16% increase in the turning distance. 5 μg/ml of DCC function blocking antibodies reduced turning in the absence of drug (D, n=24, p=0.001), as well as, turning in the presence of 10 μg/ml C3-07 (F, n=21, p<0.001) or 10 μM Y-27632 (H, n=24, p<0.001). Tukey post-hoc tests of means. Error bars = SEM. Scale bar = 100 μm.*

Rho inhibition increases the amount of plasma membrane DCC in spinal commissural neurons

Recruitment of DCC to the neuronal plasma membrane from an intracellular vesicular pool increases SCN axon outgrowth and chemoattractive turning in response to

netrin-1 (Bouchard et al., 2004;Moore and Kennedy, 2006b). In these previous studies, activation of protein kinase A (PKA) increased plasma membrane DCC. Interestingly, PKA activation has been reported to inhibit RhoA (Lang et al., 1996), raising the possibility that the inhibition of Rho signaling may lead to the recruitment of DCC to the plasma membrane, thereby enhancing netrin-1 dependent axon outgrowth and turning. We therefore determined if manipulating Rho signaling might influence plasma membrane levels of DCC. First, using biotinylation to selectively label cell surface proteins, we detected a 1.5 and a 1.8-fold increase in plasma membrane DCC 1 hour after the application of C3-07 and Y-27632, respectively (Figure 6.5A). Consistent with previous findings, application of netrin-1 alone increased plasma membrane DCC (Bouchard et al., 2004), however, application of either inhibitor together with netrin-1 did not synergize to further increase the amount of plasma membrane DCC. Plasma membrane levels of the GPI-linked membrane protein Tag-1 were unaltered by Rho inhibition, indicating that inhibiting Rho signaling did not evoke a non-specific change in the trafficking of all membrane proteins.

Using immunocytochemistry, we then extended the above findings to determine if changes in Rho signaling would influence the amount of plasma membrane DCC presented by SCN growth cones. Specifically, following fixation, to selectively label plasma membrane DCC, non-permeabilized cells were labeled using a mouse monoclonal antibody against an epitope in the extracellular domain of DCC (AF5, Calbiochem). The cells were then permeabilized and total DCC labeled with a goat polyclonal antibody (A-20, Santa Cruz) raised against an epitope in the intracellular domain of DCC. The ratio of plasma membrane DCC labeling to that of total DCC within the growth cone was compared across conditions. Consistent with findings from the biotinylation assay, application of Rho inhibitors significantly increased the amount of plasma membrane DCC detected (Figure 6.5B-H) and the amount of plasma membrane DCC was unaffected by co-application with netrin-1.

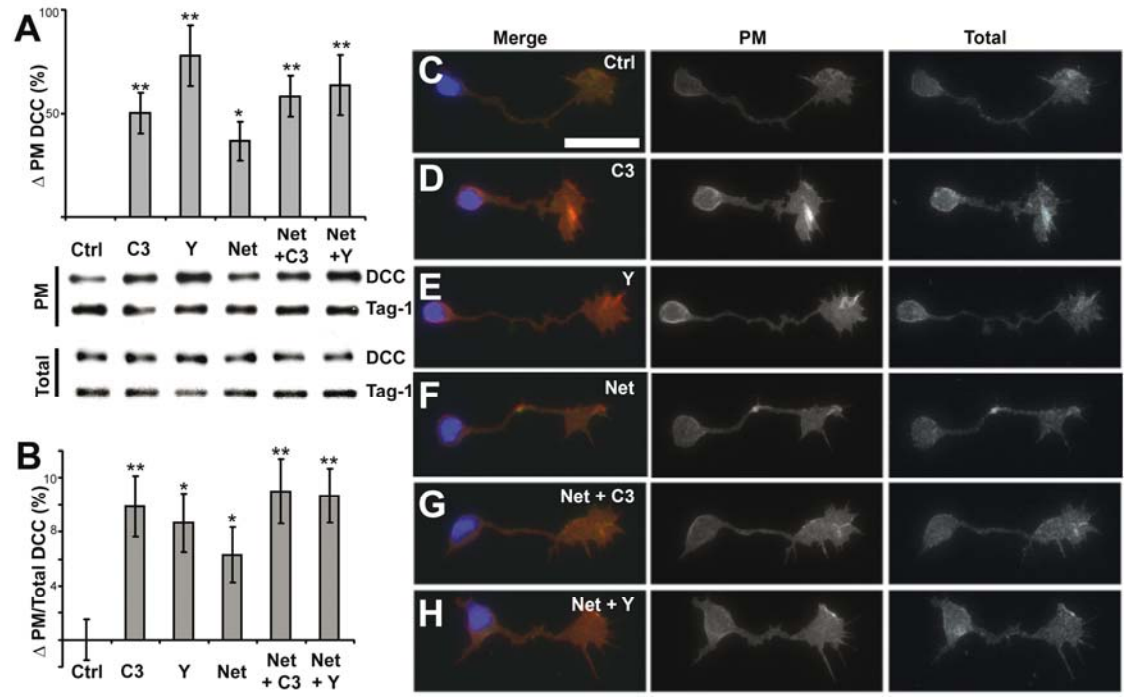

Figure 6.5 - Inhibiting Rho signaling increases the amount of plasma membrane DCC in spinal commissural neurons. *Plasma membrane levels of the netrin-1 receptor DCC in SCN in vitro were evaluated using biotinylation (A) or immunofluorescence (B-H). Cell surface levels were measured following one-hour incubations with either 10 μg/ml C3-07 (C3) or 10 μM Y-27632 (Y), and in some cases, 5 minutes following application of 50 ng/ml netrin-1 (Net), as indicated. In the biotinylation assay (A), C3-07 caused a 50% (n=8, p=0.002) and Y-27632 a 78% (n=8, p<0.001) increase in the amount of DCC in the absence of netrin-1. Netrin produced a 37% (n=8, p=0.021) increase, while the combination of both netrin and C3-07 or Y-27632 generated a 59% (n=8, p<0.001) or 64% (n=8, p<0.001) increase, respectively. No significant change in the amount of cell-surface Tag1 was detected. (B) The ratio plasma membrane DCC immunoflorescence to total cellular DCC immunoflorescence increased in the presence of C3-07 by 9.9% (n=85, p=.006), Y-27632 by 8.7% (n=87, p=0.022), netrin-1 by 6.3% (n=124, p=0.048), netrin-1/C3-07 by 11.0% (n=80, p=0.002) and netrin-1/Y-27632 by 10.7 % (n=90, p=0.001). *p<0.05, **p<0.01. Tukey post-hoc tests of means. error bars = SEM, scale bar = 20μm.*

Rho inhibition promotes growth cone expansion and adhesion to substrate bound netrin-1

Netrin-1 is a secreted protein, but the vast majority of netrin-1 protein is tightly bound to membranes or extracellular matrix *in vivo*, and not freely soluble (Serafini et al., 1994;Manitt et al., 2001;Manitt and Kennedy, 2002). We have previously reported that DCC mediates the adhesion of SCNs to substrate bound netrin-1 protein (Shekarabi et al., 2005). Based on Rho's well described role regulating the maturation of adhesive structures (reviewed in Arthur et al., 2002) and the increase in plasma membrane DCC induced by Rho inhibition (Figure 6.5), we tested the hypothesis that inhibiting Rho signaling might influence SCN adhesion to netrin-1. Cells derived from dissociated E13 spinal cord were plated on a netrin-1 substrate and we observed that Rho inhibition increased the number of adherent cells (Figure 6.6A) by a mean value of 79% with C3-07 (Figure 6.6C) and by 152% with Y-27632 (Figure 6.6D). The increased adhesion to netrin-1 induced by inhibiting Rho was blocked either by pre-incubation with netrin function blocking antibodies (anti-netrin) or by competition with a DCC receptor-body (DCC-fc, Figure 6.6A). To determine if the increased adhesion requires reorganization of filamentous actin, we applied the cell permeable reagent jasplakinolide which stabilizes actin filaments (Scott et al., 1988;Visegrady et al., 2005), and found that pretreatment with jasplakinolide reduced adhesion to netrin-1, and blocked the effects of inhibiting Rho signaling (Figure 6.6A).

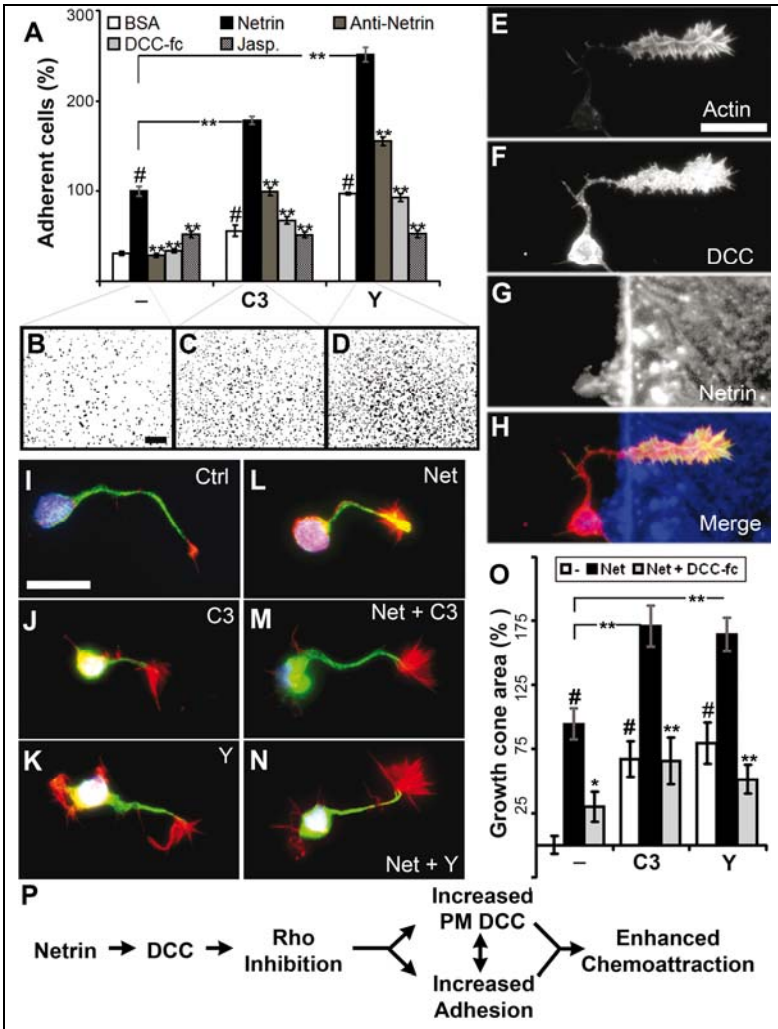

Figure 6.6 - Inhibiting Rho signaling promotes adhesion and growth cone expansion in response to netrin-1. *SCN attachment is increased on a substrate of netrin-1 (Shekarabi et al., 2005). (A) Here we show that SCN adherence to netrin-1 is increased by 79% (n=12, p<0.001) in the presence of C3-07 (C3) or by 152% (n=12, p<0.001) with Y-27632 (Y). This adhesion was reduced (n=12, p<0.001) upon pre-incubation with either: netrin function blocking antibodies (Anti-Netrin) or by competition with the extracellular domain of DCC (DCC-fc receptor-body). Stabilization of filamentous actin with Jasplakinolide (Jasp.) disrupted adhesion to netrin-1 and abolished the increased adhesion evoked by C3-07 or Y-27632. Panels B-D are representative images of cells binding to netrin-1 substrates in the absence (B) or in the presence of C3-07 (C) or Y-27632 (D). (E-H) The expansion of SCN growth cones as they encounter a boundary of immobilized netrin-1 is consistent with netrin-1 functioning as an adhesive cue. Panels I-N are SCNs labeled with phalloidin (red), beta-tubulin (green) and Hoechst (blue). The growth cones of SCNs grown on glass coverslips coated with PDL (I-K) are smaller than those grown on this same substrate with an additional coating of netrin-1 (L-N). (O) In the absence of netrin-1, the average growth cone increased by 91% (n=21, p=0.008) when treated with C3-07 and by 118%*

*(n= 20, p <0.001) when treated with Y-27632. On a substrate of netrin-1, average growth cone area increased by 84% in the absence of inhibitors. A netrin-1 substrate also increased the average area of growth cones in the presence of C3-07 by 50% (n=22, p=0.006) and Y-27632 by 38% (n=21, p=0.033). (P) Model outlining our hypothesis that netrin-1 inhibition of Rho enhances chemoattraction by facilitating DCC function, in part by recruiting additional DCC to the plasma membrane and by promoting DCC signaling mechanisms, such as increasing adhesion to immobilized netrin-1, that lead to membrane extension. Tukey post-hoc tests of means. Error bars = SEM. Scale bar = 200 μm in B-D and 20 μm in E-N. In a panels A and O, * indicates p<0.05 and **p<0.01. Above the third, fourth and fifth bar of each condition (-, C3 and Y) significance is relative to the corresponding netrin-1 alone condition (second bar). # indicates p<0.01 relative to the control condition on a substrate without netrin-1 (first bar of '-' condition).*

The assay described above addresses the adhesion of the entire cell to the substrate. To extend these findings to a context more relevant to axon guidance, we challenged extending SCN axons with a discontinuous substrate of PDL adjacent to a substrate of PDL plus an additional layer of netrin-1, and examined SCN growth cones crossing onto the netrin-1 substrate. Consistent with our previous findings examining axons on uniform substrates of either PDL alone compared to PDL plus netrin-1 (Shekarabi et al., 2005), we found that the axonal growth cones of SCN axons dramatically expanded once they had crossed onto netrin-1 (Figure 6.6 E-H). We hypothesize that the growth cone expansion observed reflects a combination of increased actin polymerization triggered by the activation of intracellular signaling events downstream of DCC and DCC mediated adhesion to substrate bound netrin-1.

To quantify the effect of inhibiting Rho signaling on growth cone surface area, SCNs were plated on uniform substrates of either PDL alone or PDL plus netrin-1. On substrates of PDL alone, treatment with C3-07 or Y-27632 induced growth cone expansion by mean values 67% and 79%, respectively (Figure 6.6 O, I-K). Consistent with Shekarabi et al. (2005) culturing SCNs on a substrate of netrin-1 increased growth

cone surface area by a mean of 94%, essentially causing them to double in size. On a netrin-1 substrate, adding Rho or ROCK inhibitors increased growth cone surface area by mean values of 155% and 107%, respectively (Figure 6.6 L-N). These increases in growth cone area were blocked by application of the DCC-fc receptor-body (Figure 6.6O). Together, these findings indicate that inhibition of Rho signaling promotes adhesive interactions between netrin-1 and its receptor DCC within the growth cones of SCNs.

DISCUSSION:

Here we provide evidence that inhibition of RhoA by netrin-1 promotes embryonic SCN axon chemoattraction. Our findings indicate that netrin-1 inhibits RhoA in SCNs through a DCC dependent mechanism, and reciprocally, that Rho signaling inhibits the sensitivity of SCN axons to netrin-1. We demonstrate that inhibition of Rho increases the amount of neuronal plasma membrane DCC and promotes DCC-dependent adhesion to immobilized netrin-1. We hypothesize that netrin-1 inhibition of Rho signaling enhances the chemoattractant response by facilitating DCC function, in part by recruiting additional DCC to the plasma membrane and by promoting DCC signaling mechanisms that lead to membrane extension (Figure 6.6P).

Rho inhibition during axon chemoattraction

Although Rho activation has been detected in response to repellent axon guidance cues (Wahl et al., 2000;Hu et al., 2001), the possible involvement of Rho family members during chemoattraction has been largely ignored. Here we demonstrate that in addition to activating Cdc42 and Rac (Shekarabi and Kennedy, 2002;Shekarabi et al., 2005), netrin-1 also inhibits RhoA. Several previous findings support a role for Rho inhibition in axonal signal transduction during chemoattraction, although these studies did not address this directly. First, transient elevation of intracellular calcium in cerebellar granule cells is both required for chemoattractant responses to BDNF (Li et al., 2005) and has been reported to inhibit RhoA (Jin et al., 2005), suggesting that RhoA inhibition may contribute to BDNF mediated chemoattraction. Secondly, expression of a constitutively active mutant of RhoA is a potent inhibitor of neurite outgrowth (Ruchhoeft et al., 1999),

suggesting that asymmetric inhibition of Rho signaling across a growth cone might evoke directed movement. Lastly, the axons of growing *X. laevis* spinal neurons migrate toward a pipette releasing Y-27632 (Yuan et al., 2003), indicating that Rho inhibition is sufficient to attract axons. As such, our current findings, in combination with these earlier studies, provide evidence that local inhibition of Rho may be a general mechanism that contributes to axonal chemoattractant responses.

Rho regulates DCC plasma membrane presentation

DCC is distributed both on the plasma membrane and sequestered in an intracellular vesicular pool in embryonic rat SCNs (Bouchard et al., 2004). We show that inhibiting Rho increases the amount of plasma membrane DCC. This could reflect described roles for Rho signaling in endocytosis (reviewed in Qualmann and Mellor, 2003) and exocytosis (reviewed in Gasman et al., 2003). Rho signaling is implicated in the transient reorganization of cortical actin, which is postulated to act as a barrier to vesicle traffic to and from the plasma membrane (Aunis and Bader, 1988;Vitale et al., 1995;Gasman et al., 1997;Sullivan et al., 1999;Gasman et al., 2003). Inhibiting Rho with C3-exoenzyme in chromaffin cells led to the dissolution of cortical actin and enhanced exocytosis (Gasman et al., 1997). Additionally, Rho signaling may influence DCC endocytosis through clathrin dependent or clathrin-independent mechanisms. Rho signaling plays a well characterized role in clathrin-independent internalization of the transmembrane interleukin-2 receptor (Lamaze et al., 2001) and clathrin-independent type-II phagocytosis by immune cells (Caron and Hall, 1998;Chimini and Chavrier, 2000). In polarized MDCK cells, expression of dominant active RhoA stimulated, while dominant negative RhoA reduced clathrin-mediated immunoglobulin receptor endocytosis (Leung et al., 1999). We are currently investigating the specific mechanisms underlying DCC trafficking in SCNs.

The result of inhibiting Rho signaling on cell surface DCC differs in several ways from our earlier findings demonstrating a role for PKA regulating plasma membrane presentation of DCC (Bouchard et al., 2004). In agreement with our current findings, Bouchard et al., (2004) found that application of netrin-1 alone produced a modest

increase in plasma membrane DCC. In contrast, activating PKA generated a larger increase in plasma membrane DCC, but this only occurred in the presence of netrin-1. We hypothesized that this was due to PKA activity enhancing the recruitment of DCC to the plasma membrane and netrin-1 stabilizing DCC at the cell surface. According to this model, in the absence of netrin-1, plasma membrane DCC is efficiently internalized. Addition of netrin-1 alone, without PKA activation, is predicted to bind DCC that would otherwise constitutively cycle on and off the cell surface and thereby stabilize DCC at the plasma membrane. Our current findings indicate that inhibition of Rho signaling generates an increase in cell surface DCC independently of added netrin-1. Furthermore, the increase was not significantly different from the increase in cell surface DCC produced by netrin-1 alone. These findings suggest that although PKA can directly inhibit RhoA (Lang et al., 1996;Forget et al., 2002;Ellerbroek et al., 2003;Qiao et al., 2003), the PKA induced recruitment of DCC to the plasma membrane described by Bouchard et al., (2004) must engage additional mechanisms beyond inhibition of Rho signaling.

Adhesion, RhoGTPase signaling, and Netrin-1/DCC interactions

Early studies indicated that axon extension requires adhesion to a substrate (Harrison, 1914) and subsequent studies have identified essential roles for mechanical coupling between the substrate and the growth cones cytoskeleton (Schmidt et al., 1995;Suter and Forscher, 2000). Importantly, however, the adhesivity of a substrate is not a reliable predictor of the guidance choices made by an extending axon (Lemmon et al., 1992;Burden-Gulley et al., 1995;Isbister and O'Connor, 1999). These findings indicate that although adhesion to a substrate is required for motility, mechanisms in addition to adhesion, such as the engagement of specific intracellular signaling pathways, are required for appropriate axon guidance. For example, we have demonstrated that DCC expressing cells adhere to a netrin-1 substrate, however depending on the context, netrin-1 can function as a chemoattractant or conversely a chemorepellent, and DCC can contribute to responses in both directions (reviewed in Huber et al., 2003;Moore and Kennedy, 2006a).

In migrating cells, two broad categories of adhesion sites can be distinguished: 'focal complexes' that support protrusion and traction of the leading edge of a cell and larger 'focal adhesions' which provide longer term anchorage (reviewed in Kaverina et al., 2002;Ridley et al., 2003). Rho GTPases are important coordinators of these adhesive structures; Rac and Cdc42 signal the assembly of focal complexes, whereas RhoA promotes the maturation of focal complexes into focal adhesions. An antagonistic relationship exists between Rac/Cdc42 and RhoA pathways. For example fibroblast migration to fibronectin inhibits RhoA while activating Rac and Cdc42 (Price et al., 1998;Ren et al., 1999;del Pozo et al., 2000). This pattern of activation is consistent with netrin-1 activating Rac and Cdc42 (Shekarabi and Kennedy, 2002;Shekarabi et al., 2005), and our finding that netrin-1 inhibits RhoA. Notably, in migrating cells inhibition of RhoA promotes the initiation of focal complexes by Rac (Rottner et al., 1999;Sander et al., 1999). Thus, in SCNs, inhibition of Rho by netrin-1 may facilitate activation of Rac and Cdc42 and therefore promote chemoattractive turning by enhancing the formation of focal complex-like transient adhesions and the extension of the leading edge of the growth cone.

Rho inhibition promotes chemoattraction to netrin-1

In contrast to our findings that Rho inhibition promotes chemoattraction to netrin-1, a recent study concluded that inhibiting Rho signaling disrupted the guidance of neurites from an explant of embryonic cerebellum toward a source of netrin-1 (Causeret et al., 2004). These findings may be reconciled with ours by considering the essential role of Rho activation in growth cone repulsion. Causeret and colleagues assayed neurite outgrowth from precerebellar explants into a collagen gel, a three-dimensional matrix that does not promote neurite extension by these cells. In the assay used, a local source of netrin-1 overcomes the inhibitory collagen, generating neurite outgrowth biased toward the netrin-1 source. In contrast, inhibiting Rho generated a radial distribution of outgrowth from the explant, consistent with the collagen no longer functioning as a non-permissive matrix for neurite extension. We interpret this finding not as a loss of the

capacity to respond to a chemoattractant, but the loss of the response to collagen as an inhibitor of neurite extension.

Rho inhibition, axon regeneration, and axon guidance

Inhibition of Rho and signaling mechanisms downstream of Rho have been used to promote axon regeneration following spinal cord injury (Dergham et al., 2002;Fournier et al., 2003). In these studies, inhibiting Rho signaling significantly enhanced axon extension in spite of growth inhibitors associated with myelin and the glial scar. While crossing an injury site involves the axon ignoring cues that would normally be effective inhibitors of axon regeneration, for successful regeneration to occur, axons must regain the ability to respond appropriately to cues that will guide them to their targets and promote synapse formation. Initiating this study, we anticipated that inhibiting Rho signaling would most likely disrupt the ability of axons to response to guidance cues, such as a chemoattractant like netrin-1. Contrary to these expectations, we determined that chemoattraction to netrin-1 was not only intact, but enhanced when Rho signaling was inhibited. Importantly, this provide evidence that while inhibiting Rho signaling leads to a loss of sensitivity to certain growth inhibitory cues, axonal growth cones retain the capacity to respond to at least some growth promoting cues. As such, these findings may be of significance for the development of strategies to promote axon regeneration and recovery of function following injury.

CHAPTER 7

Soluble adenylyl cyclase is not required for axon guidance to netrin-1

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PREFACE

A manuscript of this chapter will be submitted as a brief communication to the Journal of Neuroscience. This study was undertaken to address a recent report claiming that cAMP production through soluble adenylyl cyclase (sAC) was essential for netrin-1 mediated axon attraction. In this study we propose that for cAMP production to be included as an essential component of a guidance cue's signal transduction cascade, two criteria must be met: (1) the cue must be capable of altering cAMP production and (2) global elevation of cAMP should wash out the response to the cue. Although, the guidance cue pituitary adenylate cyclase-activating polypeptide (PACAP) meets these criteria, netrin-1 does not.

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ABSTRACT

During development, axons are directed to their targets by extracellular guidance cues. The axonal response to the guidance cue netrin-1 is profoundly influenced by the concentration of cAMP within the growth cone. In some cases, cAMP affects the sensitivity of the growth cone to netrin-1, while in others it changes the response to netrin-1 from attraction to repulsion. The effects of cAMP on netrin-1 action are well accepted, but the critical issue of whether cAMP production is activated by a netrin-1 induced signaling cascade is uncertain. A recent report has suggested that axon guidance in response to netrin-1 requires cAMP production mediated by soluble adenylyl cyclase (sAC). We have used genetic, molecular and biochemical strategies to assess this issue. Surprisingly, we found that sAC is not expressed in neurons and determined that, under conditions where netrin-1 directs axonal pathfinding, exposure to netrin-1 does not alter cAMP levels. Furthermore, although netrin-1 deficient mice exhibit major defects in axonal pathfinding, we show that pathfinding is normal in sAC null mice. Therefore, while cAMP can alter the response of axons to netrin-1, we conclude that sAC is not required for axon attraction to netrin-1, and that netrin-1 does not alter cAMP levels in neurons that are attracted by this cue.

INTRODUCTION

Cyclic AMP (cAMP) is generated by adenylyl cyclase from ATP and is degraded by phosphodiesterases (see Tasken and Aandahl, 2004). Mammals express one soluble and nine transmembrane isoforms of adenylyl cyclase. Transmembrane adenylyl cyclases are typically activated by G-proteins, whereas activity of soluble adenylyl cyclase is increased by bicarbonate ions (Chen et al., 2000b;Cooper, 2003). Testis exhibit the highest sAC expression levels, but low levels of sAC expression have been reported in adult brain, choroid plexus, kidney, and lungs (Sinclair et al., 2000;Chen et al., 2000b;Schmid et al., 2007). Mice rendered null for sAC show no gross abnormalities and the only phenotype reported for these animals is male infertility (Esposito et al., 2004).

During development, axons are directed to their targets along defined pathways by extracellular cues (reviewed in Huber et al., 2003). In the embryonic spinal cord,

commissural axons are guided ventrally by netrin-1 secreted by the floor plate (Kennedy et al., 1994;Serafini et al., 1994;Serafini et al., 1996). Netrin-1 binding to its transmembrane receptor DCC on commissural axons causes N-WASP, Pak1 and FAK to associate with the intracellular domain of DCC and induces activation of phospholipase C and the Rho GTPases Rac and Cdc42 (reviewed in Barallobre et al., 2005). These effectors collaborate to alter the axonal cytoskeleton to mediate turning in response to netrin-1.

Exposure to cAMP signaling can regulate the sensitivity of the axon to netrin-1 or cause it to switch from being an attractive to a repellent cue (Ming et al., 1997;Hopker et al., 1999;Moore and Kennedy, 2006b). Early reports suggested that netrin-1 increases cAMP in dissociated *Xenopus* retinal neurons, but we have shown that cAMP levels are not altered by netrin-1 in rat spinal commissural neurons (Hopker et al., 1999;Bouchard et al., 2004;Moore and Kennedy, 2006b). We were therefore intrigued by a recent report indicating that sAC-dependent cAMP production is activated by netrin-1 and is essential for attractant responses by embryonic dorsal root ganglion (DRG) neurons and in spinal commissural neurons (Wu et al., 2006). In this report, we demonstrate that embryonic DRG and spinal commissural neurons do not express sAC and do not show changes in intracellular cAMP levels when exposed to netrin-1. Furthermore, we report that sAC knockout mice exhibit no defect in their spinal ventral commissure – a hallmark of netrin-1 deficiency. We conclude that sAC and cAMP production are not required for axonal chemoattraction to netrin-1.

MATERIALS AND METHODS

Reagents

Forskolin, 5-fluorodeoxyuridine (FdU), 3-isobutyl-1-methylxanthine (IBMX), DNase, Hoechst 33258 and poly-D-lysine (PDL, 70-150 kD) were obtained from Sigma-Aldrich, (Missisauga, Canada). Neurobasal, fetal bovine serum (FBS), B-27 supplement, GlutaMAX-1, Penicillin-Streptomycin, Ca^{2+}/Mg^{2+}-free HBSS and goat anti-rabbit Alexa® 488 were purchased from Invitrogen Canada (Burlington, ON). Rabbit anti-NFM antibody was purchased from Chemicon (Temecula, CA). Tuj1 antibody was purchased

from Covance Research Products (Emeryville, CA). PACAP-38 was purchased from EMD Biosciences (San Diego, CA). 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). 2.5S nerve growth factor (NGF) was obtained from Cedarlane Labs Canada (Burlington, ON).

Cell Culture

Staged pregnant Sprague-Dawley rats were obtained from Charles River Canada (St-Constant, QC). Spinal commissural neuron cultures were prepared from embryonic day 14 (E14, vaginal plug = E1) Sprague-Dawley rats, as previously described (Bouchard et al., 2004). Cells were plated and cultured in 6-well culture Falcon® plates (Becton Dickinson, Franklin Lakes, NJ) previously coated with 2 μg/cm^2 PDL for 2 hrs at room temperature.

DRGs were isolated from E15 Sprague-Dawley rat embryos, as described (Hall, 2006). For explant experiments, five DRGs centered on each forelimb bud were selected and embedded in bovine dermal collagen (Inamed, Santa Barbara, CA). For dissociated cultures, DRGs between the two limb buds were used. Dissociated DRGs were plated on and cultured in 6-well culture Falcon® plates (Becton Dickinson, Franklin Lakes, NJ) previously coated at RT with 2 μg/cm^2 PDL for 2 hrs and then 1 μg/cm^2 fibronectin for another 2 hrs. Both explant and dissociated DRG cultures were cultured in Neurobasal supplemented with 2% B-27, 2 mM GlutaMAX-1, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.08-75 ng/ml NGF and 20 μM FdU. Explant cultures were fixed after 14 hours and labeled with Tug1 antibody and Hoechst 33258; outgrowth was quantified with Image J (NIH) as the difference in area between Tug1 and Hoechst 33258 labeling. For dissociated DRG cultures, the media was replaced after the first 24 hrs.

RT-PCR analysis

Total RNA was extracted from flash frozen adult rat testis (gift of Drs. Craig Mandato and Louis Hermo, McGill University), adult mouse brain, testis and lung, as well as, 2 DIV E14 rat spinal commissural neurons and 3 DIV E15 rat dorsal root

ganglion neuron cultures using TRIzol® (Invitrogen Life Technologies, Burlington, Ontario). For amplification of individual exons or ROCKI, genomic DNA was removed from RNA samples with RNase-free DNase I (Illustra RNAspin Mini RNA Isolation Kit, GE Healthcare, Buckinghamshire, UK). RNA samples were then heated to 95°C for 2-3 min to denature the DNase I. RT-PCR was performed with 0.5 μg of total RNA per reaction using the QIAGEN® OneStep RT-PCR Kit (Qiagen, Mississauga, Ontario). All primers, except for DCC, were annealed at 60°C. The primer pair for amplification of rat DCC was annealed at 55°C. Primer sequences are listed in Table 7.1. Equal volumes of RT-PCR products were separated on a 1% or 3% agarose gel.

	Forward	**Reverse**
Rat N-term	CGAGCAGCTGGTGGAGATCC	GCGTGAGTGATCTCGTCAGGGGC
Rat C-term	CCTGCTTCTCCCTGCTGTG	GCAGGAGTAAAGTCCCAGG
Rat C1	AGCAGCTGGTGGAGATCCT	TTCAATCATGCTCCGATCAC
Rat C2	TCATAGGATCAGCCATCCAAG	AAAAGTAGGCTGGCAGGTTG
Mouse C1	AACAGCTCGTGGAGATCCT	TTCAATCATGCTCCGATCAC
Mouse C2	TCATAGGCTCAGCCATCCAAG	AAAAGTAGGCTGGTAGG
Rat Exon #4	AGAAGTTCAGCACAGCCATGT	TCGCACTTATGTAGTAGTTGAGGA
Rat Exon #6	GTGGAAAGTGGAACGAAAGC	CTCCTTGGCTTCAAACAAGC
Rat Exon #7	CTTGCTCAGAACATGGCTCA	ATCCGGAATCCTCTCGATTT
Rat Exon #10	TGTGACGATCGTGTTTGTGA	TCAAGACGGAAGTGATGTGC
Rat Exon #11	CCTCTGTGTCTTCGGTTTCC	GGACCTGAGAGCAGAAGTCG
Rat Exon #12	GCCAGTGGGATTGTCTTCTG	CACAGTGTGTCCAACGATCC
Rat Exon #13	CAACCTGCCAGCCTACTTTT	TTTCTCATTGAGGCCCAGAC
Rat DCC*	CCGGAATTCCCACCTATGAGTGCA	GTCCGCTCGAGCAATGCATGTCAAAAGG
Rat ROCKI	GTAATCGGCAGAGGTGCATT	TCCAGACTTATCCAGCAGCA

Table 7.1 - *RT-PCR primers.* **The rat DCC primers contain 5' restriction sites*

cAMP detection

2 DIV commissural neuron and 3 DIV DRG cultures were stimulated with either 10 μM forskolin, 0.1 μM PACAP or various concentrations of netrin-1 and NGF. Stimulations were done for 5 min in the absence of IBMX or for 15 min in the presence of 0.5 μM IBMX. cAMP levels were measured using either Parameter cAMP ELISA (R&D Systems, Minneapolis, MN) or Cyclic AMP EIA (Assay design, Ann Arbor, MI) kits. Absorbance in each well was measured on a Model 680 microplate reader (Bio-Rad,

Hercules, CA). Concentrations of cAMP were normalized across experiments relative to the average value in culture media alone.

Immunohistochemistry

Embryos were fixed in 4% PFA in PBS overnight, 30μm cryostat sections cut, and axons visualized with antibodies against NFM and Alexa® 488 secondary antibodies.

RESULTS

Intact embryonic spinal ventral commissure in sAC knockout mice

sAC knockout mice contain an IRES-LacZ/*neomycin* cassette that replaces exons 2 to 4, deleting a portion of the C1 region (Figure 7.1A, Esposito et al., 2004). The C1 region combines with the C2 region to form the cyclase catalytic domain (Figure 7.1A, see Kamenetsky et al., 2006). In these animals, RNA transcription proceeds through the inserted IRES-LacZ/neomycin cassette and the sequence encoding C2, however, due to a frameshift introduced by the transgene, these portions of sAC are not translated and neither sAC protein nor its activity are detectable in testis and spermatozoa of knockout animals (Sinclair et al., 2000;Esposito et al., 2004;Hess et al., 2005). Consistent with this, using RT-PCR, we detected mRNA transcripts encoding the C2, but not the C1 region in testis of adult sAC null mice (Figure 7.1B).

The absence of either netrin-1 or its receptor DCC in mice results in disruption of major axon tracts and early postnatal lethality (Serafini et al., 1996;Fazeli et al., 1997). sAC null mice do not exhibit any obvious neurological deficits and are viable to adulthood, but they have not, to our knowledge, been closely examined for axonal targeting defects (Esposito et al., 2004). To explore the possibility that subtle netrin-dependent axon defects might be present, we examined the spinal ventral commissure in sAC null mice. Figure 7.1C- 7.1F shows the spinal ventral commissure in sAC null mice is normal and appears indistinguishable from wild type mice. We conclude that sAC is not required for netrin-1 mediated guidance of spinal commissural axons.

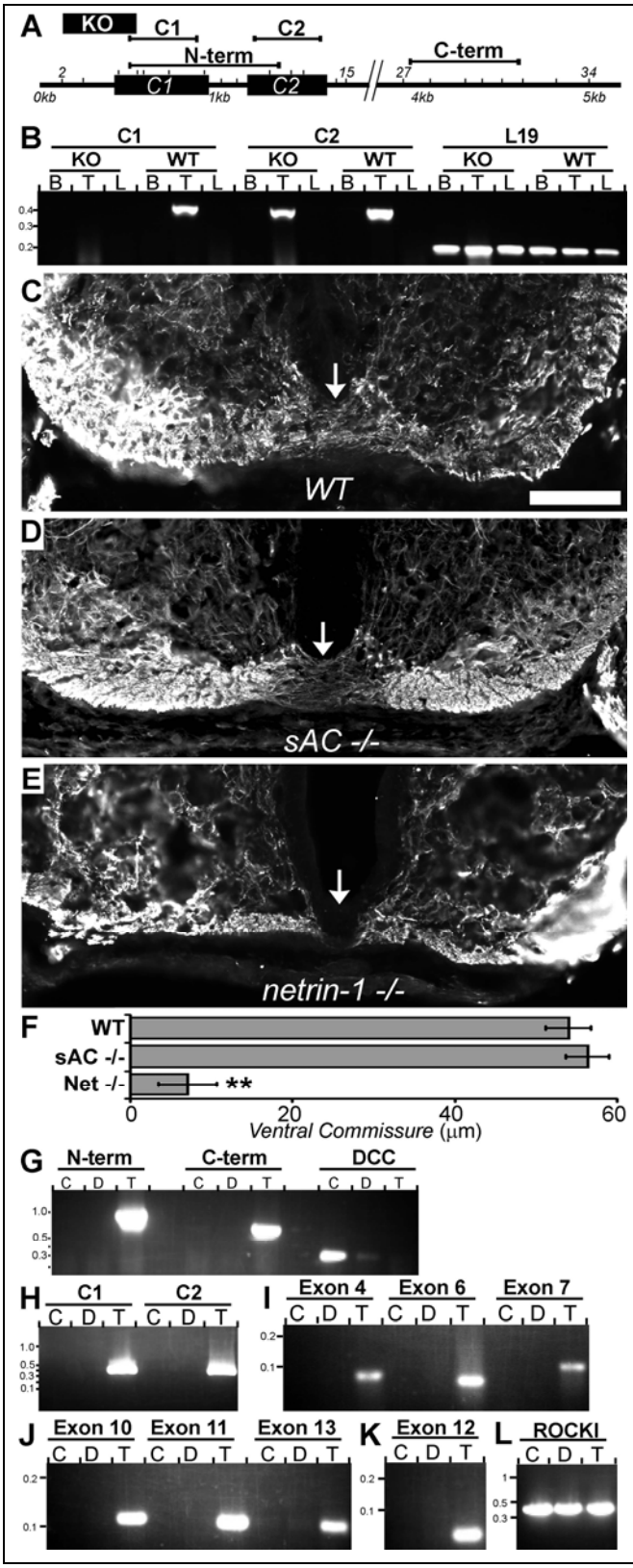

Figure 7.1 - Normal spinal ventral commissure in sAC-Deficient mice and no sAC expression in embryonic DRG or spinal commissural neurons.. *(A) Schematic of rat sAC mRNA indicating exon boundaries, the location of the sAC knockout mouse insertion, as well as, the N-term, C-term, C1 and C2 primers. (B) RT-PCR analysis of adult brain [B], testis [T] and lung [L] in wildtype [WT] and sAC knockout mice using primer sets 'C1' and 'C2', and the L19 gene as control. (C-E) Ventral spinal cords of WT (B), sAC KO (C), netrin-1 KO (D) immunolabeled for NFM; arrows indicate ventral commissure, scale bar = 100 μm. (F) Quantification of the height of the ventral commissures of wildtype and knockout mice (** $p < 0.01$). Error bars = SEM. (G,H) RT-PCR analysis of rat testis [T], as well as, embryonic spinal commissural [C] and DRG [D] neurons using the N-term, C-term, C1 and C2 primer sets (see panel A). (I-K) RT-PCR analysis of individual exons longer than 40 base pairs within the C1 and C2 domains of sAC. (L) The integrity of these DNase-treated RNA preparations in panels (I-K) was confirmed by the amplification of the ROCKI kinase.*

No expression of soluble adenylyl cyclase in the developing nervous system

The expression of sAC during development is controversial. Wu *et al.* reported immunofluorescence in embryonic DRG and spinal commissural neurons using antibodies raised against the 50kDa splice variant of human sAC (Zippin et al., 2003;Wu et al., 2006); however the immunoreactive species detected in these neurons has not been positively identified. Examination of the NCBI Unigene EST database for transcriptionally active genes reveals that netrin-1, its receptor DCC and all nine transmembrane adenylyl cyclases are expressed in the embryos of mice, rats, and humans during the period when axons are extending in response to netrin-1. Interestingly, ESTs encoding sAC have not been detected in embryos of these species during this period (Table 7.2).

Organism	**Gene**	**Unigene Cluster**	**Adult Brain (tpm)**	**Adult Testis (tpm)**	**Embryo* (tpm)**
Mouse	sAC	Mm.66952	6	131	0
	Netrin-1	Mm.39095	6	0	206
	DCC	Mm.167882	36	8	57
	AC1	Mm.259733	66	24	144
	AC2	Mm.390617	73	8	80
	AC3	Mm.71996	30	8	45
	AC4	Mm.287010	26	0	34
	AC5	Mm.41137	123	0	91
	AC6	Mm.157091	3	16	63
	AC7	Mm.288206	0	24	182
	AC8	Mm.1425	60	0	34
	AC9	Mm.439750	30	0	28
Rat	sAC	Rn.42892	0	-	0
	Netrin-1	Rn.41052	19	-	112
Human	sAC	Hs.320892	0	56	0
	Netrin-1	Hs.660885	5	0	58
	DCC	Hs.694733	2	76	19

Table 7.2 - *Summary of NCBI Unigene database information on August 1, 2007.* *For mice embryos, transcripts from post-implantation to late gestation embryos were summed. Tpm = Transcripts per million.*

To provide a robust account of sAC expression during neural development, we performed RT-PCR analysis on isolated DRG and spinal commissural neurons at ages when they use netrin-1 as a guidance cue. Using primer sets previously used to document

low levels of sAC expression in the adult brain (primer sets 'N-term' and 'C-term', Sinclair et al., 2000), sAC transcripts were detected in testis, but not in embryonic DRG or spinal commissural neurons (Figure 7.1G). To address the possibility that an alternatively spliced sAC may be present in these neurons, we amplified the sequences encoding the C1 and C2 domains of sAC but again, sAC expression was not detected in DRG or spinal commissural neurons (Figure 7.1H). As a final test, we amplified mRNA sequence from each exon greater than 40 bp in length within the C1 (exons 4, 6 and 7) and C2 (exons 10-13) regions. mRNA encoding these exon sequences were readily amplified from testis, but were not detected in embryonic DRG or spinal commissural neurons (Figure 7.1I-K). We conclude that catalytically active sAC is not expressed by DRG or spinal commissural neurons during the developmental time period when their axons utilize netrin-1 as an attractive guidance cue.

Netrin-1 does not elevate cAMP levels in embryonic DRG neurons

Wu *et al.* report that 300ng/ml netrin-1 applied for 15 min induces a 40% ($n=3$, $p<0.01$) increase in cAMP production in embryonic DRGs that have been cultured in 100 ng/ml NGF for three days (Wu et al., 2006). Importantly, this is a concentration of NGF that is approximately one hundred times that required for DRG survival and axon outgrowth (Levi-Montalcini and Angeletti, 1968;Levi-Montalcini, 1982). In attempting to replicate these findings, we observed relatively little axon outgrowth at 100 ng/ml NGF (Figure 7.2A). NGF has been reported to induce cAMP production in variety of cell types (Schubert and Whitlock, 1977;Knipper et al., 1993;Cai et al., 1999;Stessin et al., 2006). There are, however, contradictory reports as to whether it is capable of elevating cAMP in embryonic DRGs (Frazier et al., 1973;Bradshaw et al., 1974;Narumi and Fujita, 1978).

Given the excessive amount of NGF used by Wu *et al.* and the possibility that NGF itself might influence the concentration of cAMP in these cultures, we were compelled to revisit their findings. In eight independent experiments using identical culture conditions (3 DIV, PDL and fibronectin substrate, 100 ng/ml NGF) and a stimulation paradigm identical to that of Wu *et al.* (pre-treat for 5 min with 0.5 mM IBMX, stimulate for 15 min), netrin-1 consistently ($n>14$) did not alter the concentration

of cAMP (Figure 7.2B). We then examined the possibility that netrin-1 might influence cAMP production in cultured DRGs maintained in concentrations of NGF at 0.08 or 2.5 ng/ml. Again, no change in cAMP concentration was evoked following application of netrin-1 (Figure 7.2C). Given our inability to detect changes in cAMP production, we sought a suitable positive control. To this end, we tested NGF itself and pituitary adenylate cyclase-activating polypeptide (PACAP). No change in cAMP levels were detected when the concentration of NGF was doubled or an additional 100 ng/ml NGF added. In contrast, PACAP induced a robust increase in cAMP concentration: a >200% increase on its own or in the presence of the phosphodiesterase inhibitor IBMX, which inhibits the breakdown of cAMP (Figure 7.22C). We conclude that netrin-1 does not induce cAMP production in embryonic DRG neurons.

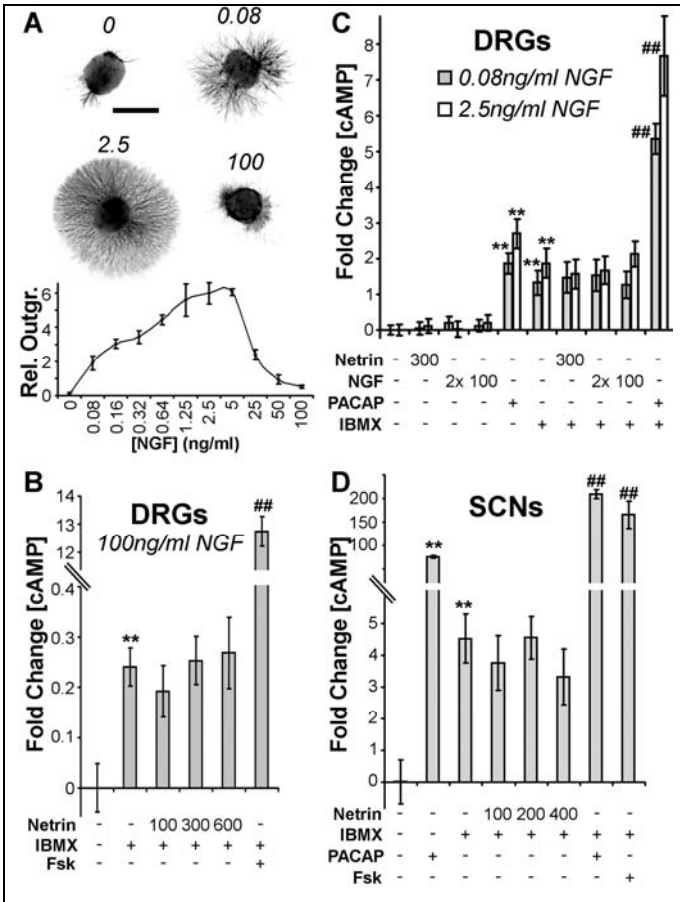

Figure 7.2 - Netrin-1 does not induce cAMP production in DRG neurons or spinal commissural neurons. *(A) NGF-induced outgrowth from E15 DRG explants peaks between 1.25 and 5ng/ml. (B) cAMP ELISAs on E15 DRG neurons cultured for 3DIV in 100 ng/ml NGF. (C) cAMP ELISAs on E15 DRG neurons cultured for 3DIV in 0.08 ng/ml or 2.5ng/ml NGF ('2x NGF' refers to a doubling of the concentration of NGF). (D) cAMP ELISAs on 2DIV E14 spinal commissural neurons (SCNs). Netrin-1 concentrations are in ng/ml. The axis in panels C and D are discontinuous. Scale bar = 0.5 mm. **p<0.01 relative to media alone, ##p<0.01 relative media with only IBMX. Error bars =*

SEM.

Netrin-1 does not induce cAMP production in embryonic spinal commissural neurons

The axonal response of DRG neurons to netrin-1 is unclear. Wu *et al.* suggest an attractive response based on morphological changes in DRG growth cones in culture; however, *in vivo* and *in vitro* evidence argue that netrin-1 functions as a repellent for these axons (Watanabe et al., 2006). In contrast, unambiguous evidence indicates that netrin-1 is a chemoattractant for embryonic spinal commissural axons (Kennedy et al., 1994;Serafini et al., 1996). Using cAMP immunocytochemistry, phospho-CREB immunoblotting, and ELISA techniques we have previously provided evidence that netrin-1 does not elevate cAMP in spinal commissural neurons (Bouchard et al., 2004;Moore and Kennedy, 2006b). These studies, however, did not apply the phosphodiesterase inhibitor, IBMX, which could enhance the detection of small cAMP transients. Here, using a stimulation strategy identical to that applied by Wu *et al.* to DRG neurons (pretreat for 5 min with 0.5 mM IBMX, stimulate for 15 min with netrin-1), netrin-1 did not alter the concentration of cAMP across a broad range of concentrations (Figure 7.2D). Application of PACAP as a positive control induced robust cAMP production in spinal commissural neurons; a 762% (±29%, n=6) increase applied on its own and a massive 2100% (±92%, n=6) increase in the presence of IBMX (Figure 7.2D).

Discussion

The importance of sAC in male fertility is supported by its robust expression in testis and by the infertility of male sAC knockout mice (Esposito et al., 2004); however a role for sAC outside of the testis has yet to be firmly established. In the adult nervous system, sAC cannot be detected by northern blot or *in situ* techniques but low level expression in the adult brain has been reported by RT-PCR (Sinclair et al., 2000;Geng et al., 2005;Schmid et al., 2007). Contradictory expression patterns are reported in expressed sequence tag (EST) databases, that provide direct evidence of transcriptionally active genes (see Nagaraj et al., 2007). For instance, while NCBI Unigene indicates sAC is predominantly expressed in the testis of adult mice and humans, sAC expression was also detected in the brains of adult mice, but not rats or humans (Table 7.2). Several reasons

may contribute to these discrepancies. sAC is thought to function as a bicarbonate sensor in the choroid plexus in the mature CNS where expression has been detected by western blot (Chen et al., 2000b). sAC expression has also been reported in leukocytes (Geng et al., 2005) and neutrophils (Han et al., 2005), therefore contamination by blood may contribute to the low levels of sAC expression reported in the mature CNS. Additionally, in humans, but not mice or rats, a fully spliced sAC pseudogene is present on chromosome 6, as opposed to sAC present on chromosome 1. Amplification of the pseudogene from contaminating genomic DNA may confound studies assaying mRNA expression in human tissues using RT-PCR. The source of positive signals suggesting low levels of sAC expression in the adult CNS requires further characterization. Critical to this discussion, in the developing nervous system, EST databanks provide no evidence of sAC expression in the embryo during axon extension, consistent with the findings we describe here.

Axons will turn toward a source of a membrane permeable cAMP analogue (Gundersen and Barrett, 1980;Lohof et al., 1992). If cAMP production is a required component of the signal transduction cascade activated by a guidance cue, the two following criteria must be met: (1) the guidance cue must be capable of inducing cAMP production and (2) global elevation of cAMP must disrupt turning. For instance, a gradient of PACAP rapidly induces robust cAMP production in a receptive axonal growth cone, and axonal attraction to a gradient of PACAP is disrupted when cAMP is elevated globally (Figure 2B&D, Guirland et al., 2003;Hashimoto et al., 2006). Neither criterion is met by netrin-1. Rather, we have shown that in cells that respond to netrin-1 as an chemoattractant, netrin-1 does not elevate cAMP levels (Figure 2D, Bouchard et al., 2004;Moore and Kennedy, 2006b). Moreover, axon turning to netrin-1 is not disrupted by global elevation of cAMP, but is in fact enhanced (Moore and Kennedy, 2006b).

In summary, the absence of axon guidance defects in sAC knockout mice, as well as the absence of sAC expression within DRG neurons and spinal commissural neurons, indicates that sAC is not required for axon guidance to netrin-1. While cAMP production is required downstream of some chemoattractant guidance cues, such as PACAP, we conclude that cAMP production is not required during axon attraction to netrin-1.

CHAPTER 8

General Discussion

PREFACE

This final chapter begins with a discussion of how models that consider netrin-1 to function as a freely soluble cue overlook evidence indicating its interaction with extracellular matrix (ECM) components, its distribution *in vivo* close to its site of production, and evidence from a variety of systems that it can function as an adhesive ligand. Possible mechanisms by which netrin-1 regulates Rho GTPases will then be briefly outlined, followed by a discussion on why spinal commissural neurons are not repelled when cAMP signaling is inhibited. The thesis concludes by exploring several issues regarding the role of cAMP in axon guidance.

NETRIN AS AN ADHESIVE CUE

Netrin-1 is a secreted glycoprotein that promotes axon outgrowth and turns embryonic spinal commissural neuron axons 250 μm away from its source (Figure 2.1D&E; Tessier-Lavigne et al., 1988;Serafini et al., 1994). Thus netrin-1, secreted by the floor plate, is capable of diffusing through collagen and the developing neuroepithelium. In this section I will outline accumulating evidence that, although a secreted cue, netrin-1 becomes attached to ECM components and may function as an adhesive cue.

Netrin is not freely soluble

The first evidence indicating that netrin-1 may not be freely soluble originates from its initial purification where high salt washes were required to extract it from membranes (Serafini et al., 1994). Later, fractionation experiments performed by Colleen Manitt, a former PhD student in our lab, confirmed that the vast majority of netrin-1 in the embryonic and adult CNS is not freely soluble, but attached to membranes (Manitt et al., 2001;Manitt and Kennedy, 2002). As discussed in literature review II, this appears to

be a largely electrostatic interaction between the positively charged C-domain of netrin-1 (see Figure 2.3) and negatively charged sugar groups, such as heparin, found on ECM proteins (Serafini et al., 1994;Kappler et al., 2000). The association of netrin-1 with heparin is strong; it requires approximately 1.4 M NaCl to disrupt (Serafini et al., 1994). By comparison, other heparin binding proteins, such as sonic hedgehog and Wnt, are broken with less than 0.8 M NaCl (Bradley and Brown, 1990;Rubin et al., 2002). Moreover, recent evidence indicates that this netrin-heparin interaction is functionally important; expression of the heparin sulfate synthesis enzyme, Ext1, by spinal commissural neurons has been shown to be required for netrin-1 induced outgrowth and signaling events (Matsumoto et al., 2007).

Netrin-1 binding to heparin opens up the possibility for interactions with a wide variety of glycosaminoglycan-containing proteoglycans and this is reflected in the distribution of netrin-1 protein *in vivo*. In mice, fruit flies and *C. elegans*, netrin protein remains in close proximity to its cellular sources of expression at the midline (Figure 8.1). Netrin-1 is also closely associated with glial cells at the optic disk where it allows retinal ganglion cells to exit the eye and enter the optic nerve (Figure 2.1F, Deiner et al., 1997). Similarly, the expression of netrin by discrete muscle targets in *Drosophila* is required for proper target selection by motoneuron axons (Mitchell et al., 1996;Winberg et al., 1998). Netrin's ability to bind ECM components is also seen outside the nervous system; restricted distributions have been reported in the proximal tubules of the developing lung (Figure 8.2A) and in the basement membranes of blood vessels, kidneys, and ovaries (Koch et al., 2000;Liu et al., 2004b).

Therefore, in a variety of contexts netrin's restricted distribution suggests that it efficiently binds ECM components *in vivo*. These observations raise the question of whether substrate-bound netrin remains functional. This issue was addressed in an elegant study by Barry Dickson's lab that replaced secreted netrin with an engineered membrane-tethered form in fruit flies (Brankatschk and Dickson, 2006). While this membrane-tethered netrin could not rescue netrin's ability to repel axons at a distance from the midline, it was sufficient to rescue the midline crossing of commissural neuron axons.

These findings indicate that while netrin's ability to diffuse may be required for it to reach its intended target, it is not required for its ability to induce axonal responses.

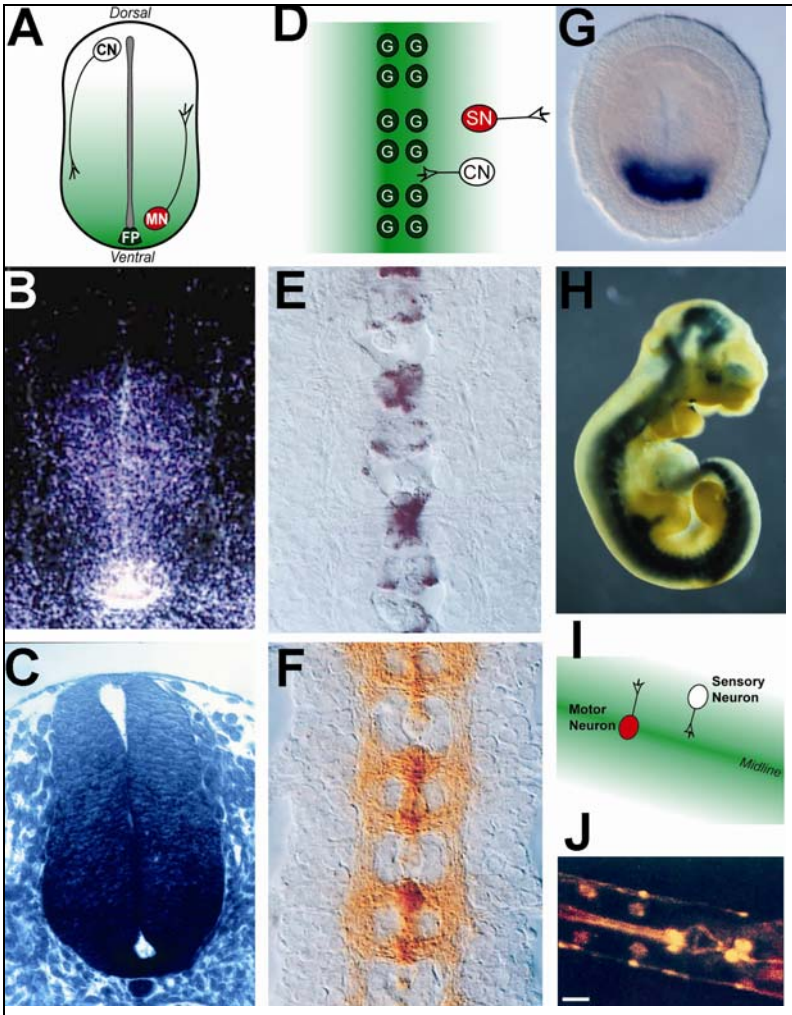

Figure 8.1 – Midline expression of netrin homologues in a variety of organisms: *The diagrams in panels A, D and I illustrate simplified models of the distributions of netrin protein at the midline in the developing mouse spinal cord, fruit fly and C. elegans nematode worm, respectively. In each case, sensory neurons extend towards, while motoneurons extend away from, the source of netrin protein at the midline. In situ hybridization illustrates floor plate cells expressing netrin-1 in the embryonic day 9.5 mouse spinal cord (B). Whole mount staining for expression of a β-galactosidase reporter gene shows netrin-1 expression in an entire E12.5 day old mouse embryo, illustrating netrin-1 expression along the full rostro-caudal extent of the spinal cord, the developing brain, and the peripheral nervous system (H). In situ hybridization reveals netrin expression in the fruit fly (E) and N. Vectensis (G) during neural development. The distribution of netrin protein in the embryonic day 9.5 mouse spinal cord (C), as well as, the fruit fly (F) and C. elegans (J) embryos are also shown. CN= commissural neuron, MN = motoneuron, SN = segmental nerve. Panel B is reprinted with permission from Serafini,T. et al. Cell 87, 1001-1014 (1996). Panels E and F are reprinted with permission from Harris,R. et al. Neuron 17, 217-228 (1996). Panel G is reprinted with permission from Matus et al. PNAS 103, 11195-11200 (2006). Panel J is*

reprinted with permission from Wadsworth,W.G. et al. Neuron 16, 35-46 (1996).

Netrin functions as an adhesive ligand

The above studies provide evidence that netrin is not freely soluble *in vivo*; they however do not directly assess netrin's ability to act as an adhesive ligand. In chapter 5 and 6 we report that spinal commissural neurons adhere to, and that their growth cones expand on, a substrate of netrin-1 (Figures 5.3 and 6.6). These findings indicate that netrin-1 may act as an adhesive ligand for spinal commissural neurons. In this section I will outline how our findings are corroborated by studies examining netrin-1 in angiogenesis, as well as, in the developing mammary gland and pancreas.

During angiogenesis, netrin-1 may act as an adhesive ligand for vascular smooth muscle cells. Using an adhesion assay very similar to the one used in our studies, it was shown that vascular smooth muscle cells, but not endothelial cells, bind a substrate of netrin-1 (Park et al., 2004). Similarly, in the developing mammary gland, terminal end buds – the growing tips of the developing ductal network of the gland – netrin-1 is expressed by a layer of luminal epithelial cells, and the netrin receptor neogenin by an adjacent layer of cap cells (Figure 8.2B, Srinivasan et al., 2003). Knockouts of either netrin-1 or neogenin produce a similar phenotype: enlarged terminal end buds containing disorganized cap cells. These phenotypes, as well as results from cell aggregation assays point towards an adhesive role for netrin-1.

A final example of an adhesive role for netrin-1 comes from the developing pancreas. Here, netrin-1 is expressed by a discrete population of cells within the ductal epithelium and localizes to basal membranes and extracellular matrix (Yebra et al., 2003). Again, using an adhesion assay similar to one used in Chapters 5 and 6, fetal pancreatic cells were shown to bind a substrate of netrin-1. This study also provided evidence that, in addition to DCC and UNC5, integrins could function as receptors for netrin-1. While this may not be surprising given the homology of the N-terminus of netrin to the integrin-ligand, laminins (see Figure 2.3), the interaction reported did not occur through this homologous region but through positively charged sequences in netrin's C-domain.

Although, further studies are needed to determine whether integrins function as netrin receptors in *vivo* and whether they contribute to netrin-mediated axon guidance, this report raises the exciting possibility of an interaction with a receptor family extensively characterized for its ability to promote adhesion of wide range of cell types (see Hynes, 1992).

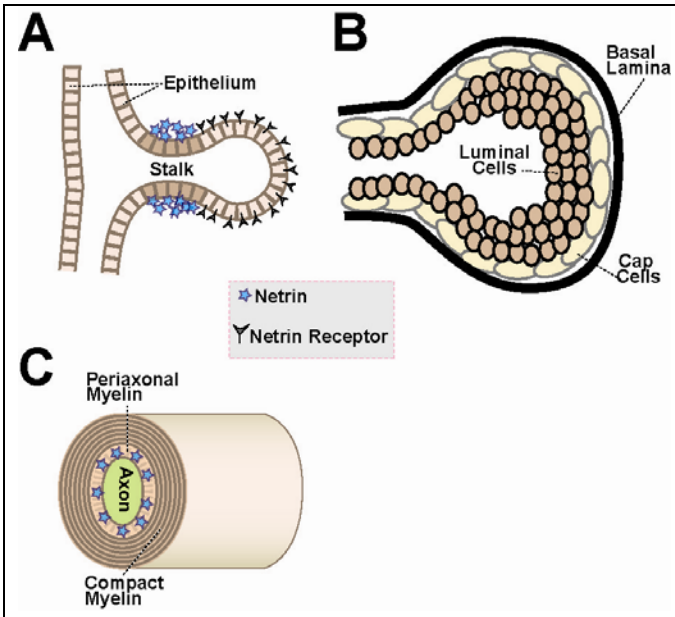

Figure 8.2 – Netrin functions outside the developing nervous system: *(A) Netrin released by epiendothelial stalk cells inhibits proximal branch formation during lung morphogenesis. (B) In the developing mammary gland, netrin released by luminal cells is essential for appropriate adhesive interactions between luminal and cap cells. (C) In the mature mammalian CNS, netrin-1 is localized to periaxonal myelin suggesting a role in regulating interactions between axonal and oligodendroglial membranes.*

Why an adhesive interaction?

Substrate attachment is not unique to netrin; physical restraint is seen with most – if not all – axon guidance cues. While this is evident for transmembrane (e.g. Ephrin-Bs and Semaphorins-1, -4, -5 & -6) and GPI-linked cues (e.g. Ephrin-As and Semaphorin-7), secreted molecules/guidance cues such as Slits and Sema-3A are, like netrin, electrostatically bound to cell surface proteins (see Figure 1.3, Koppel et al., 1997;Liang et al., 1999;Hu, 2001;Ronca et al., 2001;De et al., 2005). Physical restraint may only be necessary to preserve their graded distribution (Figure 8.1C, Braisted et al., 1997;Stubbs et al., 2000;Kennedy et al., 2006). However, it is possible that this physical restraint may also be important for initiating and/or supporting the morphological changes of a turning

axon. For instance, an extending axon generates locomotive force by mechanically coupling its cytoskeleton to the extracellular matrix (reviewed in Suter and Forscher, 2000). Studies to date have examined mechanical interactions with classical extracellular matrix components, such as laminin and fibronectin, while overlooking the possibility that axon guidance cues may themselves provide mechanical traction. In chapters 5 and 6 it was shown that: (1) DCC on the surface of spinal commissural neurons binds directly to a substrate of netrin-1 and (2) DCC has the potential of acting as the transmembrane bridge through the association of numerous intracellular proteins to the cytoskeleton, as proposed in the substrate to cytoskeleton model of axon guidance. As such, an exciting possibility is that guidance cues provide mechanical traction for the morphological changes associated with axon guidance.

RHO GTPASES IN THE GUIDANCE OF AXONS TO NETRIN-1

Another compelling argument for classifying netrin as an adhesive ligand, are the common signal transduction events seen during axon attraction to netrin-1 and in the adhesive remodeling of migrating cells. In migrating cells, two broad categories of adhesion sites can be distinguished: focal complexes support protrusion and traction of the leading edge of a cell and larger focal adhesions provide long term anchorage (reviewed in Kaverina et al., 2002;Ridley et al., 2003). Studies by others have shown that focal adhesion kinase (FAK) and Src family tyrosine kinases associate with the intracellular domain of DCC, and that netrin-mediated axon attraction is disrupted in the presence of Src kinase inhibitors or dominant negative FAK constructs (Ren et al., 2004;Li et al., 2004;Liu et al., 2004a). In migrating cells, FAK is required for adhesive complex maturation (Sieg et al., 1999), while focal adhesion turnover requires both FAK and Src (Mitra et al., 2005).

In chapter 5 we report that the Rho-family GTPases Rac and Cdc42 are activated upon netrin-1 stimulation and that they associate with the intracellular domain of DCC. In chapter 6, we report that Rho is inactivated by netrin. Rho GTPases are important coordinators of adhesive structures; Rac and Cdc42 signal the assembly of focal complexes, whereas the Rho subfamily promotes the maturation of focal complexes into

focal adhesions (see Ridley, 2001). In this section we will explore possible mechanisms by which netrin-1 regulates Rho GTPase activation.

Netrin-1 activates Rac and Cdc42, but inhibits Rho

The cycling of Rho GTPases between an inactive GDP-bound and an active GTP-bound state is mainly under the control of regulators classified into three categories: (1) guanine nucleotide exchange factors (GEFs) activate Rho GTPases by promoting their exchange of GDP for GTP; (2) GTPase activating proteins (GAPs), inhibit their activity by promoting their hydrolysis of GTP to GDP; and (3) guanine nucleotide dissociation inhibitors (GDIs) sequester Rho GTPases and prevent the dissociation of GDP (Figure 1.4B, Hall, 1998).

Interestingly, the same pattern of Rho GTPase activation we report in response to netrin-1 chemoattraction – activation of Rac and Cdc42, inactivation Rho – has been reported for fibroblast migration on fibronectin (Price et al., 1998;Ren et al., 1999;del Pozo et al., 2000). Moreover, inactivation of Rho promotes focal complex formation by Rac in migrating cells (Rottner et al., 1999;Sander et al., 1999). Thus there appears to be an inverse relationship between the activation of Rac/Cdc42 and Rho. Masoud Shekarabi, a former PhD student in our lab, found that netrin-1 induces a rapid activation of Rac and Cdc42 within 5 minutes (Figure 5.4F, Shekarabi and Kennedy, 2002); in contrast, I only saw Rho inhibition within 15 minutes (Figure 6.2). Therefore, Rac and Cdc42 activation may precede Rho inhibition. Furthermore, dominant-negative Cdc42 blocks netrin-induced Rac activation, but dominant-negative Rac doesn't affect activation of Cdc42 (Figure 5.4E). An important question, not addressed in this thesis, is whether activation of Rac and/or Cdc42 is required for netrin-induced Rho inactivation. Nevertheless, current evidence predicts that the activation of Rho GTPases in response to netrin-1 begins with Cdc42, followed by Rac and then inhibition of Rho.

Unfortunately, little is known of the GEFs/GAPs/GDIs implicated in netrin-mediated responses. There are approximately 70 GEFs, 80 GAPs and four GDIs in mammals – but only one GEF, Trio, has been implicated in netrin-mediated axon guidance. Specifically, studies have shown a genetic interaction in fruit flies and *C.*

elegans (Merz and Culotti, 2000;Forsthoefel et al., 2005;Watari-Goshima et al., 2007). In vertebrates, Trio's has two independently regulated GEF domains, one that acts on Rac and the other on Rho (see Bateman and Van, 2001;Karnoub et al., 2004). While this could suggest that netrin activates both Rac and Rho, our finding that netrin-1 inactivates Rho, as well as, reports that only the Rac-specific GEF domain of Trio is functional in *Drosophila* (Newsome et al., 2000), indicates that Trio only activates Rac in the context of netrin-mediated axon guidance.

In addition to regulation by GEFs/GAPs/GDIs, RhoA activity can be affected by phosphorylation on its Ser188 site by PKA and PKG (Lang et al., 1996;Sawada et al., 2001). This phosphorylation does not affect its ability to bind or hydrolyse GTP; rather it removes it from the membrane by associating it with a GDI. This phosphorylation site is not present on RhoB or RhoC (Ridley, 2001). The possibility that RhoA may be phosphorylated by PKA is of particular interest because, as presented in chapter 3, 4 and 6, the consequences of PKA activation and Rho inhibition are very similar – both promote DCC recruitment to the plasma membrane and enhance commissural axon outgrowth and chemoattraction to netrin-1. PKA is a kinase that is activated by cAMP production (reviewed in Cooper, 2003). Our extensive analysis of PKA activity using cAMP immunofluorescence, cAMP ELISA assays, and immunoblot analysis of phospho-CREB, has led us to conclude that netrin-1 does not activate PKA in commissural neurons (Figures 3.6, 4.1 & 7.2). As such, our findings indicate that netrin-induced inhibition of Rho does not occur through activation of PKA.

In summary, although further investigation is needed, tantalizing evidence suggests that netrin-1 induces a stepwise activation of Cdc42 and then Rac, followed by inhibition of Rho. The specific complements of proteins that mediate this response are unknown, however genetic evidence supports the involvement of the GEF Trio in the activation of Rac.

CYCLIC AMP IN AXON GUIDANCE

The production of cAMP from ATP by adenylyl cyclases is an important signal transduction mechanism employed by the most primitive bacteria and humans alike

(Berman et al., 2005). It is known to be important for cellular processes as diverse as regulating heart contractions, T-cell activation, steroid biosynthesis, sperm maturation, adipocyte metabolism, and exocytosis in a variety of cell types (see Tasken and Aandahl, 2004). In mammals, there is one soluble and nine transmembrane adenylyl cyclase isoforms. Transmembrane adenylyl cyclase (tmAC) activity is primarily regulated by stimulatory and inhibitory G proteins, but can also be influenced by calcium concentrations and the activity of other intracellular factors (see Cooper, 2003). In contrast, soluble adenylyl cyclase (sAC) is not regulated by G-proteins, but is directly stimulated by bicarbonate ions (Chen et al., 2000b).

Adenylyl cyclases are selectively expressed in different tissues. For instance, hippocampal and cerebellar neurons, as well as, secretory tissues are enriched with tmAC1 and 8, whereas tmAC5 is found in striatal neurons and cardiac tissue (see Cooper, 2003). sAC, on the other hand, is primarily expressed in sperm (Sinclair et al., 2000). In line with this distribution, the only apparent phenotype of sAC knockout mice is infertility of males due to impaired sperm motility (Esposito et al., 2004). Additional roles for sAC based on low level expression in kidney, choroid plexus and lungs may indicate that it participates in the bicarbonate sensing of these tissues (Chen et al., 2000b;Schmid et al., 2007). In chapter 7 we report that sAC is not present in embryonic DRG or spinal commissural neurons. Moreover, the absence of sAC in the genomes of fruit flies and *C. elegans* (Roelofs and Van Haastert, 2002) where orthologs of netrin-1 have a well-documented, essential function in orienting axon extension in these organisms (see chapter 2), further argues against a role for sAC in the guidance of axons to netrin.

However, given that cAMP concentrations profoundly influence axon guidance to netrin-1, an important line of research will be to determine which of the nine transmembrane cyclases, all of which are detected in the embryo (Table 7.2), may be implicated in netrin-mediated axon guidance.

Why aren't spinal commissural axons repelled by netrin-1 low cAMP concentrations?

In chapter 6 we observed that the distance over which spinal commissural neuron axons turn to netrin-1 is reduced upon inhibition of cAMP signaling, we did not,

however, observe a switch to repulsion reported in *Xenopus* retinal ganglion cells (RGCs) and spinal neurons *in vitro* (Song et al., 1997;Ming et al., 1997;Song et al., 1998;Hopker et al., 1999). The underlying reason for this likely reflects the presence of an UNC5 netrin receptor family member in neurons from the RGC and whole spinal cord, but not in our commissural neurons isolated from the dorsal spinal cord.

Attraction to a source of netrin-1 requires DCC, whereas members of the UNC5 homologue netrin receptor family (UNC5A-D) are required for the repellent response (see Chapter 2). The expression of UNC5 in the ventral spinal cord suggests that a portion of neurons in *Xenopus* spinal neurons preparations express UNC5 (Leonardo et al., 1997;Dillon et al., 2007). Similarly, RGCs have been shown to express both DCC and multiple UNC5 homologues in zebrafish, rats, and mice (Deiner et al., 1997;Petrausch et al., 2000;Ellezam et al., 2001).

Relative expression levels of DCC and UNC5 homologues influence whether axons are repelled or attracted to netrin-1. In *C. elegans,* ectopic expression of UNC5 caused axons to be repelled rather than attracted to a source of netrin (Hamelin et al., 1993). Likewise, growth cones of cultured *Xenopus* spinal neurons were repelled by netrin-1 when they were engineered to over-express UNC5 (Hong et al., 1999). In chapter 3 we report that cell surface presentation of DCC is regulated by PKA and work from Lindsay Hinck's lab has shown that PKC regulates the endocytosis of UNC5 from the cell surface (Williams et al., 2003). Thus PKA and PKC activity influence the relative amounts of DCC and UNC5 homologues present on the surface of the growth cone; this in turn influences whether axonal responses to netrin-1 are attractive or repulsive. In line with this model, it has been reported that PKC activation converts netrin-1-induced hippocampal axon repulsion to attraction (Bartoe et al., 2006). Embryonic rat spinal commissural neurons, however, do not express UNC5 homologues as they are extending to the floor plate (Leonardo et al., 1997) and therefore may lack the ability to be repelled by netrin-1. Instead only the sensitivity of DCC mediated attraction to netrin-1 is affected by changes in cAMP/PKA activity (see figure 4.4).

Is cAMP required for axon guidance to neurotrophins?

Cyclic AMP has long been implicated in the guidance of axons. In the early 1970s, it was revealed that elevating the intracellular concentration of cAMP promoted axon extension (Roisen et al., 1972;Haas et al., 1972). More recently, elevating cAMP levels has been shown to promote regeneration following injury, by inducing an injured axon to overcome inhibitory cues (Neumann et al., 2002;Qiu et al., 2002a). Similar to our findings for DCC on spinal commissural neurons, the axonal targeting of olfactory neurons is influenced by cAMP-mediated cell surface expression of the semaphorin receptor, neuropilin-1 (Figure 1.3, Imai et al., 2006).

These observations suggest an important role for cAMP as a modulator of the response to guidance cues. However, reports that local release of cAMP analogs attracts extending axons, has prompted models whereby cAMP production occurs in response to chemoattractive cues (Gundersen and Barrett, 1980;Lohof et al., 1992). Identifying an axon guidance cue that relies on cAMP production for its function has been difficult. We proposed in chapter 7 that two criteria should be met for cAMP to be included as a necessary component in the signal transduction cascade of an axon guidance cue: (1) the guidance cue must alter cAMP levels in the appropriate context and (2) global elevation of cAMP must disrupt turning. In the discussion of chapter 7 we outlined how these criteria are met by pituitary adenylate cyclase-activating polypeptide (PACAP), but not by netrin-1. Here, I will examine the possibility that cAMP production is important for axon attraction to neurotrophins.

In mammals there are four neurotrophins: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4) (see Huang and Reichardt, 2001). These four proteins are classified as neurotrophins because they originate from the same ancestral gene. NGF, BDNF and NT-3 promote axon outgrowth and steer axons (Levi-Montalcini and Angeletti, 1968;Gundersen and Barrett, 1979;Song et al., 1997). While numerous studies have explored the possibility that cAMP may be implicated in the outgrowth and guidance to NGF, a clear picture has yet to emerge. NGF can induce cAMP production in PC12 cell lines (Schubert and Whitlock, 1977;Knipper et al., 1993;Stessin et al., 2006) and in dissociated postnatal rat

cerebellar and DRG neurons (Cai et al., 1999). However, the ability of NGF to affect cAMP levels in embryonic DRG neurons is unclear. A thorough examination initially demonstrated that 0.04-40 ng/ml NGF was incapable of influencing cAMP levels after 15, 30 or 180 minutes (Frazier et al., 1973;Bradshaw et al., 1974); in contrast, a later study reported a rapid transient increase in cAMP by 2ng/ml of NGF within 10 minutes (Narumi and Fujita, 1978). Our findings agree with the former study that NGF is incapable of elevating cAMP in embryonic DRG neurons (Figure 7.2). Importantly, our assays would have detected rapid transient increases because they were done in the presence of IBMX – a phosphodiesterase inhibitor that prevents breakdown of cAMP. Furthermore, global elevation of cAMP does not disrupt turning to the neurotrophins BDNF and NT-3 (Song et al., 1997). As such, cAMP production does not appear to be necessary for axon attraction to neurotrophins.

Possible cues that regulate the response to netrin-1 through cAMP

Although we have found that netrin-1 is incapable of altering cAMP levels in spinal commissural neurons (Figures 3.6, 4.1 & 7.2), this does not rule out the possibility that concomitant cues act in parallel to activate PKA. This activation of PKA could inhibit Rho, promote plasma membrane presentation of DCC, and therefore assist spinal commissural neuron axon responses to netrin-1.

One potential regulator is laminin-1, which has been reported to switch the response of retinal ganglion axon to netrin-1 from attraction to repulsion by lowering cAMP levels (Hopker et al., 1999). Glutamate and SDF-1 are also potential candidates as both can influence cAMP levels and axon guidance (Chalasani et al., 2003;Kreibich et al., 2004). Specifically, although SDF-1 inhibits cAMP production in a number of cells (Kowalska et al., 2000;Peng et al., 2004;Dwinell et al., 2004), glutamate and SDF-1 have been reported to elevate cAMP in cultured E8 sympathetic neurons and reduce the inhibitory effects of Sema 3A and Slit-2 on embryonic DRG axons and RGC axon, respectively (Chalasani et al., 2003;Kreibich et al., 2004). Unfortunately, the distribution in the developing spinal cord, as well as, the ability of laminin, SDF-1 and glutamate to influence cAMP production in spinal commissural neurons is unknown.

On the other hand, an excellent case was made for PACAP regulating spinal commissural axon guidance to netrin-1. Although best understood for its role as a neurotransmitter, neuromodulator and neurotrophic factor (Hashimoto et al., 2006), PACAP has also been implicated in neurite outgrowth (Falluel-Morel et al., 2006). In the developing spinal cord, a striking pattern of PACAP expression is found in cells over which spinal commissural axon extend as they are being guided to netrin-1, and the PACAP receptor, PAC1, is expressed in the dorsal spinal cord where spinal commissural neuron cell bodies are located (Waschek et al., 1998). Moreover, we have shown in figure 7.2D that PACAP induces a massive elevation of cAMP concentration in spinal commissural neurons. PACAP knockout mice have neurological defects and die shortly after birth, however a close examination for axon guidance defects in PACAP knockout embryos has not been done (Gray et al., 2001;Hashimoto et al., 2001). As such, examining whether guidance defects in the trajectory of spinal commissural neuron axons exist in PACAP knockout mice could provide important insights into mechanisms that influence spinal commissural neuron axon extension to netrin-1.

In summary, while the distribution and ability of laminin, SDF-1 and glutamate to elevate cAMP in spinal commissural neurons is unknown and deserves investigation, several lines of evidence indicate that PACAP may regulate the response of spinal commissural neurons to netrin-1 in the developing vertebrate spinal cord.

CONCLUSION:

Netrin-1 associates with ECM components and can function as an adhesive ligand; this could allow it to mechanically support the morphological rearrangements required for axonal responses. Netrin-1 can also regulate several intracellular signaling cascades; evidence provided in this thesis reveals that it can regulate Rho GTPases and that this may possibly occur through a stepwise activation mechanism. But extensive analysis has shown that cAMP is not produced during a chemoattractive response to netrin-1, suggesting that cAMP elevation is not necessary for netrin-mediated attraction.

These studies are not only important for our fundamental understanding of axon guidance, but will contribute to the development of better spinal cord regeneration

strategies – both inhibition of RhoA and elevation of cAMP improve axon regeneration following injury (Neumann et al., 2002;Dergham et al., 2002;Qiu et al., 2002a;Fournier et al., 2003). This thesis investigates the cellular mechanisms altered by these manipulations and how they affect the appropriate guidance of axons. While improving regeneration requires an axon to ignore inhibitory cues at the injury site, guidance to their targets requires axons to sense and respond appropriately to cues in their environment. Results presented in this thesis suggest that axon attraction to netrin-1 is not only intact, but improved by either elevation of cAMP or inhibition of RhoA.

CHAPTER 9

APPENDIX I: Dissection and Culture of Spinal Commissural Neurons

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PREFACE

This section details the micro-dissection and culture of spinal commissural neurons. Techniques described here were used throughout the thesis. This chapter will appear as an upcoming unit in chapter 3 of Current Protocols in Neuroscience.

ABSTRACT

Spinal commissural neurons have provided substantial insight into the mechanisms that regulate axon guidance. Explants of embryonic spinal cords and isolated spinal commissural neurons have been important experimental tools for the identification and characterization of several guidance cues, including netrins, semaphorins, slits, sonic hedgehog, BMPs, and wnts. In this unit, we describe how to establish these explant assays that assess the outgrowth and turning capacity of commissural axons. We also describe how to prepare cultures highly enriched with embryonic commissural neurons, which have been used to probe the biochemical signaling mechanisms regulating axon guidance.

INTRODUCTION

In the late nineteenth century, Santiago Ramón y Cajal observed commissural neurons extending axons ventrally towards the floor plate in fixed sections of embryonic chick spinal cord (Ramón y Cajal, 1999). As part of his formulation of the chemotropic model of axon guidance – the theory that chemical cues guide axons - he proposed that the floor plate would attract these axons by secreting factors that guide their growth ventrally.

Experimental validation of this hypothesis was obtained approximately one hundred years later, in part based on the results of the assays presented in this unit (Basic Protocols 2 and 3, Tessier-Lavigne et al., 1988;Placzek et al., 1990;Kennedy et al., 1994;Serafini et al., 1994). Methods have since been developed to generate cultures highly enriched with embryonic spinal commissural neurons (Basic Protocol 1), and these have been used in biochemical studies to address the intracellular signaling mechanisms underlying axon guidance to the midline of the embryonic CNS (Bouchard et al., 2004).

NOTE: Published reports often employ an embryonic dating scheme where the day after fertilization is recorded as E0. Here, we use a more common method where the morning after fertilization is set as E1 (Bayer and Altman, 1995). Basic Protocols 1 and 2 require E14.5 rat, equivalent to Carnegie stage 17 embryos, while Basic Protocol 3 requires E12.5 rat, equivalent to stage 13 embryos (O'Rahilly et al., 1987). Although we describe explant microdissection using E12.5 and E14.5 rat embryos, it is equally possible to use E11 and E13 mice embryos, or Hamburger & Hamilton stage 17 and 25 chick embryos.

NOTE: All procedures using live animals must be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and must follow officially approved procedures for the use and care of laboratory animals.

BASIC PROTOCOL 1: SPINAL COMMISSURAL NEURON CULTURES

This procedure describes how to isolate and dissociate dorsal spinal cord from E14.5 rats, a day after most commissural neurons have been born (Bayer and Altman, 1995). When cultured overnight in the presence of 10% fetal bovine serum (FBS), over 90% of cells express Tag-1 and DCC (Bouchard et al., 2004), consistent with expression of these markers by spinal commissural neurons. .Conversely, choline acetyltransferase, ChAT, which is expressed by motoneurons, was not detected. Each dissected rat dorsal spinal cord provides approximately 750,000 cells and, with practice, takes approximately 10 min to dissect. It is informative to note that, at this embryonic stage, pioneer commissural axons have already reached the midline.

NOTE: For basic principles of cell culture, please refer to Phelan, 2007.

Materials

Antibodies:

Mouse IgM anti-Tag-1 *(4D7, Developmental Studies Hybridoma Bank, University of Iowa)*

Mouse IgG anti-DCC (*554223, BD Pharmingen^{TM})*

Goat anti-choline acetyltransferase *(552623, BD Pharmingen^{TM})*

B-27 supplement *(Invitrogen # 17504-044, Brewer et al., 1993;Podratz et al., 1998)*

Cell culture incubator, humidified, set to 37°C and 5% CO_2

Dissection tools:

Dissecting microscope, at least 5x magnification, with a transmitted light base *(e.g. Zeiss SV6 with a Diagnostic Instruments Inc. #TLB5006 base)*

Forceps (2), Dumont #5 (*Fine Science Tools #91150-20)*

Needle holder (*Insect Pin Holder: Fine Science Tools #26015-11)*

Scissors, iris (*Fine Science Tools #91460-11)*

Scissors, spring loaded (*Fine Science Tools #91501-09)*

Scissors, surgical *(Fine Science Tools # 91402-14)*

Tungsten wire, 0.5mm diameter (*Omega Engineering Inc #WW26020)*

Fetal bovine serum (FBS)

GlutaMAX *(Invitrogen #35050-061)*

Hanks' balanced salt solution, without Ca^{2+}/Mg^{2+}

Hemacytometer (*e.g. Fisher Scientific # 02-671-54)*

L15 dissection media *(Invitrogen #41300-039)*

Methanol flame

Neurobasal® media, without L-glutamine *(Invitrogen #21103-049, Brewer et al., 1993)*

Penicillin/Streptomycin *(e.g. Invitrogen # 10378-018)*

Pipettes, plastic transfer *(e.g. Fisher #13-711-7)*

Poly-D-lysine, 70-150kDa *(e.g. Sigma # P-0899)*

Rat, pregnant at E14.5 (*vaginal plug = E1*)

Syringe filters, 0.2µm

Tabletop centrifuge (*e.g. IEC Clinical*)

Trypan blue solution *(e.g. Invitrogen # 15250-061)*

Vacuum aspirator with tubing to accommodate glass pipettes

Remove uterus from mother

1. Euthanize an anesthetized E14.5 pregnant rat (see Donovan and Brown, 2005)

2. Wipe abdomen with 70% ethanol to sanitize area and weigh down fur.

3. While wearing gloves, create a C-section by gently pinching ~1cm of abdominal skin and underlying muscle and cutting the pinched tissue with a pair of surgical scissors to produce a ~2cm incision.

4. Using a pair of Dumont #5 forceps, pull on uterus between embryo sacs while cutting away, with surgical scissors, the connective tissue that restricts their withdrawal.

5. Place intact uterus into a Petri dish with L-15 on ice; typically, each uterus should contain approximately twelve embryos.

Remove embryos from uterus

6. In a 10cm Petri dish filled with ice cold L15, clasp the uterus between embryo sacs with a pair of No. 5 forceps. With the other hand cut the clear top of the sac with iris scissors

7. Expel the embryo into the ice-cold L15 by gently squeezing the opposite side of the sac

8. Use iris scissors to fully detach embryo

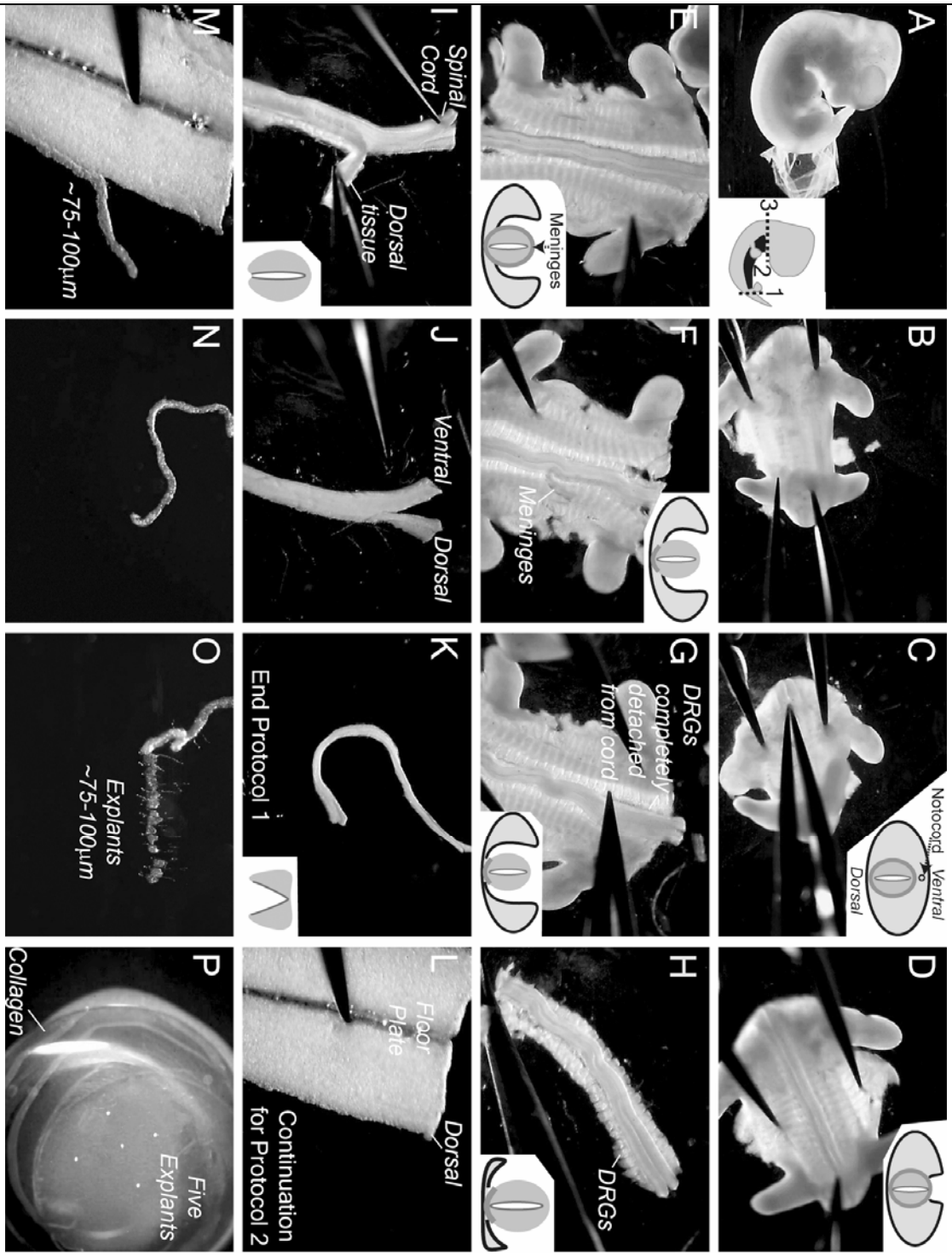

Figure 9.1 - Important dissection steps for Basic Protocol 1 (A-K) and Basic Protocol 2 (A-I, L-P). *See text for details*

9. Transfer embryos, using a cut plastic transfer pipette, into Petri dish containing fresh L15 on ice.

10. Repeat until all embryos are removed from uterus.

Isolate dorsal spinal cord

NOTE: To facilitate manipulating the embryo the inverted lids of 60mm petri dish lids are ideal for microdissection, due to the relatively low height of the edge of the dish.

NOTE: It is important to keep the medium containing the embryos on ice at all times and to dissect in cold L15 media. We generally use two 60mm Petri dish lids, alternating which is on ice and which is used to dissect.

NOTE: Unless otherwise stated, all dissection steps use two pairs of No. 5 forceps. One pair holds and orients the embryo, while the other grasps, cuts off (by pinching and pulling away) and teases apart (by gentle scraping with closed forceps).

11. Cut away tail (cut '1' in Figure 9.1A), internal organs (area '2' in Figure 9.1A) and head (cut '3' in Figure 9.1A)
    To remove head, pinch neck tightly with one pair of forceps and run the tip of the other pair of forceps along ridge of pinching forceps to cut away tissue.

12. Grab hold of notochord at rostral-most spinal cord by closing forceps on tissue above spinal cord and pulling away caudally; the notochord is clear 'string' with relatively high tensile strength that when pulled away will expose the ventral-most spinal cord (Figure 9.1B,C)

13. Using a pair of closed forceps, scrape tissue away from the top (Figure 9.1D) and sides of the spinal cord (Figure 9.1E)

14. At the rostral end, push closed forceps between meninges and spinal cord and tear away meninges that cover the spinal cord (Figure 9.1F)

15. Using a pair of closed forceps, ensure that the nerves attached to the dorsal root ganglia (DRGs), visible as repeating columns entering the spinal cord perpendicularly, are fully detached (Figure 9.1G).

    This is important to prevent excessive tissue from being torn away in the following steps.

16. Using spring loaded scissors, cut away body on either side of the cord to leave only a small amount of tissue attached to either side of the spinal cord (Figure 9.1H)

17. Poke through and hold the ventral spinal cord with one pair of forceps and pull away dorsal tissue with another pair of forceps (Figure 9.1I)

18. Position the dissected spinal cord on its side (Figure 9.1J). Using a sharpened tungsten needle (see Support Protocol 1), push down on the spinal cord to cut it into dorsal and ventral halves.

19. Transfer dorsal portion of spinal cord (Figure 9.1K) to a 1.5-ml tube containing L15. Maintain submerged in medium on ice until dissociation *(tissue can be maintained on ice for 6hrs and remain viable)*

20. Repeat with a new embryo and pool dissected dorsal spinal cords for use in step 21.

Dissociate dorsal spinal cord into individual cells

21. Transfer dorsal spinal cords to a 15-ml plastic conical tube with 10 ml of room temperature Ca^{2+}/Mg^{2+}-free HBSS.

22. Allow tissue to settle then aspirate and replace with another 10 ml of Ca^{2+}/Mg^{2+}-free HBSS.

23. Incubate at 37°C for 30 min while inverting every 5-10 min. *This is a good time to prepare Neurobasal®/FBS culture media (see Reagents and Solutions)*

24. Remove tissue from 37°C (tissue should now have broken up into small pieces)

25. Pellet tissue by spinning at 300x g for 2 min at room temperature

    If properly done, there will be no DNA released (visible as a viscous gel that does not pellet). However, in the event that DNA is present, it can be eliminated by adding 20µl of 1 mg/ml DNase I, inverting several times over a couple minutes and then spinning again at 300 x g for 2 min

26. Remove supernatant and triturate with 250µl of media.

    This is most easily accomplished with several pulses in a standard 1000µl pipette tip and then again in a 200µl pipette tip. Methanol-flamed glass Pasteur pipettes can also be used.

Count cells

27. Dilute cells with Neurobasal®/FBS culture media *(see Reagents and Solutions)* to an estimated density 1 million cells per ml (each dorsal spinal cord should give approximately 750,000 cells)

28. Remove 25 µl of cells to 1.5-ml tube containing 25 µl of 0.4% trypan blue solution

29. Wait 4 minutes

30. When visualized in a hemacytometer, there should be very few cellular aggregates and less than 1% of cells with trypan blue staining (dark blue).

NOTE: For a more detailed account of trypan blue labeling and cell counting, see Phelan, 2007.

Culture cells

31. Dilute cells in Neurobasal®/FBS culture media (*see Reagents and Solutions*).

    To generate a culture of distantly separated cells on 12mm coverslips in the wells of a 24 well-plate, seed 10 000 cells in 500µl per well.

32. Plate cells on tissue culture plastic or glass that has been coated with 2 µg/cm^2 poly-D-lysine for 2 hrs at room temperature

33. Culture overnight at 37^{o}C with 5% CO_2 before changing to defined Neurobasal®/B-27 culture media (*see Reagents and Solutions*)

    Experiments are normally done on these cultures within the first 48 hours. However, these cultures can be maintained for several weeks as long as the media (Neurobasal®/B-27) is replaced every 48hrs.

Determining purity of culture

34. Using antibodies against Tag1, DCC and choline acetyltransferase (ChAT), immunofluorecently label cells after 40hrs in culture (for more detail see Watkins, 2000).

35. Over 90% of cells should express the spinal commissural neuron markers Tag-1 and DCC, while very few, if any, should express the motoneuron marker ChAT

(Bouchard et al., 2004). If this degree of homogeneity is not reached, the most likely cause is either that the embryos were older than E14.5 or that excess ventral spinal cord tissue was dissected.

BASIC PROTOCOL 2: SPINAL COMMISSURAL NEURON AXON OUTGROWTH ASSAY

This explant assay evaluates the ability of commissural axons to extend into a matrix of type I collagen. This procedure is an extension of Basic Protocol 1, but instead of cutting the dorsal half of the spinal cord, only a 75-100μm strip is cut; this strip is then cut into explants of 75-100μm in diameter. Approximately 80 explants, 40 explants from each side, can be obtained from a single dissected spinal cord.

Materials

Cell culture incubator, set to 37°C and 5% CO_2

Type I collagen, we recommend that it be purchased as either bovine skin collagen (*Inamed #5409*) or rat tail collagen (*Sigma #C3867*); its preparation has been described elsewhere (Chandrakasan et al., 1976;Habermehl et al., 2005).

Culture plates, 4-well *(e.g. NUNC #176740). These plates are ideal because of the low height of the well wall.*

Dissection tools:

Dissecting microscope, at least 5x magnification, with a transmitted light base *(e.g. Zeiss SV6 with a Diagnostic Instruments Inc. #TLB5006 base)*

Forceps (2), Dumont #5 (*Fine Science Tools #91150-20)*

Needle Holder (*Fine Science Tools #26015-11)*

Scissors, iris (*Fine Science Tools #91460-11)*

Scissors, surgical *(Fine Science Tools # 91402-14)*

Scissors, spring Loaded (*Fine Science Tools #91501-09)*

Tungsten wire, 0.5mm diameter (*Omega Engineering Inc #WW26020)*

DMEM powder *(Sigma #D7777-1L)*

Fetal bovine serum (FBS)

GlutaMAX *(Invitrogen #35050-061)*

L15 dissection media *(Invitrogen #41300-039)*

L15 supplemented with 5% filtered serum (L15/serum)

Methanol flame

Mouth pipette with glass 100µl micropipettes (*e.g. VWR 53432-921*)

$NaHCO_3$

Neurobasal® media, without L-glutamine *(Invitrogen #21103-049)*

Penicillin/Streptomycin *(e.g. Invitrogen # 10378-018)*

Pipettes, plastic transfer *(Fisher #13-711-7)*

Rat, pregnant at E14.5 *(vaginal plug = E1)*

Syringe filters, 0.2µm

Vacuum aspirator with tubing to accommodate glass pipettes

Cut Explants

1. Dissect spinal cord as described in Basic Protocol 1, steps 1 to 17 for dissociated cells (Figure 9.1A-I)

2. Prepare a pulled glass micropipette for transferring tissue:

   Pull glass micropipettes into two over a methanol flame. Break off pulled end to generate a ~150µm diameter tip. Pass L15/serum through several times to prevent tissue from sticking to glass.

NOTE: Perform remaining dissection step in L15/serum to reduce adherence of tissue to dissection tools.

3. In L15/serum dissection media, use a sharpened tungsten needle (see Support Protocol 1) to cut a strip of tissue of approximately 75-100µm wide from the dorsal-most portion of the spinal cord *(Figure 9.1L-N)*.

   Given that there is a rostral-caudal gradient in the relative developmental maturity of the spinal cord, to minimize variations between the explants,

this strip should not exceed 3mm in rostro-caudal length. If more than one strip is required, cut a strip from approximately the same location on the opposite side or from another spinal cord.

4. Cut this strip of tissue into blocks of 75-100μm in diameter (Figure 9.1O)

5. Transfer explants, using the pulled glass micropipette, to fresh L15/serum on ice

   On occasion, explants will remain stuck within micropipette. These explants when finally expelled from pipette will most likely be flattened and irreversibly damaged; as such, they should be discarded.

Embed, culture and fix explants

6. Embed tissue in collagen (see Support Protocol 2) with at least 5mm between them (Figure 9.1P)

7. Culture in Neurobasal®/FBS in a humidified incubator set to 37°C and 5% CO_2 for 16hrs hours or until desired outgrowth is reached.

   It is also possible to culture these explants in Neurobasal®/B-27 media, however the absence of certain components (e.g. lysophosphatidic acid) could alter the outgrowth under certain conditions.

8. Fix explants with 4% PFA (*see Reagents and Solutions)* for 20min at room temperature with agitation.

   To confirm that axons bundles exiting the explants are commissural axons, immunofluorescent staining for the marker Tag-1 can be carried out as described in Support Protocol #3

BASIC PROTOCOL 3: COMMISSURAL NEURON AXON TURNING ASSAY

This procedure describes how to dissect E12.5 rat spinal cords. At this stage of embryogenesis, spinal commissural neurons are being born (Bayer and Altman, 1995). As such, sources of cues can be placed perpendicular to the normal dorsal-ventral trajectory of the commissural axons and the ability of these cues to deflect the trajectory of commissural axons assessed. This assay has been used to show that floor plate releases factors, including netrin-1 and sonic hedgehog, that attract the growth of spinal commissural axons (Placzek et al., 1990;Kennedy et al., 1994;Charron et al., 2003). It has also been used to demonstrate that roof plate releases factors, including GDF7 and BMP7, that repel spinal commissural axons (Augsburger et al., 1999;Butler and Dodd, 2003). Variations on this protocol, known as the 'open-book preparation', allow for turning assays to be performed on commissural axons that have crossed the midline (Shirasaki et al., 1998;Zou et al., 2000).

Materials

Cell culture incubator, set to 37°C and 5% CO_2

Type I collagen, we recommend that it be purchased as either bovine skin collagen (*Inamed #5409*) or rat tail collagen (*Sigma #C3867*); its preparation has been described elsewhere (Chandrakasan et al., 1976;Habermehl et al., 2005).

Culture plates, 4-well (*NUNC #176740*)

Dissection tools:

Dissecting microscope, at least 5x magnification, with a transmitted light base *(e.g. Zeiss SV6 with a Diagnostic Instruments Inc. #TLB5006 base)*

Forceps (2), Dumont #5 (*Fine Science Tools #91150-20)*

Iris Scissors (*Fine Science Tools #91460-11)*

Needle Holder (*Fine Science Tools #26015-11)*

Spring Loaded Scissors (*Fine Science Tools #91501-09)*

Tungsten wire, 0.5mm diameter (*Omega Engineering Inc #WW26020)*

DMEM powder *(Sigma #D7777-1L)*

Fetal bovine serum (FBS)

GlutaMAX *(Invitrogen #35050-061)*

L15 dissection media *(Invitrogen #41300-039)*

L15 supplemented with 5% filtered serum (L15/serum), preferably fetal bovine serum

Methanol flame

Mouth pipette with glass 100µl micropipettes (*e.g. VWR 53432-921)*

$NaHCO_3$

Neurobasal® media, without L-glutamine *(Invitrogen #21103-049)*

Penicillin/Streptomycin *(e.g. Invitrogen # 10378-018)*

Pipettes, plastic transfer *(e.g. Fisher #13-711-7)*

Rat, pregnant at E12.5 (vaginal plug = E1)

Syringe filters, 0.2µm

Trypsin, 2.5% *(e.g. Invitrogen #15090-046)*

Isolate embryos

1. Euthanize an anesthetized E12.5 pregnant rat (see Donovan and Brown, 2005)

2. Separate embryos as detailed in Basic Protocol 1, steps 1-10

Isolate a six somite long piece of dorsal tissue

3. Maintain in serum-free L15 media until after trypsinizaiton

4. Starting at the rostal-most portion of the forelimb, cut a piece of dorsal tissue containing 6 somites between the anterior portion of the forelimb and the spinal cord (Figure 9.2A-D)

5. Transfer 6 somite piece back to L15 media on ice using a plastic Pasteur pipette

6. Repeat with a new embryo and pool dissected tissue for use in step 7

Digest tissue

7. Place dorsal tissue pieces in a 35-mm petri dish containing 2 ml of L15 and 500 µl of 1:250 trypsin at room temperature. Use forceps to keep tissue from aggregating (Figure 9.2E).

8. Incubate for ~20 min at room temperature until tissue at edges begins to separate from the spinal cord (Figure 9.2F).

   This step is of particular importance; insufficient trypsinization will make it difficult to separate the spinal cord from the meninges and associated mesodermal tissue, excessive trypsinization will degrade the spinal neuroepithelium. Either outcome will adversely affect the dissection.

9. Wash tissue with two changes of L15/Serum in a 60mm dish to stop trypsinization.

Isolate spinal cord

NOTE: Perform remaining dissection step in L15/serum to inhibit trypsin and reduce adherence of tissue to dissection tools.

NOTE: It is important to keep embryos in medium on ice at all times and to dissect in cold L15 media. We generally use two 60mm Petri dish lids, alternating between one on ice and one being used to dissect.

10. Beginning at one end, poke through lateral tissue with a single forcep while scraping with a tungsten needle to expose ventral portion of the spinal cord (Figure 9.2G)

11. In short steps, tease tungsten needle between the sides of the spinal cord and the lateral tissue to separate them from each other (Figure 9.2H).

12. At rostral end, use tungsten needle to tease apart notocord and spinal cord; then remove notocord with a pair of forceps (Figure 9.2I,J)

Prepare spinal cord for culture

13. Prepare a pulled glass micropipette for transferring tissue:

    Pull glass micropipettes into two over a methanol flame. Break off pulled end to generate a ~250μm diameter tip. Pass L15/serum through several times to prevent tissue from sticking to glass.

14. Using a tungsten needle, mark each end of the spinal cord (to identify the side exposed to trypsinization) and cut in half. (Figure 9.2K,L)

15. Transfer cut spinal cords back on ice

16. Repeat with another 6 somite long piece and pool tissue for step 17.

Prepare co-explant

17. Three different co-explants are commonly used to deflect the trajectory of commissural axons: floor plates and roof plates are generated by cutting a subset of the dissected spinal cords medially into ventral and dorsal sections (Figure 9.2N). Cellular aggregates are generated from hanging drop cultures (Figure 9.2M, see Support Protocol #4).

Embed tissue

18. Embed in a collagen gel (see Support Protocol 2) by placing co-explanted tissue along the cut edge of a whole spinal cord (Figure 9.2M,O,P). If using floor plates or roof plates ensure that the ventral-most or dorsal-most portion of spinal cord halves is contacting the cut edge of the whole spinal cord.

19. Culture in Neurobasal®/FBS culture media for 40hrs.

20. Fix and label commissural neuron axons as described in Support Protocol 3.

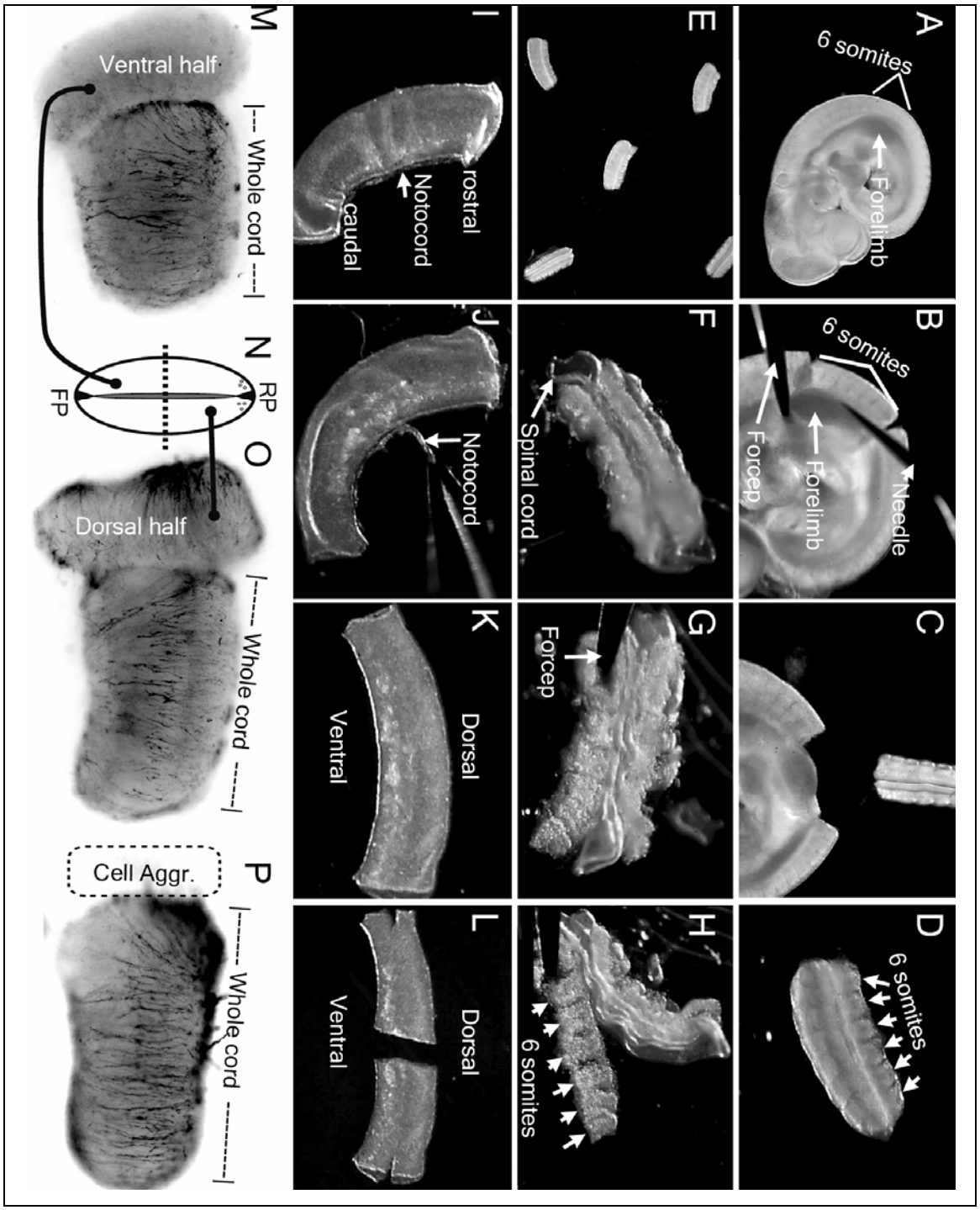

Figure 9.2 - Spinal commissural neuron axon turning assay. *Panel A-L illustrate key steps in the microdissection described in Basic Protocol 3. Panels M, O and P are fluorescent images of commissural axons labeled with an antibody against Tag-1 (image brightness was inverted, and therefore Tag-1 labeled axons appear black). In panel M, commissural axons extending within a whole spinal cord turn away from their normal dorsal-ventral trajectory and toward an ectopic floor-plate (ventral-most portion of spinal cord) that was placed along the cut edge of the whole spinal cord explant. In panel O, commissural axons turn away from an ectopic roof plate (dorsal-most portion of spinal cord) that was placed along the cut edge of the whole spinal cord explant. Explants containing floor-plate and roof-plate were generated by cutting a spinal cord medially as shown in panel N. As an alternative to ectopic floor-plate or roof plate, cellular aggregates can be used as shown in panel P. In this case, an aggregate of HEK 293 engineered to express netrin-1 attract the extension of spinal commissural axons.*

SUPPORT PROTOCOL 1: ELECTROLYTIC SHARPENING OF TUNGSTEN WIRE

This support protocol describes how to electrolytically sharpen tungsten wire into rigid sharp needles. These needles are required for microdissection of tissue in Basic Protocols 2 and 3.

Materials:

Alligator clips, mini (*e.g. RadioShack #270-378)*

Deionizied water

Dissecting microscope, at least 5x magnification, with a transmitted light base *(e.g. Zeiss SV6 with a Diagnostic Instruments Inc. #TLB5006)*

Electrode, either a paper clip or a 10cm section of metal hangar

Jar, 125ml plastic *(e.g. Fisher 02-912-028)*

Power supply, regulated direct current capable of at least 2 Amps (*e.g. Fisher Scientific #S90163)*

If no power supply is available, it is also possible to use a 9V battery

Needle holder (*e.g. Fine Science Tools #26015-11)*

Pliers, needle nose with wire cutter *(e.g. Radio Shack # 6429-57)*

Sodium hydroxide *(NaOH, e.g. Fisher Scientific #S318)*

Stage micrometer, 1mm with 10µm divisions (*e.g. Fisher #12-561-SM1*)

Tungsten wire, 0.5mm diameter (e.g. *Omega Engineering Inc #WW26020)*

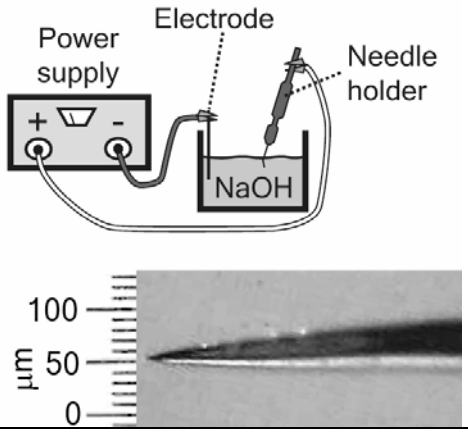

Figure 9.3 - Electrolytic sharpening of tungsten wire. *Above, diagram illustrating how the power supply, alligator clips, electrode, 50ml of 1M NaOH in a plastic jar and needle holder with tungsten wire are setup. Below, a composite image of a stage micrometer (lowest division 10µm) and a sharpened tungsten wire with a tip of <10 µm.*

Electrolytic sharpening:

1. Cut a 3-5cm piece of tungsten wire using the wire cutter portion of needle nose pliers

2. Fasten the tungsten wire in the needle holder

3. Use the needle nose pliers to bend the distal 1 cm to an angle of approximately 30°

4. Assemble power supply, electrodes, 75ml of 1M NaOH in deionized water in a 125ml plastic jar, two alligator clips and tungsten wire in its holder as shown in Figure 9.3 (for additional detail, see Conrad et al., 1993)

5. Set power supply to between 5 and 10V of direct current (DC)

6. Electrolytically sharpen the tungsten wire by immersing the bent end into the 1M NaOH. Proper assembly can be confirmed when bubbles appear on electrode after a few seconds. Immersing the tip with a steady up and down motion will

create a cone shaped tip, while fully immersing the tip will produce a thin even tip. Continue until tip itself is less than 10 μm in diameter (Figure 9.3). Gently touching the meniscus of the 1M NaOH can produce flattened, scalpel shapes (Conrad et al., 1993).

7. Rinse tungsten needle in dH_2O before using.

SUPPORT PROTOCOL 2: EMBEDDING TISSUE IN A COLLAGEN MATRIX

This support protocol is used in Basic Protocols 2 and 3 to immobilize explants in a collagen matrix.

Materials:

Cell culture incubator, set to 37°C and 5% CO_2

Dissecting microscope, at least 5x magnification, with a transmitted light base *(e.g. Zeiss SV6 with a Diagnostic Instruments Inc. #TLB5006)*

Type I collagen, we recommend that it be purchased as either bovine skin collagen (*Inamed #5409*) or rat tail collagen (*Sigma #C3867*); its preparation has been described elsewhere (Chandrakasan et al., 1976;Habermehl et al., 2005).

Culture plates, 4-well *(e.g. NUNC #176740). These plates are ideal because of the low height of the well wall.*

DMEM powder *(e.g. Sigma #D7777-1L)*

$NaHCO_3$ (*e.g. Sigma # S5761)*

Prepare first cushion

1. Mix collagen solution: for each 4-well plate, gently mix 80μl of collagen, 10μl 10X DMEM *(see Reagents and Solutions)* & 10μl of 10x $NaHCO_3$ *(see Reagents and Solutions)*

2. Place 20µl of collagen solution on the bottom each well of a 4-well plate and spread until approximately half of the bottom surface is covered, but not touching the sides of the well

3. Allow collagen solution to gel in a 37°C, 5% CO_2 incubator for 20-40 min

Embed tissue

4. Prepare a glass micropipette for transferring tissue:

   Pull glass micropipettes into two over a methanol flame. Break off pulled end to produce a ~150µm (Basic Protocol 1) or ~250µm (Basic Protocol 2) diameter tip. Pass L15/serum through several times to prevent tissue from sticking to glass

5. Prepare another 100µl/4-well plate of collagen solution

6. Using the mouth pipette, transfer tissue with a small amount of dissection media onto the surface of each hardened collagen cushion of a 4-well plate

7. Proceeding one well at a time (to prevent explants from drying out), remove excess media with mouth pipette and add 20µl of collagen solution on top.

8. Using either a single forcep tip or closed forceps, ensure explants are properly positioned in each well.

9. Allow collagen solution to gel in a 37°C, 5% CO_2 incubator for another 40 min.

   To minimize undesired tissue displacement, particularly in co-culture explant experiments, it can be useful to inspect and reposition tissue before collagen has gelled completely (i.e. after 5-10 min of incubation)

10. Add 0.5-1 ml of culture media.

If collagen solution is old or if significant dissection media remains when adding second cushion, the collagen cushions may fall apart. Adding media directly onto the top of the cushion minimizes stress on the cushion.

SUPPORT PROTOCOL 3: IMMUNOLABELING COMMISSURAL AXONS WITHIN EXPLANTS

This procedure is necessary to visualize the trajectory of spinal commissural axons within the spinal cord segments cultured as described in Basic Protocols 3 (as seen in Figure 9.2M,O,P). It can also be used to confirm that axons are Tag-1 positive and to better visualize axon bundles in Basic Protocol 2.

Materials

Antibodies:

Mouse IgM anti-Tag-1 *(4D7, Developmental Studies Hybridoma Bank, University of Iowa)*

Labeled secondary antibody against mouse IgM

Paraformaldehyde (PFA, e.g. Fisher #O4042)

Serum, horse or goat

Triton X-100 (e.g. Fischer #BP151)

Immunolabel

1. Fix explants embedded in collagen in 4% PFA (*see Reagents and Solutions*) for 20 min at room temperature

2. Block in PBS with 1% triton X-100 and 3% serum for at least 1hour at room temperature, with gentle agitation.

3. Dilute mouse anti-Tag-1 antibody to 2 µg/ml in PBS with 1% triton X-100 and 1% serum.

4. Decant blocking solution, add antibody solution and incubate for 3hrs at room temperature or overnight at 4°C, with gentle agitation.

5. Over a period of at least 3hrs, wash with at least six changes of PBS plus 1% triton X-100 and 1% serum at room temperature, with gentle agitation.

6. Dilute labeled secondary antibody against mouse IgM *(not IgG)* to 1 μg/ml in PBS with 1% triton X-100 and 1% serum. Depending on the source of the labeled secondary, it may be necessary to filter the diluted secondary antibody to remove aggregates, visible as intense dots.

7. Decant primary antibody solution, add secondary antibody solution and incubate for 3hrs at room temperature or overnight at 4°C, with agitation.

8. Over a period of at least 3hrs, wash with at least six changes of PBS with 1% triton X-100 and 1% serum at room temperature, with agitation.

Mount on a glass slide

9. Cut out collagen cushion from bottom of well using a tungsten needle (see Support Protocol 1)

10. Mount explants within cushions on glass slides using an aqueous-based mounting solution.

11. To prevent evaporation, seal coverslip onto glass slide by applying a thick coat of nail polish onto edges.

SUPPORT PROTOCOL 4: HANGING DROP AGGREGATION OF ADHERENT CELLS

This procedure is used to obtain aggregates of cells that can be placed and cultured alongside a dissected explant. These aggregates can be generated from any adherent cells and, in particular, cell lines engineered to secrete a particular gene product of interest. Since these aggregates are not attached to a substrate, they can be cut into explants and embedded in collagen.

Materials

Adherent cell line (such as HEK 293 or COS)

Dissecting microscope, at least 5x magnification, with a transmitted light base *(e.g. Zeiss SV6 with a Diagnostic Instruments Inc. #TLB5006)*

Hemacytometer *(e.g. Fisher Scientific # 02-671-54)*

L15 supplemented with 5% filtered serum (L15/serum), preferably fetal bovine serum

Mouth pipette with glass 100µl micropipettes (*e.g. VWR 53432-921)*

Petri dishes, 35mm

Sterile water

Trypsin EDTA *(e.g. Invitrogen #25300)*

Generate a sheet of adherent cells

1. From an adherent culture at 90% confluence in a 60-mm tissue culture dish, detach with a 5 minute incubation with trypsin EDTA.

2. Use a hemacytometer to make a suspension of 20 million cells per ml (for more detail on cell counting, see Phelan, 2007).

   The culture of transfected cell lines may require selection reagents to maintain an episome or plasmid; however the selection reagents should be left out of the final culture as they may adversely affect the co-cultured tissue.

3. Place 10 µl drops onto the inside of an inverted 30-mm dish lid (see Figure 9.4).

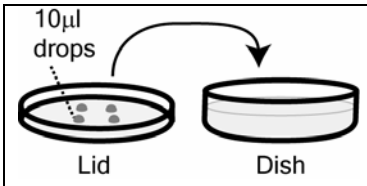

Figure 9.4 -Hanging drop culture. *10µl drops of 20 million adherent cells per milliliter are placed on the inside of a 35mm lid, which is then inverted onto the dish.*

4. To avoid evaporation, place 2 ml of sterile water or media in the bottom half of the dish

5. Gently turn over lid onto bottom of dish

6. Culture overnight in a 37°C, 5% CO_2 incubator

7. Gently invert lid onto a dissection microscope

8. The cells should form a uniform sheet

9. Gently push sheet down with closed forceps and, using the mouth pipette, transfer to L15/serum dissection media

10. Cut sheet into desired size using a tungsten needle *(see Support Protocol 1)*

REAGENTS AND SOLUTIONS

10X DMEM

80 ml dH_2O

Dissolve DMEM powder (sufficient for 1 L of 1X media)

PH to 1.8 with 10M HCl

Bring to 100 ml with dH_2O

Filter sterilize with a 0.2 μm syringe filter

Make aliquots and store at 4°C for up to 6 months.

10x $NaHCO_3$

9.0 ml dH_2O

Dissolve 0.22 g $NaHCO_3$

Add ~1 ml of 10M NaOH to produce a pink/red soln when diluted 8:1:1 (Collagen: DMEM: $NaHCO_3$)

Filter sterilize with a 0.2 μm syringe filter

Make aliquots and store at 4°C for up to 6 months

4% PFA, 25ml:

To 20ml of dH_2O

Add 1.0 g of paraformaldehyde

Add 35 μL of 1N NaOH

Cap loosely and heat in a fume hood with vigorous stirring at 60°C

Add 2.5ml of 10x PBS

PH to 1.2 with 1M HCl

Dilute to 25ml with dH_2O

Chill on ice

Remove debris by filtering with a 0.2 μm syringe filter.

Use within 24 hours

Neurobasal®/B-27 culture media:

Prepare fresh on the day of use

Neurobasal® supplemented with:

2% B-27

2 mM glutamax

100 units/ml penicillin & 100 μg/ml streptomycin

Sterilize with 0.2 μm syringe filter

Pre-heat to 37°C (>10 min)

Neurobasal®/FBS culture media:

Prepare fresh on the day of use

Neurobasal® supplemented with:

10% fetal bovine serum

2mM glutamax

100 units/ml penicillin & 100 μg/ml streptomycin

Sterilize with 0.2 μm syringe filter

Pre-heat to 37°C (>10 min)

COMMENTARY

Background Information

In rats, most spinal commissural neurons are born on the twelfth and thirteenth embryonic days (Bayer and Altman, 1995). Multiple cues contribute to guiding commissural axon , including BMPs , netrins, sonic hedge hog, Nr-CAM, B-class ephrins, Wnt4, semaphorins and slits (Figure 9.5, Moore and Kennedy, 2006a).

Critical Parameters and Troubleshooting

If axons fail to extend from dorsal spinal cord explants (Basic Protocol 2), the most common causes are that the explants are too large (well over 100μm) or that the explants were crushed during dissection. Ensuring that the tungsten dissecting needle is sharp helps resolve both these issues.

A common problem encountered during the dissection of E12.5 rat spinal cords (Basic Protocol 3) is that excess mesodermal tissue remains attached to the spinal cord. The most likely cause is insufficient trypsinization. It is also informative to note that two different turning assays are commonly used: the assay described here and another that puffs guidance cues from a micropipette onto the axons of dissociated neurons. A positive feature of the assay described here is that commissural axons extend within the native embryonic spinal neuroepithelium, migrating through an environment very similar to that

normally encountered *in vivo*. A limitation of the assay is that it relies on a cellular source for production of the cue, and as such, is limited to factors that can be produced and released by cells. Furthermore, the application of pharmacological reagents intended to act on commissural neurons may also affect release of the cue from the cellular source.

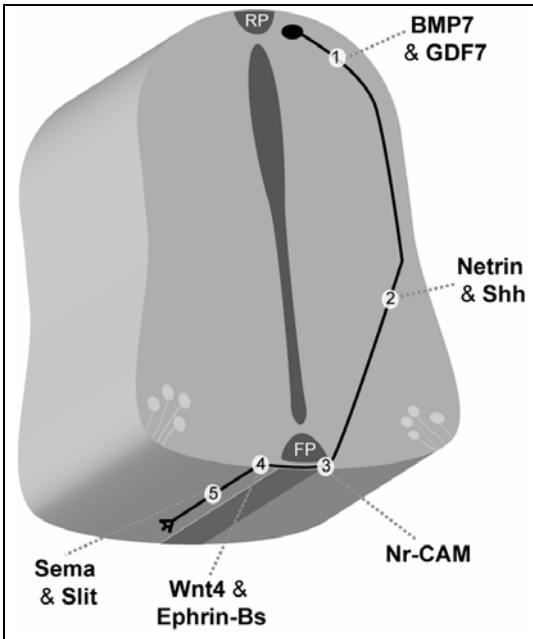

Figure 9.5 Five stages in the guidance of spinal commissural neuron axons. *(1) BMP7 and GDF7, released by the roof plate, repel commissural axons ventrally along the lateral edge of the embryonic spinal cord (Augsburger et al., 1999;Butler and Dodd, 2003). (2) Netrin-1 and sonic hedgehog (Shh), released by the floor plate, attract commissural axons to the ventral midline (Kennedy et al., 1994;Serafini et al., 1994;Charron et al., 2003). (3) A contact mediated interaction between the cell adhesion molecules tag-1 on the growth cone and Nr-CAM on floor plate cells is required for commissural axons to cross the midline (Stoeckli et al., 1997). (4) Most commissural axons then extend longitudinally towards the head; B-class ephrins and Wnt4 by the floor plate have been implicated in this turn ((Imondi and Kaprielian, 2001;Lyuksyutova et al., 2003). (5) Semaphorin and Slit family members prevent re-crossing of the midline and direct them out of the gray matter where they fasciculate into different longitudinal tracks (Zou et al., 2000;Long et al., 2004).*

Anticipated Results

Culturing dissociated dorsal spinal neurons (Basic Protocol 1) with floor plate conditioned media or purified netrin-1 will not increase commissural axon length (Placzek et al., 1990;Ming et al., 1997;Shekarabi et al., 2005), but will increase the number of filopodia and the size of the growth cone (Shekarabi et al., 2005).

When dorsal spinal cord explants (Basic Protocol 2) are cultured in standard culture media, few axons will exit the explant within 16 hours. In contrast, when cultured within ~250μm of a floor plate explant (Tessier-Lavigne et al., 1988), or in the presence of an outgrowth-promoting factor, such as netrin-1, large fasciculated bundles appear (Serafini et al., 1994). Purified netrin-1 and netrin-2 produce a bell-shaped outgrowth response, with maximal outgrowth evoked at ~150 ng/ml (Serafini et al., 1994). Netrin-3 also elicits similar outgrowth from dorsal spinal cord explants (Wang et al., 1999a). Although no other endogenous cues have been identified that promote outgrowth from dorsal spinal cord explants on their own, bone morphogenetic proteins can reduce netrin-1 induced outgrowth (Augsburger et al., 1999).

In whole spinal cord explants (Basic Protocol 3), commissural axons turn towards a floor plate explant over approximately 300μm (Placzek et al., 1990;Kennedy et al., 1994). Similarly, cells expressing netrin-1, netrin-2, or sonic hedgehog have been shown to deflect commissural axons over a similar distance (Kennedy et al., 1994;Charron et al., 2003).

Time Considerations

The spinal cords from an entire litter of E14.5 rats (approximately twelve embryos) can be isolated, dissociated and plated in less than 4 hrs. With practice, it takes less than 10 minutes to dissect each E14.5 spinal cord, while dissociation and plating takes approximately 40 minutes.

For an individual who has mastered these methods, two plates (eight wells) of dorsal spinal cord explants (Basic Protocol 2) can be dissected and plated in less than 3 hours; dissecting and cutting approximately forty explants takes less than an hour, and embedding the tissue takes an hour for the first plate and approximately 10 minutes per additional plate.

It takes about 6 hours to dissect and plate a litter of E12.5 rat spinal cords (Basic Protocol 3). Given that considerable time is saved by dissecting several simultaneously, up to four litters can be dissected in under 12 hours.

KEY REFERENCES

Goslin, K. and Banker, G. 1998. Culturing nerve cells. MIT Press, Cambridge, Mass.

Discusses both general and specific principles of neuronal culture.

Moore, S.W. and T.E.Kennedy. 2006. Axon Guidance during Development and Regeneration. *In* Textbook of Neural Repair and Rehabilitation (M. Selzer, S. Clarke, L. Cohen, P. Duncan, and F. Gage, eds.) pp 326-345. Cambridge University Press, Cambridge.

Provides an overview of axon guidance mechanisms and reviews spinal commissural axon guidance.

INTERNET RESOURCES

http://embryology.med.unsw.edu.au/embryo.htm

This website, created by Dr. Mark Hill of the University of South Wales in Sidney Australia, provides extensive information regarding the embryonic development of a variety of organisms.

CHAPTER 10

APPENDIX II: Netrin-1 is a chemorepellent for oligodendrocyte precursor cells in the embryonic spinal cord

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PREFACE

This appendix reports that netrin-1 repels oligodendrocyte precursor cells. These cells will eventually differentiate in the oligodendrocytes that myelinate axons within the central nervous system. My contribution to this paper was to generate and purify recombinant chick netrin-1 from stability transfected HEK 293 T cells using fast protein liquid chromatography (FPLC). This paper was published in the Journal of Neuroscience (Jarjour et al., 2003).

Acknowledgements

We thank Adriana Di Polo, Cecilia Flores, Alan Peterson, and Peter Braun for comments on the manuscript; and Mireille Bouchard, Talan Basmascioglu, Michel Gravel, and Laurence Simard-Edmond for technical assistance. This work was supported by the Multiple Sclerosis Society of Canada and the Canadian Institutes of Health Research. A.A.J. was supported by FCAR and Multiple Sclerosis Society of Canada studentships. T.E.K. is a CIHR Scholar.

ABSTRACT

Netrin-1, secreted by floor plate cells, orients axon extension in relation to the ventral midline of the embryonic spinal cord. Oligodendrocyte precursor (OP) cells are born

close to the ventral midline and migrate away from the floor plate. Here we show that OP cells, identified by expression of the PDGFα receptor, express the netrin receptors dcc and unc5h1, but do not express netrin-1. Using a microchemotaxis assay, we demonstrate that migrating OPs are repelled by a gradient of netrin-1 in vitro. Furthermore, application of netrin-1 to OPs in vitro triggers retraction of OP processes. In the absence of netrin-1 or DCC function in vivo, fewer OP cells migrate from the ventral to the dorsal embryonic spinal cord, consistent with netirn-1 acting as a repellent. In addition to their role regulating cell movement, DCC and UNC-5 homologues have been suggested to function as pro-apoptotic dependence receptors, triggering cell death in the absence of netrin-1. In contrast, we report no evidence of increased OP cell death in vivo or in vitro in the absence of either netrin-1 or DCC. These findings indicate that netrin-1 is a repellent cue for migrating OPs in the embryonic spinal cord.

INTRODUCTION

During the development of the central nervous system (CNS), many neural cell types migrate great distances to reach their final destinations. While neuronal migration has been studied extensively, the cues that direct the migration of oligodendrocyte precursors (OP) are not well understood. Several factors that influence OP motility in vitro have been identified. Basic fibroblast growth factor (bFGF or FGF-2) and the platelet-derived growth factor A chain are soluble chemoattractants for OPs (Armstrong et al., 1990;Milner et al., 1996;Simpson and Armstrong, 1999), and reduced numbers of Ops (Armstrong et al., 1990;Simpson and Armstrong, 1999) and oligodendrocytes have been found in PDGF-A knockout mice (Fruttiger et al., 1999). However, because PDGF-A is also a mitogen for OPs (Noble et al., 1988;Calver et al., 1998), this phenotype may be due to deficient OP migration, proliferation, or both. Substrates of the extracellular matrix (ECM) proteins laminin, fibronectin, or vitronectin promote OP migration (Milner et al., 1996) while tenascin C and collagen are non-permissive substrates for migrating OPs (Milner et al., 1996;Kiernan et al., 1996). The rate of OP migration increases in tenascin-C null mice, but tenascin C does not appear to direct OP cell migration (Garcion et al., 2001). Sugimoto et al (2001) have provided evidence that both Sema3A and netrin-1 are

chemorepellents for OP cells migrating from explants of newborn rat optic nerve (Sugimoto et al., 2001). In contrast, using a similar in vitro assay, Spassky et al (2002) conclude that netrin-1 attracts OP cells migrating from explants of embryonic optic nerve (Spassky et al., 2002). Spassky et al. (2002) also provide evidence for Sema3F attracting migrating OPs, and, in agreement with Sugimoto et al. (2002), that Sema3A is a chemorepellent for these cells. While these experiments address OP migration in vitro, if these cues direct OP migration in vivo is not clear.

In the embryonic spinal cord, OPs originate in the ventral ventricular zone, at two foci located on either side of the midline, slightly dorsal to the floor plate (Pringle and Richardson, 1993;Yu et al., 1994;Ono et al., 1995;Orentas and Miller, 1996). Beginning at ~E12.5 in the mouse, OPs disperse throughout the developing spinal cord, migrating away from the ventral midline (Calver et al., 1998). This trajectory suggests that cues produced by floor plate cells may repel migrating OP cells.

We postulated that netrin-1 might function as a repellent for OP cells in the embryonic spinal cord. Netrin-1 is produced at the ventral midline of the embryonic neural tube where it repels some types of migrating axons and attracts others (Kennedy et al., 1994;Colamarino and Tessier-Lavigne, 1995;Varela-Echavarria et al., 1997). Netrin-1 also directs the circumferential migration of neuronal precursor cells (Varela-Echavarria et al., 1997;Przyborski et al., 1998;Alcantara et al., 2000;Hamasaki et al., 2001). Here we show that netrin-1 is expressed by floor plate cells as OP cells migrate away from the ventral midline of the developing spinal cord. A netrin receptor complex composed of DCC and an UNC-5 homolog mediates the repellent response to netrin-1 (Hong et al., 1999). We report that both dcc and unc5h1 are expressed by migrating OP cells in the embryonic spinal cord. To test the hypothesis that netrin-1 might influence OP motility, we used an in vitro microchemotaxis assay and found that a gradient of netrin-1 repels the migration of cultured OP cells. Application of netrin-1 to OP cells in vitro caused the retraction of OP processes, consistent with a repellent function. Furthermore, we report that the distribution of OP cells is disrupted in the spinal cords of mouse embryos lacking DCC or netrin-1. Importantly, the change in cell distribution occurs without a change in cell number, indicating that the absence of netrin-1 or DCC does not affect cell survival.

These findings indicate that netrin-1 functions as a repellent guidance cue for OP cell migration in the embryonic spinal cord.

MATERIALS AND METHODS

Animals and oligodendrocyte precursor cell culture

Sprague Dawley rat pups and pregnant Balb/c mice were obtained from Charles River Canada (QC). Mice heterozygous for netrin-1 or DCC function were obtained from Marc Tessier-Lavigne (Stanford) and Robert Weinberg (Harvard), respectively. All procedures with animals were performed in accordance with the "Canadian Council on Animal Care" guidelines for the use of animals in research. Oligodendrocyte precursor cells were obtained from mixed glial cultures derived from the cerebral cortices of P0 rat pups as described (Armstrong, 1998).

Antibodies, immunocytochemistry, and immunohistochemical quantification

The following antibodies were used: monoclonal anti-NG2 (Chemicon, CA), anti-DCC intracellular domain (anti-DCCIN, G97-449), anti-DCC function blocking (DCCFB, AF5, Calbiochem, CA); polyclonal anti-PDGFαR (C-20, Santa Cruz Biotech, CA), and anti-netrin PN2 (Manitt et al., 2001). A2B5 hybridoma was provided by V.W. Yong (U. Calgary).

For live labeling with A2B5, cells were incubated for 30 min at 4□C with hybridoma supernatant. Unbound antibody was washed away with DMEM. Cells were then washed with PBS, fixed with 4% paraformaldehyde (PFA), permeabilized with PBS containing 0.25% Triton X-100, and blocked with 3% heat-inactivated horse serum (HS)/1% bovine serum albumin/0.1% Triton X-100. Cells were then incubated with anti-DCC or anti-netrin PN2 diluted in blocking solution. NG2 labeling was similarly carried out on fixed cells without the live labeling step. Primary antibodies were detected with secondary antibodies coupled to Cy3 or Alexa 488 (Molecular Probes). Nuclei were stained with Hoechst dye.

For immunohistochemical analyses, netrin-1 or DCC heterozygous mice were crossed and E15 embryos obtained (plug date designated E1). Embryos were frozen in 2-

methyl butane (Fisher) chilled in liquid nitrogen, then mounted individually in optimal cutting temperature compound (O.C.T. Tissue Tek, Sakura Finetek, CA), 6 μm cryostat sections of the spinal brachial enlargement cut, mounted onto slides (Superfrost Plus, Fisher) and fixed by immersion in 4% PFA, 15% picric acid (pH 8.5) in PBS (45 min, rt). The sections were rinsed in PBS, permeabilized with 0.5% Triton X-100 in PBS, and rinsed in PBS. To enhance antigenicity, sections were immersed in boiling PBS in a microwave oven for 11 min., cooled, and blocked (5% HINHS, 1% BSA in PBS; 1 hr, rt). Sections were then incubated with anti-PDGFαR (1:1000) in blocking solution (overnight, 4°C) and visualized using Alexa 488-conjugated secondary antibody. Sections were stained with Hoechst dye to visualize nuclei. Fluorescence was visualized using a Carl Zeiss Axiovert 100 microscope, a Magnafire CCD camera (Optronics, CA), and Northern Eclipse Software (Empix Imaging, ON). To analyze the distribution of OPs, each spinal cord section was photographed in four quadrants using a 20 X objective. The four images were assembled into a single image of a complete spinal cord using Photoshop (Adobe, CA). The length of the dorsal-ventral axis was measured along the midline from the top of the roof plate to the base of the ventral commissure, and each cord divided into thirds. A cell was counted only if anti-PDGFαR staining encircled a blue Hoechst-stained nucleus. Due to this criterion, fewer cells were counted in this analysis per section of cord relative to previous studies using PDGFαR mRNA in situ hybridization (Calver et al., 1998). Three sections were counted per embryo. Non-adjacent sections were counted to avoid counting the same cell more than once.

Transfilter microchemotaxis assay

OP-enriched cell suspensions were plated at a density of 1.25 x 105 cells/ml on poly-D-lysine-coated polycarbonate transwell culture inserts (6.5 mm diameter with 8 μm pore size, Corning). 100 μl of cell suspension was used per filter, and the filters placed in the wells of a 24-well tissue culture plate over 600 μl of medium. OLDEM was the base medium used for all assay conditions (DMEM, 5 μg/ml insulin, 100 μg/ml transferrin, 30 nM sodium selenite, 30 nM triiodothyronine, 6.3 ng/ml progesterone, 16 μg/ml putrescine, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamax). Cells were

allowed to migrate for 16 hrs at 37°C, cells on the upper side of the filter were then scraped off, and the cells attached to the lower side of the filter fixed with 4% paraformaldehyde/0.1% glutaraldehyde (30 min, rt). Filters were then rinsed with PBS, and cell nuclei stained with Hoechst dye. Cell nuclei were imaged using epiflorescence. For each transwell assay, a single image of the filter was captured using a 4 X objective and nuclei counted using Northern Eclipse software. Where pooled results are presented, the value 'percent migration vs control' (Figure 10.3C) for a given trial represents the number of cells migrated in that condition expressed as a percentage of the mean number of cells migrating in control conditions.

Analysis of OP morphology

Dissociated OP cells were plated in OLDEM at a density of 2.5 x 104 cells/well in an 8-well plastic chamber slide pre-coated with poly-D-lysine. Cells were maintained in culture overnight, and the medium was then replaced with either fresh OLDEM (as a control) or OLDEM plus the factors being tested. Following incubations of 30 minutes, 2 hours, or 16 hours, the cells were fixed and labeled with rhodamine-conjugated phalloidin, anti-PDGFαR (visualized using Alexa 488-conjugated secondary antibody), and Hoechst as described above. The surface area, length of longest process, and number of major processes of PDGFαR-positive OPs were measured using rhodamine-phalloidin staining and Northern Eclipse software. Surface area corresponds to the two-dimensional surface area of the entire OP cell, including the cell body and all processes. The length of the longest process is the measurement from the base of the longest process to its tip. A major process refers to a terminal process whose length exceeded the maximum diameter of the cell body.

MTT assay

Dissociated OP cells were plated in OLDEM at a density of 1 x 104 cells/well in a 96-well tissue culture plate pre-coated with poly-D-lysine. Cells were grown in culture for 16 hrs, followed by one additional hr in the presence of 0.5 mg/ml MTT (Sigma). The medium was then aspirated, and the cells dissolved in isopropanol (100 μl/well).

Absorbance of the converted dye (ΔO.D.) was measured at 570 nm with background subtraction at 630 nm (Denizot and Lang, 1986).

Statistical analyses

All comparisons were carried out by ANOVA with Fisher's Least-Significant-Difference post-hoc test, and were performed using SYSTAT software (SPSS, Il).

In situ hybridization

Sense and antisense cRNA probe pairs corresponding to netrin-1 (Manitt et al., 2001), dcc (Keino-Masu et al., 1996), unc5h1 and unc5h2 (Leonardo et al., 1997), and unc5h3 (Ackerman et al., 1997) were used. Cryostat sections of E15 mouse spinal cord were cut and fixed, synthesis of digoxigenin (DIG) labeled probes, and hybridization, were carried out as described (Manitt et al., 2001). For in situ hybridization analysis of expression in vitro, OP cells were cultured using Lab-Tek chamber slides (Fisher) and fixed with 4% PFA. Hybridization was carried out overnight at 57°C (netrin-1), 57°C (dcc), 57°C (unc5h1), 60°C (unc5h2), and 57°C (unc5h3), followed by a stringent wash in 2X SSC for 1 hr at 65°C. Slides were blocked (0.5 % blocking powder in 1 X PBS; NEN, MA) for 30 min rt. Hybridization was detected using a peroxidase-coupled antibody against DIG (Roche, QC). Incubation with anti-PDGFαR was carried out during this step. In situ signals were amplified using the TSA-Indirect (ISH) Tyramide Signal Amplification kit (NEN, MA), and visualized using Alexa 594-conjugated streptavidin. Alexa 488-conjugated anti-rabbit IgG secondary antibody was used to visualize PDGFαR immunostaining.

RESULTS

Netrin-1 is expressed at the ventral midline of the developing spinal cord during oligodendrocyte precursor migration

In the embryonic spinal cord, OP cells are born in the ventricular zone just dorsal to the floor plate. These cells then migrate away to populate all regions of the cord (Pringle and Richardson, 1993;Yu et al., 1994;Ono et al., 1995;Orentas and Miller, 1996;Diers-Fenger

et al., 2001). OP cells first appear in the embryonic mouse spinal cord at ~E12.5 and are distributed throughout the mouse brachial spinal cord by E15 (Pringle and Richardson, 1993;Calver et al., 1998). Netrin-1 is known to be expressed in the floor plate and ventral neuroepithelium of the E11.5 spinal cord (Serafini et al., 1996), but expression had not been examined later in development during OP cell migration. Using in situ hybridization analysis we show that at E15, floor plate cells continue to express netrin-1 as OPs migrate through the neuroepithelium (Figure 10.1A). Double labeling with an antibody against the PDGFαR, a marker specific for OP cells in the embryonic spinal cord, indicated that OPs do not express detectable levels of netrin-1 at E15 (Figure 10.1A, B). Thus, expression of netrin-1 is temporally and spatially consistent with it having a role as an OP repellent.

Oligodendrocyte precursor cells express the netrin receptors dcc and unc5h1, but not unc5h2 or unc5h3 in the E15 mouse spinal cord

DCC and UNC-5 homolog family members mediate the chemorepellent response of migrating neurons and axons to netrin-1 (Hedgecock et al., 1990;Hamelin et al., 1993;Przyborski et al., 1998;Hong et al., 1999;Goldowitz et al., 2000;Keleman and Dickson, 2001;Hamasaki et al., 2001). By labeling OP cells with anti-PDGFαR in combination with in situ hybridization analysis we investigated the expression of dcc, unc5h1, unc5h2, and unc5h3 by OPs in E15 spinal cord. Dcc and unc5h1 expression was detected in most, if not all, PDGFαR-positive OPs (Figure 10.1C-F), supporting the hypothesis that these cells could respond to netrin-1. Unc5h2 and unc5h3 expression was not detected in oligodendrocyte precursors at E15 (not shown).

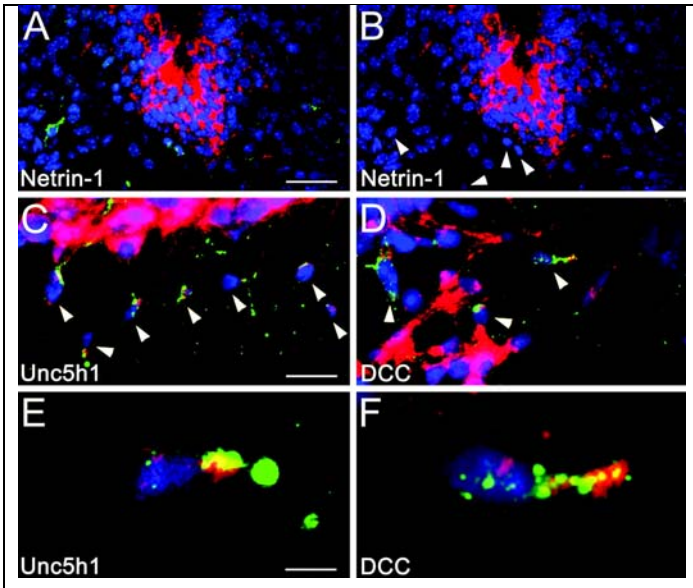

Figure 10.1 – Oligodendrocyte precursors express dcc and unc5h1, but not netrin-1, in vivo. *Double-label in situ hybridization-immunohistochemical analyses of coronal sections of E15 mouse spinal cord. Cell nuclei are stained blue with Hoechst dye. (A and B) In situ hybridization identifies netrin-1 expressing floor plate cells (red). Panel B illustrates that the PDGFαR immuno-positive OPs in panel A (green) do not express netrin-1. Panels C and D show ventral spinal cord white matter and motoneurons at the edge of the gray matter. Panel C shows that PDGFαR immuno-positive OPs (green) express unc5h1 (red, in situ hybridization). Panel E presents a four-fold magnification of one cell from panel C, illustrating double-labeling. Panel D shows that PDGFαR immuno-positive OPs (green) express dcc (red, in situ hybridization). Panel F depicts a four-fold magnification of one cell from panel D, illustrating double-labeling. No in situ hybridization signal was detected in PDGFαR positive OPs (green) using either the unc5h1 or the dcc control sense hybridization probes (not shown). Arrowheads in panels C and D indicate double-labeled cells. The large PDGFαR negative dcc and unc5h1 positive cells in the gray matter are motoneurons. Objective magnification: (A and B) 20X, (C-F) 40X. Scale bars: (A and B) 40 μm, (C and D) 20 μm, (E and F) 5 μm.*

Netrin-1 repels migrating oligodendrocyte precursors in vitro

To directly test the hypothesis that netrin-1 repels OP migration, cultures enriched for OP cells were prepared from the cerebral cortices of newborn rat brain as described (Armstrong, 1998). OP cells were identified using the A2B5 monoclonal antibody (Raff et al., 1983), polyclonal antibodies against NG2 (Stallcup and Beasley, 1987), or polyclonal antibodies against PDGFαR, all markers of OPs but not mature oligodendrocytes. Double labeling with these markers and either antibodies against netrin or DCC, or in situ hybridization for unc-5 homolog expression was carried out 24 hrs

after plating OP cells. These findings indicated that under these conditions in vitro, all OP cells express DCC (Figure 10.2A, B) and unc5h1 (Figure 10.2C, D) but not netrin-1 (Figure 10.2E). These results are consistent with our findings in the E15 spinal cord (Figure 10.1).

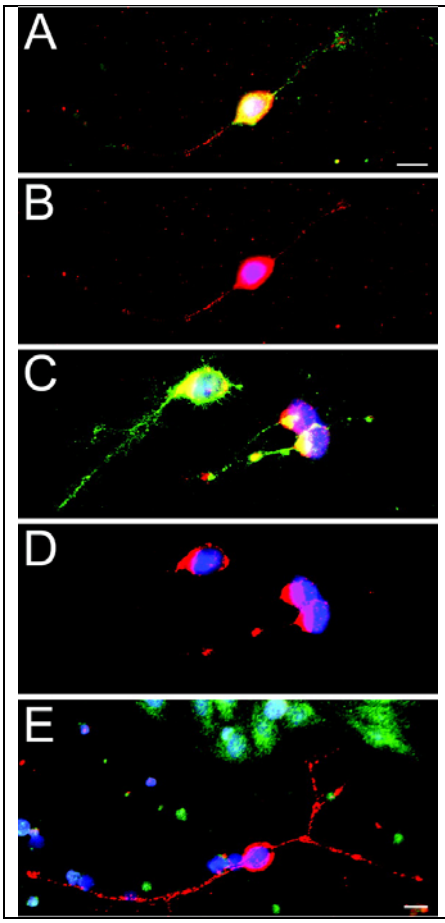

Figure 10.2 - Oligodendrocyte precursors express dcc and unc5h1, but not netrin-1, in vitro. *(A) OPs co-immunolabel with antibodies against the OP marker NG2 (green) and the netrin receptor DCC (red). Panel B illustrates the same cell as in (A), showing only the DCC immunostaining (red) and Hoechst (blue). Panel C shows cells double-labeled with anti-PDGFαR (green) and with unc5h1 anti-sense RNA probes (red). Panel D illustrates the same cell as in (C), showing only the unc5h1 anti-sense signal. The corresponding unc5h1 sense RNA probe produced no signal (not shown). Panel E illustrates that OPs, immunolabeled with the A2B5 monoclonal antibody (red) in a mixed glial culture are not labeled by an antibody against netrin-1 (green). Objective magnification: (A, B, C and D) 100X, (E) 40X. Scale bar corresponds to 10 μm in all panels.*

We then determined if netrin-1 influences OP migration using a transfilter microchemotaxis assay (described in Falk et al., 1980), an established method of analyzing OP cell motility (Armstrong et al., 1990;Simpson and Armstrong, 1999;Frost et al., 2000). Cells were plated onto the top surface of a polycarbonate filter containing pores 8 μm in diameter that was suspended in the well of a tissue culture plate (Figure 10.3A). Putative tropic factors can be tested by adding them to the medium beneath the filter, to the cell suspension prior to plating on top of the filter, or to both the top and bottom compartments. Cells migrating into a pore from the top of the filter can be

challenged with an increasing gradient of the putative cue (cue on bottom), a decreasing gradient of the cue (cue on top), or an equal concentration of the cue on both sides (cue on top and bottom). Cells initially adhere to the upper surface of the filter. During migration, either spontaneously or in response to an added factor, some cells enter a pore and move to the lower side of the filter. At the end of the assay, which lasts 16 hrs, cells that remain on the upper side of the filter are scraped off and the cells that have migrated to the lower side of the filter are fixed in place, stained, and counted. An attractant in the bottom chamber enhances migration from the top to the bottom of the filter. Conversely, a repellent cue in the lower chamber will reduce migration from the top to the bottom. A cue that has a kinetic effect will similarly influence migration irrespective of being placed in the bottom, top, or both chambers.

Figure 10.3B presents data from a single representative trial using the microchemotaxis assay. In the absence of any added cue, 187 ± 16 (mean ± SEM per 4x field) OP cells spontaneously migrated to the bottom of the filter. PDGF-AA (20 ng/mL), a known OP cell chemoattractant, increased migration (372 ± 25 cells) when added to the bottom compartment. In contrast, when OPs were challenged with 100 ng/ml netrin-1 in the bottom compartment, migration decreased significantly (100 ± 7 cells). Addition of 100 ng/ml netrin-1 to the top compartment caused an increase in the number of cells migrating to the lower side of the filter (223 ± 21 cells; Figure 10.3B). When the results from multiple experimental trials were pooled (Figure 10.3C), the increase in the number of cells migrating away from netrin-1 in the top compartment was found to be significant. Interestingly, when OPs were exposed to netrin-1 (100 ng/ml) added to both the top and bottom compartments (Figure 10.3B) migration was reduced (95 ± 8 cells) to a level not significantly different from that obtained with netrin-1 in the lower chamber alone. Immunostaining the cells plated on the top of the filter, or the cells that migrated to the lower side of the filter, demonstrated in both cases that ~90% of the cells present were A2B5 or PDGFαR positive (not shown).

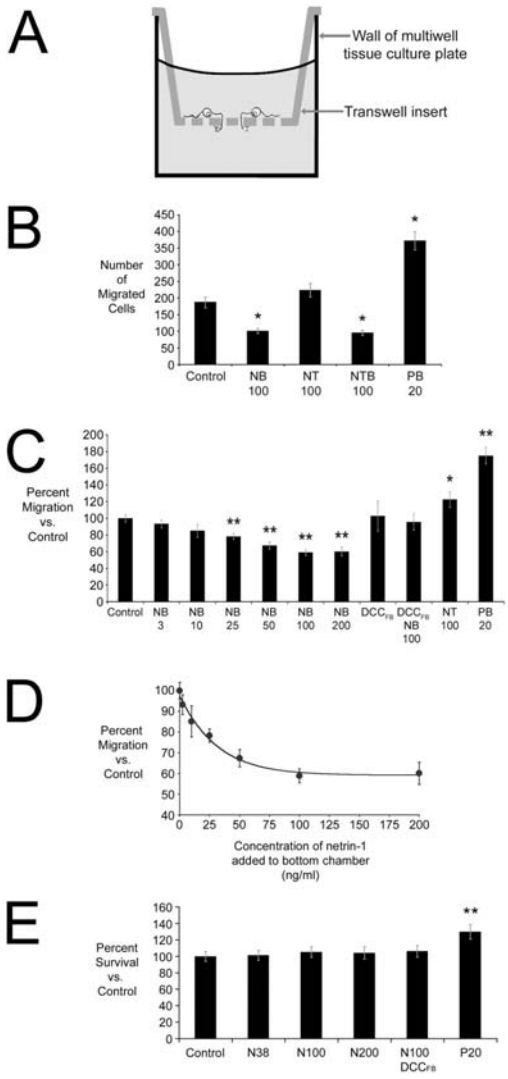

Figure 10.3 - Netrin-1 is a chemorepellent for oligodendrocyte precursor cells in vitro. *(A) Illustration of the transfilter microchemotaxis assay. (B-D) Netrin-1 repels OP cells. Panel B shows the data from one representative trial of this experiment. 100 ng/ml netrin-1 in the bottom compartment (NB100) significantly reduced cell migration compared to control. Increased migration to the bottom chamber was seen when 100 ng/ml netrin-1 was added to the top compartment (NT100, B and C). The OP chemoattractant PDGF-AA (20 ng/ml, bottom) was used as a positive control (PB20). For each condition shown in B, n=4 wells per condition. Panel C presents results pooled from multiple experimental trials and expressed as percent of control. Increasing concentrations of netrin-1 (3-200 ng/ml, NB3 to NB 200) produce a graded increase in the repellent action of netrin-1. Addition of the DCC function blocking antibody (2.7 μg/ml) to the top and bottom chambers blocked the repellent action of netrin-1 in the bottom chamber (DCCFB NB100). In the presence of DCCFB without added netrin-1 the same number of cells migrated as did in control. In panel C, n=22 for control, n=8 for NB3, NB10, NB25, NB50, NB200, DCCFB, DCCFB NB100, and NT100, n=16 for NB100, and n=21 for PB20. (D) Analysis of the results presented in (C) indicated that ~20 ng/ml netrin-1 produced a half-maximal repellent effect of netrin-1. (E) The MTT assay indicated that application of netrin-1 at 0, 38, 100, or 200 ng/ml for 16 hrs does not affect OP survival (n=6). Addition of DCCFB with 100 ng/ml netrin-1 also had no effect. PDGF-AA, an established mitogen for OP cells, resulted in an increased number of OPs after 16 hrs. Values shown are the mean ± SEM. *: $p < 0.05$; **: $p < 0.005$.*

DCC contributes to mediating both attractant and repellent responses of neuronal growth cones to netrin-1 (Hong et al., 1999). To determine if DCC is required for the repellent response of OP cells to netrin-1, OP cells were challenged with netrin-1 in the lower chamber in the presence of a DCC function blocking antibody added to the top and bottom chambers (2.7 μg/ml, DCCFB). Addition of DCCFB blocked the response to netrin-1, producing migration not significantly different from control (Figure 10.3C).

We carried out a dose response analysis of the repellent action of netrin-1 using the transfilter migration assay. Cells were challenged with increasing concentrations of netrin-1, from 3 ng/ml to 200 ng/ml, added to the bottom chamber. Increasing concentrations of netrin-1 resulted in fewer cells migrating across the filter (Figure 10.3C). These values were best fit using a sigmoidal curve and the EC50 for the repellent response of OP cells to netrin-1 determined to be ~20 ng/ml (Figure 10.3D).

Netrin-1 has been proposed to have trophic effects (Mehlen et al., 1998;Llambi et al., 2001;Forcet et al., 2001) in addition to its well-documented function as a tropic guidance cue. This raised the possibility that netrin-1 might influence transfilter migration through an effect on cell survival, and not cell motility. We directly tested if the presence or absence of netrin-1 influences the survival or proliferation of OP cells in vitro. We determined if netrin-1 influences OP survival or proliferation over a period of 16 hrs in culture, the duration of the migration assay, using MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), as previously described (Denizot and Lang, 1986;Richter-Landsberg and Vollgraf, 1998;Pang et al., 2000). Quantification of the amount of metabolized MTT indicated that there was no difference between cells cultured without netrin-1 and cells cultured with 38 ng/ml, 100 ng/ml, 200 ng/ml netrin-1, or 100 ng/ml netrin-1 and 2.7 μg/ml DCCFB (Figure 10.3E). These observations indicated that the presence or absence of netrin-1 does not affect the survival of these cells. In contrast, increased MTT conversion was detected in the presence of 20 ng/ml PDGF-AA (Figure 10.3E). However, the ~30% increase in dye production is not sufficient to account for the ~75% increase in the number of cells detected on the underside of the filter at the end of the migration assay, consistent with PDGF being both a mitogen and a chemoattractant

for OPs, as previously described ((Noble et al., 1988;Armstrong et al., 1990;Milner et al., 1996;Calver et al., 1998;Simpson and Armstrong, 1999).

Netrin-1 induces retraction of oligodendrocyte precursor cell processes

Partial collapse of the cytoskeleton has been proposed to contribute to the turning response made by axonal growth cones to repellent guidance cues (Luo et al., 1993). To test the hypothesis that the repellent action of netrin-1 might trigger cytoskeletal collapse and process retraction, OPs were incubated in vitro with 100 ng/ml netrin-1 for 30 minutes, 2 hours, and 16 hours. OPs were then labeled with an antibody against PDGFαR and for filamentous action (F-actin) using rhodamine-coupled phalloidin. Addition of netrin-1 caused a rapid and persistent decrease in OP surface area, process length, and process number at all time points. Addition of 2.7 μg/ml DCCFB blocked the effect of netrin-1 at all time points examined. Application of DCCFB alone had no effect (Figure 10.4, Table 10.1).

Aberrant distribution of oligodendrocyte precursors in mice lacking netrin-1 or DCC

To determine if netrin-1 contributes to directing OP migration in vivo, we examined the distribution of OP cells in E15 mouse embryos lacking functional netrin-1 or DCC. Although the optic nerve has been widely used as a model system to study oligodendrocyte development, in netrin-1 or DCC knockout mice the axons of retinal ganglion cell neurons do not enter the optic nerve, producing optic nerve hypoplasia (Deiner et al., 1997). We therefore focused our analysis of the distribution of OP cells in vivo on the E15 spinal cord. Heterozygous netrin-1 or DCC mice were crossed, producing litters containing wild type, heterozygote, and homozygous loss of function embryos. Quantitative comparisons were performed within litters to maintain a precise age-match between embryos.

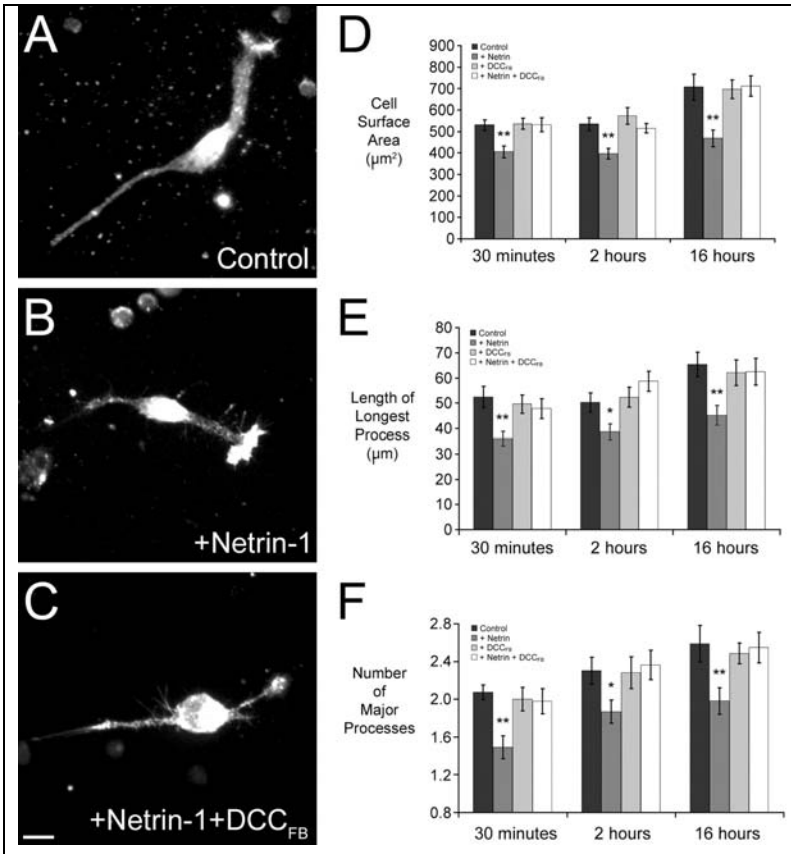

Figure 10.4 - Retraction of OP processes induced by netrin-1. *OP cultures were exposed to netrin-1, DCCFB, or both netrin-1 and DCCFB, for 30 minutes, 2 hours, or 16 hours; fixed, and then stained for PDGFαR immunoreactivity and F-actin using rhodamine-coupled phalloidin. Phalloidin staining is shown in A, B, and C. Cell surface area, length of longest process, and number of major processes were then quantified. (A, B, C) Images of a control cell (A), and cells exposed to netrin-1 (B), or netrin-1 and DCCFB (C) for 30 minutes. The cells illustrated in panels A, B, and C, have morphologies corresponding to the mean values shown in D, E, and F at the 30 min time point. Exposure of OP cells to netrin-1 for 30 minutes, 2 hours, or 16 hours results in decreased mean cellular surface area (D), length of the longest process (E), and number of major processes (F). Results obtained following incubation with DCCFB or co-incubation of OPs with netrin-1 and DCCFB did not differ significantly from control. Values shown are the mean ± SEM. *: $p < 0.05$; **: $p < 0.005$. Objective magnification is 40X. Scale bar corresponds to 10 μm.*

Surface Area (µm2)

	30 minutes	2 hours	16 hours
Control	530.3±24.9	533.6±30.8	706.1±60.9
Netrin-1	406.2±27.7	396.9±25.3	468.1±38.4
DCCFB	536.2±26.0	573.1±39.1	696.6±44.1
Net+DCCFB	531.6±33.0	515.3±22.1	712.3±46.8

Length of Longest Process (µm)

	30 minutes	2 hours	16 hours
Control	52.4±4.2	50.3±3.8	65.4±4.9
Netrin-1	36.1±2.8	38.7±3.1	45.2±3.9
DCCFB	49.7±3.6	52.3±4.0	62.1±5.1
Net+DCCFB	47.9±4.0	58.7±3.9	62.5±5.4

Number of Major Processes

	30 minutes	2 hours	16 hours
Control	2.07±0.08	2.30±0.14	2.59±0.19
Netrin-1	1.49±0.12	1.87±0.12	1.98±0.14
DCCFB	2.00±0.13	2.28±0.17	2.49±0.11
Net+DCCFB	1.98±0.13	2.36±0.16	2.55±0.16

n

	30 minutes	2 hours	16 hours
Control	54	46	34
Netrin-1	57	53	56
DCCFB	54	39	35
Net+DCCFB	50	33	42

Table 10.1 - Retraction of OP processes by netrin-1. *Netrin-1 induces a rapid and persistent retraction of OP processes, an effect that is DCC-dependant. OP surface area, process length, and process number (mean ± S.E.M.) were measured as described in Materials and Methods. n is the number of cells counted for a given condition and time point.*

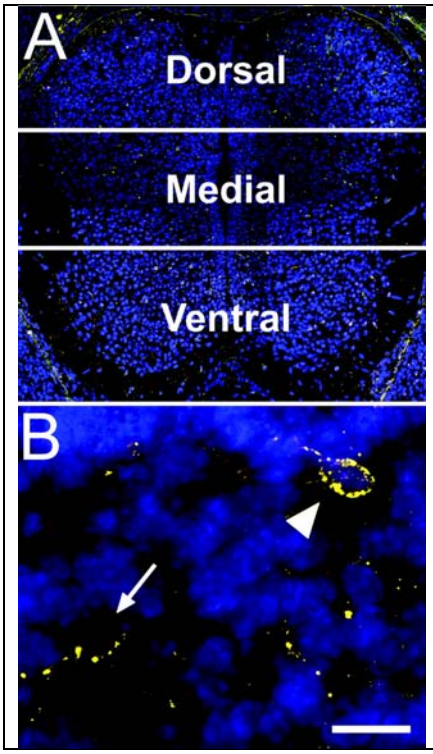

Figure 10.5 - Analysis of OP cell distribution in E15 spinal cord. *(A) Coronal section of the brachial enlargement of an E15 spinal cord immunolabeled with anti-PDGFαR (yellow), and nuclei stained blue with Hoechst dye. For each section, four images were collected and tiled into a single image. The dorso-ventral height of the cord was measured at the midline, the cord divided into dorsal, medial, and ventral thirds, and the number of PDGFαR-positive OPs in each third counted. Only cells with Hoechst-positive nuclei encircled by PDGFαR immunoreactivity (B, arrowhead) were counted. Immunoreactivity not meeting this criterion (B, arrow) was excluded.*

Because development of the oligodendroglial lineage progresses along a rostral-caudal gradient in the spinal cord (Foran and Peterson, 1992;Hajihosseini et al., 1996), tissue sections were collected exclusively from the brachial enlargement. OPs were detected with anti-PDGFαR. For quantification, the image of each cross section of the spinal cord was divided into dorsal, medial, and ventral thirds along the dorsal-ventral axis (Figure 10.5A). A cell was counted only if anti-PDGFαR staining encircled a blue Hoechst-stained nucleus (Figure 10.5B). This analysis indicated that in embryos homozygous for loss of netrin-1 or DCC function, significantly fewer OP cells were present in the dorsal third of the spinal cord. A corresponding increase was found in the number of cells present in the ventral third of the embryonic spinal cord in the absence of netrin-1 or DCC function (Figures 10.6, 10.7, Table 10.2).

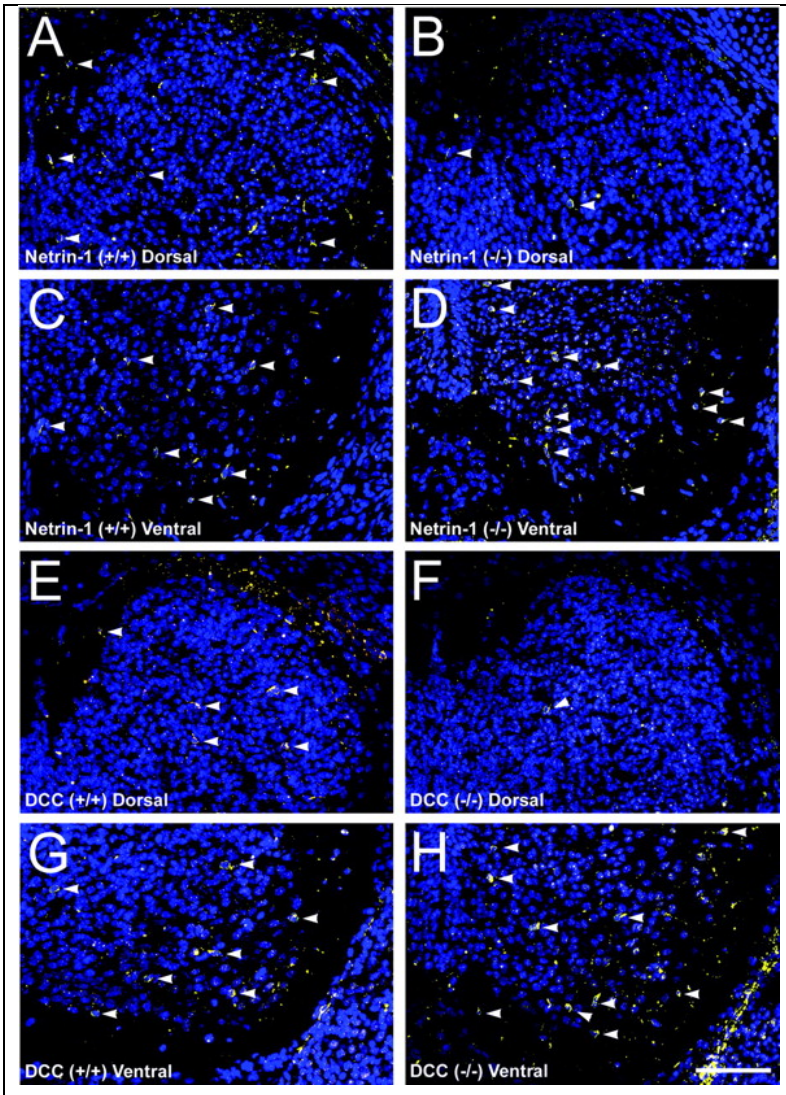

Figure 10.6 - Distribution of OP cells in E15 spinal cord sections. *The absence of netrin-1 or DCC function produces an aberrant distribution of OP cells in E15 spinal cord. Fewer PDGFαR-positive OP cells (arrowheads) were detected in the dorsal spinal cords of netrin-1 (B) or DCC (F) knockout (-/-) embryos compared to wild-type (+/+) littermates (A, E). Conversely, more OP cells were detected in the ventral spinal cords of netrin-1 (D) or DCC (H) knockout (-/-) embryos compared to their wild-type (+/+) littermates (C, G). 20 X objective, scale bar is 100 µm.*

Mean # of OP / region of s.c

Genotype	Dorsal	Medial	Ventral	Total	n
Netrin-1+/+	13.9±0.8	15.1±2.62	2.9±3.0	51.9±3.7	9
Netrin-1+/-	13.2±3.2	15.6±2.4	23.8±3.6	52.5±7.2	18
Netrin-1-/-	7.4±1.7	13.6±3.5	28.4±4.0	49.4±8.3	9
DCC+/+	11.3±1.6	14.8±2.2	24.5±2.4	50.7±4.3	12
DCC+/-	9.5±1.4	14.1±2.2	23.3±4.1	47.0±4.8	15
DCC-/-	5.6±1.7	12.4±1.4	30.3±2.1	48.3±3.6	12

Table 10.2 - Distribution of PDGFαR-positive OP cells in wild-type, netrin-1, or DCC-deficient E15 mouse spinal cord. *Spinal cord sections from E15 mouse embryos were collected and processed as described in Materials and Methods. Images of spinal cord sections were divided into dorsal, medial and ventral thirds, and the number of OPs in each third counted. An OP was only counted if PDGFαR immunostaining surrounded a Hoechst-stained nucleus. OP number is mean ± SD. n is tissue sections analyzed per condition.*

Importantly, the total OP cell number in sections of homozygous netrin-1 or DCC loss of function embryos was not significantly different from their heterozygote or wild-type littermates (Figure 10.7B, D). To age match the embryos as precisely as possible, the data presented in Figure 10.7 and Table 10.2 is restricted to embryos derived from a single litter. The same total number of PDGFαR positive cells was not always found in E15 spinal cords when compared between litters, likely reflecting the lack of a precise age match. However, analysis of multiple litters always revealed the phenotype reported in Figure 10.7 and Table 10.2. These results indicate that the absence of netrin-1 or DCC function produces a dramatic change in the distribution of these cells, consistent with DCC being required to mediate a repellent response of OP cells to netrin-1.

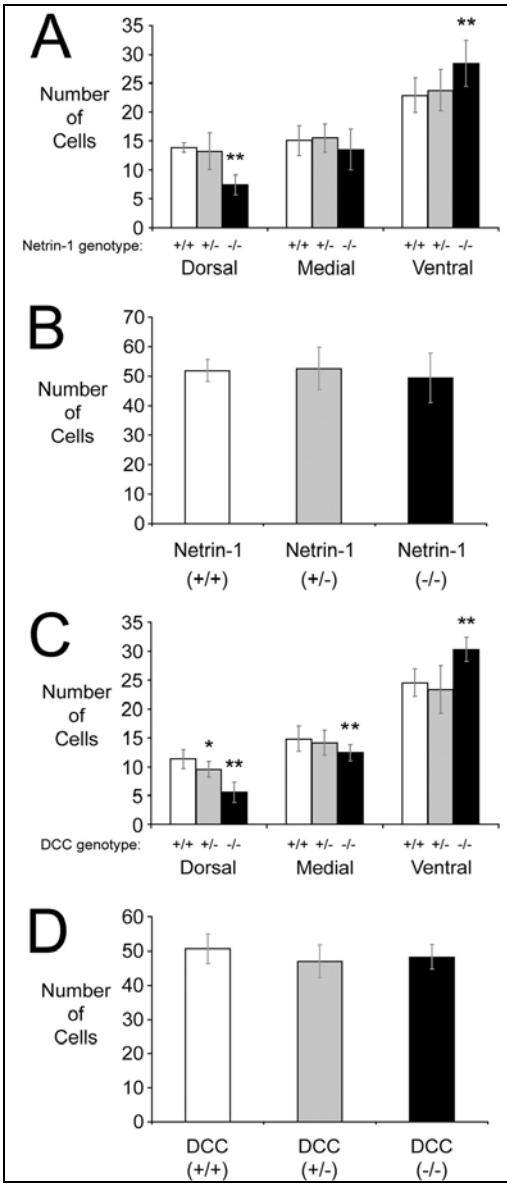

Figure 10.7 - OP cell number is reduced in the dorsal spinal cord and increased in the ventral spinal cord of E15 mice lacking netrin-1 or DCC function. *(A, C) Quantification of the number of OP cells in the dorsal, medial, and ventral thirds of the spinal cords of netrin-1 (A) and DCC (C) wild-type, heterozygote, and homozygous loss of function embryos. As shown in Figure 10.5, fewer OPs were detected in the dorsal spinal cords of the homozygous loss of function embryos, while a greater number of OPs were detected in the ventral spinal cords of these animals. (B, D) Total number of OPs counted in the spinal cords of netrin-1 (B) and DCC (D) wild-type, heterozygote, and mutant spinal cord sections. Values shown are the mean ± SD. *: $p < 0.05$; **: $p < 0.005$.*

DISCUSSION

Netrins are a family of secreted proteins that function as tropic guidance cues directing cell and axon migration. We have recently reported that netrin-1 is expressed by mature myelinating oligodendroglia in the adult spinal cord (Manitt et al., 2001). This prompted us to investigate the possibility that netrin-1 might contribute to oligodendrocyte development. Here, we show that migrating OP cells in the embryonic spinal cord express dcc and unc5h1. Furthermore, we report that in the absence of netrin-

1 or DCC function, fewer OP cells are found in the dorsal embryonic spinal cord, with a corresponding increase in the ventral spinal cord. The total number of OP cells present in a section of spinal cord remains the same, supporting the conclusion that this phenotype is the result of disrupted OP migration, and not due to altered proliferation or cell death. The repellent response to netrin often involves both DCC and an UNC-5 homolog family member (Colavita and Culotti, 1998;Hong et al., 1999;Keleman and Dickson, 2001). Our analysis of the effect of loss of DCC function, either due to gene knockout in vivo or using a function blocking antibody in vitro, indicates that OPs require DCC to be repelled by netrin-1.

In contrast, Spassky et al. (2002) have recently reported that an aggregate of cells expressing netrin-1 exerts a modest chemoattractive effect on OP cells migrating from explants of embryonic rat optic nerve in vitro. Several possibilities may account for the discrepancy between these results and our conclusion that netrin-1 functions as a repellent for OPs. Multiple lineages of OP cells have been described (Spassky et al., 1998;Fu et al., 2002) and it may be the case that OP cells migrating from explants of optic nerve are different from OP cells in the embryonic spinal cord. Secondly, cues presented with netrin-1 can influence the response to netrin-1 (Hopker et al., 1999). Such cues present in the optic nerve explant, or secreted by the netrin-1 producing cells, may switch the response of the OP cells to netrin-1 from repulsion to attraction. Importantly, the results of the transfilter migration assays reported here minimize the contributions of additional environmental factors by challenging OP cells with purified netrin-1 protein. In agreement with our findings, Sugimoto et al. (2001) have provided evidence that netrin-1 is a repellent cue for glial precursor cells, including OPs, migrating out of explants of newborn rat optic nerve. Spassky et al. (2002) suggest that the age of the explanted optic nerve may account for the discrepancy between these results, but this remains to be resolved. No direct evidence had been provided that netrin-1 influences OP cell migration in vivo. Here, the results of our in vitro analysis are consistent our findings in vivo, indicating that netrin-1 is a repellent for migrating OP cells in the embryonic spinal cord.

Relatively little is known about the migratory paths taken by OPs as they disperse throughout the developing spinal cord. While radial glia may contribute to directing OPs

laterally (Diers-Fenger et al., 2001), the ventro-dorsal migration of OP cells is poorly understood. It was suggested that OPs might migrate dorsally along commissural axons (Miller, 1996) however, the absence of migrating streams of OPs along these axons argues against this (Miller et al., 1997;Calver et al., 1998). Alternatively, it has been proposed that OPs might be directed by cues that either attract them dorsally or repel them from the ventral embryonic spinal cord (Miller et al., 1997). Our finding that netrin-1 repels OP migration in vitro supports the conclusion that a repellent action of netrin-1 directs migrating OP cells into the dorsal embryonic spinal cord.

Loss of DCC or netrin-1 function does not induce OP cell death

Both DCC and UNC-5 homologues have been suggested to function as pro-apoptotic dependence receptors, causing cell death in the absence of netrin-1 (Mehlen et al., 1998;Llambi et al., 2001;Forcet et al., 2001). Convincing evidence; however, has not been provided that cells expressing physiologically relevant levels of netrin receptors die, either in vitro or in vivo, as a result of the absence of netrin. We show that OP cells express dcc and unc5h1, but not netrin-1. The absence of netrin-1 or DCC caused a defect in migration, but no effect on cell survival was observed in vivo or in vitro, indicating that neither DCC nor UNC5H1 functions as a pro-apoptotic dependence receptor in these cells.

Tropism, repulsion, and collapse

Using the transfilter microchemotaxis assay, we found that netrin-1 placed in the bottom chamber reduced the number of OPs migrating to the lower side of the filter, suggesting that netrin-1 is a repellent for OP cells. Consistent with this, netrin-1 in the upper chamber increased the number of OP cells migrating through the filter, indicating that these cells preferentially migrate down a gradient of netrin-1. Interestingly, an equal concentration of netrin-1 in the top and bottom compartments reduced migration to a level similar to that produced by netrin-1 on the bottom alone. If netrin-1 produced a purely tropic effect on OP migration, it might be expected that surrounding the cells with a uniform concentration of netrin-1 would have no effect on motility. The observation

that a uniform concentration of netrin-1 causes the cells to become less motile indicates that netrin also exerts a kinetic effect on OP motility. However, when netrin-1 is placed only in the upper compartment and a high concentration of netrin-1 surrounds the cells, the cells do not freeze, but given the opportunity to escape from netrin-1, they migrate to the lower side of the filter. These results show that netrin-1 inhibits OP motility, but also suggest that if an OP cell finds itself in the midst of a gradient of netrin-1, its movement will be asymmetrically inhibited, and the cell will move in the direction of less inhibition.

These findings suggest that asymmetric limited collapse of the OP cytoskeleton and withdrawal of OP cell processes may underlie the repellent response of OP cells to a gradient of netrin-1. Partial collapse has been proposed as a mechanism underlying turns made by axonal growth cones in response to repellent guidance cues. For example, semaphorins were first identified in vertebrates on the basis of their ability to cause growth cone collapse (Luo et al., 1993). Although a repellent cue can cause the complete collapse of a neuronal growth cone (Luo et al., 1993), encountering a local source of the same repellent, such as a microscopic bead coated with the cue, may only induce partial collapse (Fan and Raper, 1995). In this case, the edge of the growth cone contacting the bead may withdraw, but the growth cone will continue to extend away from the cue (Luo and Raper, 1994). Our demonstration that netrin-1 induces a rapid and persistent retraction of OP cell processes is consistent with the hypothesis that a gradient of netrin-1 may direct OP cell migration by triggering asymmetric collapse the OP cytoskeleton.

In the chemotaxis assay, although migration toward netrin-1 is significantly reduced when compared with control, migration was not completely blocked. Our findings are consistent with a model in which netrin-1 reduces, but does not completely suppress spontaneous oligodendrocyte motility. If a cell is to move from a high concentration toward a lower concentration of a repellent cue, the collapsing action of the cue cannot be so potent that it inhibits motility entirely, otherwise the cell will never escape the high concentration of the cue. This interpretation is in agreement with the observations that netrin-1 induces partial, but not complete, withdrawal of OP cell processes, that netrin-1 does not completely block OP migration in the transfilter assay, and that a uniform concentration of netrin-1 presented on the top and bottom of the

chemotaxis chamber inhibits migration to an extent similar to presentation of netrin-1 in the bottom chamber alone.

Oligodendrocytes, but not oligodendrocyte precursors, express netrin-1 in vivo

Here we show that netrin-1 is not expressed by OP cells. We have recently reported that netrin-1 is expressed by myelinating oligodendrocytes in the adult mammalian spinal cord (Manitt et al., 2001). While OPs are capable of migrating great distances and remyelinating axons when transplanted into either demyelinated lesions or mutant animals lacking normal myelination (Gumpel et al., 1989;Groves et al., 1993;Warrington et al., 1993), OPs transplanted into appropriately myelinated regions migrate very little (O'Leary and Blakemore, 1997). Furthermore, in a study using co-culture of oligodendroglia and the CG4 OP-like cell line (Louis et al., 1992), the extending processes of CG4 cells collapsed when they contacted oligodendrocyte processes (Jefferson et al., 1997). These findings suggest that netrin-1 produced by mature oligodendroglia in vivo may inhibit the migration of OPs into regions where sufficient numbers of oligodendrocytes are present and locally contribute to appropriately spacing them along the axon.

Myelination is essential for proper CNS function. In demyelinating diseases, such as multiple sclerosis (MS), even focal myelin loss can result in impairment (Orentas and Miller, 1996). The evidence presented here indicates that netrin-1 is an essential cue that directs migrating OP cells during neural development. Further understanding the fundamental mechanisms that direct the development and maturation of oligodendrocytes will provide insight into developing strategies that aim to promote remyelination in the context of demyelinating diseases.

CHAPTER 11

Appendix III: Animal use protocols and permit to use biohazardous materials

MCGILL UNIVERSITY ANIMAL USE PROTOCOL

McGill University
Animal Use Protocol – Research
Guidelines for completing the form are available at www.mcgill.ca/rgo/animal

Protocol #: 4617
Investigator #: 875
Approval End Date: Aug. 31, 2003
Facility Committee: MNI

☐ **Pilot** ☒ **New Application** ☐ **Renewal of Protocol #** ____

Title Cellular and Molecular Mechanisms of Netrin Mediated Chemoattraction
(must match the title of the funding source application)

B level

1. Investigator Data:

Principal Investigator:	Timothy E. Kennedy, Ph.D.	**Office #:**	398-7136
Department:	Neurology and Neurosurgery	**Fax#:**	398-1319
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2. Emergency Contacts: Two people must be designated to handle emergencies.

Name:	Masoud Shekarabi	**Work #:**	398-8409	**Emergency #:**	731-4688
Name:	Simon Moore	**Work #:**	398-8409	**Emergency #:**	845-1265

3. Funding Source:

External ☒	**Internal** ☐
Source (s): CIHR	**Source (s):** ____
Peer Reviewed: ☒ YES ☐ NO**	**Peer Reviewed:** ☐ YES ☐ NO**
Status : ☒ Awarded ☐ Pending	**Status:** ☐ Awarded ☐ Pending
Funding period: 10.2002 to 9:2005	**Funding period:** ____

ACTION	✓	DATE
CCs	J	Sept 9 02
DB		
APPROVED		

** All projects that have not been peer reviewed for scientific merit by the funding source require 2 Peer Review Forms to be completed . e.g. Projects funded from industrial sources. Peer Review Forms are available at www.mcgill.ca/fgsr/rgo/animal/

Proposed Start Date of Animal Use (d/m/y):	Oct 1, 2002	**or ongoing** ☐
Expected Date of Completion of Animal Use (d/m/y):	Sept 30, 2005	**or ongoing** ☐

Investigator's Statement: The information in this application is exact and complete. I assure that all care and use of animals in this proposal will be in accordance with the guidelines and policies of the Canadian Council on Animal Care and those of McGill University. I shall request the Animal Care Committee's approval prior to any deviations from this protocol as approved. I understand that this approval is valid for one year and must be approved on an annual basis.

Principal Investigator's signature: Tim E. Kennedy **Date:** July 24. 2002

Approval Signatures:

Chair, Facility Animal Care Committee:	Gladi Pappius	**Date:** Sept. 4 2002
University Veterinarian:	J Maitenya	**Date:** Sept 4, 2002
Chair, Ethics Subcommittee(as per UACC policy):		**Date:**
Approved Period for Animal Use	**Beginning:** Sept 1, 2002	**Ending:** Aug. 31, 2003

☐ This protocol has been approved with the modifications noted in Section 13.

SEP 06 2002

4. Research Personnel and Qualifications: List the names of all individuals who will be in contact with animals in this study (including the Principal Investigator) and their employment classification (investigator, technician, research assistant, undergraduate/graduate student, fellow). Indicate any training received (e.g workshops, lectures, etc.). The PI certifies that all personnel listed here have suitable training and/or experience, or will be provided with the specific training which qualifies them to perform the procedures described in the protocol. Each person listed in this section must sign to indicate that s/he has read this protocol. (Space will expand as needed.)

Name	Classification	Training Information	Signature
Tim Kennedy	PI	rodent handling experience 3 years McMaster University 7 years Columbia University 4 years UCSF 7 years McGill will attend upcoming McGill Workshop	
Nicolas Tritsch	Graduate Student	by PI, 2 years McGill, completed McGill Animal Methods Workshop	
Simon Moore	Graduate Student	by PI, 3 years McGill will attend upcoming McGill Workshop	
J-F Bouchard	Post-Doc	5 years, U de Montreal by PI, 3 years McGill will attend upcoming McGill Workshop	

* Enter the first name, press 'enter', then the 2nd name… complete the first column, then the 2nd, then the 3rd
** If an undergraduate student is involved , the role of the student and the supervision received must be described.

5. Summary (In language that will be understood by members of the general public)

a) RATIONALE: Describe, in a short paragraph, the overall aim of the study and its potential benefit to human/animal health or to the advancement of scientific knowledge.

We are interested in identifying the mechanisms that axons use to navigate in the embryo. Netrins are a family of proteins secreted by axonal targets during development. We have found that a receptor for netrin called DCC has a powerful effect on the organization of the cytoskeleton. We believe that it is this effect on the cytoskeleton that drives the movement of the axon in the presence of netrin. We propose to identify the molecular players in the axon that are regulated by DCC and cause the reorganization of the nerve cell cytoskeleton. The proposed studies will provide insight into a fundamental mechanism that regulates cell motility in the embryo. Our findings may also have implications for developing stratagies that aim to promote nerve regeneration in adults.

b) SPECIFIC OBJECTIVES OF THE STUDY: Summarize in point form the primary objectives of this study.

1. To determine if netrin-1 and the netrin receptor DCC are an adhesive ligand-receptor pair.
2. To characterize the role of the Rho GTPases in commissural axon extension by netrin and DCC.
3. To indentify proteins interacting with DCC.

c) PROGRESS REPORT: If this is a renewal of an ongoing project, BRIEFLY summarize what was accomplished during the prior approval period and indicate if and how the current goals differ from those in the original application.

The study is progressing well and is ongoing.

d) SUMMARY OF PROCEDURES FOR ANIMAL USE REPORT TO THE CCAC: Using KEY WORDS ONLY, list the procedures used (e.g. anaesthesia, breeding colony, injection IP, gavage, drug administration, major survival surgery, euthanasia by exsanguination, behavioural studies). Refer to Appendix 1of the Guidelines for a more complete list of suggested key words.

euthanasia, tissue collection.

6. Animals To Be Used

a) Purpose of Animal Use (Check one):

1. ☒ **Studies of a fundamental nature/basic research**
2. ☐ **Studies for medical purposes relating to human/animal diseases/disorders**
3. ☐ **Regulatory testing**
4. ☐ **Development of products/appliances for human/veterinary medicine**

McGill University
Animal Use Protocol – Research
Guidelines for completing the form are available at www.mcgill.ca/rgo/animal

Protocol #: 4617
Investigator #: 875
Approval End Date: Aug 31, 2004
Facility Committee: MNI

☐ **Pilot** ☐ **New Application** ☒ **Renewal of Protocol #** 4617

Title Cellular and Molecular Mechanisms of Netrin Mediated Chemoattraction
(must match the title of the funding source application)

B

1. Investigator Data:

Principal Investigator: Timothy E. Kennedy, Ph.D. **Office #:** 398-7136
Department: Neurology and Neurosurgery **Fax#:** 398-1319
Address: MNI, 3801 University, MTL, QC, H3A 2B4 **Email:** timothy.kennedy@mcgill.ca

2. Emergency Contacts: Two people must be designated to handle emergencies.

Name: Nicolas Tritsch **Work #:** 398-8409 **Emergency #:** 288-5402
Name: Simon Moore **Work #:** 398-8409 **Emergency #:** 845-1265

3. Funding Source:

External ☒
Source (s): CIHR
Peer Reviewed: ☒ YES ☐ NO**
Status : ☒ Awarded ☐ Pending
Funding period: 10.2002 to 9:2005

Internal ☐
Source (s): ____
Peer Reviewed: ☐ YES ☐ NO**
Status: ☐ Awarded ☐ Pending
Funding period: ____

ACTION	✓	DATE
CCs		
DB	✓	Sept 24/03
APPROVED		

** All projects that have not been peer reviewed for scientific merit by the funding source require 2 Peer Review Forms to be completed . e.g. Projects funded from industrial sources. Peer Review Forms are available at www.mcgill.ca/fgsr/rgo/animal/

Proposed Start Date of Animal Use (d/m/y): ____ **or ongoing** ☒

Expected Date of Completion of Animal Use (d/m/y): ~~Sept 30, 2005~~ **or ongoing** ☒

Investigator's Statement: The information in this application is exact and complete. I assure that all care and use of animals in this proposal will be in accordance with the guidelines and policies of the Canadian Council on Animal Care and those of McGill University. I shall request the Animal Care Committee's approval prior to any deviations from this protocol as approved. I understand that this approval is valid for one year and must be approved on an annual basis.

Principal Investigator's signature: Timothy E. Kennedy **Date:** Aug 5-2003

Approval Signatures:

Chair, Facility Animal Care Committee:	Hla Le Peppins	**Date:** 8/8/2003
University Veterinarian: B	Anatsuya	**Date:** Sept 18, 2003
Chair, Ethics Subcommittee(as per UACC policy):		**Date:**
Approved Period for Animal Use	**Beginning:** Sept. 1, 2003	**Ending:** Aug 31, 2004

☐ This protocol has been approved with the modifications noted in Section 13.

November 2001

< SEP 0 2 2003

Guidelines for completing the form are available at www.mcgill.ca/rgo/animal/

McGill University
Animal Use Protocol – Research

Protocol #: 5032
Investigator #: —
Approval End Date: April 30, 2006
Facility Committee: MNI

Title: Role of the Netrin Receptor DCC in Synaptogenesis and Synaptic Plasticity
(must match the title of the funding source application)

☒ New Application ☐ Renewal of Protocol # ____ ☐ Pilot **Category** *(see section 11)*: B

1. Investigator Data:

Principal Investigator: Timothy E. Kennedy, Ph.D. **Phone #:** 398-7136
Unit/Department: Neurology and Neurosurgery **Fax#:** 398-1319
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Name: Bin Xu **Work #:** 398-8409 **Emergency #:** (514) 939-9697
Name: Nathalie Marcal **Work #:** 398-8409 **Emergency #:** (514) 526-4962

3. Funding Source:

External ☒
Source (s): CIHR 30307
Peer Reviewed: ☒ YES ☐ NO**
Status : ☒ Awarded ☐ Pending
Funding period: 04.05 - 03.10

Internal ☐
Source (s): ____
Peer Reviewed: ☐ YES ☐ NO**
Status: ☐ Awarded ☐ Pending
Funding period: ____

For Office Use Only:

ACTION		DATE
CCs		
DB	✓	May 11 '05
APPROVED		

** All projects that have not been peer reviewed for scientific merit by the funding source require 2 Peer Review Forms to be completed e.g. Projects funded from industrial sources. Peer Review Forms are available at www.mcgill.ca/rgo/animal

Proposed Start Date of Animal Use (d/m/y): 04.05 **or ongoing** ☐
Expected Date of Completion of Animal Use (d/m/y): ____ **or ongoing** ☒

Investigator's Statement: The information in this application is exact and complete. I assure that all care and use of animals in this proposal will be in accordance with the guidelines and policies of the Canadian Council on Animal Care and those of McGill University. I shall request the Animal Care Committee's approval prior to any deviations from this protocol as approved. I understand that this approval is valid for one year and must be approved on an annual basis.

Principal Investigator's signature: Tim E Kennedy **Date:** April 7, 2005

Approved by:

Chair, Facility Animal Care Committee:	Ken Hastings	**Date:** April 28, 2005
University Veterinarian: DH	David Hamilton	**Date:** May 6, 2005
Chair, Ethics Subcommittee (as per UACC policy):	—	**Date:** —
Approved Animal Use	**Beginning:** MAY 1, 2005	**Ending:** April 30, 2006

☐ This protocol has been approved with the modifications noted in Section 13.

December 2003

02 MAI 2005

APPROVED

www.mcgill.ca/research/compliance/animal/forms/

For Office Use Only:
Protocol #: 5032
Approval end date: ~~Sept 30, 2007~~
Facility Committee: MNI
Renewal#: 1st 2nd 5
EXTENSION: MAY 31, 2007

McGill University Animal Care Committee
RENEWAL of Animal Use Protocol

For: Research ☒ Teaching ☐ project

Principal Investigator:	Timothy E Kennedy, Ph.D.	**Protocol #**	5032
Protocol Title:	Role of the netrin receptor DCC in syaptogenesis and synaptic plasticity	**Phone:**	398-7136
Unit, Dept. & Address:	Centre for Neuronal Survival, Montreal Neurological Inst Room F116, 3801 University St.	**Fax:**	398-1319
Email:	timothy.kennedy@mcgill.ca	**Level:** B	**Funding source:** CIHR 90307
Start of Funding:	April 1, 2005	**End of Funding:**	March 31, 2010
Emergency contact #1 + work AND home phone #s:	Nathalie Marcal: work 398-8409, home 526-4962		
Emergency contact #2 + work AND home phone #s:	Sonia Rodrigues: work 398-8409, home 843-9729		

1. Personnel and Qualifications

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

Name	Classification	Animal Related Training Information	Occupational Health Program *	Signature *"Has read the original full protocol"*
Tim Kennedy, PhD	principal investigator	more than 20 years working with rodents passed advanced theory training		
Sonia Rodrigues	graduate student	more than 5 years working with rodents passed McGill workshop for mice and theory course		
Simon Moore	graduate student	more than 5 years working with rodents Passed McGill workshop for mice and theory course.		
Jennifer Goldman	graduate student	trained by PI passed McGill workshop for mice and theory course.		
Tamarah Luk	graduate student	more than 2 years working with rodents passed McGill workshop for mice and theory course		
Bin Xu, PhD	post-doc	more than 10 years working with rodents passed McGill workshop for mice and theory		
Nathalie Marcal, MSc	technician	more than 5 years working with rodents passed McGill workshop for mice and theory course		

* Indicate for each person, if participating in the local Occupational Health Program, see http://www.mcgill.ca/research/compliance/animal/occupational/ for details.

25 MAR 2005

MCGILL UNIVERSITY PERMIT TO USE BIOHAZARDOUS MATERIALS

McGill University

University Biohazards Committee

APPLICATION TO USE BIOHAZARDOUS MATERIALS*

No project should be commenced without prior approval of an application to use biohazardous materials. Submit this application to the Chair, Biohazards Committee, one month before starting new projects or expiry of a previously approved application.

1. PRINCIPAL INVESTIGATOR: Timothy E. Kennedy TELEPHONE: 514-398-7136

   ADDRESS: Montreal Neurological Institute, 3801 University, H3A 2B4 FAX NUMBER: 514-398-1319

   E-MAIL: timothy.kennedy@mcgill.ca

   DEPARTMENT: Neurology and Neurosurgery

   PROJECT TITLE: Cellular and molecular mechanisms of netrin mediated chemoattraction

2. FUNDING SOURCE: CIHR X NSERC ☐ NIH ☐ FCAR ☐ FRSQ ☐

   INTERNAL ☐ OTHER (specify)

   Grant No.: 100366

   Beginning date 10.2002 End date 09.2007

3.. Indicate if this is a

Renewal use application: procedures have been previously approved and no alterations have been made to the protocol.
Approval End Date

New funding source: project previously reviewed and approved under an application to another agency.

* New project: project not previously reviewed or procedures and/or microorganism altered from previously approved application.
Agency CIHR
Approval End Date 09.2005

CERTIFICATION STATEMENT: The Biohazards Committee approves the experimental procedures proposed and certifies with the applicant that the experiment will be in accordance with the principles outlined in the "Laboratory Biosafety Guidelines" prepared by Health Canada and the MRC, and in the "McGill Laboratory Biosafety Manual".

Containment Level (circle 1): 1 (2) 3 4

Principal Investigator or course director: _____ SIGNATURE date: 05 (day) 03 (month) 02 (year)

D. BRIEDIS

Chairperson, Biohazards Committee: _____ SIGNATURE date: 05-03-02 (day month year)

Approved period: _____ beginning 01-10-2002 (day month year) ending 30-09-2007 (day month year)

* as defined in the "McGill Laboratory Biosafety manual"

2nd REVISION, JANUARY 1996

Name	Department	Check appropriate classification				Fellow
		Investigator	Technician & Research Assistant	Student		
				Undergraduate	Graduate	
Dr. Timothy E. Kennedy	Neurology	X				
Dr. Cecilia Flores	Neurology					X
Mr.. Simon Moore	Neurology				X	
Ms. Jean-Francois Bouchard	Neurology					X
Mr. Masoud Shekarabi	Neurology				X	

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

Name: Dr. Tim Kennedy Phone No: work: 514-398-7136 home: 514-527-2187

Name: Mr. Masoud Shekarabi Phone No: work: 514-398-8409 home: 514-731-4688

6. Briefly describe:

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

a) Non-Pathogenic Bacterial Waste

b) Broken Glass/Sharps

c) Organic Solvents

d) Replication-defective adenovirus

ii) the procedures involving biohazards

a) Biohazardous waste will be disposed of separately from regular garbage. Bacterial culture waste will be placed in biohazard autoclave bags and autoclaved prior to disposal; liquid waste will be neutralized with 0.1% Roccal detergent or bleach.

b) Containers/equipment leaving the lab will be decontaminated with either 1% bleach and /or 70% ethanol.

c) Working areas will be regularly wiped with 70% ethanol.

d) Chloroform and phenol will be disposed of as toxic waste.

e) Sharps will be disposed of in plastic containers; glass in sealed cardboard boxes.

f) Organic/caustic chemicals will be stored in a reinforced cabinet, and used in a fume hood.

g) Equipment/disposables in contact with recombinant adenovirus will be soaked in 10% bleach prior to placement in biohazard bags and autoclaving.

iii) the protocol for decontaminating spills

Spills will be decontaminated by:

- allowing aerosols to settle.

- covering spill with paper towel and then applying 1% bleach from the periphery inwards.

- after a 30 minute incubation period in the applied bleach, the paper towel will be disposed in a biohazard garbage bag and subsequently autoclaved.

- spills onto clothing will be decontaminated by autoclaving.

7. Does the protocol present conditions (e.g. handling of large volumes or high concentrations of pathogens) which could increase the hazards of the infectious agent(s)? **NO**
8. Do the specific procedures to be employed involving genetically engineered organisms have a history of safe use? **YES**
9. What precautions are being taken to reduce production of infectious droplets and aerosols? **Not applicable.**

10. List the biological safety cabinets to be used.

Building	Room No.	Manufacturer	Model No.	Serial No.	Date Certified
MNI Centre for Neuronal Survival	F 104	Forma	1184	16068-1473	04.12.01
MNI Centre for Neuronal Survival	F 104	Forma	1184	14517-327	11.02.02
MNI Centre for Neuronal Survival	F 104	Forma	1184	16069-1477	05.12.02

McGill University

APPLICATION TO USE BIOHAZARDOUS MATERIALS

Projects involving potentially biohazardous materials should not be commenced without approval from Environmental Health & Safety. Submit applications before 1) starting new projects, 2) renewing existing projects, or 3) changing the nature of the biohazardous materials within existing projects.

1. PRINCIPAL INVESTIGATOR: Timothy E Kennedy PHONE: 514-398-7136
DEPARTMENT: Neurology and Neurosurgery FAX: 514-398-1319
ADDRESS: MNI, 3801 University St. E-MAIL: timothy.kennedy@mcgill.ca

PROJECT TITLE(S): Cellular and Molecular Mechanisms Regulating Axon Guidance

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

Name: Tim Kennedy Phone No: work: 514-398-7136 home: 514-739-1448
Name: Nathalie Marcal Phone No: work: 514-398-8409 home: 514-526-4962

3. FUNDING SOURCE OR AGENCY: list all sources when information in Sections 5-12 is identical:

Source	CIHR	Grant No.	MOP-79513	Start date	01/01/2006	End date	31/03/2011
Source		Grant No.		Start date		End date	
Source		Grant No.		Start date		End date	

4. Indicate if this is

☐ Renewal: procedures previously approved without alterations.
Approval End Date: _____

☐ New funding source: project previously reviewed and approved under an application to another agency.
Agency: _____ Approval End Date: _____

☒ New project: project not previously reviewed.

☐ Approved project: change in biohazardous materials or procedures.

☐ Work/project involving biohazardous materials in teaching/diagnostics.

CERTIFICATION STATEMENT: Environmental Health & Safety approves the experimental procedures proposed and certifies with the applicant that the experiment will be in accordance with the principles outlined in the Public Health Agency of Canada's "Laboratory Biosafety Guidelines" and in the "McGill Laboratory Biosafety Manual".

Containment Level (select one): ☐ 1 ☒ 2 ☐ 2 with additional precautions ☐ 3

Principal Investigator or course director: _____ (SIGNATURE) date: 6 (day) 6 (month) 2006 (year)

Approved by Environmental Health & Safety: _____ (SIGNATURE) date: 09 (day) 06 (month) 06 (year)

Expiry: 31 (day) 03 (month) 11 (year)

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

Name	Department	Job Title/Classification	Trained in the safe use of biological safety cabinets within the last 3 years? If yes, indicate training date.
Tim Kennedy	Neurology and Neurosurgery	Principal Investigator	no, more than 3 yrs experience
Simon Moore	Neurology and Neurosurgery	graduate student	no, more than 3 yrs experience
Nathalie Marcal	Neurology and Neurosurgery	technician	no, more than 3 yrs experience
Adam Baker	Neurology and Neurosurgery	post-doc	no, more than 3 yrs experience

6. Briefly describe:

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

a) Non-pathogenic bacterial waste
b) broken glass/sharps
c) organic solvents

ii) the procedures involving biohazards

a) Biohazardous waste will be disposed of separately from regular garbage. Bacterial culture waste will be placed in biohazard autoclave bags and autoclaved prior to disposal; liquid waste will be neutralized with 0.1% Roccal detergent or bleach.
b) Containers/equipment leaving the lab will be decontaminated with either 1% bleach and /or 70% ethanol.
c) Working areas will be regularly wiped with 70% ethanol.
d) Chloroform and phenol will be disposed of as toxic waste.
e) Sharps will be disposed of in plastic containers; glass in sealed cardboard boxes.
f) Organic/caustic chemicals will be stored in a reinforced cabinet, and used in a fume hood.

iii) the protocol for decontaminating spills

Spills will be decontaminated by:
- allowing aerosols to settle.
- covering spill with paper towel and then applying 1% bleach from the periphery inwards.
- after a 30 minute incubation period in the applied bleach, the paper towel will be disposed in a biohazard garbage bag and subsequently autoclaved.
- spills onto clothing will be decontaminated by autoclaving.

7. Does the protocol present conditions (e.g. handling of large volumes or high concentrations of pathogens) that could increase the hazards?

No

8. Do the specific procedures to be employed involving genetically engineered organisms have a history of safe use?

Yes

9. What precautions will be taken to reduce production of infectious droplets and aerosols?

Not applicable

10. Will the biohazardous materials in this project expose members of the research team to any risks that might require special training, vaccination or other protective measures? If yes, please explain.

No

11. Will this project produce combined hazardous waste – i.e. radioactive biohazardous waste, biohazardous animal carcasses contaminated with toxic chemicals, etc.? If yes, please explain how disposal will be handled.

No

12. List the biological safety cabinets to be used.

Building	Room No.	Manufacturer	Model No.	Serial No.	Date Certified
Montreal Neuro Inst	F 104	Forma	1184	16068-1473	April 6 2005
Montreal Neuro Inst	F 104	Forma	1184	14517-327	April 6 2005
Montreal Neuro Inst	F 104	Forma	1184	16069-1477	April 7 2005

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