Mechanisms underlying netrin-1 mediated chemoattraction

Karen Lai Wing Sun

Integrated Program in Neuroscience
Montreal Neurological Institute
Faculty of Medicine

McGill University
Montreal, Quebec, Canada

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ABSTRACT

Establishing synaptic connections between neurons that make up the circuitry of the brain is a crucial part of early neural development. During this process, neurons must extend their axons over long distances until they reach their appropriate synaptic targets. The trajectories taken by extending axons are determined by molecular cues that guide axon growth. Through its receptor Deleted in colorectal cancer (DCC), netrin-1 is a secreted guidance molecule that can act as an attractant for migrating cells and axons in the developing nervous system. Although both netrin-1 and DCC are required for normal development, the precise molecular mechanisms that are responsible for netrin-1 mediated axon guidance remain poorly understood.

This thesis examines the mechanisms involved in the attractive response to netrin-1 signalling. We begin by addressing the role of Src family kinases (SFKs) in the extension of spinal commissural axons to the ventral midline in the developing spinal cord, a process that is highly dependent on netrin-1. Although SFKs have previously been implicated in netrin-1 signalling downstream of DCC, we report that deleting the expression of either SFK members Fyn or Src does not disrupt normal commissural axon guidance *in vivo*. We also examined the signal transduction mechanisms involved in activating the Rho GTPases Cdc42 and Rac1 in the guidance of axons to netrin-1. Here, we demonstrate that the guanine nucleotide exchange factor (GEF) βPix, which can activate Cdc42 and Rac1, is involved in filopodia extension and growth cone expansion induced by netrin-1. We also implicate βPix function in commissural axon extension to the ventral midline. Finally, we investigated the functional significance of the netrin-1 gradient in the proper guidance of spinal commissural axons during embryogenesis. By genetically manipulating netrin-1 expression *in vivo*, we determined that a graded distribution of netrin-1 is required to accurately attract spinal commissural neurons to the ventral midline and form the spinal commissure early in development. Together, these studies provide insight into the mechanisms that regulate netrin-1 chemoattraction and may provide strategies to promote axon regeneration following injury.

RÉSUMÉ

Établir des connexions synaptiques entre les neurones qui composent les circuits du cerveau est une partie cruciale du développement neuronal précoce. Au cours de ce processus, les neurones doivent étendre leurs axones sur de longues distances jusqu'à ce qu'ils atteignent les cibles synaptiques appropriées. Les trajectoires prises par les axones sont déterminées par des signaux moléculaires qui guident la croissance axonale. Grâce à son récepteur « Deleted in colorectal cancer (DCC) », la molécule de guidage sécrétée nétrine-1 fonctionne en tant que signal attractif pour les cellules et les axones en migration lors du développement du système nerveux. Bien que la nétrine-1 et le récepteur DCC soient nécessaires pour le développement normal, les mécanismes moléculaires précis, responsables pour le guidage de la croissance axonale médié par la nétrine-1, restent mal compris.

Cette thèse porte sur l'étude des mécanismes impliqués dans les fonctions attractives de la nétrine-1. Nous commençons par aborder le rôle des kinases de la famille Src (SFK) dans le prolongement des axones commissuraux à la ligne médiane de la partie ventrale de la moelle épinière en développement, un processus qui est très dépendant de la nétrine-1. Bien que d'autres études aient impliqués les SFK dans la signalisation de la nétrine-1 par l'entremise de DCC, nous démontrons que la suppression de l'expression des SFK Fyn ou Src ne perturbe pas le guidage normal des axones commissuraux *in vivo*. Nous avons également examiné les voies de signalisation impliquées dans l'activation des GTPases Rho Cdc42 et Rac1 dans le guidage des axones à la nétrine-1. Nous démontrons que le facteur d'échange de nucléotide guanine (GEF) βPix, qui peut activer Cdc42 et Rac1, est impliqué dans l'extension de filopodes et l'expansion du cône de croissance induites par la nétrine-1. Nous établissons également un rôle de βPix dans le prolongement des axones commissuraux à la ligne médiane ventrale. Enfin, nous avons étudié l'importance fonctionnelle du gradient de nétrine-1 dans le guidage des axones commissuraux de la colonne vertébrale au cours de l'embryogenèse. En manipulant génétiquement l'expression de la nétrine-1 *in vivo*, nous apportons la preuve qu'une distribution graduelle de nétrine-1 est nécessaire pour attirer précisément les neurones

commissuraux de la colonne vertébrale à la ligne médiane et pour former la commissure de la colonne vertébrale tôt dans le développement. Collectivement, ces données permettent de mieux comprendre les mécanismes qui régulent la chimioattraction de la nétrine-1 et peuvent fournir des stratégies visant à promouvoir la régénération axonale suite à une lésion.

TABLE OF CONTENTS

RESULTS

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LIST OF FIGURES AND TABLES

LIST OF ABBREVIATIONS

80s: eukaryotic ribosomes

ADF: actin depolymerizing factor

Akt: RAC-alpha serine/threonine-protein kinase

APP: amyloid precursor protein

ARF: adenosine diphosphate-ribosylation factor

Arp2/3: complex of actin-related proteins ARP2 and ARP3

Aβ: amyloid-beta

βPix: beta p21-activated kinase-interacting exchange factor

BMP: bone morphogenic protein

Boc: biregional Cdon-binding protein

BSA: bovine serum albumin

C. elegans*: Caenorabditis elegans*

Ca^{2+}: calcium

CaMKII: calcium-calmodulin-dependent protein kinase II

cAMP: cyclic adenosine monophosphate

CaN: calcineurin

capZ: capping protein muscle Z-line, alpha 1

Cdc42: cell division cycle 42

cdk5: cyclin-dependent kinase 5

cDNA: complementary deoxyribonucleic acid

cGMP: cyclic guanosine monophosphate

CNS: central nervous system

CO_2: carbon dioxide

Comm: Commissureless

CRIB: Cdc42/Rac interactive binding domain

Csk: C-terminal Src Kinase

D. melanogaster*: Drosophila melanogaster*

DA: dopaminergic

DAG: diacylglycerol

DB: DCC-binding

Dbl: diffuse B-cell lymphoma

DCC: Deleted in colorectal cancer

DD: death domain

DH: diffuse B-cell lymphoma homology

DIV: days in vitro

DOCK180: dedicator of cytokinesis

DRG: dorsal root ganglion

Drosophila*: Drosophila melanogaster*

DSCAM: Down syndrome cell adhesion molecule

E13: embryonic day 13

ECM: extracellular matrix

EDTA: Ethylenediaminetetraacetic acid

Ena/VASP: Enabled/vasodilator-stimulated phosphoprotein

ERK: extracellular signal-regulated kinase

ERM-M: ezrin/radixin/moesin and merlin

Ext1: exostosin 1

F-actin: filamentous actin

FAK: focal adhesion kinase

FNIII: fibronectin type III domain

FRQS: Fonds de recherche du Québec - Santé

G-actin: globular actin

GAP: GTPase activating protein

GDF: growth differentiation factor

GDI: guanine nucleotide-dissociation inhibitor

GDP: guanosine diphosphate

GEF: guanine nucleotide exchange factor

GIT: G protein-coupled receptor kinase interacting protein

GPI: glycophosphatidylinositol

GSK3: glycogen synthase kinase 3

GTPγS : guanosine 5'-O-[gamma-thio]triphosphate

GTP: guanosine triphosphate

GTPase: guanosine triphosphatase

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HH: Hamilton-Hamburg stage

HI-FBS: heat inactivated fetal bovine serum

HI-HS: heat inactivated horse serum

HRP: horse radish peroxidase

HSPG: heparan sulfate proteoglycan

Ig: immunoglobulin

IgG: immunoglobulin G

IP_3: inositol 1,4,5-triphosphate

KO: knockout

LCC: L-type calcium channel

LIM-K: LIM kinase

LRR: leucine rich repeats

LTD: long-term depression

LTP: long-term potentiation

MAPK: mitogen-activated protein kinase

Max1: Motor axon guidance PH/MyTH4/FERM domain cytoplasmic protein

MEK1/2: mitogen-activated protein kinase kinase 1/2

MIG-10: Abnormal cell MIGration family member 10

MLC: myosin light chain

mTOR: mammalian target of rapamycin

NaCl: sodium chloride

Nck1: non-catalytic region of tyrosine kinase adaptor protein 1

NFM: neurofilament M

NGL: netrin-G ligand

NMDAR: N-methyl-D-aspartate-type glutamate receptor

NPF: nucleation promoting factor

Nrp: neuropilin

NTR: netrin-like

N-WASP: neuronal Wiskott-Aldrich Syndrome protein

OPC: oligodendrocyte precursor cell

P/S: penicillin streptomycin

PAK1: p21-activating kinase 1

PCR: polymerase chain reaction

PDL: poly-D-lysine

PFA: paraformaldehyde

PH: pleckstrin homology

PI(4,5)P_2 or PIP_2: Phosphatidylinositol 4,5-bisphosphate

PI3K: phosphatidylinositol 3-kinase

PICK1: protein interacting with C kinase-1

PIP: phosphatidylinositol phosphate

PIP_3: phosphatidylinositol (3,4,5)-trisphosphate

PKA: protein kinase A

PKC: protein kinase C

PLCγ: phospholipase C gamma

PMSF: phenylmethylsulfonyl fluoride

PSD-95: post-synaptic density 95

PTPδ: protein-tyrosine phosphatase δ

PTPσ: protein-tyrosine phosphatase σ

PVDF: polyvinyldiene fluoride

Rac: ras-related C3 botulinum toxin substrate

RGC: retinal ganglion cell

RGMa: repulsive guidance molecule

Rho: ras homolog gene family

RIPA: Radio-immunoprecipitation assay buffer

RNA: ribonucleic acid

Robo: Roundabout

ROCK: Rho-associated protein kinase

RT-PCR: reverse transcriptase polymerase chain reaction

SDS: sodium dodecyl sulfate

Sema: semaphorin

SFK: Src family kinase

SH2: Src homology 2

SH3: Src homology 3

Shh: Sonic hedgehog

Shp2: Src homology region 2 domain-containing phosphatase-2

siRNA: small interfering ribonucleic acid

Smo: Smoothened

Src: tyrosine kinase sarcoma

TAG-1: transient axonal glycoprotein-1

TBST: Tris buffered saline with Tween 20

TIMP: tissue inhibitor of metalloproteinase

TRP: transient receptor potential

t-SNARE: target soluble N-ethylmaleimide attachment protein receptor

TSP-1: thrombospondin type I domain

Tyr: tyrosine

UNC:Uncoordinated

VEGF: vascular endothelial growth factor

WASp: Wiskott-Aldrich syndrome protein

WAVE: WASp family Verprolin-homologous protein

Wnt: Wingless/Integrase-1

X. laevis or Xenopus*: Xenopus laevis*

ZU-5: ZO-1 and UNC5 domain

CONTRIBUTIONS OF AUTHORS

CHAPTER 1: Literature Review I - Axon guidance and the actin cytoskeleton

- **Karen Lai Wing Sun**: Wrote the chapter and drew the figure.
- **Timothy E. Kennedy**: Edited the text.

CHAPTER 2: Literature Review II - Netrins and their functions

- **Karen Lai Wing Sun**: Wrote the manuscript for the review article "Netrins: versatile extracellular cues with diverse functions", published in Development (Lai Wing Sun et al. 2011), and revised the text for this chapter.
- **James P. Correia**: Drew the figures for the review article "Netrins: versatile extracellular cues with diverse functions", published in Development (Lai Wing Sun et al. 2011).
- **Timothy E. Kennedy**: Reviewed and edited the manuscript for the review article "Netrins: versatile extracellular cues with diverse functions", published in Development (Lai Wing Sun et al. 2011).

CHAPTER 3: The Src family kinases Fyn and Src are not essential for commissural axon guidance in the embryonic spinal cord

- **Karen Lai Wing Sun**: Designed and performed all the experiments, assembled the figures, and wrote the manuscript.
- **Timothy E. Kennedy**: Designed the experiments, edited the manuscript.

CHAPTER 4: Role for the Cdc42 and Rac1 guanine nucleotide exchange factor βPix in netrin-1 mediated chemoattraction

- **Karen Lai Wing Sun**: Performed experiments and analysis pertaining to figure 4.1A, B, C, D, F, G, figure 4.2, figure 4.4, and figure 4.5, and wrote the manuscript.
- **Sonia P. Rodrigues**: Designed experiments, performed CRIB binding assay and experiments for figure 4.1C and figure 4.3.
- **Nicolas Stifani**: Performed the *in ovo* chick electroporation assay in figure 4.4.
- **Stefano Stifani**: Provided material and equipment for the *in ovo* chick electroporation assay in figure 4.4.
- **Timothy E. Kennedy**: Designed experiments and edited the manuscript.

CHAPTER 5: Assessing the functional significance of graded expression of netrin-1 in the developing spinal cord

- **Karen Lai Wing Sun**: Developed experimental design, collected embryos, sectioned netrin-1 deleted embryos at E10.5 and E12.5, sectioned all netrin-1 βgeo embryos, dissected and homogenized tissue, ran Western blots for figure 5.1S-U, performed all immunohistochemical assays and analyses, assembled the figures and wrote the manuscript.
- **Jenea M. Bin**: Participated in the experimental design, set up the breedings, collected embryos, sectioned netrin-1 deleted E12.5 and E14.5 embryos, homogenized tissue, ran Western blot for figure 5.1V.
- **Dong Han**: Provided the netrin-1 flox mice and guidance with the breedings.
- **Timothy E. Kennedy**: Designed experiments and edited the manuscript.

CHAPTER 6: General discussion and conclusion

- **Karen Lai Wing Sun**: Wrote the chapter and modified the figure from the article "Netrins: versatile extracellular cues with diverse functions", published in Development (Lai Wing Sun et al. 2011).
- **Timothy E. Kennedy**: Edited the text.

INTRODUCTION

CHAPTER 1

Literature Review I

Axon guidance and the actin cytoskeleton

PREFACE

The adult nervous system is an intricate information processing network composed of neuronal connections that control biological functions. The development of a functional central nervous system (CNS) relies on the exquisite specificity of CNS wiring between neurons and their proper targets during embryogenesis. Formation of this complex circuitry requires neurons to project their axons and navigate through the extracellular environment, often over long distances, to reach their synaptic counterparts. This introductory chapter presents an overview of mechanisms used by axons to navigate to their destination and the molecules that direct them, with a particular focus on the guidance of spinal commissural neurons. An introduction to the growth cone and a review of several regulatory molecules involved in the reorganization of the actin cytoskeleton during axon pathfinding is also presented.

AXON GUIDANCE

During development, axons must travel considerable lengths through a complex environment to reach their final destination and synapse with the proper targets. To simplify this overwhelming task, extending axons utilize intermediate targets to break up long and difficult trajectories into a series of smaller, sequential segments. These intermediate targets are generally specialized cells that serve as transient guideposts for the sensory tip of growing axons, dubbed growth cones, to gather and integrate guidance information to allow the axons to make important navigational decisions (Tessier-Lavigne and Goodman 1996). Additionally, axons are directed along a precise trajectory by molecular cues that line their path. These guidance cues may be permissive or instructive, with a permissive or non-permissive signal positively or negatively modulating axon outgrowth, and an instructive cue providing directional information.

Instructive signals are generally categorized into attractive and repulsive cues, although they can be bifunctional, attracting some cells and repelling others. These axon guidance molecules can be further subdivided into substrate-associated and diffusible forms to mediate short-range and long-range guidance, respectively (Goodman and Shatz 1993; Tessier-Lavigne and Goodman 1996). Short-range guidance refers to molecules that associate or remain close to the cells that produced them and therefore function locally. In contrast, long-range factors are secreted proteins that can modulate axonal growth at a significant distance from their source. Occasionally, these guidance molecules are presented in the form of gradients, either through the graded expression of non-diffusible short-range cues, or the diffusion of soluble chemotropic cues over a certain distance. These gradients can play important roles in regulating growth cone motility, such as in the developing optic tectum or the embryonic spinal cord (Serafini et al. 1994; Charron et al. 2003; McLaughlin et al. 2003; Kennedy et al. 2006).

The study of the ventral midline of the developing spinal cord is a relatively simple model system that has provided much insight into the mechanisms of axonal pathfinding. In this system, a population of sensory interneurons called spinal commissural neurons differentiate in the dorsal regions of the spinal cord and extend their axons ventrally along the

lateral margins before circumferentially migrating towards the ventral midline (Fig. 1.1). Once they have reached the floor plate, commissural axons cross to the contralateral side of the spinal cord and extend longitudinally in the white matter tracts of the ventral funiculus. The extension of commissural axons across the ventral midline forms what is known as the ventral commissure of the spinal cord. Once on the contralateral side, the commissural neurons do not recross again. Multiple families of guidance molecules participate in the proper navigation of spinal commissural neurons.

Axon guidance molecules

Netrins

Netrins are a small family of extracellular chemotropic molecules that are structurally similar to laminins (Kennedy et al. 1994; Serafini et al. 1994; Yurchenco and Wadsworth 2004). Four secreted members, netrin-1 through -4, and two membrane-associated proteins, netrin-G1 and -G2, are expressed in vertebrates. Netrins are bifunctional cues, acting as chemoattractants or chemorepellents depending on the receptor profile of cells. Several receptors for netrins have been identified, including Deleted in colorectal cancer (DCC), neogenin, Down's syndrome cell adhesion molecule (DSCAM) and the UNC5 family of receptors (Fearon et al. 1990; Keino-Masu et al. 1996; Leonardo et al. 1997; Ly et al. 2008). The most-studied member of the family is netrin-1, which plays roles in cell and axon migration, tissue morphogenesis and synapse formation (Kennedy et al. 1994; Colamarino and Tessier-Lavigne 1995a; Alcantara et al. 2000; Srinivasan et al. 2003; Goldman et al. 2013). In the embryonic spinal cord, it is secreted by floor plate cells and diffuses to form a netrin-1 gradient emanating from the ventral midline (Kennedy et al. 2006) (Fig. 1.1). It is this gradient that directs the DCC-expressing growth cones of extending spinal commissural neurons towards the floor plate. Removing the expression of netrin-1 or DCC in the embryonic spinal cord results in a severe disruption in commissural axon migration to the floor plate, with the majority of axons not reaching the midline (Serafini et al. 1996; Fazeli et al. 1997).

Morphogens

Best known for their role in cell fate determination and tissue patterning, some morphogens are also involved in axon guidance during embryogenesis. These include members of the bone morphogenic protein (BMP) family, the Wnt family and Hedgehog family. In the developing spinal cord, roof plate cells express BMP-7 and another BMP, growth differentiation factor-7 (GDF-7), which act as dorsal chemorepellents and drive the initial projection of commissural axons ventrally (Augsburger et al. 1999; Butler and Dodd 2003; Yamauchi et al. 2013) (Fig. 1.1). On the other hand, a gradient of Sonic hedgehog (Shh) originating at the floor plate assists netrin-1 in attracting the commissural axons to the ventral midline, without influencing their growth (Charron et al. 2003). This attraction occurs via the expression of the Shh receptors Smoothened (Smo) and biregional Cdon-binding protein (Boc) by commissural growth cones (Charron et al. 2003; Okada et al. 2006). Lastly, Wnt-4 and its receptor Frizzled-3 are involved in the longitudinal turning of commissural axons following midline crossing (Lyuksyutova et al. 2003).

Slits

Slits have functions in neuronal migration, cell adhesion and axon guidance (Kidd et al. 1998; Hu 1999; De Bellard et al. 2003; Andrews et al. 2008b). Vertebrates express three homologs, Slit-1 to -3, and four receptors, Roundabout (Robo) 1-4. Expressed at the midline, Slits act as chemorepellents for ipsilateral spinal axons to inhibit them from crossing the floor plate, and for contralateral axons to expel them from the floor plate and prevent them from recrossing (Kidd et al. 1999; Rajagopalan et al. 2000; Simpson et al. 2000) (Fig. 1.1). The responsiveness to Slits needs to be spatially and temporally regulated in order to allow crossing commissural neurons to enter the midline before being repelled by Slits. This is achieved through the differential expression of Robo receptors in commissural neurons and collaborative function of Slits expressed at the ventral midline (Long et al. 2004). In *Drosophila melanogaster*, pre-crossing commissural spinal nerves present low levels of Robo receptors on the surface of growth cones, leading to a suppressed Slit response at the midline. This reduction of Robo is

mediated by Commissureless (Comm), a transiently expressed protein that targets Robo for endosomal degradation (Keleman et al. 2002). As axons reach and cross the midline, Comm expression is downregulated, thereby relieving the inhibition on Robo and allowing the receptor to accumulate on the growth cone surface. Axons now display an increased sensitivity to Slit and are propelled to exit the midline (Kidd et al. 1998). Considering that vertebrates do not express a Comm homolog, it still remains to be determined how Slit/Robo signalling mechanisms are regulated in other systems. More recent studies suggest that splice variants of Robo3 may be involved in the process, with Robo3.1 expressed by pre-crossing commissural neurons and having an equivalent function to Comm in *Drosophila*, and Robo3.2 expressed after crossing (Sabatier et al. 2004; Chen et al. 2008). In addition to acquiring a responsiveness to Slits, commissural neurons reaching the midline need to simultaneously lose their sensitivity to midline-derived attractants in order to cross. In *Xenopus laevis*, the binding of Robo to DCC through their cytoplasmic domains is reported to silence the netrin receptor, thereby impeding netrin-mediated attraction (Stein and Tessier-Lavigne 2001).

Ephrins

Ephrins have been implicated in a multitude of developmental processes such as cell migration, morphogenesis and axon guidance, in addition to functions in adulthood like synaptic plasticity (Winning et al. 1996; Krull et al. 1997; Wang and Anderson 1997; Chan et al. 2001). Generally functioning as inhibitory or repulsive molecules, they are membrane-bound ligands and are divided into two classes: ephrin-As, which are anchored to the membrane through a glycophosphatidylinositol (GPI) linkage, and ephrin-Bs that are transmembrane proteins with cytoplasmic domains (Pandey et al. 1995; Gale et al. 1996). Ephrins signal through Eph receptors, a family of receptor tyrosine kinases which are also cell surface-associated proteins. In most cases, ephrin-As mediate their function through EphA receptors and ephrin-Bs via EphB receptors, although interactions across classes can occur. Ephrin/Eph signalling is particularly interesting because it can be bidirectional, inducing distinct signalling mechanisms through the receptor and the ligand, referred to as "forward" and "reverse" signalling

respectively. During commissural axon pathfinding, ephrin-B3 is expressed by the floor plate (Fig. 1.1), while ephrin-B1 and ephrin-B2 are found in the rest of the spinal cord (Imondi et al. 2000; Imondi and Kaprielian 2001; Jevince et al. 2006). Complementary to this, commissural neurons express several EphB receptors on decussated axonal segments that contribute to mediating their orthogonal turn after crossing the midline (Imondi and Kaprielian 2001; Jevince et al. 2006). Although the precise input of ephrin signalling in spinal commissural migration is still unclear, the loss of ephrin-B3 or multiple EphB receptors results in several relatively mild commissural axon guidance defects in mice (Kadison et al. 2006).

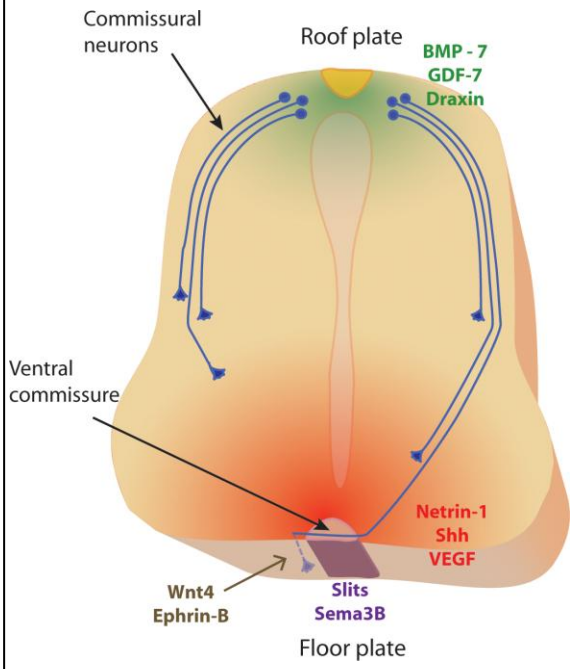

Figure 1.1 - Commissural axon guidance in the embryonic spinal cord. Spinal commissural axons are repelled by BMP-7, GDF-7 and Draxin (green) expressed by the roof plate. Concurrently, commissural axons are attracted to the ventral midline by netrin-1, Shh and VEGF (red) secreted by the floor plate. As the axons enter the floor plate, they are expelled out through the repellent actions of Slits expressed at the ventral midline. This midline crossing of axons forms the spinal ventral commissure. Expression of Slits and semaphorin 3B (purple) at the midline also prevent the axons from recrossing. Once on the contralateral side of the spinal cord, commissural axons make a sharp turn and extend longitudinally, a process that is regulated by ephrin-Bs and Wnt4.

Semaphorins

Semaphorins constitute a large family of structurally-diverse secreted and membrane-bound members that can be subdivided into eight subclasses, with classes 3 through 7 being expressed in vertebrates. With a shared ~500 amino-acid extracellular domain, class 3 semaphorins are secreted, class 4-6 are transmembrane proteins, and class 7 are GPI-linked to the membrane (Yazdani and Terman 2006). Classically recognized as inhibitory guidance factors

because of their functions in fasciculation, branching and axon steering, semaphorins can also mediate attractive responses such as promoting central and peripheral axon outgrowth (Chen et al. 2000; Giger et al. 2000; Fenstermaker et al. 2004). Several receptors have been identified to mediate semaphorin signalling, with neuropilins, plexins, integrins and neural cell adhesion molecule L1 forming receptor complexes (Castellani et al. 2000; Fenstermaker et al. 2004). After crossing the floor plate, spinal commissural axons acquire a repellent responsiveness to semaphorins and Slits, preventing them from recrossing the midline. Semaphorin 3B (Sema3B) is expressed at the floor plate (Fig. 1.1) while Sema3F is distributed throughout the rest of the spinal cord, and both act as repellents to regulate midline crossing of commissural axons in collaboration with neuropilin-2 (Nrp2) and PlexinA1 (Zou et al. 2000; Nawabi et al. 2010). Pre-crossing commissural axons are insensitive to semaphorins due to calpain-1 processing of PlexinA1, but become responsive to the repulsive cue through the actions of the cell adhesion molecule NrCAM and Shh expressed at the midline (Nawabi et al. 2010; Parra and Zou 2010). Mice lacking Sema3B, PlexinA1 or Nrp2 exhibit stalled commissural growth at the floor plate and aberrant turning post-crossing (Zou et al. 2000; Nawabi et al. 2010).

Other axon guidance cues

More recently, another secreted roof plate cue called dorsal repulsive axon guidance protein or Draxin was identified as exhibiting a repellent influence on spinal commissural axon extension (Islam et al. 2009). Commissural tracts continue to migrate ventrally in Draxin-null mice, however these projections appear defasciculated (Islam et al. 2009). The angiogenic factor vascular endothelial growth factor (VEGF) has also been reported to contribute to commissural axon guidance in the embryonic spinal cord. Secreted at the floor plate, VEGF acts as another midline attractant for extending commissural neurons via the Flk1 receptor (Ruiz de Almodovar et al. 2011). Loss-of-function studies demonstrated that removing VEGF expression resulted in abnormal and defasciculated commissural trajectories in mice (Ruiz de Almodovar et al. 2011).

Although more molecules are likely to be identified in the future, axon guidance cues play significant roles in mediating axon pathfinding, whether it be by steering the growth cone in a particular direction or simply by regulating adhesion and growth.

GROWTH CONE

Initially identified by Santiago Ramón y Cajal over 120 years ago, the growth cone is a highly motile structure at the tip of an extending axon that can probe its local environment and respond to positive and negative extracellular signals by activating signalling mechanisms that modify its cytoskeleton (Ramón y Cajal 1890). Microtubules and filamentous actin (F-actin) are the main cytoskeletal elements that regulate growth cone morphology. Microtubules are long polarized polymers that form the backbone of the axon and the central domain of the growth cone, while F-actin assembles into networks and bundles in the growth cone periphery to provide shape and movement. Microtubules and F-actin are dynamic polymers, with tubulin and globular actin (G-actin) subunits constantly undergoing assembly and disassembly. This provides the cell with the ability to form, alter and dismantle cytoskeletal structures as needed.

The growth cone explores its surroundings by forming sheet-like protrusions called lamellipodia and rod-like projections called filopodia at its leading edge. The constant reorganization and turnover of actin filaments in these dynamic sensory structures directs growth cone motility. In particular, filopodia localize several axon guidance receptors at their tips that allow them to sense ligands in the environment and direct the cytoskeletal reorganization that triggers the growth cone to retract, extend or turn depending on the signal.

Regulation of the actin cytoskeleton

During axon pathfinding, growth cones integrate inputs from a variety of axon guidance cues to orient the axon in a given direction. Directional growth cone movements are powered by the temporal and spatial regulation of actin polymerization and by retrograde flow. This

section will focus on a variety of factors involved in the organization and modification of the actin cytoskeleton.

Actin-binding proteins

1. Actin nucleating proteins

Actin filaments are formed through the polymerization of actin monomers in a polarized manner. Actin nucleators are molecules that initiate filament assembly by forming a stable actin "nucleus" composed of 3 actin subunits, and include proteins such as the formins and the Arp2/3 complex. Formins catalyze the formation of long linear actin filaments, such as those found in the filopodia (Mellor 2010). On the other hand, the Arp2/3 complex is comprised of 7 subunits, including the actin-related proteins Arp2 and Arp3, and binds to the side of pre-existing actin filaments to facilitate the nucleation of new branches. The polymerization of these branched actin networks is often associated with the formation of lamellipodia (Suraneni et al. 2012). Furthermore, the activity of the Arp2/3 complex requires the association of nucleation promoting factors (NPFs), like the Wiskott-Aldrich syndrome protein (WASp) or WASp family Verprolin-homologous protein (WAVE), to deliver actin subunits to the complex.

2. Actin monomer binding proteins

Following nucleation, actin filaments extend through the rapid addition of actin monomers at its fast-growing end, termed the plus end. The availability of subunits from the G-actin pool is determined by actin monomer binding proteins, such as thymosin β4 and profilin. Thymosin β4 binds and sequesters G-actin, impeding actin polymerization and maintaining the pool of monomeric actin (Safer et al. 1991). Conversely, profilin forms a complex with G-actin and shuttles it to the plus end of filaments to facilitate F-actin elongation (Pring et al. 1992). Since profilin cannot intrinsically transfer the actin monomer to the plus end, it collaborates with formins and WASp family members to accelerate actin assembly (Pantaloni and Carlier 1993; Romero et al. 2004). Once the actin subunit is incorporated into the filament, profilin detaches and is free to bind to another monomer.

3. Capping and anti-capping proteins

Capping proteins such as CapZ tightly bind to the plus end of filaments and prevent the addition of new actin subunits, thereby ceasing elongation (Wear and Cooper 2004). On the other hand, anti-capping proteins interact with the growing end of actin filaments to promote actin assembly and recruit profilin-bound monomeric actin (Ferron et al. 2007). Although the exact mechanisms of how they mediate actin elongation are unclear, anti-capping proteins like members of the Enabled/vasodilator-stimulated phosphoprotein (Ena/VASP) family are involved in cell migration and axon guidance (Bear et al. 2002; Drees and Gertler 2008). Together, capping and anti-capping proteins regulate actin filament organization and dynamics.

4. Actin severing proteins

An important part of actin cytoskeletal remodeling is the disassembly and turnover of actin filaments. This is in part carried out by actin severing proteins that bind and destabilize F-actin, resulting in filament severing and actin depolymerization at its slow-growing or minus end. These proteins are required to promote rapid actin remodeling as well as replenish the pool of monomeric actin. The most well-studied actin severing proteins are gelsolin and actin depolymerizing factor (ADF)/cofilin (Janmey et al. 1985; Aizawa et al. 1995). In particular, cofilin is found at the leading edge of growth cones and has been implicated in several axon guidance pathways (Aizawa et al. 2001; Marsick et al. 2010; Marsick et al. 2012).

5. Actin bundling and cross-linking proteins

Once actin filaments are formed, they are organized into large, stable structures that create the framework for cell morphology. Actin bundling proteins like fascin and α-actinin, assemble actin filaments into tightly packed parallel bundles (Maruyama and Ebashi 1965; Bryan et al. 1993). These proteins have been reported to play a role in the formation of membrane ruffles, filopodia and stress fibers (Lazarides and Burridge 1975; Yamashiro-Matsumura and Matsumura 1986; Sasaki et al. 1996). Alternatively, cross-linking proteins such as filamin cross-link newly polymerized filaments to form branched networks, and are often

found in stress fibers and lamellipodia (Wang et al. 1975; Pavalko et al. 1989; Flanagan et al. 2001).

Rho GTPases

The Rho family of small guanosine triphosphatases (GTPases) are key signalling molecules that regulate cytoskeletal dynamics. They act as molecular switches to control signal transduction pathways that link extracellular signals to the cytoskeleton. Rho GTPases are inactive when in a GDP-bound form and active when GTP-bound. Cycling between these states is regulated by guanine nucleotide-dissociation inhibitors (GDIs), guanine nucleotide exchange factors (GEFs), and GTPase-activating proteins (GAPs). When inactive, Rho GTPases are sequestered in the cytoplasm through the association with GDIs. GEFs activate Rho GTPases by promoting the release of GDIs and catalyzing the exchange of GDP for GTP. Inactivation of these signalling molecules occurs with the hydrolysis of GTP, a process that is stimulated by GAPs.

The best characterized members of the Rho GTPase family are Rho, Rac and Cdc42, specifically for their role in the organization of the actin cytoskeleton. The activation of Rho is implicated in the formation of focal adhesions, stress fibers and myosin contractility, which is required for process retraction (Ridley and Hall 1992; Mitchison and Cramer 1996). Rho has been shown to directly promote actin polymerization by activating formins (Kohno et al. 1996; Watanabe et al. 1997). Moreover, it can prevent actin depolymerization through the activation of its downstream effector, Rho-associated protein kinase (ROCK) and LIM kinases (LIM-K), which in turn inactivate cofilin (Maekawa et al. 1999). On the other hand, Rac and Cdc42 activity lead to the assembly of actin networks at the cell periphery to produce lamellipodia and membrane ruffles, and filopodia, respectively (Nobes and Hall 1995). This is mediated via the interaction of Rac and Cdc42 with WASp family members, which subsequently activate the Arp2/3 complex (Rohatgi et al. 1999; Miki et al. 2000). Rac can also stimulate actin polymerization by promoting the uncapping of actin filaments (Tolias et al. 2000). Furthermore, studies demonstrated that Rac and Cdc42 activate the serine/threonine kinase PAK, which stimulates the activity of LIM-K and leads to the inhibition of cofilin (Edwards et al. 1999). The

activity of these three Rho GTPases is suggested to be sequential, with the activity of Rho being dependent on Rac activation, which itself requires Cdc42 activation (Ridley et al. 1992; Nobes and Hall 1995).

A number of axon guidance molecules have been demonstrated to regulate the activity of Rho GTPases. Netrin and DCC mediate outgrowth by increasing Rac and Cdc42 activity, and inhibiting RhoA in spinal commissural neurons (Shekarabi et al. 2005; Moore et al. 2008). Through Robo receptors, Slits induce the activation of Rac and Rho to mediate axon repulsion at the CNS midline in *Drosophila*, and inhibit Cdc42 to mediate repulsion of migrating cells in the forebrain (Wong et al. 2001). Ephrins can activate Rho through Eph receptors in retinal ganglion cells to induce growth cone collapse, while reverse ephrin/Eph signalling can activate Rac and Cdc42 to direct repulsive pruning of hippocampal mossy fibers (Wahl et al. 2000; Xu and Henkemeyer 2009). Another example is the action of semaphorins and plexins, which enhance RhoA signalling while simultaneously inhibiting Rac activity in motor axon guidance in *Drosophila* (Hu et al. 2001).

Src family kinases

Tyrosine phosphorylation is a regulatory mechanism involved in a multitude of intracellular signalling events. Src family kinases (SFKs) are a large group of cytoplasmic protein tyrosine kinases that also participate in the regulation of the actin cytoskeleton. There are 11 members of the SFK family: Src, Yes, Fyn, Fgr, Lck, Lyn, Hck, Blk, Frk, Srm and Brk (Manning et al. 2002). Their function is dependent on their expression patterns, which varies greatly for each individual member. Src, Yes and Fyn are ubiquitously expressed in most of tissues, whereas Blk, Fgr, Hck, Lck and Lyn are expressed primarily in hematopoietic cells (Thomas and Brugge 1997). The highly divergent members Srm, Brk and Frk are predominantly found in epithelial-derived cells (Serfas and Tyner 2003).

Since they are involved in a vast array of cellular processes, the activity of SFKs needs to be tightly controlled. All SFKs share the Src homology 3 (SH3) and SH2 domains for protein-

protein interactions, a protein kinase domain and a regulatory C-terminal tail. Their N-terminal domains can also undergo lipid modifications to target them to cell membranes. SFK activity is regulated through two conserved tyrosine residues: phosphorylation of Tyr-416 found in the activation loop of the kinase domain leads to enhanced kinase activity, while phosphorylation of Tyr-527 in the regulatory tail maintains the protein in an inactive conformation (Thomas and Brugge 1997). C-terminal Src kinase (Csk) is a tyrosine kinase that inhibits SFK activity by specifically phosphorylating members at Tyr-527 (Okada et al. 1991). In general, SFKs are found in their inactive conformation, which is destabilized through interactions with tyrosine-phosphorylated proteins via the SH2 domain and ligands containing proline-rich motifs via the SH3 domain (Songyang et al. 1993). This initiates the dephosphorylation of Tyr-527 by phosphatases and the auto-phosphorylation of Tyr-416 to hold the kinases in the active form (Thomas and Brugge 1997). These active SFKs can now phosphorylate downstream effectors, creating new binding sites for other SFKs and triggering a positive feedback loop. After the signal transduction cascade is over, SFKs are inactivated by Tyr-527 phosphorylation or rapidly degraded by a ubiquitin-proteosome pathway (Hakak and Martin 1999).

SFKs are involved in cytoskeletal organization by activating several cytoskeletal proteins. Src can interact with cortactin, an actin binding protein that promotes actin polymerization by recruiting the Arp2/3 complex to existing actin filaments (Wu and Parsons 1993). The Src kinase has also been demonstrated to phosphorylate the severing protein gelsolin, resulting in its inhibition (De Corte et al. 1997). SFKs play a significant role in the formation of focal adhesions, dynamic sites of adhesion between the cytoskeleton and the underlying extracellular matrix. Src and Fyn can phosphorylate several focal adhesion molecules like focal adhesion kinase (FAK), ezrin and paxilin to induce the formation of focal adhesion complexes downstream of integrin signalling (Schaller et al. 1994; Richardson et al. 1997; Heiska and Carpen 2005). Src activation is also linked to the formation of podosomes or invadopodia, dynamic actin-rich structures involved in cell motility (Gatesman et al. 2004; Destaing et al. 2008).

SFKs can also regulate the activity of Rho GTPases. Src phosphorylation of Rho GDIs induces the release of GDP-Rho GTPases from their inhibitory complex with GDIs, allowing

them to be activated by GEFs (DerMardirossian et al. 2006). Additionally, SFKs have been demonstrated to activate GEFs and GAPs for Rho GTPases downstream of many cellular processes, including integrin mediated adhesion and growth-factor receptor signalling (Bromann et al. 2004; Huveneers and Danen 2009).

Lastly, SFKs are essential mediators of axon guidance pathways. They are involved downstream of ephrin/Eph signalling for the repulsion of retinal ganglion and cortical cells, spinal motor axon projection in limb development, and topographic map formation of retinal ganglion cells (Knoll and Drescher 2004; Zimmer et al. 2007; Lim et al. 2008; Kao et al. 2009). Netrin signalling also requires the activation of SFKs to mediate the attractive turning of spinal commissural and cortical neurons, and to direct cell migration of distal tip cells in *Caenorhabditis elegans* (Li et al. 2002a; Liu et al. 2004a; Itoh et al. 2005). Fyn can mediate semaphorin repellent responses through PlexinA to guide cortical dendritic projections (Sasaki et al. 2002). Shh also engages Fyn and Src to induce commissural axon turning in a Smo dependent-manner (Yam et al. 2009).

Phosphoinositides

Members of the phosphoinositide family also play a significant role in the regulation of actin dynamics. Phosphatidylinositol and its derivative phosphoinositides are a family of lipids that are involved in the regulation of many signal transduction events. Seven derivatives of phosphatidylinositol exist, all generated through the phosphorylation of phosphatidylinositol at different positions of its inositol ring by specific phosphatidylinositol kinases. These molecules associate with actin binding proteins to regulate their activity or subcellular localization to modulate their interaction with actin. Phosphatidylinositol 4,5-bisphosphate, also known as $PI(4,5)P_2$ or PIP_2, typically inhibits proteins involved in actin filament disassembly such as cofilin, and activates proteins that induce actin polymerization like WASp (Rohatgi et al. 2000; Hilpela et al. 2004; van Rheenen et al. 2007). Phosphoinositides also control the activity of Rho GTPases. Several GEFs and GAPs contain binding sites for phosphoinositides, which therefore

can regulate the activation/inactivation of GTPases. Interestingly, Rho and Rac can also induce the synthesis of PIP_2 (Oude Weernink et al. 2000; Chatah and Abrams 2001).

CONCLUDING REMARKS

The study of the actin cytoskeleton has been a fervent topic of research in recent years and has unraveled multiple mechanisms underlying growth cone movements, axon outgrowth, axon guidance and synapse formation. The coordination of all of these events is required to properly guide neurons to their correct targets during development. Understanding these processes is vital to appreciate the complexity of the nervous system, and more importantly, can provide insight into the development of strategies to promote axon regeneration and functional recovery following injury to the CNS or as a result of neurodegenerative diseases.

CHAPTER 2

Literature Review II

Netrins and their functions

PREFACE

Netrins are secreted proteins that were first identified as guidance cues, directing cell and axon migration during neural development. Subsequent findings have demonstrated that netrins can influence the formation of multiple tissues, including the vasculature, lung, pancreas, muscle, and mammary gland by mediating cell migration, cell-cell interactions and cell-extracellular matrix adhesion. Recent evidence also implicates the ongoing expression of netrins and netrin receptors in the maintenance of cell-cell organization in mature tissues. This chapter will review the mechanisms involved in netrin signalling in vertebrates and invertebrate systems and discuss the functions of netrin signalling during the development of neural and non-neural tissues. This section is adapted from the review article "Netrins: versatile extracellular cues with diverse functions", published in Development (Lai Wing Sun et al., 2011).

Netrins are a family of extracellular, laminin-related proteins that function as chemotropic guidance cues for migrating cells and axons during neural development. They act as chemoattractants for some cell types and chemorepellents for others. Loss-of-function mutations in netrin-1 or certain netrin receptors are lethal in mice, highlighting the importance of netrin signalling during development. Insights into the functions of netrins have arisen from studies across a wide range of animal species, including invertebrates such as the nematode worm *Caenorabditis elegans* and the fruit fly *Drosophila melanogaster*; non-mammalian vertebrates such as the frog *Xenopus laevis*; and mammals including rats, mice, and humans.

Since its discovery in the early 1990s, it is now becoming clear that the netrin gene family exhibits a rich biology, with significance beyond neural development, and contributes to the organization of multiple tissues. Along with a number of other identified axon guidance cues (Hinck 2004), secreted netrins influence organogenesis outside the central nervous system (CNS), directing cell migration and mediating cell-cell adhesion in the lung, pancreas, mammary gland, vasculature and muscle (Srinivasan et al. 2003; Yebra et al. 2003; Kang et al. 2004; Liu et al. 2004b; Lu et al. 2004; Lejmi et al. 2008). Here, we discuss the cell biology of netrin and netrin receptor functions and review the downstream signal transduction mechanisms that they activate. We also provide an overview of netrin function during development, both within the nervous system and within other developing organs and tissues.

NETRIN FAMILY MEMBERS

The first reported member of the netrin family, uncoordinated-6 (UNC-6), was identified in a search for gene products that regulate neural development in *C. elegans* (Ishii et al. 1992). Netrins have since been identified and studied in multiple vertebrate and invertebrate species (Table 2.1), including *X. laevis*, *D. melanogaster* and the sea anemone *Nematostella vectensis*, an animal that exhibits early hallmarks of the origins of bilateral symmetry (Harris et al. 1996; Mitchell et al. 1996; de la Torre et al. 1997; Matus et al. 2006). In mammals, four secreted netrins, netrin-1, -3, -4, and two membrane-tethered glycophosphatidylinositol (GPI)-linked netrins, netrin-G1 and -G2, have been identified (Table 2.1). Orthologs of netrin-1, which have

been identified in all bilaterally symmetrical animals studied so far, play a highly conserved role directing cell and axon migration in the embryonic nervous system. Among the secreted netrins, netrin-1 expression and function has been best characterized. Netrin-1 is expressed in regions of both the developing and adult nervous systems, including the optic disc, forebrain, cerebellum, and spinal cord (Kennedy et al. 1994; Deiner et al. 1997; Livesey and Hunt 1997; Hamasaki et al. 2001). Netrin-1 is also highly expressed in various tissues outside of the nervous system, including the developing heart, lung, pancreas, intestine and mammary gland (Srinivasan et al. 2003; Yebra et al. 2003; Liu et al. 2004b; Shin et al. 2007; Zhang and Cai 2010).

All netrins are composed of ~600 amino acids and belong to the superfamily of laminin-related proteins (Yurchenco and Wadsworth 2004). N-terminal netrin sequences are homologous to domains VI and V found at the N-termini of laminins; the N-terminal domains of netrin-1 and netrin-3 show most similarity to the laminin γ1 chain (Serafini et al. 1994; Wang et al. 1999), and those of netrins-4, -G1 and -G2 are most similar to the laminin β1 chain (Nakashiba et al. 2000; Yin et al. 2000; Nakashiba et al. 2002) (Fig. 2.1A). In secreted netrins, these domains (VI and V) are linked to a C-terminal domain, termed “domain C” or the netrin-like (NTR) module. This module is not homologous to any laminin domain, but exhibits sequence similarity to the tissue inhibitor of metalloproteinases (TIMPs), is rich in basic amino acid residues and can bind heparin (Serafini et al. 1994; Banyai and Patthy 1999; Kappler et al. 2000). Although all netrins include laminin-like domains, a clear functional distinction can be made between the secreted netrins and the GPI-linked netrin-G proteins, due to the engagement of different sets of receptor proteins.

NETRIN RECEPTORS

In mammals, receptors for the secreted netrins include Deleted in colorectal cancer (DCC), the DCC paralog neogenin, the UNC5 homologs UNC5A-D, Down syndrome cell adhesion molecule (DSCAM) and amyloid precursor protein (APP) (Table 2.1). The GPI-linked netrins, by contrast, function by binding to the netrin-G ligands (NGLs), NGL-1 and NGL-2, which belong to a family of transmembrane proteins that are structurally and functionally distinct from the

secreted netrin receptors. Most of the netrin receptors identified thus far are single-pass type I transmembrane proteins and are members of the immunoglobulin (Ig) superfamily (Fig. 2.1B).

DCC receptor family

DCC was originally identified in humans as a candidate tumor suppressor associated with an allelic deletion of chromosome 18q21 in colon cancer (Vogelstein et al. 1988; Fearon et al. 1990). The most commonly studied members of the DCC family include DCC and neogenin in mammals (Cho et al. 1994; Vielmetter et al. 1994), UNC-40 in *C. elegans* (Chan et al. 1996), and Frazzled in *D. melanogaster* (Kolodziej et al. 1996) (Table 2.1). Extracellularly, all members of the DCC family are composed of four Ig domains and six fibronectin type III domains (FNIII) (Fig. 2.1B), with evidence to suggest that netrin-1 binds to the fourth and fifth FNIII repeats (Geisbrecht et al. 2003; Kruger et al. 2004; Xu et al. 2014). Intracellularly, DCC does not encode any obvious catalytic domain but contains three highly conserved sequences, termed the P1-3 motifs (Keino-Masu et al. 1996). DCC mediates chemoattractant responses to netrin-1 through -4, and also contributes to chemorepellent signalling (Kennedy et al. 1994; Colavita and Culotti 1998; Hong et al. 1999; Wang et al. 1999; Jarjour et al. 2003; Qin et al. 2007). Neogenin, another member of the DCC family, shares ~50% amino acid identity with DCC (Vielmetter et al. 1994). Although less well-studied than DCC, recent reports have provided insight into neogenin function and signalling (De Vries and Cooper 2008). Interestingly, neogenin appears to act as an attractive axon guidance receptor in response to netrin-1, but also as a repellent receptor when bound to repulsive guidance molecule (RGMa), an alternative ligand that does not belong to the netrin family (Rajagopalan et al. 2004; Wilson and Key 2006). In addition to their roles in axon guidance, both DCC and neogenin regulate cell-cell adhesion and tissue organization through interactions with the secreted netrins (Srinivasan et al. 2003; Kang et al. 2004; Park et al. 2004; Lejmi et al. 2008; Krauss 2010).

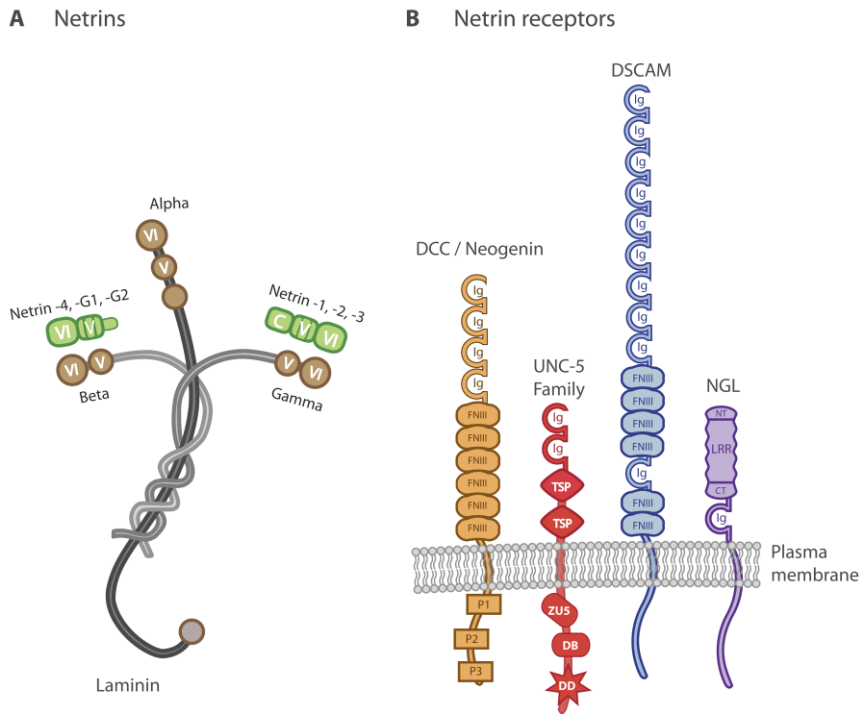

Figure 2.1 - Netrin proteins and their receptors. (**A**) Netrins are members of the laminin superfamily. N-terminal netrin sequences encode domains VI and V (green), which are homologous to the N-terminal domains VI and V of laminins (brown). These domains in netrins-1, -2, and -3 are most similar to the laminin γ chain, whereas those in netrins-4, -G1 and -G2 are most similar to the laminin β chain. The C- terminal C domain (C) of netrins-1 to -4, -G1 and -G2 are not homologous to laminin, nor are the C domains of netrins 1-4 homologous to the C domains of the netrin-G proteins. (**B**) The netrin receptors illustrated are all single-pass transmembrane proteins and members of the Ig superfamily. They include Deleted in colorectal cancer (DCC), the DCC paralog neogenin found in vertebrates, members of the UNC5 homolog family, DSCAM and the netrin-G ligands (NGLs). (CT, C-terminal cysteine-rich capping structure; DB, DCC-binding domain; DD, death domain; FNIII, fibronectin type III domain; Ig, immunoglobulin domain; LRR, leucine-rich repeat; NT, N-terminal cysteine-rich capping structure; P1, P2 and P3, conserved regions in the cytoplasmic domain of DCC; TSP, thrombospondin domain; ZU5, zona occludens 5 domain).

The UNC5 receptor family

Four orthologs of *C. elegans* UNC-5, UNC5A-D, have been characterized in vertebrates (Table 2.1). Extracellularly, they are composed of two Ig domains and two thrombospondin type I domains (TSP-1) (Fig. 2.1B), with the Ig repeats required for netrin-1 binding (Leonardo et al.

1997; Geisbrecht et al. 2003; Krauss 2010). The UNC5 intracellular domain encodes a ZU-5 domain, a DCC-binding (DB) motif, and a death domain (DD) (Hofmann and Tschopp 1995; Leonardo et al. 1997).

Netrin-1 and netrin-3 are chemorepellents for axons of *Xenopus* spinal neurons and rodent trochlear motoneurons, which express UNC5 homologs (Colamarino and Tessier-Lavigne 1995a; Hong et al. 1999; Wang et al. 1999). Although these chemorepellent responses require expression of an UNC5 protein, in some cases this response also depends on the co-expression of DCC with UNC5 (Colavita and Culotti 1998; Hong et al. 1999). In fact, many neurons in vertebrates and invertebrates express both UNC5 homologs and DCC. However, in contrast to DCC-dependent chemorepulsion, genetic analyses in *C. elegans* and *D. melanogaster* have provided examples of UNC5-dependent repellent responses that occur in the absence of the DCC homologs UNC-40 and Frazzled (Keleman and Dickson 2001; Merz et al. 2001).

The extracellular and intracellular domains of DCC and UNC5 exhibit a remarkable modularity of function. For example, expression in cultured *Xenopus* spinal neurons of a chimeric receptor composed of the extracellular domain of rat DCC fused to the intracellular domain of an UNC5 homolog is sufficient to elicit an axonal chemorepellent response to netrin-1 that is similar to that of full-length UNC5 (Hong et al. 1999). Conversely, a chimera composed of the intracellular domain of DCC with an UNC5 extracellular domain signals chemoattraction (Hong et al. 1999; Keleman and Dickson 2001). An important generalization drawn from these studies is that the intracellular domain of netrin receptors is crucial for their ability to mediate attractant or repellent responses to netrin.

Down syndrome cell adhesion molecule (DSCAM)

DSCAM was originally identified as a gene that is duplicated in Down syndrome and was recently reported to function as a netrin receptor (Yamakawa et al. 1998; Andrews et al. 2008a; Ly et al. 2008; Liu et al. 2009). In mammals, DSCAM is expressed by a specific population of interneurons, the embryonic spinal commissural neurons, and contributes to guiding these

axons to the floor plate of the developing spinal cord (Ly et al. 2008; Liu et al. 2009). In *Drosophila*, DSCAM and DSCAM3 similarly promote midline crossing by axons in response to netrin-A and -B (Andrews et al. 2008a). The DSCAM extracellular domain is composed of ten Ig domains and six FNIII repeats (Fig. 2.1B), with netrin-1 proposed to bind to the Ig loops (Yamakawa et al. 1998; Ly et al. 2008). Current findings suggest that DSCAM evokes chemoattractant responses to netrin-1 independently of DCC (Ly et al. 2008).

Netrin-G ligands

NGL-1 and NGL-2 bind to netrin-G1 and netrin-G2, respectively, and are thus considered to be receptors for netrin-G proteins (Nakashiba et al. 2002; Lin et al. 2003; Kim et al. 2006). The NGL transmembrane proteins are composed of leucine rich repeats (LRRs) and Ig domains (Lin et al. 2003; Kim et al. 2006) (Fig. 2.1B). NGL receptors and the netrin-G proteins are enriched at synapses and regulate glutamatergic synaptogenesis (Woo et al. 2009b). Notably, NGL-2 also interacts with the post-synaptic intracellular scaffolding protein PSD-95 (Kim et al. 2006). A third member of the NGL family, NGL-3, does not bind to netrin-G1 or netrin-G2, but contributes to the regulation of glutamatergic synaptogenesis through interactions with the transmembrane receptor tyrosine phosphatases LAR, protein-tyrosine phosphatase δ (PTPδ) and PTPσ (Woo et al. 2009a; Kwon et al. 2010).

Amyloid precursor protein (APP)

Initially characterized as a protein involved in the formation of amyloid plaques associated with Alzheimer disease, APP was recently identified as a novel netrin-1 receptor (Lourenco et al, 2009). APP is a transmembrane protein that generates amyloid-β (Aβ) peptides following proteolytic cleavage of its transmembrane region. The binding of netrin-1 to APP suppresses the production of Aβ in the adult brain of Alzheimer mouse models (Lourenco et al, 2009). APP is also expressed by spinal commissural neurons and acts as a co-receptor for DCC to mediate netrin-1 signalling during commissural axon pathfinding (Rama et al, 2012).

Table 2.1 - Netrin and netrin receptor homologs

Species	Latin name	Netrin	DCC family	Unc5 family
Human	*Homo sapiens*	Netrin-1	Neogenin	UNC5A
		Netrin-3	DCC	UNC5B
		(netrin 2-like)		
		Netrin-4		UNC5C
		Netrin-G1		UNC5D
		Netrin-G2		
Mouse	*Mus musculus*	Netrin-1	Neogenin	UNC5A
		Netrin-3	DCC	UNC5B
		Netrin-4		UNC5C
		Netrin-G1		UNC5D
		Netrin-G2		
Rat	*Rattus norvegicus*	Netrin-1	Neogenin	UNC5A
		Netrin-3	DCC	UNC5B
		Netrin-4		UNC5C
		Netrin-G1*		UNC5D
		Netrin-G2		
Chicken	*Gallus gallus*	Netrin-1	Neogenin	UNC5A*
		Netrin-2	DCC	UNC5B
		Netrin-4*		UNC5C
		Netrin-G1*		UNC5D*
		Netrin-G2*		
Zebrafish	*Danio rerio*	Netrin-1a	Neogenin	Unc5a*
		Netrin-1b	Dcc	Unc5b
		Netrin-2		Unc5c*
		Netrin-4		Unc5d*
		Netrin-G1*		
Clawed frog	*Xenopus laevis*	Netrin-1	DCC	UNC5b
Fruit fly	*Drosophila melanogaster*	Netrin-A	Frazzled	UNC-5
		Netrin-B		
Nematode	*Caenorhabditis elegans*	UNC-6	UNC-40	UNC-5
Lamprey	*Petromyzon marinus*	Netrin	Neogenin	UNC-5
Medicinal leech	*Hirudo medicinalis*	Netrin	-	-
Amphioxus	*Branchiostoma floridae*	AmphiNetrin	-	-
Sea squirt	*Ciona intestinalis*	Ci-netrin	-	-
Sea urchin	*Hemicentrotus pulcherrimus*	HpNetrin	-	-
Sea anemone	*Nematostella vectensis*	Netrin	-	-

The four vertebrate UNC-5 homologs UNC5A-D are sometimes described as UNC5H1-4. To date, receptors have not been identified for the medicinal leech, amphioxus, sea squirt, sea urchin and sea anemone. * Sequences identified in GenBank but not published in the literature.

Human: netrin-1 (Meyerhardt et al. 1999), netrin-3 (Van Raay et al. 1997), netrin-4 (Koch et al. 2000), netrin-G1 (Nakashiba et al. 2000), netrin-G2 (Nakashiba et al. 2002), neogenin (Meyerhardt et al. 1997), DCC (Fearon et al. 1990), UNC5A (Tanikawa et al. 2003; Thiebault et al. 2003), UNC5B (Komatsuzaki et al. 2002; Tanikawa et al. 2003), UNC5C (Ackerman and Knowles 1998), UNC5D (Wang et al. 2008a).

Mouse: netrin-1 (Serafini et al. 1996), netrin-3 (Wang et al. 1999), netrin-4 (Koch et al. 2000; Yin et al. 2000), netrin-G1 (Nakashiba et al. 2000), netrin-G2 (Nakashiba et al. 2002), neogenin (Keeling et al. 1997), DCC (Cooper et al. 1995), UNC5A (Leonardo et al. 1997; Engelkamp 2002), UNC5B (Leonardo et al. 1997; Engelkamp 2002), UNC5C (Ackerman et al. 1997), UNC5D (Engelkamp 2002).

Rat: netrin-1 (Manitt et al. 2001), netrin-3 (Manitt et al. 2001), netrin-4 (Zhang et al. 2004), netrin-G2 (Pan et al. 2010), neogenin (Keino-Masu et al. 1996), DCC (Fearon et al. 1990; Keino-Masu et al. 1996), UNC5A (Leonardo et al. 1997), UNC5B (Leonardo et al. 1997), UNC5C (Kuramoto et al. 2004), UNC5D (Zhong et al. 2004).

Chicken: netrin-1 (Serafini et al. 1994), netrin-2 (Serafini et al. 1994), neogenin (Vielmetter et al. 1994), DCC (Chuong et al. 1994), , UNC5B (Bouvree et al. 2008), UNC5C (Guan and Condic 2003).

Zebrafish: Netrin-1a (Lauderdale et al. 1997), Netrin-1b (Strahle et al. 1997), Netrin-2 (Park et al. 2005), Netrin-4 (Park et al. 2005), Neogenin (Shen et al. 2002), Dcc (Hjorth et al. 2001),Unc5b (Lu et al. 2004; Kaur et al. 2007).

Frog: netrin-1 (de la Torre et al. 1997), DCC (Pierceall et al. 1994), UNC5b (Anderson and Holt 2002; Karaulanov et al. 2009).

Fruit fly: Netrin-A (Harris et al. 1996; Mitchell et al. 1996), Netrin-B (Harris et al. 1996; Mitchell et al. 1996), Frazzled (Kolodziej et al. 1996), UNC-5 (Keleman and Dickson 2001).

Nematode: UNC-6 (Ishii et al. 1992), UNC-40 (Chan et al. 1996), UNC-5 (Leung-Hagesteijn et al. 1992).

Lamprey: netrin (Shifman and Selzer 2000b), neogenin (Shifman et al. 2009), UNC-5 (Shifman and Selzer 2000a). Leech: netrin (Gan et al. 1999). Amphioxus: AmphiNetrin (Shimeld 2000). Sea squirt: Ci-netrin (Hotta et al. 2000). Sea urchin: HpNetrin (Katow 2008). Sea anemone: netrin (Matus et al. 2006).

Other netrin receptors and binding proteins

Secreted netrins and DCC also interact with heparin, suggesting that they bind heparan sulfate proteoglycans (HSPGs) (Serafini et al. 1994; Bennett et al. 1997; Shipp and Hsieh-Wilson 2007). The positively charged C domain of secreted netrins interacts tightly with heparan sulfate, perhaps localizing and multimerizing netrin in the extracellular matrix (ECM) (Kappler et al. 2000; Geisbrecht et al. 2003; Shipp and Hsieh-Wilson 2007). The conditional ablation of the mouse *exostosin* 1 (*Ext1*) gene, which encodes an enzyme required for heparan sulfate synthesis, has revealed a cell autonomous function for heparan sulfate in embryonic spinal commissural neurons (Matsumoto et al. 2007). EXT1 is required for axonal chemoattraction to netrin-1, providing evidence for a functional interaction between HSPGs and at least one netrin receptor.

Netrins can also bind to integrins, a large family of transmembrane receptors that link the actin cytoskeleton to ECM proteins (Nikolopoulos and Giancotti 2005). Netrin-1 binds to $\alpha6\beta4$ and $\alpha3\beta1$ integrins, and this is suggested to regulate epithelial cell adhesion and migration (Yebra et al. 2003). Although netrin domains VI and V are homologous to laminins, and certain integrins function as laminin receptors, $\alpha6\beta4$ and $\alpha3\beta1$ integrins do not bind to these domains in netrins. Instead, they bind to a sequence of positively charged amino acids found at the C-terminus of netrin-1 (Yebra et al. 2003). Interestingly, however, the C-terminal domain does not appear to be required for axon chemoattraction, as a VI-V-Fc chimeric protein that lacks domain C is sufficient to promote outgrowth from rat embryonic spinal commissural axons *in vitro* (Keino-Masu et al. 1996). Although this suggests that $\alpha6\beta4$ and $\alpha3\beta1$ integrins are not essential for chemotropic responses to netrins, it does not rule out the possibility that integrins may functionally interact with netrins in other contexts.

Laminins are known to multimerize through their VI domains (Yurchenco and Wadsworth 2004). Netrin-4, but not netrin-1, -3, -G1 or -G2, can be incorporated into basement membranes of various tissues through the interaction of its domain VI with domain VI of laminin (Schneiders et al. 2007). Netrin-4 thereby inhibits basement membrane assembly by interfering with laminin multimerization, and also inhibits branching morphogenesis in the

developing lung and salivary gland (Koch et al. 2000; Liu et al. 2004b; Schneiders et al. 2007). As such, netrin-4 may directly influence organogenesis by signalling to cells from the basement membrane or by modifying the structure of the basement membrane itself.

NETRIN FUNCTION IN THE NERVOUS SYSTEM

The development of a functional nervous system depends on the establishment of precise connections between neurons. This requires migration of neural precursors to appropriately position cell bodies, and the projection of axons to synaptic targets. Studies of knockout mice have provided substantial insight into netrin and netrin receptor function in the nervous system. Mice lacking netrin-1 exhibit severe neurodevelopmental defects and die within a few hours after birth (Serafini et al. 1996), highlighting the importance of netrin signalling during development. Deficits in these mice include the disruption of multiple CNS commissures, including the ventral spinal commissure, the corpus callosum, and the anterior and hippocampal commissure (Serafini et al. 1996). Mice lacking DCC phenocopy the netrin-1 null mice remarkably closely (Fazeli et al. 1997), highlighting the role of DCC as a key netrin-1 receptor. *Unc5a* null mice are viable and live to adulthood, but exhibit reduced neuronal apoptosis (Williams et al. 2006). *Unc5b* knockout mice die during embryogenesis due to heart failure and substantial disruption of their vasculature (Lu et al. 2004). *Unc5c* null mice survive to adulthood, but are ataxic and exhibit cell migration defects in the cerebellum (Ackerman et al. 1997; Goldowitz et al. 2000). The ventral-dorsal trajectories of axons that normally project away from netrin-1 expressed at the ventral midline are also disrupted in *Unc5* nulls, including the axons of trochlear motoneurons (Burgess et al. 2006) and of hindbrain cerebellar, inferior olivary, and pontine axons (Kim and Ackerman 2011). These findings provide evidence that UNC5 homologs direct axon extension in the mammalian CNS. These studies, together with studies of netrin function in other model species, have revealed that netrins direct cell and axon migration and subsequently influence axon arborization and synapse formation during neural development. In the mature CNS, recent findings provide evidence that netrins also regulate

cell-cell interactions, including maintaining the organization of oligodendroglial paranodal junctions (Jarjour et al. 2008).

Neuronal precursor cell migration

Netrin function has been extensively studied during cerebellar development. Netrin-1 attracts migrating progenitor cells that originate from the lower rhombic lip towards the ventral midline to form the pontine nuclei in the hindbrain (Alcantara et al. 2000). This process depends on the expression of netrin-1 by midline cells and on the expression of DCC by migrating progenitors. Netrin-1 promotes precerebellar neuron migration in mice, which is disrupted by inhibiting RhoA-dependent nucleokinesis (Causeret et al. 2004). Netrin-1 directed migration and subsequent axon outgrowth by precerebellar neurons requires phosphorylation of the microtubule-associated protein MAP1B through the activation of the serine/threonine kinases cyclin-dependent kinase 5 (cdk5) and glycogen synthase kinase 3 (GSK3) (Del Rio et al. 2004). Consistent with this, MAP1B-deficient mice exhibit defects in the pontine nuclei and in several forebrain axon tracts, similar to the phenotypes of *netrin-1* or *dcc* mutants (Bloch-Gallego et al. 1999; Del Rio et al. 2004). Interestingly, during post-natal maturation, netrin-1 repels migrating cerebellar granule cell precursors, which regulate UNC5 expression (Alcantara et al. 2000). Netrin-1 has also been implicated as a chemorepellent for migrating adult neural stem cells at sites of injury in the mature nervous system, highlighting similar functions for netrin in directing cell migration during development and in adulthood (Petit et al. 2007).

Axon guidance

Although netrins are widely expressed in a range of tissues, they have largely been studied for their role as axon guidance cues during neural development. Substantial evidence supports the notion that netrins function as long-range chemotropic guidance cues in the embryonic vertebrate CNS. Floor plate cells express the *netrin-1* gene*,* and a gradient of netrin-1 protein is present in the embryonic spinal cord as commissural axons extend to the ventral

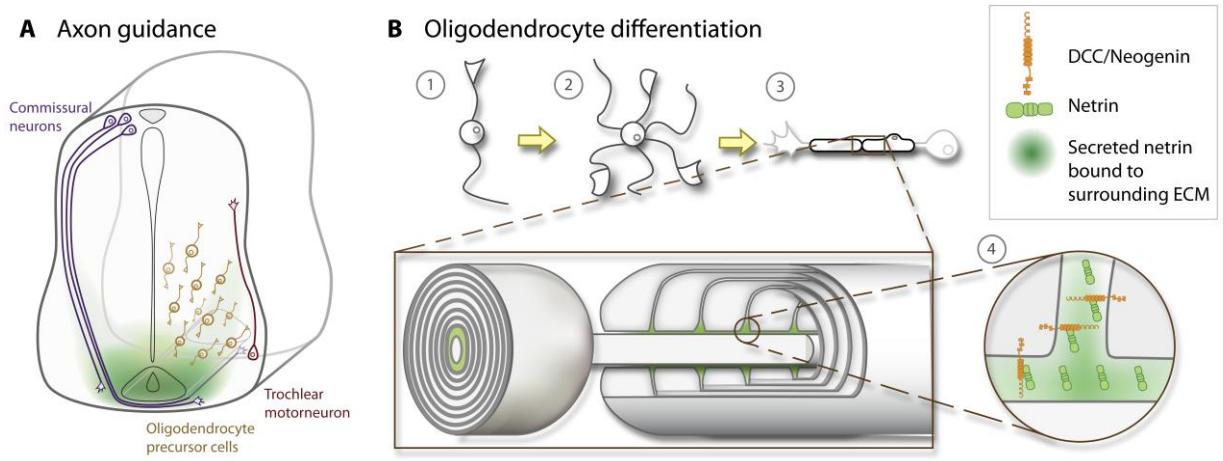

Figure 2.2 - Netrin function in the nervous system. (**A**) Within the developing spinal cord, netrin-1 (green) secreted by floor plate cells forms a gradient emanating from the ventral midline. The netrin-1 gradient is bifunctional, attracting the migration of some cells, such as spinal commissural axons (purple), and repelling others, such as migrating oligodendrocyte precursor cells (OPCs) in the spinal cord (orange) and the axons of trochlear motoneurons in the brainstem (red). (**B**) Netrin-1 influences oligodendrocytes at several stages of their differentiation: bipolar migrating OPCs (1) express DCC and UNC5A and are repelled by a gradient of netrin-1. Multipolar post-mitotic differentiating oligodendrocytes (2) express netrin-1, DCC and UNC5 homologs. Netrin-1 protein, from autocrine and paracrine sources, promotes process branching and myelin-like membrane sheet formation (3). Netrin-1 and DCC expressed by mature myelinating oligodendrocytes (4) are enriched at paranodal junctions, which are specialized junctions formed between non-compacted oligodendroglial membranes and the axon. The paranode flanks the node of Ranvier. Netrin-1 and DCC are required to maintain the organization of paranodal junctions.

midline (Tessier-Lavigne et al. 1988; Placzek et al. 1990; Kennedy et al. 1994; Serafini et al. 1996; Kennedy et al. 2006) (Fig. 2.2A). *In vitro* axon-turning assays have demonstrated that recombinant netrin-1 mimics the capacity of the floor plate to promote commissural axon outgrowth from explants of dorsal neural epithelium (Serafini et al. 1994). Similarly, a cellular source of netrin-1 attracts extending commissural axons, deflecting them from their dorsoventral trajectory in the embryonic neural tube (Kennedy et al. 1994). In this axon-turning assay, growth cones turned up to 250 µm away from the floor plate, revealing the capacity of

netrin-1 protein to diffuse at least this distance through the embryonic neural epithelium (Placzek et al. 1990; Kennedy et al. 1994). In a further reduced axon turning assay that utilized *Xenopus* retinal ganglion cells (RGCs) in dispersed culture, axonal growth cones could be attracted up a gradient of netrin-1 ejected from a pipette (de la Torre et al. 1997). In addition to chemoattraction, netrin-1 functions as a repellent for other cell types, such as the trochlear motoneurons and oligodendrocyte precursor cells (OPCs) (Colamarino and Tessier-Lavigne 1995a; Jarjour et al. 2003) (Fig. 2.2A). Subsequent studies have demonstrated that secreted netrins direct axon extension in many different parts of the developing nervous system.

Axon branching, innervation and synaptogenesis

Once an axon has reached its target, appropriate innervation often involves axon branching. Secreted netrins regulate branching and, similar to their roles in axon guidance, this contribution of netrins to neural development is evolutionarily conserved. For example, increased expression of the DCC homolog UNC-40, or the misexpression of the N-terminal domain of the netrin homolog UNC-6, increases axon branching by motoneurons in *C. elegans* (Lim et al. 1999; Wang and Wadsworth 2002; Gitai et al. 2003). UNC-40 promotion of axon branching in *C. elegans* requires MADD-2, a tripartite motif protein that recruits the actin regulatory protein MIG-10, the homolog of lamellipodin in vertebrates (Hao et al. 2010). Studies using mammalian neocortical neurons *in vitro* have demonstrated that local application of netrin-1 promotes *de novo* axon branch formation by rapidly inducing calcium (Ca^{2+}) transients, polymerization of F-actin, and the formation of filopodial protrusions that may become a branch point (Dent et al. 2004). The induced increase in intracellular Ca^{2+} appears to be crucial because inhibiting the netrin-1 mediated Ca^{2+} signalling pathway disrupts axon branch formation induced by netrin-1 (Tang and Kalil 2005).

Initial evidence in support of a role for netrins in synaptogenesis came from genetic analyses of *Drosophila* motoneurons, which form glutamatergic synapses on body wall muscles. Upregulating the expression of Netrin by muscle cells results in the increased formation of synaptic connections, whereas fewer synapses are established in the absence of Frazzled

expression by the motoneuron (Kolodziej et al. 1996; Mitchell et al. 1996; Winberg et al. 1998). Interestingly, the axon guidance cue Semaphorin was found to have an opposite effect to Netrin at this synapse (Winberg et al. 1998). When the expression of Netrin and Semaphorin were either simultaneously upregulated or absent, synaptic innervation was normal, suggesting that these factors are not required for the axon to find the muscle but rather that they modulate the number of connections that are made between motoneurons and muscles. In *C. elegans*, UNC-6 regulates synaptogenesis by organizing the subcellular distribution of presynaptic proteins and activating cytoskeletal regulators at pre-synaptic sites (Colon-Ramos et al. 2007; Poon et al. 2008; Stavoe and Colon-Ramos 2012; Stavoe et al. 2012). These findings have also been extended to vertebrates; perfusion of netrin-1 into the *Xenopus* optic tectum during development results in a DCC-dependent increase in RGC axon branching and the formation of additional presynaptic puncta (Manitt et al. 2009).

These data support a role for netrin during the early stages of synaptogenesis, but also raise the possibility that netrin-1 and DCC influence synapse structure and function in the mature nervous system. DCC is highly expressed, particularly by dopaminergic (DA) neurons during development and in adulthood (Livesey and Hunt 1997; Volenec et al. 1998). Mice heterozygous for the loss of *Dcc* function are viable but express reduced levels of DCC protein (Fazeli et al. 1997). Intriguingly, adult *Dcc* heterozygous mice exhibit a blunted response to amphetamine and do not develop behavioural sensitization to repeated doses of this drug (Flores et al. 2005; Grant et al. 2007). An examination of newborn *Dcc* heterozygous and null mice revealed defects in DA precursor cell migration, axon guidance, and terminal arborization (Xu et al. 2010). In particular, increased DA innervation was present in the medial prefrontal cortex of *Dcc* heterozygotes, a brain region that is associated with drug addiction (Steketee 2003; Xu et al. 2010). Although increased innervation was detected in adult *Dcc* heterozygotes, newborns were indistinguishable from wild-type littermates (Xu et al. 2010). This indicates that the increase in DA axon arborization occurs during post-natal development and is consistent with findings demonstrating that the response of *Dcc* heterozygotes to amphetamine changes during maturation (Grant et al. 2009). Another recent study demonstrated that DCC is implicated in synaptic plasticity of forebrain pyramidal neurons in the adult brain (Horn et al.

2013). Selectively removing DCC expression in these neurons after development resulted in shorter dendritic spines, impaired long-term potentiation (LTP) but intact long-term depression (LTD), and deficits in spatial and recognition memory (Horn et al. 2013). This occurred through the downregulation of the tyrosine kinase Src, which regulates N-methyl-D-aspartate-type glutamate receptor (NMDAR) function. These findings support the conclusion that DCC expression regulates the extent of axonal and terminal arborizations, as well as synaptic plasticity in the mammalian brain.

Oligodendroglial development and maturation

Netrin-1 makes key contributions to several stages of the maturation of oligodendrocytes, the myelinating cells of the CNS. In the embryonic spinal cord, OPCs, which express DCC and UNC5A, are repelled by the gradient of netrin-1 that emanates from the floor plate (Jarjour et al. 2003; Tsai et al. 2003; Tsai et al. 2006; Tsai et al. 2009) (Fig. 2.2A). This directs OPCs away from the ventricular zone where they were born and toward axons at the edge of the neural tube (Jarjour et al. 2003; Tsai et al. 2006). Upon reaching the nascent white matter, post-mitotic oligodendrocytes elaborate highly branched processes that then extend in search of axons (Kirby et al. 2006; Haber et al. 2009) (Fig. 2.2B). At this point in their differentiation, oligodendrocytes express netrin-1, and both autocrine and paracrine sources promote process branching and the elaboration of myelin-like membrane sheets (Rajasekharan et al. 2009). In migrating OPCs, netrin-1 activates the Rho GTPase RhoA, and requires DCC and the RhoA effector ROCK to mediate chemorepulsion (Rajasekharan et al. 2010). By contrast, netrin-1 inhibits RhoA in differentiating post-mitotic oligodendrocytes and this is required for netrin-1-dependent oligodendroglial process branching (Rajasekharan et al. 2010). These findings indicate that differential regulation of RhoA contributes to the distinct responses made by OPCs and post-mitotic oligodendrocytes to netrin-1.

In the mature CNS, myelinating oligodendrocytes continue to express netrin-1, DCC, and UNC5 homologs (Manitt et al. 2001; Manitt et al. 2004). Netrin-1 and DCC are particularly enriched at oligodendroglial paranodal junctions (Jarjour et al. 2008) (Fig. 2.2B). Paranodes

become disorganized in the absence of DCC or netrin-1, which results in the disruption of the nodes of Ranvier (Jarjour et al. 2008; Bull et al. 2014). DCC localized to the oligodendroglial membrane loops binds to netrin-1 on the axon surface to mediate oligo-axonal adhesion and organize the cytoskeleton within the oligodendrocyte (Bull et al. 2014). Together, these findings identify three distinct roles for netrin-1 at various points during development of the oligodendrocyte lineage: repelling precursor cell migration, promoting process elaboration during differentiation, and maintaining specialized cell-cell junctions in the mature cell. Although implicated at each stage of differentiation, how netrin-1 and DCC fulfil these different roles, and the unique signalling mechanisms involved in these events, are not fully understood.

NETRIN FUNCTION OUTSIDE THE NERVOUS SYSTEM

Netrins and netrin receptors are also expressed in a number of tissues outside of the nervous system and play key roles during development by regulating cell adhesion and tissue morphogenesis (Fig 2.3). In the developing mammary gland, terminal end buds are the growing tips of the ductal network and consist of two layers: the luminal epithelial cells and the cap cells. Netrin-1 secreted by the luminal cells binds to the DCC homolog neogenin, which is expressed by the adjacent cap cells. This mediates adhesion between the two cell layers, an event required for proper terminal end bud formation (Srinivasan et al. 2003) (Fig. 2.3A). Another example of a non-neuronal role for netrins occurs during branching morphogenesis of the embryonic lung, where netrin-1 and -4 are expressed by epithelial stalk cells and inserted into the basement membrane surrounding the developing endoderm buds. This sheath of netrin around the developing bud functions to constrain DCC- and UNC5B-expressing distal tip cells, thereby preventing excessive branching and ectopic bud formation (Liu et al. 2004b) (Fig. 2.3B). Pancreatic development also requires netrin-1, which is produced by epithelial ductal cells and associated with collagen IV and fibronectin in the local ECM (Yebra et al. 2003). In this context, interactions between netrin-1 and the $\alpha 6\beta 4$ and $\alpha 3\beta 1$ integrins are thought to contribute to epithelial cell-matrix adhesion (Yebra et al. 2003).

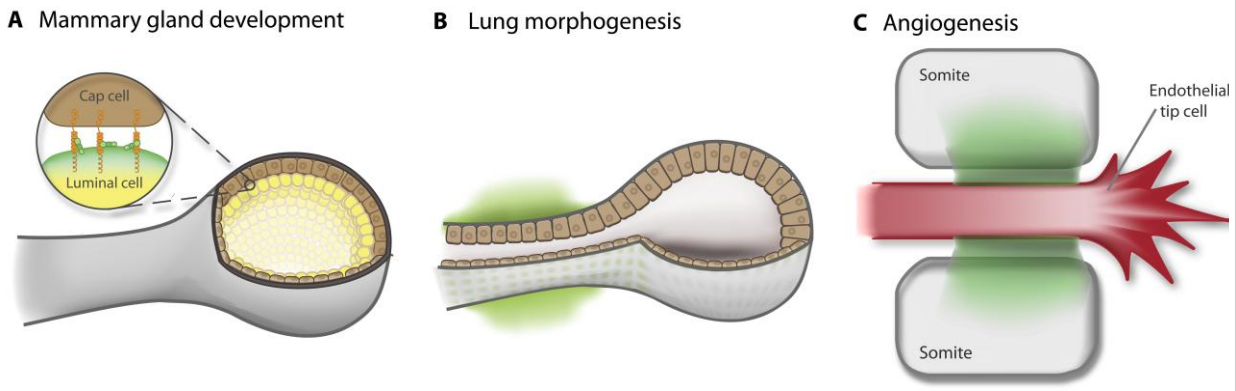

Figure 2.3 - Netrin function in other developing organs and tissues. (**A**) In mammary gland morphogenesis, the terminal end buds of ductal branches consist of two layers of cells – cap cells and luminal cells. The luminal cells express netrin-1 (green), which binds neogenin (orange) expressed by the cap cells and provides a stable adhesive interaction between the two layers. (**B**) During lung morphogenesis and the development of the bronchial tree, epithelial stalk cells secrete netrin-1 and netrin-4 into the surrounding basal lamina to inhibit inappropriate proximal branching and bud formation. (**C**) Endothelial tip cells that pioneer vascular formation are highly motile protrusive cells, similar to axonal growth cones. During angiogenesis, somites secrete netrin (green) that inhibits vascular branching via a mechanism that is dependent on UNC5B expression by endothelial tip cells.

Netrins also contribute to the elaboration of vascular networks (Fig. 2.3C). Vascular endothelial tip cells exhibit highly motile protrusions that are reminiscent of axonal growth cones. These cells express UNC5B and their motility is inhibited by netrin-1, thereby limiting endothelial cell migration and blood vessel branching (Lu et al. 2004; Larrivee et al. 2007; Lejmi et al. 2008). Controversy exists, however, with regard to the precise role of netrins during vascular development, as other studies have reported that netrin promotes angiogenesis (Park et al. 2004; Wilson et al. 2006; Epting et al. 2010), perhaps reflecting differences in the populations of endothelial cells examined or experimental conditions employed. A recent study describes a role for netrin-4 in the development of the lymphatic vascular system (lymphangiogenesis) and implicates netrin-4 activation of ERK, Akt and S6 kinase in vessel formation (Larrieu-Lahargue et al. 2010). Notably, these findings provide multiple examples of netrins directing the formation of branched networks by promoting or constraining elongation and branching in different contexts. Netrin-1 can also inhibit leukocyte migration (Ly et al.

2005), and recent findings provide intriguing evidence that upregulation of netrin expression provides protection against the deleterious effects of inflammation in several tissues (Wang et al. 2008b; Rosenberger et al. 2009; Mirakaj et al. 2010; Tadagavadi et al. 2010; Podjaski et al. 2015).

NETRIN SIGNALLING MECHANISMS

Studies investigating the signal transduction mechanisms engaged by secreted netrins have focused largely on netrin-1, and relatively little is known about the specific signalling mechanisms activated by other netrin family members. Functions described for netrin-1 include the regulation of cell migration, axon extension and guidance, cell-cell and cell-substrate adhesion, cell survival, and cellular differentiation. As we discuss below, recent studies have identified a number of molecular signalling components that function downstream of netrin-1, although the molecular details of how these elements interact to generate specific cellular responses are not well understood.

Chemoattractant signal transduction cascades

In vertebrate species, studies of the axonal projections made by embryonic spinal commissural neurons and RGCs have been particularly useful for investigating the mechanisms that underlie netrin-1 mediated axon chemoattraction. The growth cone, which is found at the tip of an extending axon, projects filopodia and lamellipodia that probe the extracellular environment for guidance cues. Cytoplasmic signal transduction molecules in the growth cone link the activation of receptors for guidance signals to the reorganization of the actin cytoskeleton (Huber et al. 2003). For example, growth-promoting extracellular guidance cues induce the formation of adhesive complexes that then enhance membrane extension on one side of the growth cone by locally restricting the retrograde flow of F-actin, resulting in directional extension (Dickson 2002; Huber et al. 2003). Recent studies have shown that netrin-1 can activate multiple downstream signal transduction molecules that regulate cytoskeletal

dynamics and process extension, including SFKs and members of the Rho GTPase family (Huber et al. 2003).

In neurons that respond to netrin-1 as a chemoattractant, the intracellular domain of DCC is constitutively bound to the adaptor protein Nck1 and to focal adhesion kinase (FAK) (Li et al. 2002a; Li et al. 2004; Ren et al. 2004). The binding of netrin-1 to DCC triggers the dimerization of DCC via its P3 intracellular domain (Stein et al. 2001), as well as FAK autophosphorylation and tyrosine phosphorylation of the DCC intracellular domain (Ren et al. 2004). This initiates the recruitment of several intracellular signalling components to the DCC/Nck1/FAK complex (Fig. 2.4A), which subsequently act to regulate SFK signalling, Rho GTPase activation, the release of Ca^{2+} stores, protein translation, and rearrangements of the cytoskeleton.

Netrin-1 induces the recruitment and activation of Fyn, which binds to DCC between P2 and P3 (Li et al. 2004; Meriane et al. 2004). Fyn is thought to then regulate the activity of Rho GTPases: Rac1 and Cdc42 become activated, whereas RhoA is inhibited (Li et al. 2002b; Shekarabi and Kennedy 2002; Shekarabi et al. 2005; Moore et al. 2008) (Fig. 2.4A). Consistent with these findings, which were obtained using mammalian neurons, the Rac-like GTPase in *C. elegans* CED-10, was shown to be required for netrin-dependent axon guidance in this organism (Gitai et al. 2003). Two guanine nucleotide exchange factors (GEF) for Rac1, Trio and DOCK180, have since been reported to function downstream of DCC in vertebrate neurons (Briancon-Marjollet et al. 2008; Li et al. 2008). These GEFs regulate the activation of Rho GTPases by promoting the exchange of GDP for GTP. Genetic analysis in *Drosophila* has also identified a role for Trio in netrin signalling (Forsthoefel et al. 2005), whereas in *C. elegans*, the Trio homolog UNC-73 is required for appropriate localization of UNC-40 to the cell surface (Watari-Goshima et al. 2007). However, the mechanisms responsible for regulating Cdc42 and RhoA downstream of DCC in neurons remain unclear. Neither *Trio* nor *Dock180* knockout mice (Briancon-Marjollet et al. 2008; Laurin et al. 2008) phenocopy the severity of the neural developmental defects found in *Dcc* or *netrin-1* knockouts (Serafini et al. 1996; Fazeli et al.

1997), indicating that additional mechanisms must contribute to DCC signalling during chemotropic axon guidance.

Netrin-1 also activates the serine/threonine kinase PAK1 and, in embryonic rat spinal commissural neurons, promotes its recruitment into a complex with DCC (Shekarabi et al. 2005) (Fig. 2.4A). PAK1 is a downstream effector of Cdc42 and Rac1, and functions as an adaptor that links Nck1 to Cdc42 or Rac1 (Bagrodia and Cerione 1999). Disruption of PAK1 binding to Nck1 blocks netrin-1 induced recruitment of PAK1 to DCC and inhibits netrin-1 induced growth cone expansion (Shekarabi et al. 2005). Additional downstream effectors of Cdc42 that are activated by DCC include the actin-binding proteins Enabled/vasodilator-stimulated phosphoprotein (Ena/VASP) and neuronal Wiskott-Aldrich syndrome protein (N-WASP), which are both modulators of actin polymerization (Lebrand et al. 2004; Shekarabi et al. 2005).

DCC-dependent commissural axon chemoattraction also involves the activation of the mitogen-activated protein kinase (MAPK) cascade (Forcet et al. 2002; Campbell and Holt 2003; Ma et al. 2010). The extracellular signal-regulated kinases 1 and 2 (ERK1 and ERK2) are phosphorylated following netrin receptor activation, which results in the activation of specific transcription factors such as Elk-1, implicating netrin-1 upstream of transcriptional activation (Forcet et al. 2002). The binding of netrin-1 to DCC also promotes the synthesis of the phosphoinositide phosphatidylinositol (4,5) biphosphate (PIP_2), which is phosphorylated by phosphatidylinositol-3 kinase (PI3K) and results in phosphatidylinositol (3,4,5) triphosphate (PIP_3) production (Xie et al. 2005). Notably, PIP_3 facilitates the binding of GTPases to their effectors, thereby enhancing signalling (Di Paolo and De Camilli 2006). Netrin-1 also induces PIP_2 hydrolysis by phospholipase C-γ (PLCγ) to generate diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3), which in turn activate protein kinase C (PKC) and stimulate the release of Ca^{2+} from intracellular stores, respectively (Ming et al. 1999; Xie et al. 2006). PKC additionally mediates cytoskeletal rearrangements and translational control (Larsson 2006), whereas increased levels of intracellular Ca^{2+} are required for the axons of *Xenopus* spinal neurons to turn toward a source of netrin-1 (Hong et al. 2000). In addition to the release of Ca^{2+} from intracellular stores, netrin-1 also activates transient receptor potential (TRP) channels to trigger

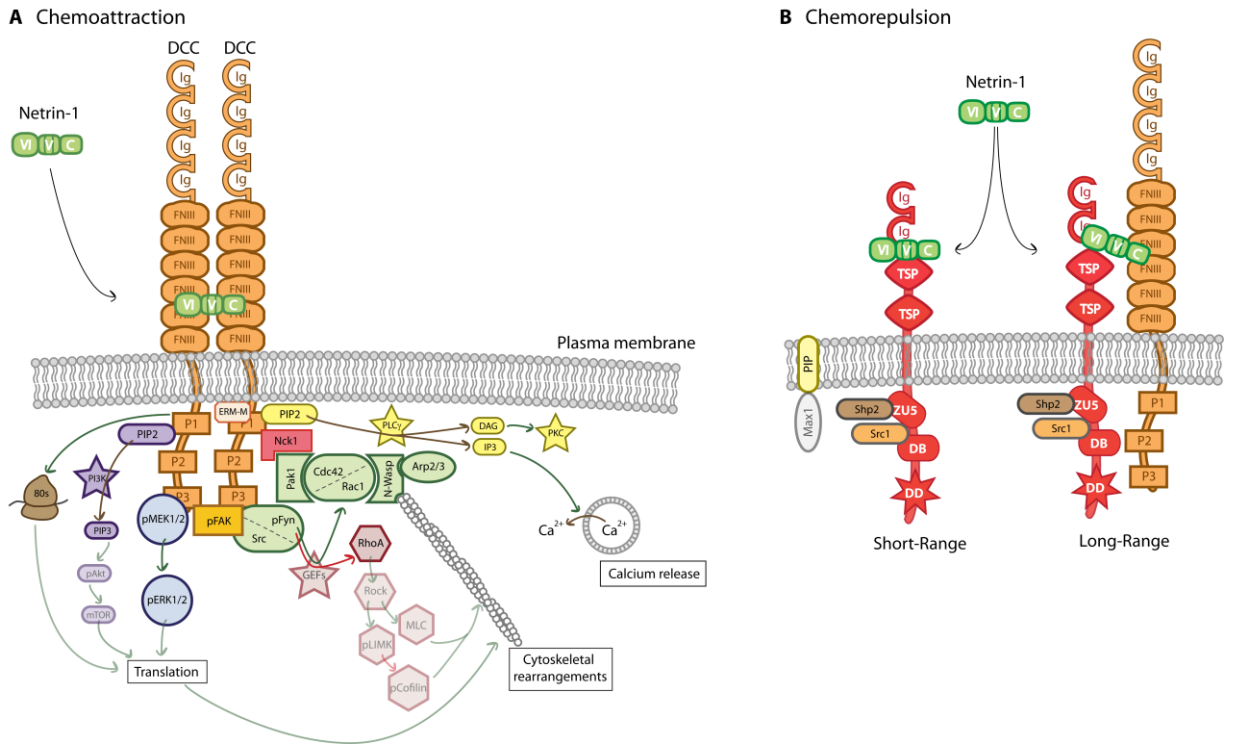

Figure 2.4 - Netrin signalling. As a guidance cue, netrin-1 dictates either an attractive or repellent response depending on the receptors expressed by the migrating cell and the signal transduction mechanisms activated. (**A**) During chemoattraction, netrin-1 binding triggers DCC homodimerization, and activation of constitutively bound Nck1 and FAK. This initiates the recruitment of a number of intracellular signalling components that activate Src family kinases, Rho GTPases, the release of Ca^{2+} stores, protein translation and, ultimately the rearrangement of the actin cytoskeleton. (**B**) During the generation of a chemorepellent response, netrin-1 signals through UNC5/DCC heterodimers, which are thought to facilitate long-range responses by increasing sensitivity to relatively low netrin concentrations, or through UNC5 in the absence of DCC to mediate relatively short-range repellent responses. Although multiple proteins are known to be required for netrin-1-mediated chemoattraction and chemorepulsion, how these proteins work together to regulate chemotropic turning by a growth cone remains poorly understood. Signal transduction components illustrated as 'faded' are speculative and direct evidence for their involvement has not been obtained. (80s, eukaryotic ribosomes; Arp2/3, complex of ARP2 and ARP3; DAG, diacylglycerol; ERM-M, ezrin/radixin/moesin and merlin protein family; GEFs, guanine exchange factors; IP3, inositol 1,4,5-triphosphate; Max1, Motor axon guidance PH/MyTH4/FERM domain cytoplasmic protein; MLC, myosin light chain; mTOR, mammalian target of rapamycin; N-WASP, neuronal Wiskott-Aldrich Syndrome protein; Nck1, non-catalytic region of tyrosine kinase adaptor protein 1; pAkt, phosphorylated RAC-alpha serine/threonine-protein kinase; pCofilin, phosphorylated cofilin; pERK1/2, phosphorylated extracellular signal-regulated kinase 1/2; pFAK, phosphorylated focal adhesion kinase; pFyn, phosphorylated Fyn; pLIMK, phosphorylated LIM domain kinase 1; pMEK1/2, phosphorylated mitogen-activated protein kinase kinase 1/2; PAK1, p21-activating kinase 1; PI3K, phosphatidylinositol 3-kinase; PIP, phosphatidylinositol phosphate; PIP2, phosphatidylinositol 4,5-bisphosphate; PIP3, phosphatidylinositol (3,4,5)-trisphosphate; PKC, protein kinase C; PLCγ, phospholipase C gamma; ROCK, RhoA kinase; Shp2, Src homology region 2 domain-containing phosphatase-2.)

a Ca^{2+} influx across the plasma membrane of *Xenopus* spinal neurons, which is required for axon chemoattraction to netrin-1 (Wang and Poo 2005).

In contrast to the signal transduction cascades activated downstream of DCC, little is known regarding signal transduction downstream of DSCAM in vertebrate species. It is known from *in vitro* studies that the intracellular domain of human DSCAM interacts with PAK1 (Li and Guan 2004), and netrin-1 binding triggers the activation of PAK1 and Fyn (Liu et al. 2009), which are downstream signalling molecules shared with DCC (Meriane et al. 2004; Shekarabi et al. 2005). In *Drosophila*, DSCAM binds DOCK, a homolog of Nck1 in mammals, and activates PAK1 (Schmucker et al. 2000), reminiscent of DCC signalling in mammalian neurons (Li et al. 2002a; Shekarabi et al. 2005). Curiously, the extracellular domains of mammalian and *Drosophila* DSCAM are well conserved, but their intracellular domains are not (Schmucker et al. 2000).

Chemorepellent signal transduction cascades

The signalling mechanisms that underlie netrin-1 induced chemorepulsion are considerably less well understood than those underlying chemoattraction, although they appear to be mediated primarily by UNC5 and UNC5-DCC signalling. Studies carried out in *Drosophila in vivo* and in mammalian cell lines *in vitro* support the conclusion that expression of an UNC5 homolog in the absence of DCC mediates short-range repulsion to netrin, whereas long-range netrin-induced repulsion requires multimerization of UNC5 with DCC, mediated by the interaction between the DB domain of UNC5 and the P1 domain of DCC (Hong et al. 1999; Keleman and Dickson 2001) (Fig. 2.4B). Interestingly, studies in *Drosophila* indicate that the DB domain of UNC-5 is also required for short-range repulsion, which does not require the DCC ortholog Frazzled (Keleman and Dickson 2001), perhaps revealing the contribution of an alternative UNC5 co-receptor. In *C. elegans,* neurons that express both UNC-5 and UNC-40 are repelled by the netrin homolog UNC-6 (Hedgecock et al. 1990) (Table 2.1). Initial evidence for UNC-40-dependent and -independent functions of UNC-5 in *C. elegans* came from studies demonstrating that, in *unc-5* null worms, defects in neuronal migration away from an UNC-6 source are almost as severe as those observed in *unc-6* nulls, whereas the deficits in

chemorepulsion that are present in *unc-40* nulls are not as severe (Hedgecock et al. 1990). This indicates that UNC-40 is not essential for UNC-5 function in all cells. Subsequent studies in *C. elegans* demonstrated that ectopic expression of UNC-5 in neurons that normally project ventrally was sufficient to direct their axons dorsally, away from the UNC-6 source *in vivo*, and that this response required UNC-40 function, supporting a role for both UNC-5 and UNC-40 in chemorepulsion to UNC-6 (Hamelin et al. 1993; Colavita and Culotti 1998). This conclusion is also consistent with studies of cell and axon migration in vertebrates, which have demonstrated that genetic ablation of *Dcc* or disruption of DCC function compromises the repellent responses of neurons to netrin-1 (Hong et al. 1999; Jarjour et al. 2003).

UNC5 function is absolutely dependent on its cytoplasmic domain (Hong et al. 1999; Keleman and Dickson 2001; Killeen et al. 2002). Remarkably, expression of the UNC5B cytoplasmic domain alone is sufficient to trigger repulsion in *Xenopus* spinal neurons through its association with the intracellular domain of DCC (Hong et al. 1999). Deletion analyses of UNC5 revealed a functional contribution of the cytoplasmic juxtamembrane domain to axon guidance in *C. elegans*, whereas deletion of the cytoplasmic ZU-5 domain disrupted function in both *Drosophila* and *C. elegans*, confirming that the cytoplasmic domain of UNC5 is crucial for its function (Keleman and Dickson 2001; Killeen et al. 2002). Both long- and short-range repellent response of axons to netrin secreted from the *Drosophila* embryo midline require an intact DD (Keleman and Dickson 2001). By contrast, the DD was found to be dispensable in *Xenopus* for chemorepellent axon turning to netrin-1, perhaps owing to a species difference or to the reduced complexity of the *in vitro* assay employed (Hong et al. 1999).

Studies of the signal transduction pathway downstream of UNC5 have identified a limited number of components. Netrin-1 induces phosphorylation of UNC5 (on Y482) in a DCC-dependent manner, through the actions of Src and FAK (Killeen et al. 2002; Li et al. 2006). This leads to the binding of the tyrosine phosphatase Shp2 to UNC5 (Tong et al. 2001) (Fig. 2.4B). Studies in *C. elegans* have also identified roles for the PAK family member Max-2 and the adaptor protein Max-1 as modulators of UNC-5 mediated axon repulsion (Huang et al. 2002; Lucanic et al. 2006).

Cell adhesion pathway signalling

When DCC binds to immobilized netrin-1 *in vitro*, it mediates cell-substrate adhesion (Shekarabi et al. 2005; Moore et al. 2008). The importance of this has been highlighted in recent studies showing that axon chemoattraction requires that DCC adheres to immobilized netrin-1 so as to transduce force across the plasma membrane, and that this mechanotransduction requires FAK (Moore et al. 2009; Moore et al. 2012). These findings support the idea that, during netrin-induced chemoattraction, DCC has two simultaneous functions: as a transmembrane bridge that links extracellular netrin-1 to the actin cytoskeleton, and as the core of a protein complex that directs the reorganization of F-actin. In further support of this mechanism of action, it has been shown that netrin-1 and DCC, when expressed by mature myelinating rodent oligodendrocytes, are both required to maintain axo-oligodendroglial paranodal junctions (Jarjour et al. 2008; Bull et al. 2014). Outside of the nervous system, netrin-1, netrin-3, netrin-4, DCC, and neogenin have been shown to regulate epithelial morphogenesis in the mammary gland, pancreas, lung, and lymphatic vasculature, in part by influencing cell-cell adhesion (Slorach and Werb 2003; Srinivasan et al. 2003; Yebra et al. 2003; Hebrok and Reichardt 2004; Liu et al. 2004b; Larrieu-Lahargue et al. 2010). Furthermore, during muscle development, myoblasts express neogenin and netrin-3, and myoblast fusion to produce myotubes requires neogenin and is enhanced by the addition of netrin (Kang et al. 2004). The intracellular domain of DCC also contains a proposed ezrin/radixin/moesin and merlin (ERM-M)-binding domain to which the ERM proteins ezrin and merlin can bind (Martin et al. 2006). ERM proteins are ubiquitous cytoplasmic adaptors that function as links between transmembrane adhesion proteins and the actin cytoskeleton, and influence protein trafficking and signal transduction to regulate tissue organization (Tepass 2009). Interestingly, ectopic expression of DCC in a colon cancer cell line increased cell-cell adhesion, while reducing cell-matrix adhesion, increasing the number of desmosomes between cells and reducing focal adhesions that link cells to the substrate (Martin et al. 2006). Overall, these findings suggest a role for netrins and their receptors in modulating cell-cell and cell-matrix adhesion; however, the details of these interactions and their full functional significance remain to be investigated.

Netrin signalling modulation by cAMP, receptor trafficking and calcium

Cyclic adenosine monophosphate (cAMP) is a well-characterized second messenger that exerts a profound influence on axon guidance and axon regeneration. Increasing cAMP activates protein kinase A (PKA), which in turn regulates Rho GTPase activation (Lang et al. 1996) and Ena/VASP function (Gertler et al. 1996; Krause et al. 2003), both of which signal downstream of netrin-1 to direct cytoskeletal rearrangements (Shekarabi and Kennedy 2002; Gitai et al. 2003; Lebrand et al. 2004; Moore et al. 2008). Importantly, it has been shown that, in response to PKA inhibition, the axons of *Xenopus* spinal neurons can shift their response to a netrin-1 gradient from attraction to repulsion (Ming et al. 1997). These findings led to the hypothesis that PKA activation regulates the direction of axon turning by altering signal transduction pathways downstream of netrin-1. More recently, it was demonstrated that PKA activation in embryonic rat spinal commissural neurons or neocortical neurons causes the relocation of DCC from an intracellular vesicular pool to the plasma membrane of the growth cone (Bouchard et al. 2004; Bouchard et al. 2008). This increased DCC presented by growth cones enhances axon outgrowth and the turning responses of these axons to netrin-1 (Bouchard et al. 2004; Moore and Kennedy 2006; Bouchard et al. 2008). The inhibition of RhoA, which is a downstream consequence of PKA activation, also causes DCC to be recruited to the plasma membrane and promotes commissural axon outgrowth to netrin-1 (Moore et al. 2008). Interestingly, PKA inhibition did not result in embryonic rat spinal commissural axons switching their response to repulsion, but instead reduced the extent of their attraction to a gradient of netrin-1 (Moore and Kennedy 2006). These findings indicate that PKA regulates the sensitivity of embryonic spinal commissural neurons to a gradient of netrin-1 by modulating the trafficking of DCC (Fig. 2.3A). Furthermore, axon extension requires the insertion of membrane to extend the leading edge of the growth cone. Netrin-1 induces membrane insertion through the interaction of DCC and the target-SNARE (t-SNARE) protein syntaxin-1, leading to synaptic vesicle exocytosis (Cotrufo et al. 2012). Membrane extension is thus hypothesized to be driven by the insertion of DCC and membrane at the leading edge of growth cones, the stabilization of DCC in the plasma membrane, the linking of DCC to actin filaments, and by signalling mechanisms that are activated by DCC to promote actin polymerization (Fig. 2.5B).

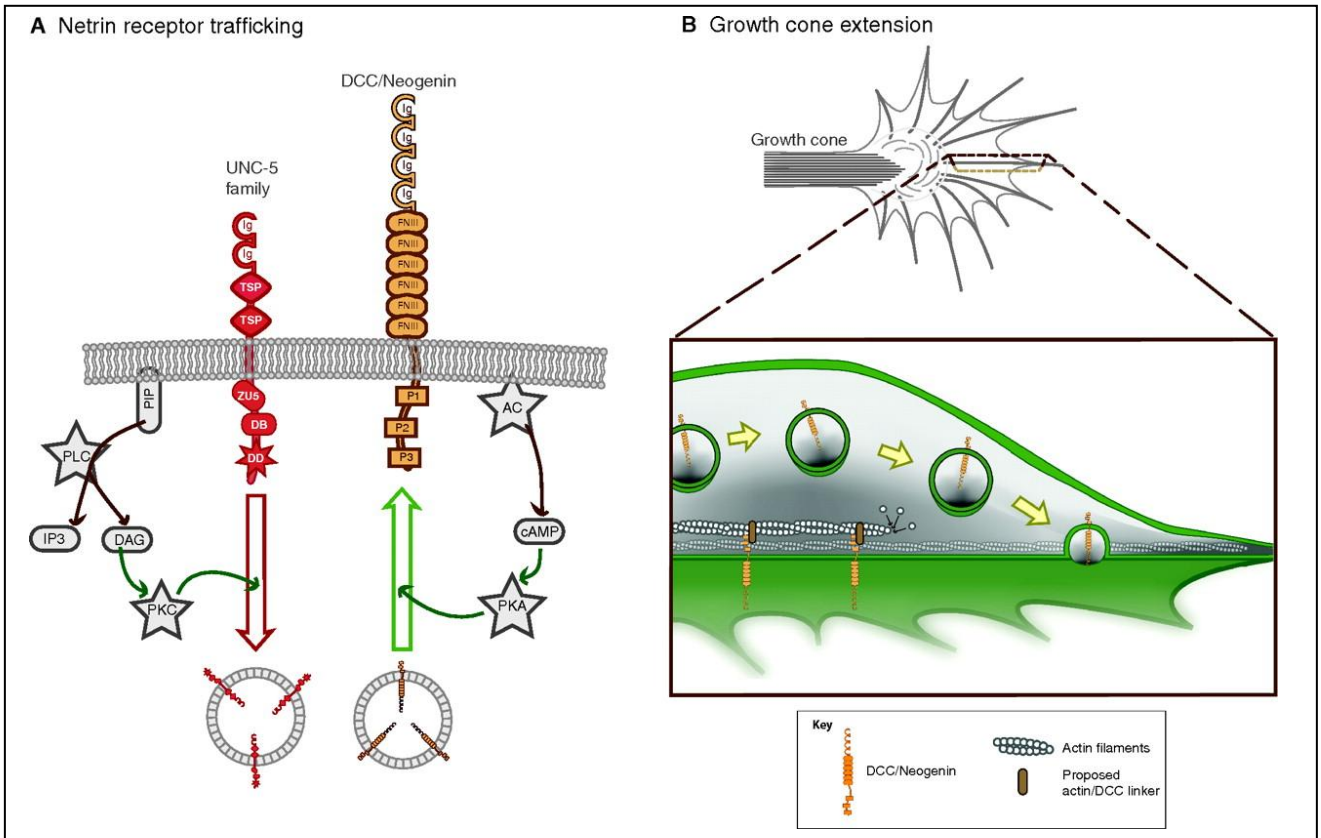

Figure 2.5 - Regulation of netrin receptor trafficking and membrane recruitment. (**A**) In axonal growth cones, the activation of protein kinase A (PKA) recruits DCC from an intracellular pool of vesicles to the plasma membrane, which enhances the axon outgrowth evoked by netrin-1. Activation of protein kinase C (PKC) activates endocytosis of UNC5A, causing neurons to switch from chemorepellent to chemoattractant responses to netrin-1. (**B**) DCC is proposed to function in axonal growth cones simultaneously as a transmembrane bridge that links extracellular netrin to the F-actin cytoskeleton and as the core of a protein complex that directs the reorganization of F-actin. Membrane extension is hypothesized to be driven by insertion of DCC and membrane at the leading edge of the growth cone, DCC stabilization in the plasma membrane through binding to immobilized matrix-associated netrin, and linkage of DCC to polymerizing filaments of F-actin. (AC, adenylate cyclase; cAMP, cyclic adenosine monophosphate).

Conversely, the activation of PKC triggers endocytosis of UNC5 homologs (Fig. 2.5A), resulting in cultured cerebellar granule cell neurons switching from repellent to attractant responses to netrin-1 (Bartoe et al. 2006). In rat hippocampal neurons, activating PKCα recruits the adaptor protein interacting with C kinase-1 (PICK1) to the UNC5A intracellular domain, triggering the internalization of UNC5A but not DCC, thereby keeping DCC on the cell surface

and switching the response of these cells to netrin-1 mediated attraction (Williams et al. 2003). Together, these findings identify the regulation of netrin receptor trafficking as a key determinant of the migratory response made by axonal growth cones.

Decreasing cytoplasmic Ca^{2+} levels, by blocking Ca^{2+} release from intracellular stores or by inhibiting its influx through Ca^{2+} channels, can also convert *Xenopus* spinal neuron responses to netrin-1 from attraction to repulsion (Hong et al. 2000). This requires calcium-calmodulin-dependent protein kinase II (CaMKII) and calcineurin (CaN) phosphatase, with high local Ca^{2+} concentrations favouring CaMKII-induced attraction and moderate levels of Ca^{2+} activating CaN to mediate repulsion (Wen et al. 2004). Cyclic guanosine monophosphate (cGMP) signalling also influences the response to netrin-1: a high intracellular ratio of cAMP to cGMP promotes attraction of *Xenopus* spinal neuron growth cones to netrin-1 by activating Ca^{2+} entry through L-type calcium channels (LCCs), whereas a low ratio results in decreased Ca^{2+} influx and netrin-1-mediated repulsion (Nishiyama et al. 2003).

Effects of netrin signalling on localized protein translation

Extending axons contain a subpopulation of transported mRNAs and the machinery for local protein translation, providing the growth cone with a substantial level of functional autonomy from the cell body during embryogenesis (Lin and Holt 2008). Recent studies suggest that localized binding of netrin to its receptors can activate translation and can function to restrict new protein synthesis to specific subdomains of a cell or growth cone, thereby influencing axon growth. In *Xenopus* RGCs, for example, the application of netrin-1 rapidly activates translation initiation factors and increases the local synthesis of proteins, such as β-actin (Leung et al. 2006). Importantly, activation of translation is required for netrin-mediated growth cone turning of *Xenopus* RGCs and is regulated by the ERK and p38 MAPK pathways (Campbell and Holt 2001; Campbell and Holt 2003). Finally, a recent study provides evidence that DCC binds directly to large and small ribosomal subunits, eukaryotic initiation factors, and monosomes, suggesting that DCC can act to anchor the translation machinery to the plasma membrane and spatially restrict protein synthesis (Tcherkezian et al. 2010).

CONCLUDING REMARKS

Netrins are essential chemotropic cues for migrating cells and axons during neural development. Although the majority of studies thus far have focused on this guidance role in the embryonic nervous system, it is now apparent that netrin family members and their receptors participate in a range of functions in several tissues, both throughout development and in adulthood. Tremendous advances have been made in identifying the signal transduction components required for netrin function. Determining how netrin receptors and signal transduction proteins function as an ensemble in the axonal growth cone to regulate motility remains a major challenge for current studies. Netrins and netrin receptors have now also been demonstrated to regulate adhesion in several cellular contexts; however, how the signalling mechanisms that direct motility during development subsequently switch during maturation to regulate cell-cell interactions and adhesion remains to be determined. In mature tissues, exciting recent findings implicate netrins in the regulation of adult stem cell migration, in tumor cell survival and as modulators of inflammation, suggesting potentially novel means of promoting recovery from injury or disease. In this regard, netrins, netrin receptors and the downstream signalling mechanisms involved are promising targets for the development of treatments for neurodegenerative disease, vascular disease, and cancer.

THESIS RATIONALE AND OBJECTIVES

RATIONALE:

Accurate axon pathfinding requires growth cones to interpret extracellular signals and modify their cytoskeleton accordingly. The signalling pathways engaged by axon guidance cues to reorganize the actin cytoskeleton and influence growth cone morphology are beginning to be understood. The primary goal of this thesis is to elucidate the mechanisms regulating chemoattractive axon guidance mediated by netrin-1 and its receptor, Deleted in colorectal cancer (DCC), during neural development.

The specific objectives of this thesis are:

1. **Examine the role of Src family kinases in commissural axon navigation (Chapter 3).**
   The Src family kinases, and specifically Fyn, have been reported to play a crucial role in the netrin-1 signalling pathway downstream of DCC (Li et al. 2004; Liu et al. 2004a; Meriane et al. 2004; Ren et al. 2004). However, the Fyn knockout mouse does not phenocopy the drastic defects seen in netrin-1 or DCC deficient mice (Serafini et al. 1996; Fazeli et al. 1997; Meriane et al. 2004). In this chapter, we investigated the role of Fyn and another Src family kinase member, Src, in commissural axon extension in the developing spinal cord.

2. **Identify a guanine nucleotide exchange factor that activates the Rho GTPase Cdc42 in netrin-1 mediated commissural axon guidance (Chapter 4).**
   Netrin-1 activates the Rho GTPases Rac1 and Cdc42 downstream of DCC in spinal commissural neurons (Shekarabi et al. 2005). These signalling molecules are regulated by guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs). Two Rac-specific GEFs have previously been implicated in netrin-1 signalling (Briancon-

Marjollet et al. 2008; Li et al. 2008), but none that activates Cdc42 downstream of DCC have been identified to date. We identify a GEF capable of activating Cdc42 and Rac1 and characterize its function in netrin-1 chemoattraction.

3. **Characterize the functional contribution of the netrin-1 gradient for commissural axon guidance (Chapter 5).**

   A gradient of netrin-1 in the ventral spinal cord is proposed to guide spinal commissural neurons to the ventral midline (Serafini et al. 1996; Kennedy et al. 2006). Although the expression of netrin-1 at the floor plate is necessary for the normal extension of commissural axons, the role of the gradient *in vivo* has not been examined. In chapter 5, we investigate the functional significance of a graded distribution of netrin-1 to commissural axon guidance.

RESULTS

CHAPTER 3

The Src family kinases Fyn and Src are not essential for commissural axon guidance in the embryonic spinal cord

Karen Lai Wing Sun and Timothy E. Kennedy

Montreal Neurological Institute, Department of Neurology and Neurosurgery, McGill University, Montreal, Quebec, Canada, H3A 2B4.

PREFACE

The axon guidance molecule netrin-1 is able to induce cytoskeletal rearragements through its receptor DCC and, consequently, direct axon outgrowth and turning of developing neurons. The Src family kinase member Fyn has previously been reported to be a critical signalling molecule involved in netrin-1 chemoattractive responses. However, Fyn null mice have not been reported to have defects comparable to netrin-1 deficient mice (Stein et al. 1992; Serafini et al. 1996; Meriane et al. 2004). The purpose of this study is to determine if mice lacking Fyn and Src function exhibit axon guidance phenotypes *in vivo*. We report that the Src family kinases Fyn and Src are not essential for netrin-1 mediated guidance in the developing spinal cord. A manuscript of this chapter is in preparation for publication.

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ABSTRACT

Netrin-1 is a chemotropic guidance molecule required for the proper guidance of spinal commissural neurons in the developing spinal cord. Netrin-1 signals through its receptor Deleted in colorectal cancer (DCC) to activate several intracellular molecules, including the Src family kinase (SFK) Fyn, to mediate chemoattraction. Although Fyn is reported to play a crucial role in DCC mediated netrin-1 signalling *in vitro*, Fyn null animals are viable and only minor neural defects have been reported. This is in stark contrast to netrin-1 and DCC knockout mice, which die shortly after birth and exhibit severe deficits in corpus callosum, hippocampal commissure and spinal ventral commissure formation. In order to directly address the role of Fyn in spinal commissural axon guidance, we examined the trajectory of commissural tracts in the developing spinal cords of Fyn null animals. We determined that Fyn is not required for commissural axons to extend to the midline in the embryonic spinal cord. To further investigate the function of SFKs in netrin-1 mediated chemoattraction, we demonstrated that Src can also interacts with DCC; however, we provide evidence that Src, like Fyn, is also not essential for commissural axon guidance in the embryonic spinal cord.

INTRODUCTION

The developing nervous system is an intricate network of synaptic connections that requires axons to travel over long distances to reach their synaptic counterparts. The precise trajectory of extending axons is determined by the orchestrated expression of chemoattractive and chemorepulsive cues. Netrins are a family of well-conserved diffusible axon guidance molecules that have a bifunctional role, attracting some groups of cells and repelling others depending in part on which receptors are expressed. In particular, netrin-1 and its receptor Deleted in colorectal cancer (DCC) play crucial roles in the attractive turning of spinal commissural neurons towards the ventral midline of the developing spinal cord (Tessier-Lavigne et al. 1988; Placzek et al. 1990; Serafini et al. 1996; Kennedy et al. 2006). In these cells, netrin-1 binding to DCC leads to the activation and recruitment of a signalling complex at the

cytoplasmic domain of the receptor, which in turn induces cytoskeletal rearrangements in the commissural growth cone for netrin-1 mediated axon outgrowth (Li et al. 2002a; Ren et al. 2004; Shekarabi et al. 2005). Netrin-1 is secreted by floor plate cells and attracts commissural neurons, which express DCC, causing the commissural axons to extend towards the ventral midline (Kennedy et al. 1994; Serafini et al. 1994; Keino-Masu et al. 1996). Activation of Src family kinases (SFKs) has been implicated in this signalling pathway downstream of DCC (Li et al. 2004; Liu et al. 2004a; Meriane et al. 2004; Ren et al. 2008). SFKs are an important family of tyrosine kinases that act as key cell signalling regulators for a wide range of cellular events, including proliferation, differentiation, migration and survival (Thomas and Brugge 1997). It has been proposed that, following netrin-1 activation, the SFK member Fyn phosphorylates tyrosine residues on the receptor DCC, leading to the activation of the Rho GTPase Rac1 required for growth cone chemoattraction to netrin-1 (Meriane et al. 2004). It has been argued that Fyn, specifically, is essential for commissural axon extension in response to netrin-1, and that the function of Fyn in promoting neurite outgrowth cannot be substituted by other SFKs such as Src (Meriane et al. 2004). However, this study did not examine commissural axon guidance in Fyn null nice, and the studies that described Fyn knockout (KO) mice did not report any gross axon guidance defects *in vivo* (Stein et al. 1992; Meriane et al. 2004). In particular, the possibility that Fyn null mice might phenocopy the profound commissural guidance deficits seen in netrin-1 or DCC mutant mice has not been addressed (Serafini et al. 1996; Fazeli et al. 1997).

In this study, we examine spinal commissural axon guidance in *fyn* deficient mice *in vivo*. We provide genetic evidence that, in contrast to previous reports, Fyn is not essential for netrin-1 mediated commissural axon guidance to the embryonic spinal floor plate. To better understand the signalling events occurring in commissural outgrowth, we also address the possibility that other SFK members may contribute to netrin-1 mediated chemoattraction in the embryonic spinal cord.

METHODS AND MATERIALS

Animals

For spinal commissural neuron cultures, staged pregnant Sprague-Dawley rats were obtained from Charles Rivers Laboratory (St-Constant, QC, Canada) (vaginal plug = E0).

For immunohistological assays, mice heterozygous for the beta-galactosidase netrin-1 recombinant allele (*ntn1* $^{-/+}$) (Serafini et al. 1996) were obtained from Marc Tessier-Lavigne (Rockefeller University, NYC, USA), and *fyn* knockout mice (*fyn*^{tm1Sor}) were purchased from Jackson Laboratory (Bar Habor, ME, USA). Conditional *c-src* mice (*c-src* $^{L/L}$) (Marcotte et al. 2012) were kindly provided by William J. Muller (McGill University, QC, Canada) and crossed to *nestin-cre* mice, obtained from Jean-François Cloutier (Montreal Neurological Institute, QC, Canada), to generate *c-src* deleted mice (through germline crossing). All procedures involving animals were performed in accordance with the Canadian Council on Animal Care guidelines for the use of animals in research.

Antibodies and cell culture reagents

The following antibodies were used in this study: polyclonal rabbit anti-Fyn (gift from André Veillette, Institut de recherches cliniques de Montréal, QC, Canada); polyclonal rabbit anti-Src, -Fyn, -Lyn, -Yes, -Lck (Cell Signaling Technology, Danvers, MA, USA); goat anti-contactin-2/TAG-1 (R & D Systems, Mineapolis, MN, USA); mouse anti-DCC (BD Pharmingen, Mississauga, ON, Canada); mouse IgG and horseradish-peroxidase (HRP)-conjugated secondary antibodies (Jackson Immuno Research Laboratories, West Grove, PA, USA); Alexa-Fluor conjugated secondary antibodies (Novex by Life Technologies, Carlsbad, CA).

For cell culture experiments, the following reagents were used: poly-D-lysine (PDL) from Sigma-Aldrich (Oakville, ON, Canada); Leibovitz's L15 medium, Neurobasal media, B27 supplement, GlutaMAX supplement and penicillin-streptomycin (P/S) from Gibco by Life Technologies (Carlsbad, CA); heat inactivated fetal bovine serum (HI-FBS) from PAA Laboratories (Piscataway, NJ, USA).

Recombinant netrin-1 protein used in the immunoprecipitation assay was purified from a HEK293T cell line secreting netrin-1 as previously described (Serafini et al. 1994; Shirasaki et al. 1996).

Embryonic dorsal spinal commissural neuron culture

Dorsal spinal cords were microdissected from embryonic day 13 (E13) rat embryos and dissociated as previously described (Moore and Kennedy 2008). Neurons were plated on culture dishes coated with 2 µg/ml PDL and cultured in Neurobasal media supplemented with 10% HI-FBS, 1% P/S, and 1% GlutaMAX in a humidified incubator at 37^{o}C and 5% CO_2. After 18-20 hr in culture, the media was changed to Neurobasal supplemented with 2% B-27, 1% P/S, and 1% GlutaMAX for another day.

Reverse-transcription PCR

RNA was isolated from E13 rat brain and spinal cord, adult rat spinal cord, 3 days *in vitro* (3 DIV) cultures of E15 dorsal root ganglion (DRG) neurons, and 2 DIV E13 spinal commissural neurons using TRIzol® (Ambion by Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. cDNA was synthesized from RNA using the Superscript™ III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA) and oligo(dT)$_{20}$ primers (R&D Systems, Mineapolis, MN, USA). Transcripts for *fgr, fyn, lyn variants 1* and *2, lck, src, yes* and *dcc* were amplified by PCR using the following primers:

fyn (5'-AGTCGTGGCAAAAGGTCAGT-3', 5'-GCTTCCCACCAGTCTCCTTC-3');
fgr (5'-CAGAACAGAGGCGACCACAT-3', 5'-GGCTGTCCACTTGATGGGAA-3');
lck (5'-TGATGCTGTGCTGGAAGGAG-3', 5'-CCCCAGAAAGGCAGTGGTAG-3');
lyn v.1 (5'-TGTGAGAGATCCAACGTCCA-3', 5'-GGCTTCTGTGGTTTGGGACT-3');
lyn v.2 (5'-CTGGAAGGAATCAGCAGAGG-3', 5'-GCTCCAGTTCAGCAAAGGTC-3');
src (5'-CATCCAAGCCTCAGACCCAG -3', 5'- TGCCGAGCTGTGTATTCGTT-3');
yes (5'-CCCTCCTCCTCAAGTAACGC-3', 5'-GCTTCCCACCAGTCTCCTTC-3');
dcc (5'-CCGGAATTCCCACCTATGAGTGCA-3', 5'-GTCCGCTCGAGCAATGCATGTCAAAAGG-3').

The dcc primers contain 5' restriction sites.

Immunoprecipitation

2 DIV spinal commissural neuron cultures were stimulated with 200 ng/ml of recombinant netrin-1 for 10 min, then lyzed in 1% Triton X-100 lysis buffer (50 mM HEPES pH 7.4, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM EDTA with protease inhibitors) and incubated with 1 μg of mouse anti-DCC or control mouse IgG for 1 hr for co-immunoprecipitation assays. The lysates were then incubated with protein A/G agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 45 min. Samples were washed with lysis buffer, solubilized with SDS sample buffer, and boiled for 5 min. The proteins were then separated by SDS-PAGE, and analysed by immunoblotting with antibodies against Fyn, Src, Lck, Yes and DCC.

Western blot analysis

Proteins were separated by SDS-PAGE and transferred to polyvinyldiene fluoride (PVDF, Biorad) membranes. The membranes were blocked with 5% BSA in Tris buffered saline with Tween 20 (TBST) for 1 hr, and then incubated with primary antibodies overnight. After washes with TBST, the membranes were then incubated with HRP-conjugated secondary antibodies for 1 hr. The proteins were visualized using Immobilon Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA).

Immunohistochemistry

E12.5 embryos were collected from timed pregnant *netrin-1*, *fyn* or *src* mice and fixed in Carnoy's fixative solution (60% ethanol, 10% acetic acid, 30% chloroform) for 2 hrs at room temperature, and then washed twice with 100% ethanol for 20 min. The embryos were then cleared in toluene for 1 hr and transferred to paraffin wax at 60^{o}C in a vacuum oven overnight. The following day, the embryos were embedded in fresh paraffin wax and 10 μm sections cut on a microtome. Prior to staining, tissue sections were rehydrated as follows: 2 x xylene for 3 min, 2 x 100% ethanol for 3 min, 2 x 95% ethanol for 3 min, 1 x 70% ethanol for 5 min, 1 x PBS 5

min. Antigen retrieval was also performed by boiling the sections in citrate buffer (10 mM citric acid, pH 6.0) for 10 min. Sections were then blocked in 3% bovine serum albumin (BSA*;* Fisher Scientific, Waltham, MA), 3% heat inactivated horse serum (HI-HS, Gibco by Life Technologies, Carlsbad, CA) and 0.1% Triton X-100 in PBS for 1 hr, and incubated with the indicated primary antibodies overnight at 4^{o} C. Following washes, sections were incubated with Alexa-Fluor secondary antibodies and coverslipped with Fluoromount-G (SouthernBiotech, Birmingham, AL, USA). All immunofluorescent images were obtained using a Zeiss Axiovert S100TV inverted microscope and a Magnafire CCD camera (Optronics).

RESULTS

Fyn is not required *in vivo* for commissural axon pathfinding to the floor plate

Several studies have identified SFKs as playing a central role in growth cone chemoattraction to netrin-1, with a particular emphasis on the family member Fyn as being responsible for the tyrosine phosphorylation of DCC required for axon extension (Li et al. 2004; Liu et al. 2004a; Meriane et al. 2004; Ren et al. 2008). For this reason, it is compelling to think that *fyn* KO animals would phenocopy the drastic neuronal defects seen in the netrin-1 loss-of-function mutant mice (Serafini et al. 1996). Although previous work has described *fyn* KOs as exhibiting disruptions in the developing and adult brain, no overt axon guidance defect was reported in these mice (Grant et al. 1992; Stein et al. 1992; Sasaki et al. 2002; Yuasa et al. 2004). To further investigate this, we examined the trajectory of spinal commissural axons extending to the floor plate *in vivo* in the developing spinal cords of these KO mice, a process that is severely disrupted in netrin-1 mutants.

Commissural growth cones express DCC and are attracted to netrin-1. These neurons originate in the dorsal portion of the spinal cord and extend their axons towards the floor plate, which secretes netrin-1 (Colamarino and Tessier-Lavigne 1995b). Here, immunohistochemistry was performed with an antibody against TAG-1 to label pre-crossing commissural neurons in E12.5 *netrin-1* and *fyn* deficient mice (Dodd et al. 1988). As expected, the stereotypical trajectory of commissural neurons is disrupted in netrin-1 deficient animals (Serafini et al. 1996) (Fig. 3.1B). Commissural axon extension is disorganized and many axons fail to reach the floor plate. Furthermore, the ventral commissure of the spinal cord is greatly reduced in these mice (Fig. 3.1B) compared to the wild-types (Fig. 3.1A). Surprisingly, when *fyn* null animals were analyzed, none of these severe defects in spinal commissural guidance were observed, with the KO animals (Fig. 3.1D) being essentially indistinguishable from wild-type littermates (Fig. 3.1C). Importantly, a robust spinal ventral commissure was intact in *fyn* null mice (Fig. 3.1D), indicating that *fyn* is not essential for commissural axons to reach and cross the ventral midline. Since SFKs have been reported to exhibit redundant functions (Stein et al. 1994), these findings suggest that other SFKs may compensate in commissural neurons for the lack of Fyn expression.

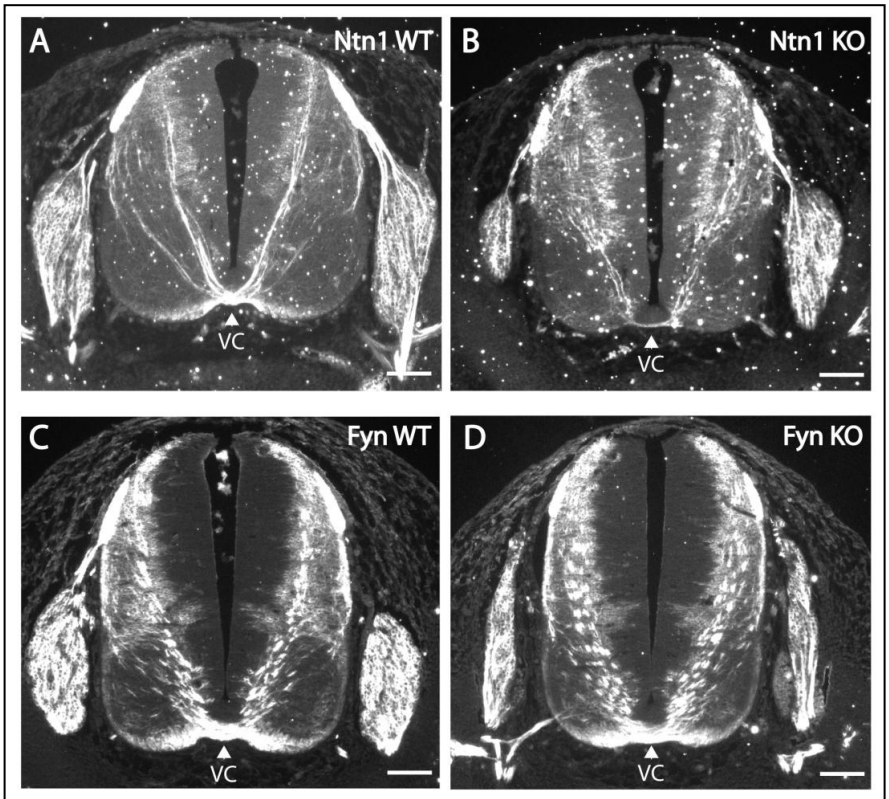

Figure 3.1 - Commissural axons reach the midline and form a normal ventral commissure in *fyn* knockout embryos. Immunohistochemical analysis of E12.5 wild-type (A) and *netrin-1* mutant (B) mice spinal cords. 10 µm transverse sections of paraffin-embedded mice embryos were stained for TAG-1 to visualize commissural neuron projections at the brachial level. Representative images of E12.5 wild-type (C) and *fyn* null (D) spinal cord sections stained for Tag-1 to visualize commissural neurons and the spinal ventral commissure (VC). Scale bar = 100µm.

Src family kinase members are expressed in the CNS and by spinal commissural neurons

The SFK family is comprised of 11 members: Src, Yes, Fyn, Fgr, Lck, Lyn, Hck, Blk, Frk, Srm and Yrk (Sen and Johnson 2011). They are widely expressed in various cell types, with Fyn, Src, Lck and Yes being especially enriched in the developing central nervous system (Cotton and Brugge 1983; Martinez et al. 1987; Sudol et al. 1988; Cooke and Perlmutter 1989; Zhao et al. 1991; Umemori et al. 1992). To identify which SFK members are expressed in the embryonic spinal cord and correlate this with CNS expression, we performed reverse transcriptase

polymerase chain reaction (RT-PCR) for several SFK family members from embryonic and adult rat tissues. cDNAs were obtained from total RNA isolated from E13.5 brain and spinal cord, adult rat spinal cord, and cultures of E13 spinal commissural neurons and E15 dorsal root ganglion neurons. PCR was then performed to detect transcripts of *lyn variant 1, lyn variant 2*, *lck*, *yes*, *fgr*, *src* and *fyn.* Amplification of *gapdh* was used as a positive control. We detected the expression of multiple SFKs in these tissues, with the cultured embryonic spinal commissural neurons in particular expressing all SFKs examined (Fig. 3.2). We also amplified *dcc* transcripts, demonstrating DCC expression by these tissues and cell types.

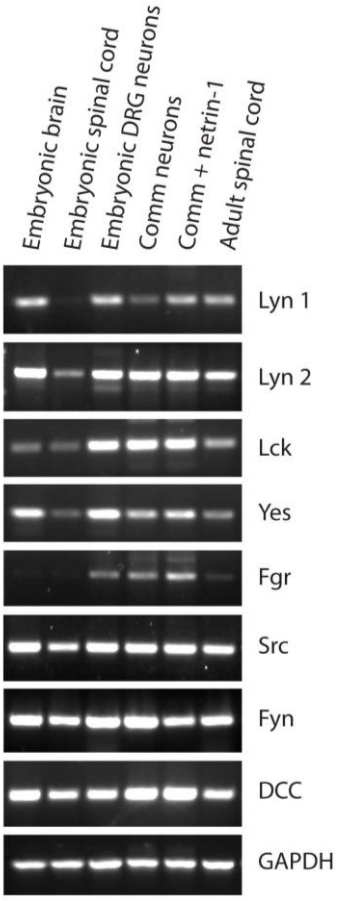

Figure 3.2 - Expression of Src family kinases in the developing central nervous system, adult spinal cord, and by cultured embryonic rat spinal commissural neurons and dorsal root ganglion neurons. Amplification of *lyn variant 1* and *2, lck, yes, fgr, src, fyn* and *dcc* transcripts by reverse transcriptase PCR from embryonic E13 rat brain and spinal cord tissues, adult rat spinal cord, as well as from 3 DIV E15 dorsal root ganglion (DRG) neuron cultures and 2 DIV E13 spinal commissural (comm) neurons. GAPDH was amplified as a positive control.

Src associates with DCC in spinal commissural neurons

It has been demonstrated biochemically that Fyn can associate with DCC following netrin-1 treatment (Li et al. 2004; Liu et al. 2004a). To assess the possibility that other SFKs might also interact with the receptor, we immunoprecipitated DCC from lysates of commissural neurons treated with recombinant netrin-1 for 10 minutes, and then immunoblotted for Lck, Yes, Fyn and Src, which are highly expressed in neural tissues (Cotton and Brugge 1983; Sudol et al. 1988; Umemori et al. 1992) (Fig. 3.3). Of all the SFKs tested, only Fyn (Fig. 3.3C) and Src (Fig. 3.3D) were detected to co-immunoprecipitation with DCC in spinal commissural neuron lysates. The robust co-immunoprecipitation of Src with DCC is unexpected, since previous studies

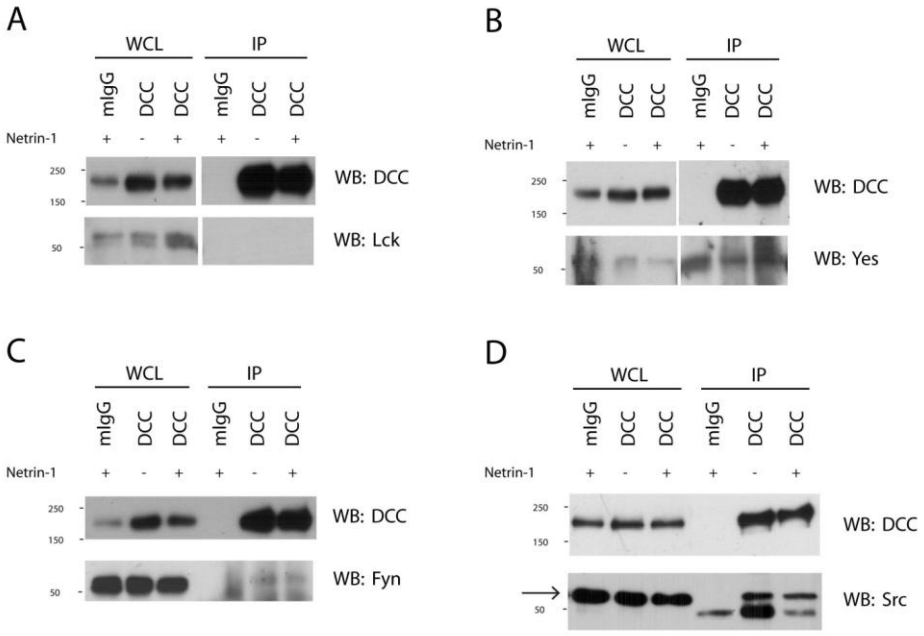

Figure 3.3 - Fyn and Src associate with DCC in dissociated embryonic rat spinal commissural neurons. DCC immunoprecipitation was performed from 2 DIV commissural neurons dissected from E13 rat spinal cord with and without a 10 min stimulation with 200 ng/ml netrin-1. Western blots for various SFKs demonstrate a failure to co-immunoprecipitate DCC with Lck (A) or Yes (B), but an interaction with Fyn (C) and Src (D) in spinal commissural neurons. The arrow in (D) identifies the band corresponding to Src.

implicated Fyn only, and not Src, in DCC signalling (Li et al. 2004; Meriane et al. 2004). Additionally, it is interesting to note that Src interacts with DCC independently of netrin-1 stimulation (Fig 3.3D). These data indicate that, at least in spinal commissural neurons, Fyn is not the only SFK associated with DCC, and that Src interacts constitutively in a complex with DCC.

Src is not essential in commissural axon guidance in the developing spinal cord

A key mediator of cell signalling, Src regulates remodeling of the actin cytoskeleton and cell migration (Thomas and Brugge 1997). Netrin-1 and DCC exert a potent influence on the organization of F-actin in spinal commissural growth cones (Shekarabi et al. 2005). We therefore set out to investigate the functional contribution of Src to commissural axon guidance. To do so, we examined the trajectory of commissural axons in embryonic *src* null mice. These mice are viable and display no severe neuro-anatomical defects, but they do develop osteopetrosis (Soriano et al. 1991). Examination of sections of the brachial spinal cord immunostained for TAG-1 to specifically reveal commissural axons (Dodd et al. 1988), we detected no discernible difference in the trajectory of commissural axons in wild-type and *src* null animals at E12.5, an age when most commissural neurons have reached the floor plate and crossed the midline to form the ventral commissure (Colamarino and Tessier-Lavigne, 1995) (Fig. 3.4A, B). Akin to the *fyn* KO mice, these *src* null KOs also exhibit a thick ventral commissure, indicating that there is no overt commissural guidance defect in the absence of Src function. This is in marked contrast to netrin-1 or DCC KO mice in which very few spinal commissural neurons reach the ventral midline and cross (Serafini et al., 1996; Fazeli et al, 1997). The lack of a loss-of-function phenotype argues that Src alone is not required for commissural projections to reach the floor plate, a known netrin-1 mediated event.

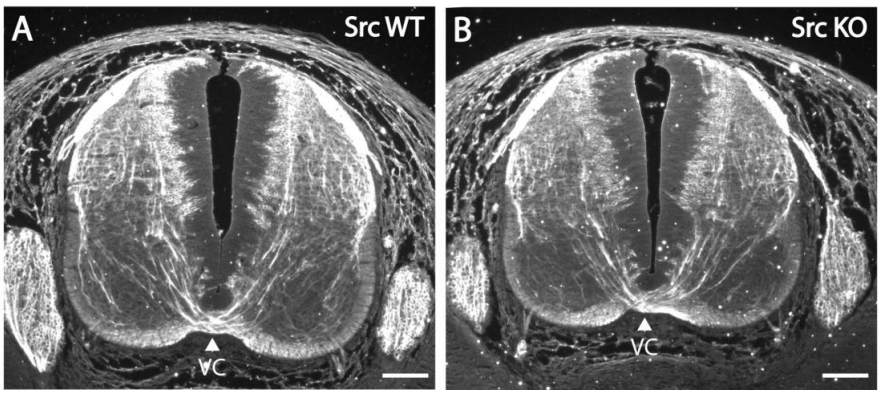

Figure 3.4 - Commissural axons reach the midline and form a normal ventral commissure in *src* knockout embryos. Immunohistochemical analysis of E12.5 wild-type and *src* null mouse spinal cords. Sections at the level of the brachial spinal cord were immunostained for TAG-1 to visualize commissural neuron projections and the ventral commissure (VC). Scale bar = 100µm.

DISCUSSION

SFKs have well-established functions in signal transduction in a variety of cellular processes. In the last few years, Fyn has been reported to be the major SFK contributing to axon outgrowth and turning in response to netrin-1, but the validity of this conclusion had not been examined *in vivo* (Li et al. 2004; Liu et al. 2004a; Meriane et al. 2004; Ren et al. 2008). Based on the findings described here, we have determined that deleting *fyn* expression does not impair commissural axon extension to the ventral midline of the embryonic spinal cord. Furthermore, we also demonstrate that although Src can be co-immunoprecipitated with DCC from cultured spinal commissural neurons, it too is not essential for proper commissural axon pathfinding *in vivo*. Together, these findings imply that, at least individually, Fyn and Src functions are dispensable for netrin-1 dependent attraction of commissural neurons.

One possible explanation for these results is that members of the SFK family may compensate in the absence of Fyn or Src function in netrin-mediated signalling in spinal commissural neurons. The plausibility of redundant SFK function is supported by previous

demonstrations of similar mechanisms of activation with overlapping functional redundancy (Stein et al. 1994; Lowell and Soriano 1996). Furthermore, targeted deletion of *src*, *fyn* or *yes* in mice displayed very few obvious phenotypic defects, whereas knocking out *src* and *fyn* or *src, fyn* and *yes* in mice resulted in substantially abnormal phenotypes (Soriano et al. 1991; Stein et al. 1992; Stein et al. 1994). These include severe consequences for survival, with *src/fyn* double mutants and *src/fyn/yes* triple mutant animals dying perinatally and early in gestation, respectively (Stein et al. 1994). The absence of more severe phenotypes in single knockouts supports the conclusion that there is functional compensation between SFK members. Although no axon guidance abnormalities have been described in *src/fyn* double mutant embryos, an in-depth examination of commissural axon trajectories in these mice is needed to determine if Src and Fyn together are required for commissural axon guidance *in vivo*. Moreover, even though SFKs have been shown to exhibit functionally redundant roles, it remains to be seen if Src and Fyn play similar roles in netrin-mediated signalling in spinal commissural neurons, or perhaps if another SFK family member may also contribute.

An alternative, not mutually exclusive interpretation is that other factors, such as the cellular environment, contribute to modify SFK signalling. The minimal substrate presented to neurons in cell culture is drastically different than that encountered *in vivo*, which may influence the intracellular signalling requirements of migrating commissural neurons. For example, *Xenopus* retinal ganglion cells switch from an attractive response to netrin-1 when cultured on glass, poly-D-lysine or fibronectin, to chemorepulsion when plated on laminin-1 (Hopker et al. 1999). As such, the conditions encountered by commissural neurons migrating through the embryonic spinal neural epithelium *in vivo* may influence netrin-1 mediated signal transduction in ways that are critically different from these neurons grown in isolation in cell culture.

Similar to netrin-1, the morphogen Sonic hedgehog (Shh) secreted by floor plate cells at the ventral midline of the embryonic spinal cord also functions as a chemoattractant for extending commissural axons (Charron et al. 2003). Shh was demonstrated to rapidly activate Src and Fyn signalling in commissural axon growth cones (Yam et al. 2009). Chemoattractive

axon turning up a gradient of Shh *in vitro* was also disrupted with the pharmacological SFK inhibitor PP2 or with the expression of C-terminal Src Kinase (Csk), which binds to and inhibits SFKs (Yam et al. 2009). Furthermore, commissural neurons turned down a gradient of the SFK inhibitor PP2, indicating that differential inhibition across the growth cone by PP2 is sufficient to trigger a directed turn *in vitro* (Yam et al. 2009). Disruption of Shh signalling *in vivo* produces relatively modest defects in spinal commissural axon guidance compared to the loss of netrin-1 or DCC function (Serafini et al. 1996; Fazeli et al. 1997; Charron et al. 2003). While we have focused on the role of netrin-1, our findings that neither Src nor Fyn signalling are essential for commissural axon guidance to the ventral midline of the embryonic spinal cord suggest commissural axon guidance by Shh *in vivo* may be similarly independent of SFK function. Interestingly, a study carried out in the *Drosophila* embryonic central nervous system (CNS) demonstrated that SFK signalling is not required for axon attraction to the midline (O'Donnell and Bashaw 2013). This study also revealed that tyrosine phosphorylation of DCC or of its *Drosophila* ortholog Frazzled is not required to mediates netrin dependent chemoattraction in the *Drosophila* CNS (O'Donnell and Bashaw 2013). These findings demonstrate that a SFK independent mechanism that is sufficient for commissural axon guidance exists in *Drosophila*, and raise the possibility that a similar mechanism may function in the mammalian spinal cord.

CHAPTER 4

Role for the Cdc42 and Rac1 guanine nucleotide exchange factor βPix in netrin-1 mediated chemoattraction

Karen Lai Wing Sun, Sonia P. Rodrigues, Nicolas Stifani, Stefano Stifani, Timothy E Kennedy

Montreal Neurological Institute, Department of Neurology and Neurosurgery, McGill University, Montreal, Quebec, Canada, H3A 2B4.

PREFACE

In the previous chapter, we examined the role of SFKs, and more particularly members Fyn and Src, in guiding spinal commissural neurons in the developing spinal cord. Our findings demonstrated that, at least individually, the expression of Fyn or Src was not necessary to guide commissural axons to the ventral midline (Chapter 3). Despite this, SFKs are still essential components of the signalling cascade required for the attractive response to netrin-1. Through phosphorylation, SFKs can control the activity of guanine nucleotide exchange factors, GTPase-activating proteins and guanine nucleotide-dissociation inhibitors, which in turn are regulators of the GTPase family of signalling molecules (Thomas and Brugge 1997; Huveneers and Danen 2009). The following chapter looks into identifying a guanine nucleotide exchange factor that activates the Rho GTPases Cdc42 and Rac1 downstream of DCC in netrin-1 mediated chemoattraction. These Rho GTPases have previously been demonstrated to be crucial for netrin-1 signalling through DCC (Shekarabi et al, 2005). Here, we identify βPix as a candidate regulator of Cdc42 and Rac1 in this pathway and examine its role in DCC signalling in spinal commissural neurons. This chapter is presented as a manuscript in preparation for submission.

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ABSTRACT

Netrin-1 is a chemotropic guidance molecule that attracts extending spinal commissural neurons towards the ventral midline of the developing spinal cord. Through its receptor Deleted in colorectal cancer (DCC), netrin-1 promotes cytoskeletal rearrangement and changes in growth cone morphology necessary for commissural axon extension. The exact mechanisms by which this occurs are however not fully understood. Netrin-1 chemoattraction in commissural neurons requires the activation and recruitment of the Rho GTPases Rac1 and Cdc42, as well as the serine-threonine kinase PAK1 to the intracellular domain of DCC. To further our understanding of the mechanisms that regulate Rac1 and Cdc42 downstream of DCC, we have demonstrated that the Rho guanine nucleotide exchange factor (GEF) βPix, along with the adaptor protein GIT2, associate with DCC in commissural neurons. We also report that a complex of βPix with GIT and PAK are essential to induce growth cone expansion by netrin-1 in spinal commissural neurons, and that downregulating βPix expression in these neurons abolishes netrin-1 mediated changes in the growth cone. Additionally, we provide evidence that βPix function is required for proper guidance of commissural axons to the ventral midline in the embryonic spinal cord. Together, these findings provide evidence that βPix is implicated in the DCC signalling pathway in netrin-1 dependent commissural axon chemoattraction.

INTRODUCTION

In the developing nervous system, axons must travel over long distances to reach their synaptic targets. To do so, they are guided towards their final destination by guidance cues which can be attractive or repulsive. An important guidance molecule is netrin-1, a secreted chemotropic cue that can act as an attractant or a repellent depending on which receptors are expressed by the cells (Kennedy et al. 1994; Serafini et al. 1994; Colamarino and Tessier-Lavigne 1995a). In particular, netrin-1 plays a crucial role in the guidance of spinal commissural neurons, a population of interneurons that project axons across the midline to the contralateral side of the spinal cord. These neurons originate in the dorsal portion of the spinal cord and their axons are attracted to netrin-1 secreted by the floor plate, causing them to extend

towards the ventral midline and cross it to form the ventral spinal commissure (Kennedy et al. 1994; Serafini et al. 1994; Colamarino and Tessier-Lavigne 1995b).

Netrin-1, binding to its receptor Deleted in colorectal cancer (DCC), activates the Rho GTPases Cdc42 and Rac1 in embryonic spinal commissural neurons (Shekarabi et al. 2005). This was initially demonstrated using a GTPγS loading assay, where GTPγS is not converted to GDP and binds irreversibly to Rho GTPases to maintain them in their active state. Lysates of commissural neurons were incubated with GTPγS and GTPγS-bound Cdc42 and Rac1 were isolated using the GST-PAK-CRIB fusion protein. This revealed an increase in GTPγS binding to the endogenous Rho GTPases using antibodies specific for Cdc42 and Rac1 (Shekarabi et al. 2005). Importantly, because this assay measures the accumulation of GTPγS bound to Cdc42 and Rac1, the increased GTPγS binding implicates DCC in the activation of a guanine nucleotide exchange factor (GEF) for Cdc42 and Rac1 in commissural neurons. GEFs function as activators for small GTPases by promoting the dissociation of GDP and allowing for the binding of GTP.

Recently, two GEFs were reported to be involved in netrin-1 mediated chemoattraction: Trio and DOCK180 (Briancon-Marjollet et al. 2008; Li et al. 2008). Although biochemical studies implicate both as potentially functioning downstream of DCC, Trio and DOCK180 knockout animals do not phenocopy the severe defects present in DCC or netrin-1 deficient mice. Trio knockout mice have a corpus callosum, a hippocampal commissure and a spinal ventral commissure, structures that are all absent in netrin-1 and DCC mutants (Serafini et al. 1996; Fazeli et al. 1997; Briancon-Marjollet et al. 2008). As for DOCK180, even though the null animals die shortly after birth like DCC and netrin-1 deficient mice, no neural defects were reported for these mice (Serafini et al. 1996; Fazeli et al. 1997; Laurin et al. 2008). These observations indicate that, although Trio and DOCK180 may contribute to DCC signalling, they are not essential for netrin-1 mediated axon extension. Furthermore, Trio and DOCK180 have been identified as GEFs for Rac1, and have little or no reported activity on Cdc42 (Bellanger et al. 1998; Brugnera et al. 2002; Backer et al. 2007). Previous studies have shown that endogenous Cdc42 is activated by netrin-1 downstream of DCC in embryonic rat commissural neurons, and

proposed that Rac1 activation in these cells requires activation of Cdc42 (Shekarabi et al. 2005). Together, this lead us to search for a GEF for Cdc42 that acts downstream of DCC.

The p21-activated kinase interacting exchange factor (Pix) family of GEFs are able to activate both Rac1 and Cdc42 (Manser et al. 1998; Bagrodia et al. 1999). They bind to p21-activated kinase (PAK) and contain an N-terminal SH3 domain, a diffuse B-cell lymphoma (Dbl) homology (DH) domain, and a pleckstrin homology (PH) domain (Manser et al. 1998). Two isoforms of Pix have been identified, αPix and βPix. Oligomerization of Pix proteins is required for their physiological function, and disrupting their ability to form homodimers or heterodimers leads to subcellular mislocalization and diffuse protein expression (Kim et al. 2001; Koh et al. 2001). Additionally, Pix can interact with G-protein-coupled receptor kinase-interacting proteins (GITs), which function as GTPase-activating proteins (GAPs) for ADP-ribosylation factor (ARF) small GTP-binding proteins (Premont et al. 2000; Premont et al. 2004). Together with GIT, Pix proteins form the core of a large multimeric signalling complex to regulate processes such as cell polarity, cell migration, and synaptogenesis (Zhao et al. 2000; Manabe et al. 2002; Saneyoshi et al. 2008).

Here, we examine the role of βPix in the signalling pathway downstream of DCC. We report that βPix can associate with activated Cdc42 and Rac1 and that this association is increased following netrin-1 stimulation. We demonstrate that βPix is essential to induce growth cone expansion by netrin-1 in spinal commissural neurons. We also demonstrate that βPix function is required for proper guidance of commissural axons to the ventral midline in the embryonic spinal cord. Together, these findings provide evidence that βPix activates Cdc42 downstream of netrin-1 during commissural axon chemoattraction to ventral midline in the embryonic spinal cord.

MATERIALS AND METHODS

Antibodies and reagents

The following primary antibodies were used in this study: monoclonal mouse anti-DCC from BD Pharmingen (San Diego, CA); polyclonal goat anti-DCC and polyclonal anti-Cdc42 from Santa Cruz Biotechnology (Santa Cruz, CA); monoclonal mouse anti-βPix from BD Transduction Laboratories (Mississauga, ON); polyclonal rabbit anti-βPix from Enzo Life Sciences (Farmingdale, NY); polyclonal rabbit anti-βPix SH3 domain, monoclonal mouse anti-Neurofilament M (NFM) from EMD Millipore (Billerica, MA); polyclonal chicken anti-NFM from Aves Labs (Tigard, OR); monoclonal mouse anti-GST, monoclonal mouse anti-GIT1 and monoclonal mouse anti-GIT2 from UC Davis/NIH NeuroMab Facility (Davis, CA); monoclonal anti-Rac1 from Transduction Laboratories (Lexington, KY); polyclonal goat anti-myc from Abcam (Cambridge, UK); polyclonal rabbit anti-FLAG from Sigma-Aldrich (St. Louis, MO); polyclonal rabbit anti-GFP from Life Technologies (Carlsbad, CA). Poly-D-lysine (PDL) was purchased from Sigma-Aldrich (St. Louis, MO). Neurobasal media, penicillin/streptomycin (P/S), GlutaMAX, B27 supplement, heat inactivated horse serum (HI-HS), TRIzol® reagent, Superscript™ III First-Strand Synthesis System for RT-PCR, Lipofectamine® 2000 transfection reagent, and Lipofectamine® RNAiMAX transfection reagent were obtained from Life Technologies (Carlsbad, CA). Bovine serum albumin (BSA) was purchased at Fisher Scientific, (Waltham, MA). Protein A/G agarose beads were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Oligo$(dT)_{20}$ primers were obtained from R&D Systems (Mineapolis, MN). Heat-inactivated fetal bovine serum (HI-FBS) was purchased from PAA Laboratories (Piscataway, NJ). Recombinant netrin-1 protein was purified from a HEK293T cell line secreting netrin-1 as previously described (Serafini et al., 1994).

Embryonic dorsal spinal commissural neuron culture

Embryos were removed from a staged pregnant female Sprague-Dawley rat purchased from Charles Rivers Laboratory (St-Constant, QC) at embryonic day 13 (E13, vaginal plug is E0). Dorsal spinal cords were microdissected and dissociated as described in Moore and Kennedy (2008), and cultured in Neurobasal media supplemented with 10% HI-FBS, 1% P/S, and 1%

GlutaMAX in a humidified incubator at 37 ^{o}C and 5% CO_2. Neurons were plated on plasma-cleaned coverslips or culture dishes coated with 2 µg/ml PDL for 2 days in vitro (DIV). After 18-20 hr in culture, the media was changed to Neurobasal supplemented with 2% B27 supplement, 1% P/S, and 1% GlutaMAX. Cultures were treated with 200 ng/ml of purified recombinant netrin-1 for 10 min before the fixing or lysis.

Reverse-transcription PCR

Total RNA was isolated from various tissues using TRIzol® reagent according to the manufacturer's directions. RNA was then transcribed into cDNA using the Superscript™ III First-Strand Synthesis System for RT-PCR and oligo$(dT)_{20}$ primers. Transcripts for βPix, GIT1, GIT2, and DCC were amplified by RT-PCR using the following primers:

	Forward primer (5'- 3')	Reverse primer (5'- 3')
βPix	GGTCTTCAAAAGGCAAGCAG	ACAGGGCCAAGATACACCAG
GIT1	AAGCCCCAAGGATGGGAATG	GGGGAGAGATGGAGGGAAGT
GIT2	TGGGAGACGGAAAGCCAATC	ACCGGAAGAAAGGGAACGAC
DCC*	CCGGAATTCCCACCTATGAGTGCA	GTCCGCTCGAGCAATGCATGTCAAAAGG

* The DCC primers contain 5' restriction sites.

PCR products were separated on an ethidium bromide containing agarose gel and visualized using UV illumination.

Immunoprecipitation

2 DIV spinal commissural neuron cultures were serum-starved for 6 hr before being treated with 200 ng/ml of recombinant netrin-1 for 10 min. Neurons were then lysed in 1% Triton X-100 lysis buffer (50 mM HEPES pH 7.4, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 10 ng/ml leupeptin, 10 ng/ml aprotinin), and incubated with indicated primary antibodies or control IgG. Protein A/G agarose beads were then added to the lysates and incubated for 45 min. After 3 washes with lysis buffer, the immunocomplexes were solubilized with SDS sample buffer and

boiled for 5 min. The proteins were then separated by SDS-PAGE, and analysed by immunoblotting with antibodies against βPix, DCC, and GIT2.

Recombinant DNA and siRNA

cDNAs encoding GST-βPix WT, FLAG-βPix Δ80, and FLAG-βPix 1-459 were graciously provided by Dr. Edward Manser (Institute of Molecular and Cell Biology, Singapore), and the construct encoding the myc-βPix DH mutant by Dr. Richard Cerione (Cornell University, New York). The pEGFP-C1 plasmid used in chick electroporations was obtained from Clontech Laboratories (Mountain View, CA).

3 Stealth RNAi small interfering RNA oligonucleotides (siRNA) against βPix (Arhgef7 RSS300268, RSS300269, RSS350242) and siRNA-negative control were obtained from Life Technologies (Carlsbad, CA).

Immunohistochemistry and immunocytochemistry

Fresh embryos were collected and fixed in Carnoy's fixative solution (60% ethanol, 10% acetic acid, 30% chloroform) for 2 hrs at room temperature, and then washed twice with 100% ethanol for 20 min. The embryos were cleared in toluene for 1 hr and embedded in paraffin wax before being cut by microtome in 10 μm thick sections. Prior to staining, tissue sections were rehydrated as follows: 2 x xylene 3 min, 2 x 100% ethanol 3 min, 2 x 95% ethanol 3 min, 2 x 70% ethanol, 1 x PBS 5 min. Sections were boiled in citrate buffer (10 mM citric acid, pH 6.0) for 10 min for antigen retrieval before immunohistochemical analyses were performed.

For growth cone expansion and knockdown assays, dissociated spinal commissural neurons were isolated as described above, but maintained in P/S-free medium. After 1 DIV, neurons were transfected with either βPix mutant constructs using Lipofectamine® 2000 transfection reagent according to the manufacturer's recommendations, or βPix siRNAs using Lipofectamine® RNAiMAX transfection reagent according to the manufacturer's instructions. The neurons were incubated for another 1-2 DIV prior to treatment with 200 ng/ml of recombinant netrin-1 for 10 min. Cells were then fixed with 4% paraformaldehyde (PFA) for 20 min and immunocytochemical analyses were performed.

All immunofluorescent images were obtained using a Zeiss Axiovert S100TV inverted microscope and a Magnafire CCD camera (Optronics).

In ovo chick electroporation

Fertilized White Leghorn chicken eggs (Couvoir Simetin) were incubated to Hamburger-Hamilton (HH) stages 14-16 of chick development. The embryos were injected and electroporated *in ovo* at the brachial neural tube level with pEGFP-C1 alone or together with a mutant βPix construct (described above). The embryos were incubated for approximately 48 hr post-electroporation to HH stages 27-28, after which the spinal cords were dissected out of the embryos as open book preparations and fixed in 4% PFA for 1 hr for subsequent immunohistochemical analysis.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism software. Tests used were two-tailed Student's t-test, one-way ANOVA with a Tukey's post-hoc test, or two-way ANOVA with a Bonferroni post-hoc test. All data is presented as mean +/- standard error of the mean.

RESULTS

βPix, GIT1, GIT2, and DCC are expressed in the developing spinal cord and localized to spinal commissural neuron growth cones

To begin to study the function of βPix in netrin-1 mediated outgrowth, we initially examined the expression of βPix in the developing rat nervous system. We amplified βPix transcripts by reverse transcriptase PCR (RT-PCR) from mRNA isolated from E13 rat brain and spinal cord, as well as from cultures of dissociated E15 dorsal root ganglion neurons (DRGs) and E13 spinal commissural neurons (Fig. 4.1A). The amplification products indicate that βPix is expressed in the developing nervous system and by commissural neurons *in vitro*. This is consistent with previous studies describing βPix as being ubiquitously expressed in most rat tissues (Manser et al. 1998). We also successfully amplified the ARF-GAPs GIT1 and GIT2, and DCC transcripts in these different tissues and cell types. To further investigate βPix expression, we immunoblotted 2 DIV spinal commissural neuron homogenates for βPix protein, revealing the expression of several isoforms of βPix in these cells (Fig. 4.1B). Previous studies have identified these isoforms as βPix-a/b1-Pix, βPix-d/b2Pix, βPix-b, βPix-bL, and βPix-c (Kim et al. 2000; Kim and Park 2001; Rhee et al. 2004).

During embryonic development, commissural neurons that originate in the dorsal portion of the spinal cord extend axons that are attracted by netrin-1 secreted by floor plate cells (Kennedy et al. 1994; Serafini et al. 1994). These axons continue to extend until they reach the ventral midline and cross it to form the ventral spinal commissure. Immunohistochemical analysis of βPix expression in the developing rat spinal cord at E12.5, an age when these commissural axons are actively extending axons towards the midline, detected βPix protein associated with commissural neuron cell bodies as well as their projecting axons (Fig. 4.1C, D). We also observed βPix immunoreactivity in the ventral commissure, motor column and dorsal root entry zone of the spinal cord. Neurofilament M (NFM) staining was used to visualize the neurons.

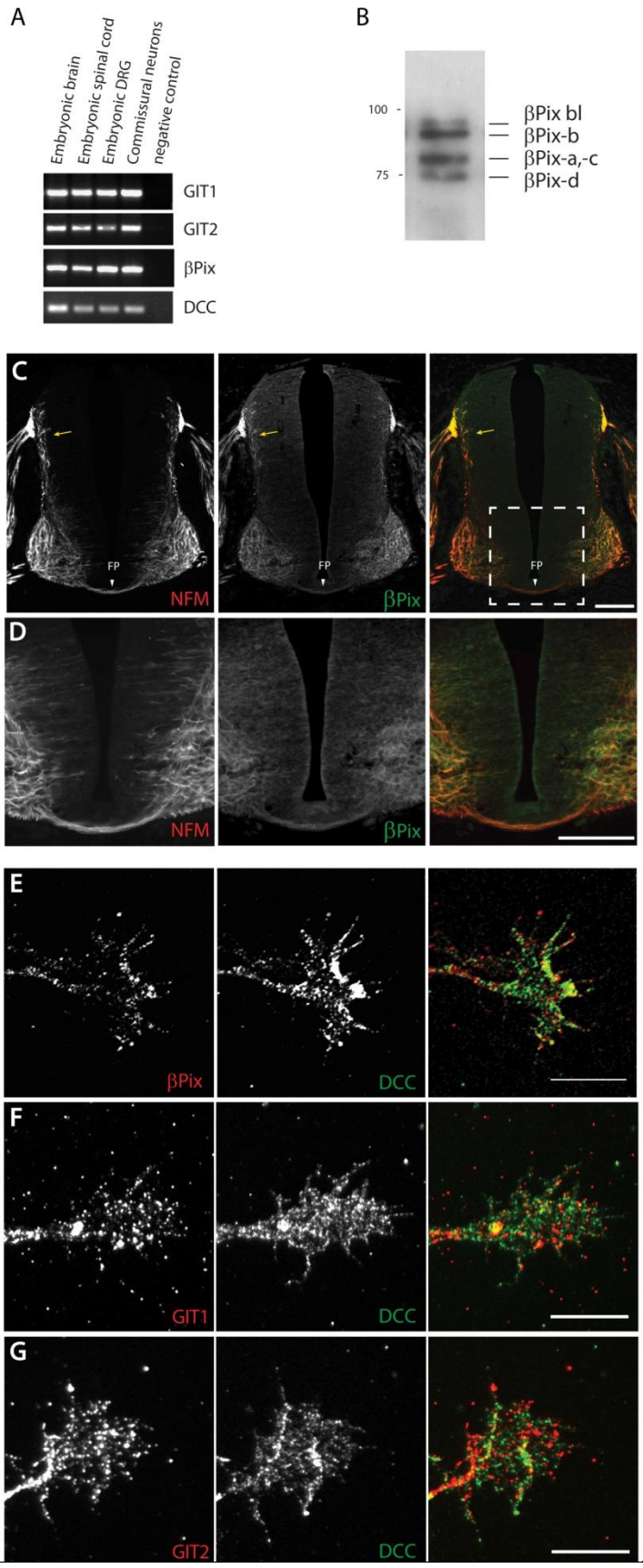

Figure 4.1 - Expression of βPix, GIT1, GIT2 and DCC in the developing central nervous system and by embryonic rat cultures. (**A**) Amplification of βPix, GIT1, GIT2 and DCC transcripts by reverse transcriptase PCR from embryonic rat brain and spinal cord tissues, as well as in dissociated dorsal root ganglion (DRG) neurons and spinal commissural neurons cultured for 2DIV. (**B**) Western blot of βPix isoforms expressed by 2DIV dissociated commissural neurons. (**C-D**) Expression pattern of βPix in embryonic rat spinal cord sections. 10 µm sections of paraffin-embedded rat embryos were stained for NFM and βPix. Arrows indicate the location of spinal commissural neuron cells bodies. Dashed box illustrates the region that is magnified in (D). (FP, floor plate of spinal cord). Scale bars = 100µm. (**E-G**) Distribution of βPix (E), GIT1 (F), GIT2 (G) and DCC immunoreactivity in the growth cone of a spinal commissural neuron *in vitro*. Cells were treated with 200 ng/ml of netrin-1 for 10 minutes. Scale bar = 10µm

The localization of a GEF contributes to determining where in the cell it may function. βPix is widely expressed by cells in many tissues, including the nervous system (Manser et al. 1998). Furthermore, DCC can be co-immunoprecipitated with the kinase PAK1, a βPix interacting protein, and PAK1 function has been implicated downstream of netrin-1 in spinal commissural neurons (Shekarabi et al. 2005). We therefore wanted to determine whether βPix is involved in netrin-1 signalling. To do this, we examined the subcellular localization of βPix and DCC in E13 spinal commissural neurons stimulated with 200 ng/ml netrin-1 for 10 min. Immunocytochemical analysis with antibodies against DCC and βPix revealed overlapping distributions in the soma, axon and growth cone (Fig. 4.1E). Furthermore, the expression of βPix interacting proteins GIT1 and GIT2 also overlapped with DCC in these cellular regions (Fig. 4.1F, G).

Netrin-1 promotes the association of βPix with Cdc42 and Rac1 in spinal commissural neurons

βPix can activate the Rho GTPases Cdc42 and Rac1 (Manser et al. 1998; Bagrodia et al. 1999). Netrin-1 activates Cdc42 and Rac1, and their downstream effector the serine/threonine kinase PAK1, in spinal commissural neurons (Shekarabi et al. 2005). To determine whether βPix may similarly regulate Rho GTPase activation in commissural neurons, we determined if βPix associates with endogenously activated Cdc42 and Rac1. To do this, a pulldown assay was performed using a recombinant GST protein fused to the Cdc42/Rac1 interacting binding (CRIB) motif of PAK1 in homogenates of commissural neurons that had been treated with netrin-1 for 10 min. This CRIB fusion protein selectively interacts with activated Rho GTPases and is therefore used to isolate GTP-bound Cdc42 and Rac1. Rho GTPase pulldown and βPix binding were determined by Western blot. Previous work reported a ~1.2 fold increase in the levels of GTP-Rac1 and a ~1.3 fold increase in the levels of GTP-Cdc42 binding to GST-CRIB peptide following netrin-1 stimulation (Rodrigues 2011). Similarly, the levels of βPix protein associated with activated Rac1 or Cdc42 showed a ~1.2 fold increase. These results suggest that βPix proteins binds activated Rac1 and Cdc42 in commissural neurons and that netrin-1 induced activation of the Rho GTPases is associated with increased binding of βPix to GTP-Rac1/Cdc42.

DCC associates with βPix and GIT2

Netrin-1 activates Rac1 and Cdc42, as well as the downstream effectors PAK1 and N-WASP, resulting in the reorganization of the F-actin cytoskeleton (Shekarabi et al. 2005). To further characterize the role of βPix in netrin-1 mediated signalling, we performed DCC co-immunoprecipitations from 2 DIV dissociated commissural neurons with and without a 10 min stimulation with netrin-1. Indeed, we observed an interaction between endogenous DCC and βPix, as well as a DCC-GIT2 association in these cells, and that these interactions occur independently of netrin-1 treatment (Fig. 4.2A, B). We also probed for GIT1, but did not detect GIT1 binding to DCC in these cultures (data not shown). These results suggest a constitutive association between DCC, βPix and GIT2 in commissural neurons, and that βPix and GIT2 may form a complex with DCC.

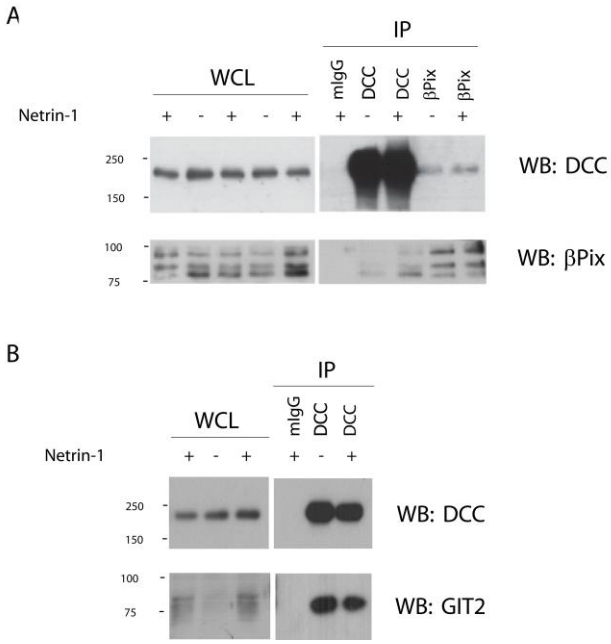

Figure 4.2 - βPix and GIT2 associate with DCC in dissociated embryonic rat spinal commissural neurons.

(**A**) Co-immunoprecipitation of DCC and βPix from 2DIV commissural neuron lysates. Commissural cells were treated with netrin-1 (200ng/ml) for 10 minutes before being lyzed. Proteins were immunoprecipitated with 1μg of either anti-DCC or anti-βPix antibodies. Western blot analyses were also performed using antibodies against DCC and βPix. (**B**) Co-immunoprecipitation assays for DCC and GIT2 from 2DIV commissural lysates. Commissural neurons were stimulated with netrin-1 (200ng/ml) for 10 minutes and lyzed. DCC was immunoprecipitated using 1μg of antibody, and then analyzed by Western blot with anti-DCC and anti-GIT2 antibodies.

βPix function requires PAK binding and GIT-multimerization to mediate netrin-1 induced filopodial outgrowth and growth cone expansion

To establish the functional relevance of the βPix/GIT2 interaction with DCC in commissural neuron axon guidance, we expressed various βPix mutant constructs in commissural neurons and observed their impact on netrin-1 function. These include a wild-type βPix (GST-βPix WT), an N-terminal deletion mutant that can no longer bind PAK (FLAG-βPixΔ80), and a C-terminal deletion mutant that can no longer multimerize with GIT proteins (FLAG-βPix 1-459). Netrin-1 induces increases in the number of filopodia in commissural neuron growth cones and in growth cone surface area (Shekarabi et al. 2005). We confirmed this finding following bath application of 200 ng/ml of netrin-1 to commissural neurons for 10 min (Fig. 4.3B), measuring increases in filopodial number (Fig. 4.3E), filopodial length (Fig. 4.3F), and growth cone surface area (Fig. 4.3G). Ectopic expression of wild-type βPix was sufficient to induce a partial increase in the number and length of growth cone filopodia, but only to ~50% of that induced by netrin-1 (Fig. 4.3E, F). This suggests that although βPix influences the reorganization of F-actin in the growth cone, netrin-1 likely engages additional cytoskeletal machinery that also contributes to growth cone expansion. In contrast, expression of the βPixΔ80 or βPix 1-459 mutants severely compromised netrin-1 induced growth cone expansion (Fig. 4.3E, F, G). These findings provide evidence that the association of βPix with PAK and GIT is required for netrin-1 induced growth cone expansion. Interestingly, total axon length did not change when these mutants were expressed, nor when neurons were treated with netrin-1, consistent with previous studies demonstrating that netrin-1 does not influence the rate of commissural axon extension *in vitro* in dispersed cell culture (Shekarabi et al. 2005) (Fig. 4.3H). Together, these findings reveal a role for an influence of βPix on growth cone dynamics but not axonal growth *in vitro* in these conditions.

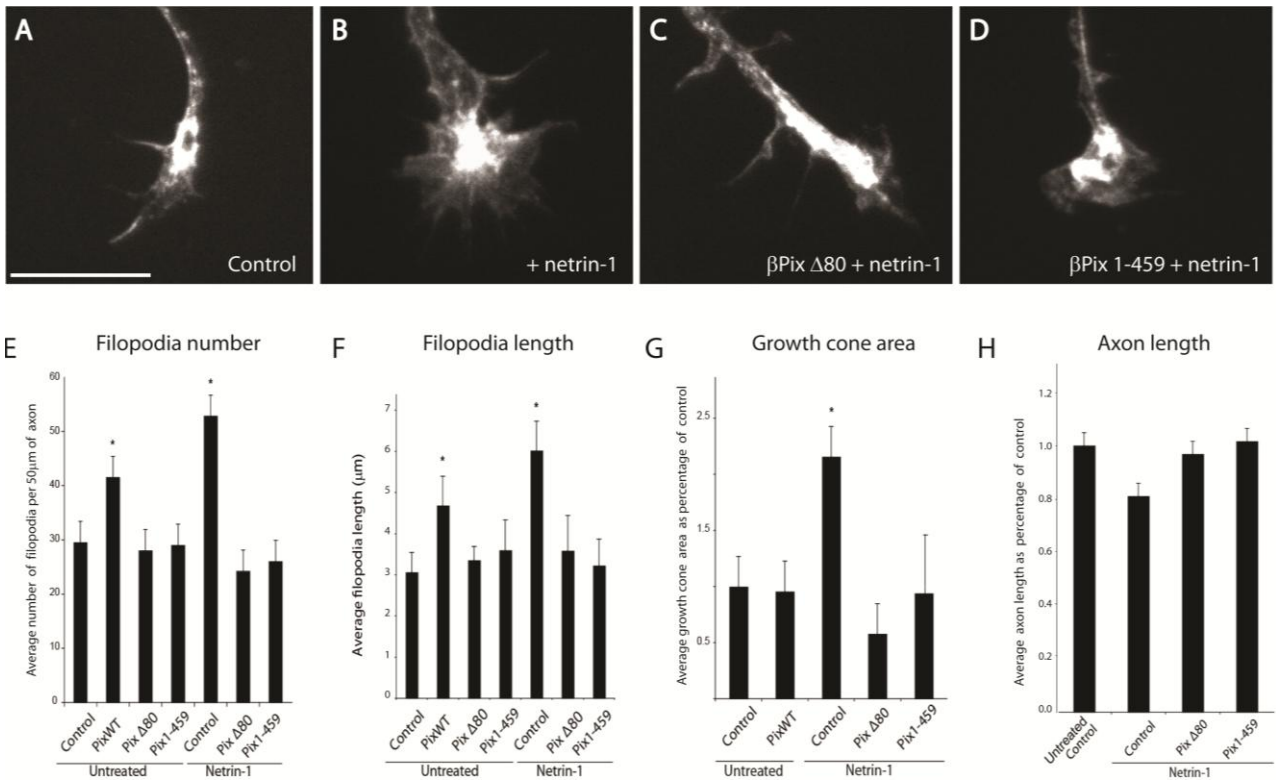

Figure 4.3 - Recombinant βPix mutants inhibit netrin-1 induced growth cone expansion and filopodia formation in commissural neurons. βPixΔ80 is a mutant βPix construct that is unable to associate with PAK, while βPix 1-459 is a βPix mutant lacking its coiled-coil domain, rendering it unable to form multimers with GIT. (**A-D**) Representative images of growth cones from untransfected commissural neurons (A), untransfected neurons treated with 200ng/ml of netrin-1 for 10 minutes (B), commissural neurons transfected with βPixΔ80 and treated with netrin-1 (C), and commissural neurons transfected with βPix 1-459 and stimulated with netrin-1 (D). The cells were then stained for F-actin. Scale bar = 10μm. (**E-G**) Quantification of filopodia number (E), filopodia length (F) and growth cone area (G) of transfected commissural neurons following netrin-1 treatment. (**H**) Diagram demonstrating the total axon length of transfected commissural neurons with and without netrin-1 stimulation. (n = 20-46 growth cones/condition, * = $p \leq 0.01$).

Disrupting βPix function leads to defective commissural axon guidance to the midline *in vivo*

Netrin-1 is required for commissural axons to extend to the ventral midline of the embryonic spinal cord (Kennedy et al. 1994; Serafini et al. 1994). To determine the functional contribution of βPix to commissural axon guidance *in vivo*, chick embryos at Hamilton Hamburg stage (HH) 14-16 were co-electroporated with GFP and a mutant βPix construct injected into

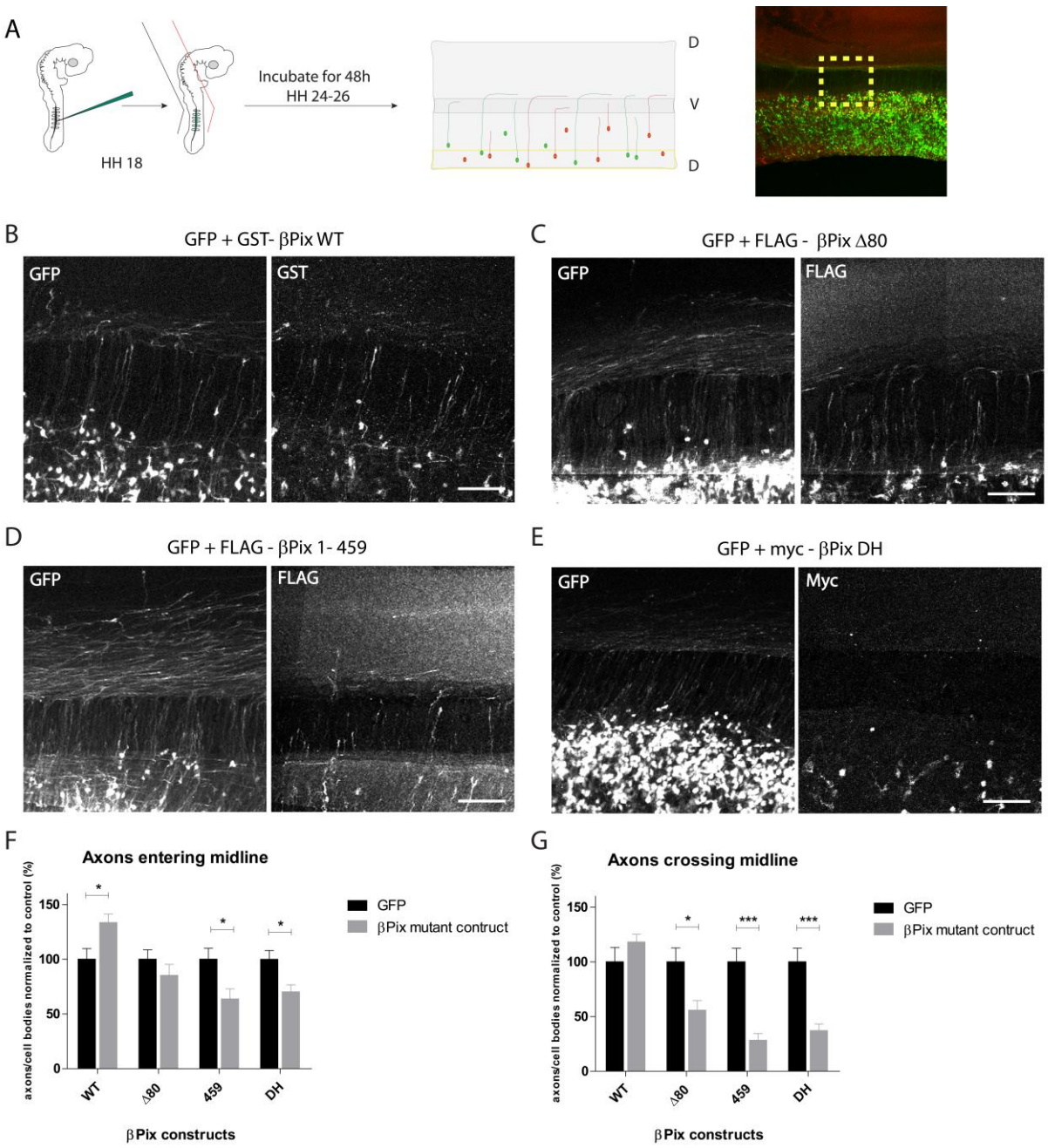

Figure 4.4 - βPix mutants electroporated into embryonic chick spinal cords disrupt commissural axon extension toward the floor plate. Stage 18 chick embryos were co-electroporated with GFP and either βPix WT, βPix Δ80, βPix 1-459 or βPix DH, a catalytically inactive βPix mutant. Eggs were incubated for 48 hours post-electroporation before spinal cords were isolated and analyzed as open-book preparations. (**A**) Diagram illustrating chick electroporation and analysis. (D, dorsal spinal cord; V, ventral spinal cord). (**B-E**) Chick spinal cords electroporated with βPix WT (B) βPix Δ80 (C), βPix 1-459 (D) and βPix DH (E). Scale bars = 100μm. (**F**) Ratio of axons entering the floor plate in spinal cords expressing βPix mutants. (**G**) Ratio of axons crossing the ventral midline in spinal cords expressing βPix mutants. (n = 258-543 axons/condition, 3-5 spinal cords/construct, * = $p \leq 0.05$, *** = $p \leq 0.001$).

the lumen of the spinal cord (Fig. 4.4A). These constructs are GST-βPix WT, FLAG-βPixΔ80, FLAG-βPix 1-459, and myc-βPix DH, which is mutant βPix containing two point mutations in its catalytic DH domain to inhibit its guanine nucleotide exchange ability (Hart et al. 1994; Manser et al. 1998). After a 48 hr incubation, the spinal cords of these embryos were microdissected and wholemount stained for GST, FLAG or Myc to identify neurons that were successfully electroporated and expressing the mutant constructs. Normally, commissural neurons extend towards the floor plate and once they reach the midline, cross and make a stereotypical 90 degree rostral turn (Bovolenta and Dodd 1990). Interestingly, when GST-βPix WT was overexpressed in theses neurons, we observed an increase in the number of axons that reached the midline (Fig. 4.4B, F). On the other hand, overexpressing FLAG-βPixΔ80 (Fig. 4.4C), FLAG-βPix 1-459 (Fig. 4.4D) and myc-βPix DH (Fig. 4.4E) reduced the number of axons that reached and/or crossed the midline (Fig. 4.4F, G). These findings provide evidence that disrupting βPix leads to defective commissural axon crossing at the midline in the embryonic spinal cord.

βPix knockdown impairs netrin-1 induced morphological changes in commissural growth cones

Using dominant negative constructs, we have implicated βPix function downstream of DCC (Fig. 4.3, 4.4). However, off-target effects are a common artifact that can confound the interpretation of results derived from over-expressing mutant proteins. To extend our functional studies, we opted for a loss-of-function approach using siRNAs that target rat βPix. To assess the capacity of the siRNAs to knockdown βPix expression, we transfected dissociated commissural neurons with βPix or control siRNAs and analyzed whole cell homogenates by Western blot. We detect a substantial reduction in βPix protein levels in neurons transfected with the βPix siRNAs but not with the control siRNA (Fig. 4.5A). GAPDH was used to control for protein loading.

To directly assess the contribution of βPix to netrin-1 induced growth cone dynamics, commissural neurons were again transfected with βPix or control siRNAs. After 2 DIV, the cultures were treated with 200 ng/ml netrin-1 for 10 min, fixed and stained with a fluorescently

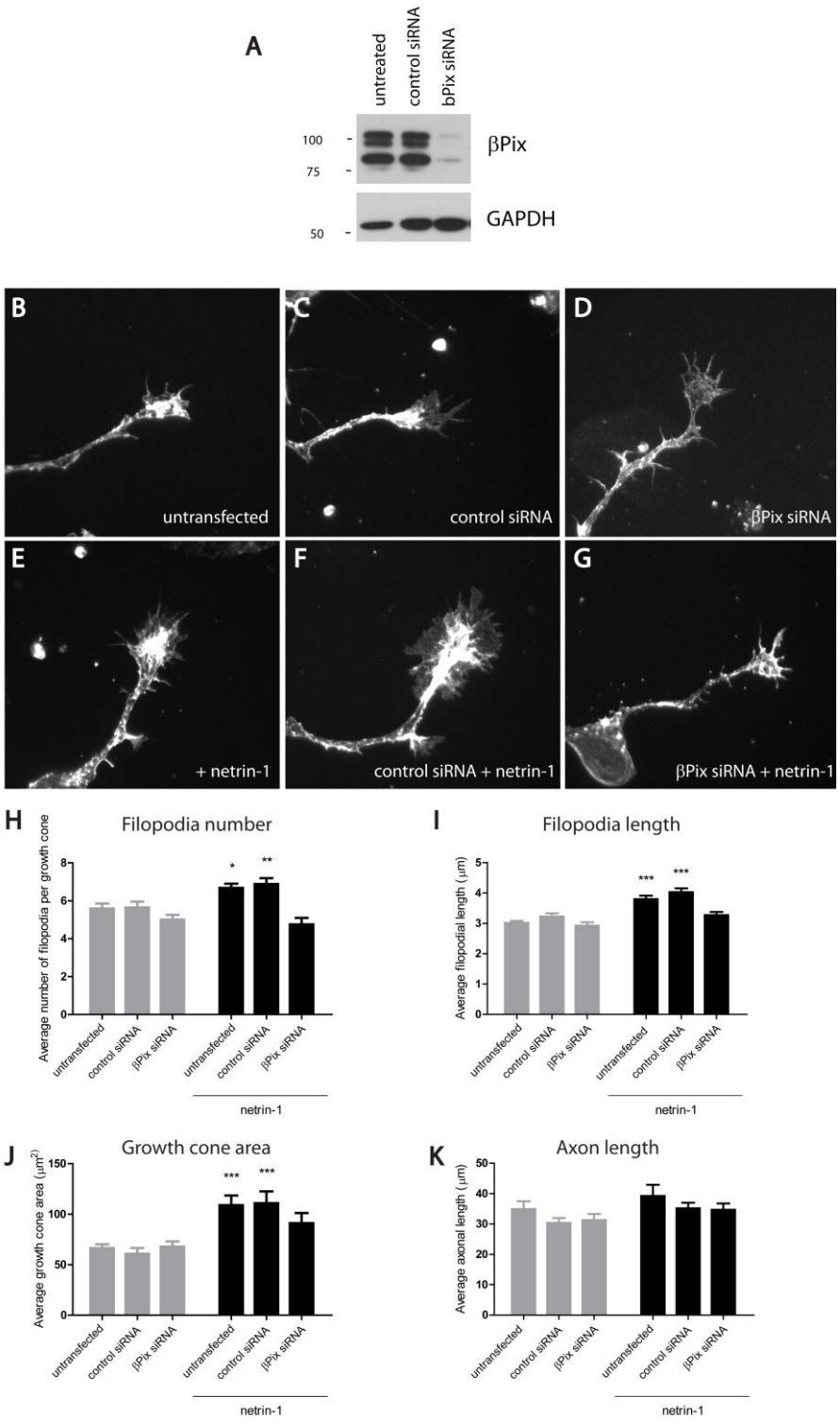

Figure 4.5 - βPix knockdown inhibits netrin-1 induced growth cone expansion and filopodia formation in commissural neurons.

(**A**) Western blot of lysates from 3DIV commissural neurons transfected with βPix or control siRNA and immunoblotted for βPix and GAPDH as a loading control. (**B-G**) Representative images of growth cones from untransfected commissural neurons (B, E), commissural neurons expressing control siRNA (C, F) and commissural neurons expressing βPix siRNA (D, G). Neurons were stimulated with 200ng/ml of netrin-1 for 10 minutes. (E,F,G). The cells were then stained for F-actin. Scale bar =10μm. (**H-K**) Quantification of filopodia number (H), filopodia length (I), growth cone area (J), and total axon length (K) of βPix knockdown commissural neurons following netrin-1 treatment. (n = 58-105 growth cones/condition , * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$).

labelled phalloidin to visualize F-actin (Fig. 4.5B-G). Non-transfected neurons and neurons transfected with the control siRNA exhibited increases in the number and length of filopodia, and growth cone surface area (Fig. 4.5H-J). In contrast, neurons in which βPix expression was downregulated displayed no changes in growth cone expansion when stimulated with netrin-1 (Fig. 4.5H-J). These findings support the conclusion that βPix mediates filopodia formation and growth cone expansion induced by netrin-1.

DISCUSSION

Although a number of molecules have been identified to act downstream of DCC, the precise signal transduction mechanisms by which netrin-1 influences growth cone dynamics remain incompletely understood. Earlier work demonstrated that netrin-1 promotes the formation of a protein complex that includes the intracellular domain of DCC, the adaptor Nck1, the Rho GTPases Cdc42 and Rac1, and their effector PAK1 (Li et al. 2002a; Shekarabi et al. 2005). Netrin-1 was also shown to activate Cdc42, Rac1, and PAK1 in embryonic spinal commissural neurons, with strong evidence implicating DCC in the activation of a GEF and suggesting that the activation of Rac1 is dependent on the prior activation of Cdc42 (Shekarabi et al. 2005).

Here, we identify the Cdc42 and Rac1 RhoGEF βPix as a major effector in the netrin-1 activated signal transduction pathway in commissural axon extension. We demonstrate that βPix is expressed in the developing nervous system, along with GIT1, GIT2 and DCC. Previous findings revealed that βPix interacts with GTP-bound Cdc42 and Rac1 in commissural neurons, and that this association is enhanced by netrin-1 (Rodrigues 2011). Furthermore, we show that a complex consisting of βPix and GIT2 constitutively associates with DCC in commissural neurons. Finally, we provide evidence that disrupting βPix function, using dominant negative mutants of βPix or by siRNA knockdown, acutely compromises netrin-1 induced cytoskeletal rearrangements in the growth cone and hinders commissural axon guidance in the developing spinal cord. Together, these findings implicate βPix function in chemoattractant signalling in

response to netrin-1. Analysis of commissural axon trajectories in βPix knockout mice would further validate the requirement for βPix in netrin-1 chemoattraction. Although βPix nulls have been generated, unfortunately they exhibit severe defects in neural tube closure and die at ~E8.5, making it impossible to assess the trajectory of commissural axons in these mice (Dr. D. Park, Korea, personal communication). Although a floxed βPix allele is being generated (Dr D. Park, Korea, personal communication), it is not yet available.

Previous studies have proposed Trio and DOCK180 as candidate GEFs acting downstream of DCC (Briancon-Marjollet et al. 2008; Li et al. 2008). However, analysis of the Trio and DOCK180 null animals does not support an essential role for either of these proteins in commissural neuron axon guidance as the severe defects exhibited by DCC and netrin-1 deficient mice are not recapitulated in Trio and DOCK180 knockouts (Serafini et al. 1996; Fazeli et al. 1997; Briancon-Marjollet et al. 2008; Laurin et al. 2008; Li et al. 2008). Moreover, both of these GEFs appear to be specific for Rac1, while evidence suggests that netrin-1 regulation of Cdc42 may function upstream of Rac1 activation (Shekarabi et al. 2005). These findings suggest that a Cdc42 specific GEF will be a critical downstream component of netrin-1 signalling through DCC. It is plausible that more than one GEF contributes to netrin-1 mediated chemoattraction. Many signalling components are required to regulate cell migration, axon extension and growth cone turning. It is therefore possible that several GEFs are regulated by DCC to activate distinct Rho GTPases for various cellular processes. The activation of different GEFs may also be dependent on cell types or temporal factors.

In summary, we propose a model where netrin-1 binding to DCC induces the recruitment and activation of several signalling molecules into a complex with the cytoplasmic domain of DCC. This signalling complex includes the Src family kinase Fyn, which is known to regulate Rho GTPase activity, Cdc42, Rac1 and PAK1 (Meriane et al. 2004; Shekarabi et al. 2005). PAK1, acting as an adaptor, then recruits the Cdc42/Rac1 GEF βPix. Based on current models of PAK function, we hypothesize that GIT2, which regulates βPix activity, is phosphorylated by Fyn and induces βPix activation (Bagrodia et al. 1999; Premont et al. 2004). βPix can subsequently promote the activation of Cdc42 and Rac1, which then leads to the

recruitment of the actin binding proteins N-WASP and the Arp2/3 complex to mediate the reorganization of F-actin. Identifying the circumstances in which a specific GEF is activated and how βPix coordinates with other GEFs to enable netrin-1 chemoattraction remains to be established.

CHAPTER 5

Assessing the functional significance of graded expression of netrin-1 in the developing spinal cord

Karen Lai Wing Sun, Jenea M. Bin, Dong Han and Timothy E. Kennedy

Montreal Neurological Institute, Department of Neurology and Neurosurgery, McGill University, Montreal, Quebec, Canada, H3A 2B4.

PREFACE

The two previous chapters of this thesis investigated the signalling mechanisms involved in netrin-1 mediated chemoattraction and how they contribute to commissural axon guidance in the embryonic spinal cord (Chapters 3 and 4). This next chapter looks at the role of netrin-1 as a guidance cue, examining the importance of the netrin-1 gradient in the developing spinal cord for the extension of spinal commissural axons. Netrin-1 is indispensible for proper commissural pathfinding and it has been shown to have growth-promoting and guidance activities *in vitro*, but the contribution of the gradient of netrin-1 to commissural axon extension *in vivo* is still unclear. This section aims to better understand what function netrin-1 and its graded distribution play in the developing spinal cord. The material in this chapter corresponds to a manuscript in preparation for submission.

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ABSTRACT

Netrin-1 is a secreted chemotropic molecule that contributes to multiple processes, including cell migration, tissue morphogenesis and synapse formation. Its best-described function is as an axon guidance cue, particularly in the developing spinal cord where it is expressed by floor plate cells and promotes the extension of spinal commissural axons. Netrin-1 diffuses from the floor plate and forms an extracellular gradient. It is this netrin-1 gradient that is proposed to direct the spinal commissural axons in a well-defined trajectory towards the floor plate. Here, we demonstrate that the amount of netrin-1 expressed in the embryonic mouse spinal cord can be regulated genetically. We also provide evidence that spinal commissural neurons respond to a graded distribution of netrin-1 early in development.

INTRODUCTION

Netrin-1 is a secreted cue that plays important roles throughout development and into adulthood (Lai Wing Sun et al. 2011). In particular, the role of netrin-1 in midline guidance has been very well-studied in the developing spinal cord. In this context, netrin-1 is secreted by floor plate cells and diffuses dorsally into the surrounding neural epithelium to create a gradient of netrin-1 emanating from the ventral midline of the embryonic spinal cord (Kennedy et al. 1994; Serafini et al. 1996; Kennedy et al. 2006). This netrin-1 gradient is thought to repel the migration of trochlear motor neurons and oligodendrocyte precursor cells, and attract the axons of spinal commissural neurons (Colamarino and Tessier-Lavigne 1995b; Jarjour et al. 2003). Spinal commissural neurons initiate axon extension in the embryonic dorsal spinal cord and extend their projections circumferentially towards the ventral midline, where they cross and form the ventral commissure. This stereotypical trajectory depends on netrin-1 expression at the midline and that of its receptor Deleted in colorectal cancer (DCC) by the commissural axon growth cones (Kennedy et al. 1994; Serafini et al. 1994; Keino-Masu et al. 1996). In netrin-1 deficient mice, in which a truncated form of netrin-1 fused to a β-galactosidase transgene is expressed (netrin-1 βgeo), spinal commissural axons migrate in aberrant directions and few

reach the midline (Serafini et al. 1996). Netrin-1 βgeo homozygotes are however not complete netrin-1 nulls, but rather severely hypomorphic animals because some wild-type *netrin-1* mRNA is still expressed due to alternative splicing (Serafini et al. 1996).

While multiple studies have confirmed that netrin-1 directs commissural axon outgrowth *in vitro* and is indispensable for accurate commissural axon outgrowth *in vivo*, it remains unclear whether netrin-1 functions *in vivo* as a permissive cue that promotes axonal growth without imparting directional guidance information, or if it plays a more instructive role by attracting commissural neurons along a defined path. Some evidence supports the conclusion that a netrin-1 gradient can elicit directed growth cone extension and turning *in vitro*: commissural axons in dorsal spinal cord explants reorient their growth when placed next to floor plate tissue (Serafini et al. 1996); *Xenopus* retinal ganglion cells turn towards a source of netrin-1 ejected from a pipette (de la Torre et al. 1997); shallow gradients of the morphogen Sonic hedgehog (Shh) and netrin-1 synergistically guide dissociated commissural neurons using an *in vitro* microfluidics guidance assay (Sloan et al. 2015). Despite all of this, it has not been demonstrated that the graded distribution of netrin-1 present *in vivo* is required for commissural axon guidance to the ventral midline in the developing spinal cord. In the current study, we address this question by genetically manipulating the levels of netrin-1 expression, thereby altering the distribution of netrin-1 in the spinal cord. For this purpose, we make use of the previously characterized netrin-1 βgeo mutant mice and a new netrin-1 deleted mouse in which netrin-1 expression has been completely abolished (Serafini et al. 1996; Bin et al. in press). Based on the genetic manipulation of netrin-1 gene dosage, our findings support the conclusion that the graded distribution of netrin-1 in the embryonic spinal cord functions as an attractive signal that directs embryonic spinal commissural axons to the ventral midline.

METHODS AND MATERIALS

Animals

Mice heterozygous for the β-galactosidase netrin-1 recombinant allele (*netrin-1*$^{\beta geo}$) (Serafini et al. 1996) were obtained from Marc Tessier-Lavigne (Rockefeller University, NYC, USA). The netrin-1 deleted mice were generated as described (Bin et al. in press). All animal procedures were performed in accordance with the Canadian Council on Animal Care guidelines for the use of animals in research.

Antibodies

The following primary antibodies were used in this study: monoclonal rabbit anti-netrin-1 (EPR5428) from Abcam (Cambridge, UK); monoclonal mouse anti-pan-axonal neurofilament (SMI-312) from Covance (Princeton, NJ); polyclonal rabbit anti-GAPDH (FL-335) from Santa Cruz Biotechnology (Santa Cruz, CA); polyclonal goat anti-contactin-2/TAG-1 from R & D Systems (Mineapolis, MN).

Western blot analysis

Spinal cords from embryonic day 12.5 (E12.5) and E14.5 embryos were microdissected and homogenized in ice-cold RIPA buffer (10 mM phosphate buffer pH 7.2, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) with protease and phosphatase inhibitors (2 μg/ml aprotinin, 5 μg/ml leupeptin, 2 mM EDTA, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1 mM PMSF). Proteins were separated by SDS-PAGE and transferred to polyvinyldiene fluoride (PVDF) membranes (Bio-Rad Laboratories, Hercules, CA). The membranes were probed with primary antibodies overnight. Immunoreactivity was visualized using Immobilon Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA).

Immunohistochemistry

E10.5, E12.5 and E14.5 embryos were collected from timed pregnant netrin-1 βgeo and netrin-1 deleted mice, fixed in Carnoy's fixative solution (60% ethanol, 10% acetic acid, 30% chloroform) for 2 hrs at room temperature, and then washed twice with 100% ethanol for 20 min. The embryos were cleared in toluene for 1 hr and transferred to paraffin wax at 60 ^{o}C in a vacuum oven overnight. The following day, the embryos were embedded in fresh paraffin and 10μm sections cut on a microtome. Prior to staining, tissue sections were rehydrated as follows: 2 x xylene 3 min, 2 x 100% ethanol 3 min, 2 x 95% ethanol 3 min, 2 x 70% ethanol 3 min, 1 x PBS 5 min. Antigen retrieval was also performed on these sections by boiling the slides in citrate buffer (10 mM citric acid, pH 6.0) for 10 min. Sections were blocked in PBS containing 3% bovine serum albumin (BSA) and 0.1% Triton X-100 for 1 hr, and incubated with the primary antibodies overnight at 4^{o}C. Following washes, sections were incubated with Alexa secondary antibodies and coverslipped with Fluoromount-G (SouthernBiotech, Birmingham, AL). All immunofluorescent images were obtained using a Zeiss Axiovert S100TV inverted microscope and a Magnafire CCD camera (Optronics).

Measurement of ventral commissure thickness

All ventral commissures were quantified from images of spinal cords taken using a 20X objective lens. Commissural axons were visualized by immunohistochemistry using goat anti-TAG-1 antibody (R & D Systems) on sections from E10.5 and E12.5 embryos, and mouse anti-pan neurofilament (SMI-312, Covance) on E14.5 sections. The images were first thresholded to obtain binary images of the axonal trajectories. A 18.75 μm x 80.21 μm box was drawn over the centre of the ventral commissure in each image and the number of pixels above threshold were counted. To account for the variable sizes of the embryos, the number of pixels was normalized to the height of the spinal cords. The results were presented as percentages of the WT netrin-1 βgeo values. Statistical analyses using two-tailed Student's t-test were performed using GraphPad Prism 5. The data is presented as mean +/- standard error.

RESULTS

Genetic manipulation of netrin-1 expression in the developing spinal cord

Homozygous netrin-1 mutant animals (netrin-$1^{\beta geo/\beta geo}$) are not complete netrin-1 nulls but actually netrin-1 hypomorphs that express low levels of full-length netrin-1 (Serafini et al. 1996). Conversely, netrin-1 expression is completely abolished in the recently developed netrin-1 deleted line ($Ntn1^{-/-}$) described by Bin et al. (in press). Based on the prediction that heterozygous animals, which express only one copy of the mutant or deleted allele, should produce less netrin-1 than their wild-type littermates, we were interested in determining what amount of netrin-1 was detected in homozygotes, heterozygotes and wild-types of the netrin-1 βgeo and netrin-1 deleted mouse lines. To begin, we sectioned spinal cords of netrin-1 βgeo and netrin-1 deleted embryos at different stages during development and immunostained for netrin-1 protein (Fig. 5.1 A-R). In mice, the first spinal commissural neurons begin to extend axons at approximately E9.5 in the gestational period, making E10.5 an ideal time to observe the early trajectory of pioneering commissural neurons (Colamarino and Tessier-Lavigne 1995b). By E12.5, many commissural axons have reached the midline and crossed to the opposite side to form the ventral commissure. At E14.5, commissural axon extension and crossing are complete, with the commissural neurons making their stereotypical longitudinal turn on the contralateral side of the spinal cord. Netrin-1 is expressed in the floor plate and ventral neural epithelium in wild-type E10.5 (Fig. 5.1A and J), E12.5 (Fig. 5.1D and M) and E14.5 animals (Fig. 5.1G and P). In contrast, we detected no netrin-1 expression in homozygous netrin-1 βgeo (netrin-$1^{\beta geo/\beta geo}$) (Fig. 5.1C, F, I) or netrin-1 deleted ($Ntn1^{-/-}$) mice at any age by immunohistochemistry (Fig. 5.1L, O, R). A modest reduction in the level of detectable netrin-1 immunoreactivity was observed in heterozygous netrin-$1^{+/\beta geo}$ (Fig. 5.1B, E, H) and $Ntn1^{+/-}$ embryos (Fig. 5.1K, N, Q) when compared to wild-type littermates in both mouse lines. To further explore the effect of gene dosage on netrin-1 expression, we homogenized E12.5 and E14.5 spinal cords from wild-type, heterozygous and homozygous netrin-1 βgeo and netrin-1 deleted mice. Western blot analysis for netrin-1 confirmed that a small amount of netrin-1 protein is present in the spinal cords of netrin-$1^{\beta geo/\beta geo}$ mutant mice, whereas no netrin-1 was

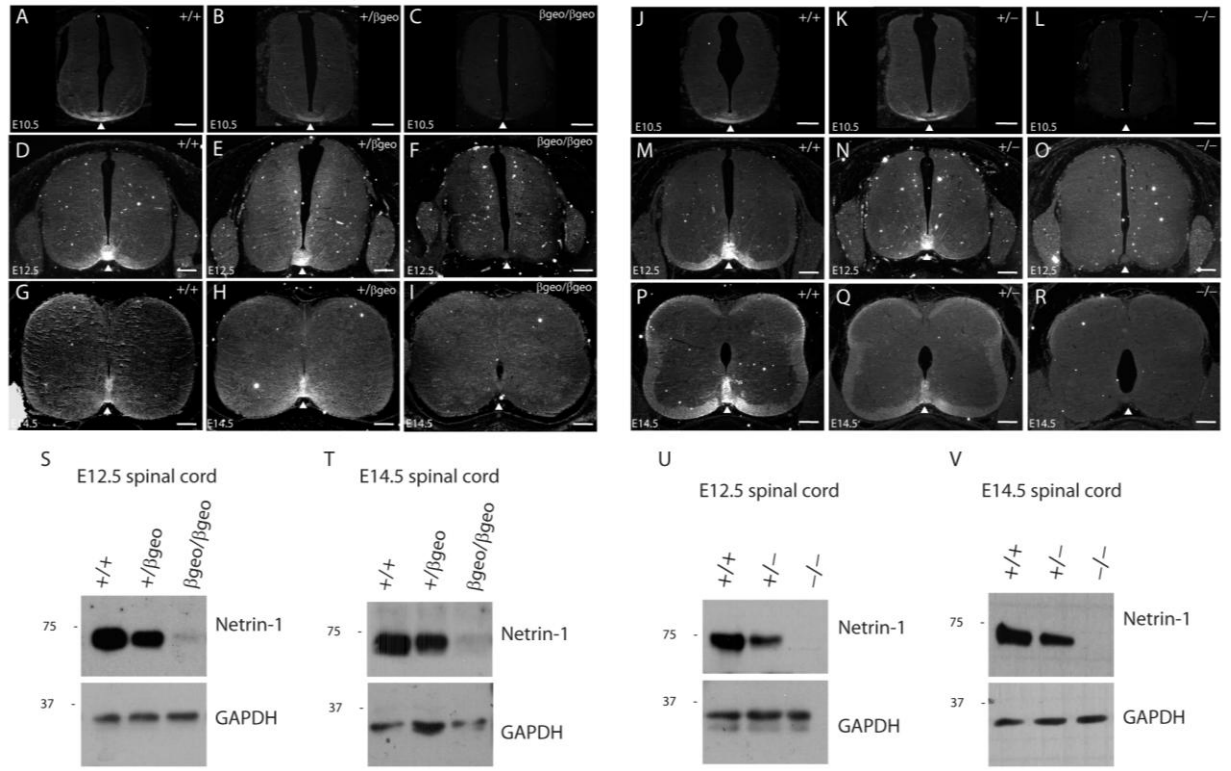

Figure 5.1 - Manipulation of netrin-1 expression in the developing spinal cord. (**A-R**) Analysis of netrin-1 expression in wild-type, heterozygous and homozygous embryos from netrin-1 βgeo (A-I) and netrin-1 deleted mice (J-R). Sections of E10.5, E12.5 and E14.5 spinal cords from netrin-1 βgeo and netrin-1 deleted mice were immunostained for netrin-1. Representative images are presented as follows: (A-C) E10.5 netrin-1 βgeo mice; (D-F) E12.5 netrin-1 βgeo mice; (G-I) E14.5 netrin-1 βgeo mice; (J-L) E10.5 netrin-1 deleted mice; (M-O) E12.5 netrin-1 deleted mice; (P-R) E14.5 netrin-1 deleted mice. Scale bars = 100 μm. (**S-T**) Western blots of protein homogenates collected from spinal cords of E12.5 (S) and E14.5 (T) netrin-1 βgeo mice. (**U-V**) Western blots of spinal cord homogenates collected from E12.5 (U) and E14.5 (V) netrin-1 deleted mice. No netrin-1 protein is detected in the KO, and reduced levels of netrin-1 are detected in heterozygotes.

detected in Ntn1 $^{-/-}$ spinal cord homogenates (Fig. 5.1 S-V). The western blots also provide a clear visualization of the decreased levels of netrin-1 protein being made by netrin-1$^{+/\beta geo}$ and Ntn1 $^{+/-}$ animals. Notably, we detected a greater difference in netrin-1 levels between Ntn1 $^{+/-}$ and wild-type spinal cords than between netrin-1$^{+/\beta geo}$ mice and their wild-type littermates (Fig. 1 S-V), consistent with the conclusion that expression of the netrin-1 βgeo allele contributes

some additional full-length netrin-1 protein. These findings indicate that the distribution of netrin-1 protein in the developing spinal cord can be genetically manipulated using these mice.

Graded expression of netrin-1 influences commissural axon extension

Following the demonstration that different genotypes express varied amounts of netrin-1 at the spinal cord floor plate, we investigated what effect this would have on the guidance of spinal commissural neurons *in vivo*. To do this, we collected embryos from netrin-1 βgeo and netrin-1 deleted mice at E10.5, E12.5 and E14.5 and analyzed their commissural axon trajectories. For E10.5 embryos (Fig. 5.2A-F), pre-crossing commissural axons were visualized by staining the sections with an antibody against TAG-1 (Dodd et al. 1988). We observe that the trajectories of netrin-$1^{\beta geo/\beta geo}$ and Ntn1 $^{-/-}$ homozygotes were significantly different than their wild-type and heterozygous counterparts, with commissural axons initially projecting ventrally, but then stalling or migrating aberrantly into the epithelium in various directions (Fig. 5.2C, F). We also observe a drastic reduction in the thickness of the ventral commissure of homozygous animals, indicating that a decreased number of commissural neurons are reaching and crossing the midline. To quantify this, we measured the area of fluorescent TAG-1 immunoreactivity at the ventral midline of the spinal cord of each animal as a correlate of commissure size, and normalized it to the height of the spinal cord to account for any size differences between embryos. Consistent with previous findings, the netrin-1 deficient mice exhibit a considerable reduction in the size of the spinal ventral commissure compared to that of wild-type and heterozygous littermates (Serafini et al. 1996; Bin et al. in press) (Fig. 5.2G). Notably, the netrin-$1^{+/\beta geo}$ and Ntn1 $^{+/-}$ embryos also display a large difference in commissure heights. Based on the demonstration that netrin-$1^{+/\beta geo}$ mice express more netrin-1 than Ntn1 $^{+/-}$ mice (Fig. 5.1S-V), the difference in commissure thickness between these mice is consistent with the conclusion that the amount of netrin-1 expressed in the spinal cord directly influences the extension of spinal commissural neurons to the ventral midline and their crossing at this age.

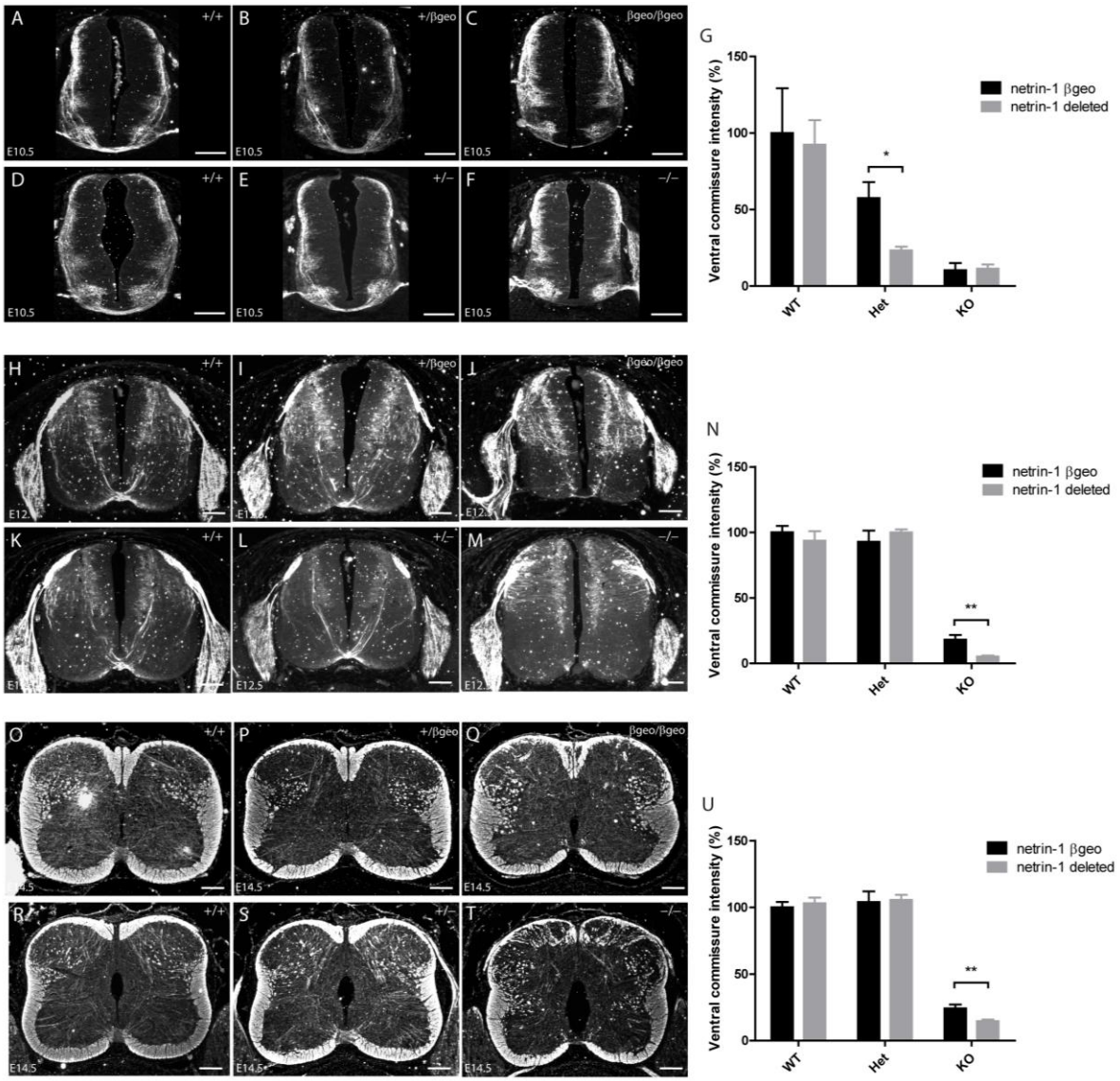

Figure 5.2 - Commissural trajectories of netrin-1 βgeo and netrin-1 deleted mice.

(**A-F**) Representative sections of E10.5 netrin-1 βgeo spinal cords (A-C) and netrin-1 deleted spinal cords (D-F) stained with a TAG-1 antibody to visualize commissural neurons. (**G**) The fluorescence intensity of the ventral commissures of E10.5 embryos were quantified (n = 4-5 embryos/genotype). (**H-M**) Representative images of TAG-1 positive commissural neurons in E12.5 netrin-1 βgeo (H-J) and netrin-1 deleted (K-M) spinal cords. (**N**) Quantification of fluorescence intensity of E12.5 ventral commissures (n = 4-5 embryos/genotype). (**O-T**) Representative sections of E14.5 netrin-1 βgeo (O-Q) and netrin-1 deleted (R-T) spinal cords stained for neurofilaments. (**U**) Quantification of F14.5 commissure fluorescence (n = 5-6 embryos/genotype). (* = $p \leq 0.05$, ** = $p \leq 0.01$) Scale bars = 100 μm.

TAG-1 immunohistochemistry was used to visualize the commissural trajectories in E12.5 netrin-1 βgeo and netrin-1 deleted embryos (Fig. 5.2H-M). At this stage of development, a large population of commissural neurons remain misguided in the homozygotes (Fig. 5.2J, M). However, we note that significantly more axons were able to reach the midline and form a commisure in netrin-$1^{\beta geo/\beta geo}$ deficient mice than in Ntn1 $^{-/-}$ nulls (Fig. 5.2N). Conversely, wild-types and heterozygotes of both mouse lines were indistinguishable from each other, appearing to have normal commissural trajectories and similar commissure thicknesses (Fig. 5.2H,I, K, L and N). In E14.5 embryos, where commissural neurons were labelled with a neurofilament antibody (SMI-312, Covance), analogous observations were made: homozygotes have disrupted commissural tracts, netrin-$1^{\beta geo/\beta geo}$ mice have thicker commissures than Ntn1 $^{-/-}$ mice, and there is no detectable difference in the commissures of netrin-$1^{+/+}$, netrin-$1^{+/\beta geo}$ and Ntn1 $^{+/-}$ spinal cords (Fig. 5.2O-U). Together, these results imply that manipulation of netrin-1 levels in the spinal cord can alter the extension of commissural axons to the midline early in embryogenesis, but that the graded distribution of netrin-1 in spinal cord is less pertinent later in development.

DISCUSSION

Netrin-1 is secreted by floor plate cells in the developing spinal cord and is pivotal to the guidance of spinal commissural axons to the ventral midline (Serafini et al. 1996). In this study, we demonstrated that the amount of netrin-1 expressed in the spinal cord is partial to genotype in netrin-1 βgeo and netrin-1 deleted mice. We also established that commissural neurons are sensitive to the graded distribution of netrin-1 at the floor plate in E10.5 embryos, but less so in E12.5 and E14.5 animals. These results provide evidence that the graded distribution of netrin-1 is important early in spinal cord development to attract commissural neurons to the floor plate, but that varying netrin-1 levels are not as critical for commissural axon attraction and ventral commissure formation later in embryogenesis as long as a sufficient amount of netrin-1 is expressed.

A plausible explanation for this finding is that the graded distribution of netrin-1 is critical to direct the circumferential trajectory of pioneering spinal commissural axons early in embryogenesis, when the network is first being established. When the commissural axons first start to extend, they are initially propelled ventrally through the repellent actions of bone morphogenic proteins (BMPs) and Draxin expressed at the roof plate of the spinal cord (Augsburger et al. 1999; Butler and Dodd 2003; Islam et al. 2009). As they continue along their path, these pioneering commissural axons respond to low levels of netrin-1 and are attracted up the gradient towards the floor plate until they reach the ventral midline. In this scenario, netrin-1 acts as a directional cue, guiding the axons towards the midline. Once the pioneering neurons have extended to the midline, the newer population of commissural axons may simply follow the trajectory set out earlier by the pioneers and fasciculate along their tracts (Colamarino and Tessier-Lavigne 1995b). Consequently, we can imagine that as long as a small number of pioneering neurons project to the midline normally, the later developing axons can travel relatively easily along the path laid out by the pioneers, regardless of the amount of netrin-1 that is expressed at the floor plate. This would account for why there is a significant difference in the thickness of the ventral commissure between wild-type, heterozygous and homozygous mice early in development, but not as the animal progresses through development.

Alternatively, we cannot dismiss the contribution of other molecules that are implicated in the guidance of spinal commissural axons. The morphogen Shh and the angiogenic factor vascular endothelial growth factor (VEGF) are two additional midline-derived chemoattractants that direct commissural extension (Charron et al. 2003; Ruiz de Almodovar et al. 2011). The relative contributions of this ensemble of factors to appropriate commissural pathfinding *in vivo* remains to be determined; however the substantial deficiency detected in the absence of netrin-1 is consistent with an essential contribution of the netrin-1 gradient to commissural axon guidance to the floor plate.

DISCUSSION

CHAPTER 6

General Discussion and Conclusion

PREFACE

This final chapter reviews the signalling events activated downstream of the receptor DCC in netrin-1-dependent commissural axon extension, and discusses how the results obtained in chapters 3, 4 and 5 of this thesis enhance our understanding of the mechanisms underlying netrin-1 mediated chemoattraction and its contribution to axon guidance in the developing nervous system.

DCC SIGNALLING IN NETRIN-1 MEDIATED CHEMOATTRACTION

Netrin-1 is a secreted glycoprotein involved in cell migration and adhesion, axon extension, cell survival, and synapse formation (Lai Wing Sun et al. 2011). Its best characterized function however still remains its role as an axon guidance molecule, particularly as a chemoattractant for embryonic spinal commissural neurons in the developing spinal cord (Kennedy et al. 1994; Serafini et al. 1994; Serafini et al. 1996). In commissural growth cones, netrin-1 induces actin cytoskeleton remodeling that results in the formation of lamellipodia and filopodia, and eventually to axon outgrowth and attractive growth cone turning. However, the precise signalling mechanisms downstream of DCC that regulate cytoskeletal dynamics during netrin-1 chemoattraction are not fully understood. Studies have demonstrated that the binding of netrin-1 to DCC triggers the receptor to dimerize, which is thought to lead to the recruitment and activation of multiple intracellular signalling molecules into a complex with the cytoplasmic domain of DCC. These include Src family kinases (SFKs), members of the Rho GTPase family, the serine/threonine kinase PAK1, and actin-binding proteins (Li et al. 2004; Liu et al. 2004a; Shekarabi et al. 2005). More specifically, Fyn has been argued to be the critical SFK member recruited into this signalling complex with DCC and demonstrated to activate focal adhesion kinase (FAK), a tyrosine kinase that is constitutively bound to the intracellular domain of DCC and required to mediate netrin-1 induced axon outgrowth (Li et al. 2004; Liu et al. 2004a; Ren et al. 2004). Additionally, Fyn is required for the tyrosine phosphorylation of DCC, an event critical for netrin signalling (Li et al. 2004; Meriane et al. 2004; Ren et al. 2008). Contrary to these findings, our results, presented in chapter 3, indicate that Fyn is not required for commissural axon extension to the ventral midline in the embryonic spinal cord, a process strongly dependent on netrin-1 (Serafini et al. 1996; Bin et al. in press). *Fyn* null mice exhibited normal spinal commissural tracts and a properly formed ventral spinal commissure, whereas *netrin-1* and *DCC* deficient animals display misguided commissural projections and severely disrupted ventral commissures (Serafini et al. 1996; Fazeli et al. 1997) (Fig. 3.1). Furthermore, *fyn* knockout mice are viable, while *netrin-1* and *DCC* deficient mice die shortly after birth (Stein et al. 1992; Serafini et al. 1996; Fazeli et al. 1997). This data demonstrate that Fyn is not critical for commissural axon guidance in the spinal cord, as concluded by previous studies. SFK

members have similar and overlapping functions in various processes, raising the possibility that they might functionally compensate for one another (Kypta et al. 1990; Burkhardt et al. 1991; Courtneidge et al. 1993). To address this, we investigated if other SFK members were able to associate with DCC in spinal commissural neurons. Co-immunoprecipitation assays demonstrated that DCC interacts with Fyn and Src in these cells (Fig. 3.3), suggesting a possible role for Src in commissural axon extension. Interestingly, *Src* knockout mice also displayed normal commissural axon trajectories and a robust ventral spinal commissure, consistent with normal chemoattraction to netrin-1 (Fig. 3.4). Taken together, the results obtained in chapter 3 support the conclusion that, individually, Fyn and Src are not essential for normal commissural axon guidance in the developing spinal cord. Whether they function together, or with another SFK member, in mediating netrin-1-dependent extension to the ventral midline still needs to be determined.

Even though our findings question the significance of SFK function in netrin-1 chemoattraction, activation of the Rho GTPases Cdc42 and Rac1 following netrin-1 stimulation is well-established (Shekarabi and Kennedy 2002; Shekarabi et al. 2005). Rho GTPases are important regulators of actin dynamics, with Rho mediating the formation of stress fibers, and Cdc42 and Rac1 inducing the formation of filopodia and lamellipodia, respectively (Hall and Lalli 2010). Cdc42 and Rac1, which are active when bound to GTP and inactive when bound to GDP, are required for netrin-1 induced outgrowth and turning of commissural axons (Shekarabi et al. 2005). Moreover, dominant-negative Cdc42 was shown to block netrin-1 induced activation of Rac1, whereas a dominant-negative form of Rac1 had no effect on Cdc42 activation following netrin-1 stimulation (Shekarabi et al. 2005). This suggests that netrin-1 initially triggers the activation of Cdc42, which is then necessary to activate Rac1. For their activation, Rho GTPases require the function of guanine nucleotide exchange factors (GEFs), which catalyze the exchange of the GDP for a GTP. To date, two Rac-specific GEFs have been identified to act downstream of DCC: Trio and DOCK180 (Briancon-Marjollet et al. 2008; Li et al. 2008). However, analysis of knockout mice lacking Trio or DOCK180 has failed to phenocopy the deficits found in netrin-1 and DCC nulls. Previous work demonstrated that the spinal ventral commissure, corpus callosum and hippocampal commissure are all present in Trio nulls, but are

absent in netrin-1 and DCC knockout mice (Serafini et al. 1996; Fazeli et al. 1997; Briancon-Marjollet et al. 2008). As for DOCK180, *in ovo* electroporation of cDNAs encoding dominant negative mutants or of siRNAs into the embryonic chick spinal cord resulted in defective commissural axon projections (Li et al. 2008). However, no abnormal neural phenotype has been reported in DOCK180 null mice (Laurin et al. 2008). These findings suggest that, while Trio and DOCK180 may contribute to DCC signaling, they are not essential for axon chemoattraction to netrin-1 and may have redundant functions. Furthermore, although DOCK180 and Trio are GEFs for Rac, DOCK180 does not exhibit GEF activity for Cdc42 and it is unclear if Trio exhibits GEF activity for Cdc42 (Bellanger et al. 1998; Brugnera et al. 2002; Cote and Vuori 2002; Estrach et al. 2002). In chapter 4, we identify βPix, a PAK-interacting GEF capable of activating Cdc42 and Rac1, as playing a significant role in filopodia formation and growth cone expansion induced by netrin-1 (Fig. 4.3, Fig. 4.5). Furthermore, our evidence suggests that it does so by forming a multimeric complex with the ARF-GTPase activating protein (ARF-GAP) and scaffolding protein GIT2 (Fig. 4.2). Interestingly, oligomerization of βPix is required for its GEF activity, with previous studies suggesting that dimerization of Pix specifically activates Rac while the interaction of PAK with monomeric Pix exhibits GEF activity for Cdc42 (Kim et al. 2001; Zhu et al. 2001; Feng et al. 2004). We also implicate βPix function in the extension of spinal commissural neurons to the ventral midline in chick embryos (Fig. 4.4). Overall, the evidence presented in this chapter supports a role for βPix, in association with GIT2, in the activation of Cdc42 and Rac1 in netrin-1 induced chemoattraction.

In spinal commissural neurons, the activation of Cdc42 and Rac1 by netrin-1 is accompanied by the downregulation of RhoA activity, also induced by netrin-1 through DCC (Moore et al. 2008). Members of the Rho subfamily have been implicated in signalling downstream of chemorepellent cues and in growth cone collapse (Hu 1999; Wahl et al. 2000; Driessens et al. 2001). Inhibiting RhoA activity resulted in the enhanced chemoattractive turning responses of growth cones to netrin-1 by increasing the levels of DCC inserted into the plasma membrane (Moore et al. 2008). Once activated by netrin-1, Cdc42 and Rac1 subsequently activate downstream effectors such as the serine/threonine kinase p21-activated kinase 1 (PAK1) and the actin-binding proteins Enabled/vasodilator-stimulated phosphoprotein

(Ena/VASP) and neuronal Wiskott-Aldrich syndrome protein (N-WASP), which modulate actin polymerization (Lebrand et al. 2004; Shekarabi et al. 2005). In addition to being an important regulator of cytoskeletal dynamics, PAK1 can also interact with the GEF βPix (Manser et al.

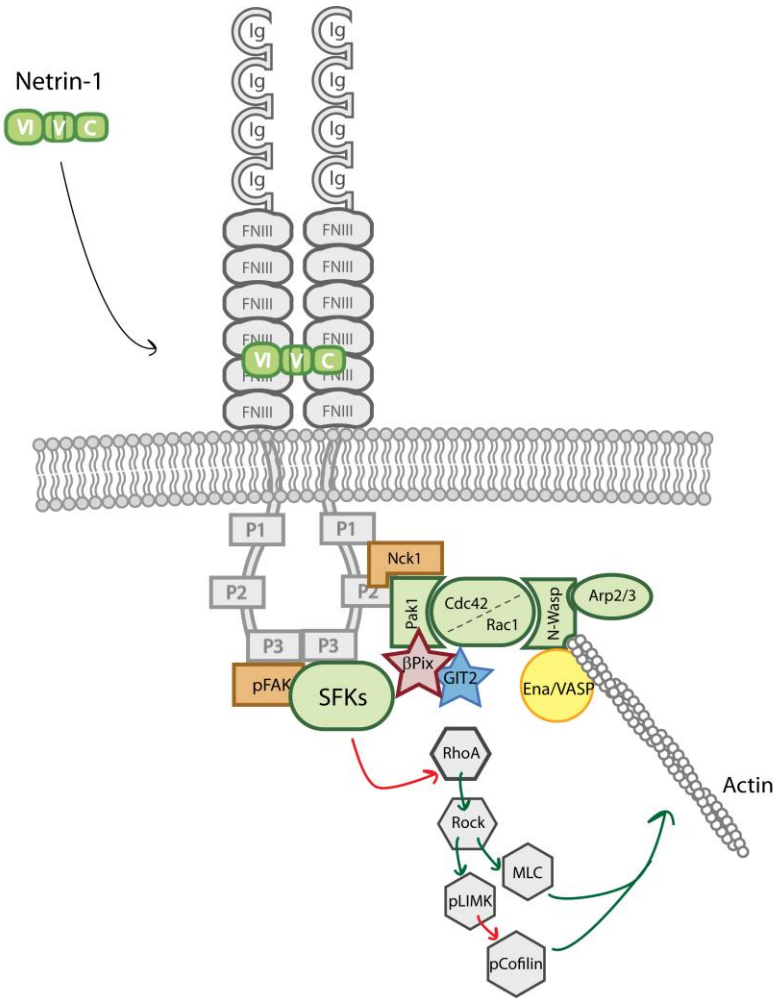

Figure 6.1 - Netrin-1 chemoattraction signalling model. Netrin-1 binding to DCC triggers the receptor to dimerize and activates FAK and Nck1. This leads to the recruitment of SFKs and the phosphorylation of the cytoplasmic domain of DCC. More signalling molecules are then activated and recruited into the complex, including βPix/GIT2, which can associate with PAK1 and activate Cdc42 and Rac1. Actin binding proteins, such as N-WASP and the Arp2/3 complex, are subsequently activated, leading to actin cytoskeleton remodelling. Furthermore, LIM kinase is also activated and inhibits the actin severing functions of cofilin.

1998; Bokoch 2003). PAKs phosphorylate and activate Pix proteins, which in turn activate Cdc42 and Rac1 (Manser et al. 1998; Frank and Hansen 2008). Furthermore, previous work suggests that GTP-bound Rac1/Cdc42 induces a conformational change in PAK to expose its Pix binding region and allow it to associate with Pix/GIT oligomers, which leads to the autophosphorylation and activation of PAK (Brown et al. 2002; Loo et al. 2004). The possible interactions between Cdc42/Rac1, PAK1, βPix and GIT2 suggest that a positive feedback loop may be triggered to stimulate their activities and induce actin remodelling downstream of DCC. This type of signalling complex involving GTPases, PAK, Pix and GIT molecules occurs in other processes, such as cell spreading, cell motility, dendritic spine motility and synapse formation (Manabe et al. 2002; Zhang et al. 2005; Jones and Katan 2007; Wilson et al. 2014).

In summary, we present a model (Fig. 6.1) where netrin-1 activates the constitutively bound FAK, which may recruit one or several SFK proteins, and induces the phosphorylation of multiple tyrosine residues in the intracellular domain of DCC. This tyrosine phosphorylation of DCC provides novel binding sites for other signalling molecules to associate with the signalling complex, including the Cdc42/Rac1/PAK1/βPix/GIT2 complex. Along with Ena/VASP and N-WASP, PAK1 can induce cytoskeletal changes through its activation of LIM kinase and subsequent downregulation of the actin severing protein cofilin (Edwards et al. 1999). This actin remodelling of the growth cone is pivotal to promote directional axon extension.

FUNCTIONAL RELEVANCE OF THE NETRIN-1 GRADIENT

Netrin-1 is a secreted molecule and can diffuse through the embryonic neural epithelium to function as a long-range cue. Substantial evidence also indicates that it can also interact with extracellular matrix (ECM) components and mediate short-range adhesion (Kennedy et al. 1994; Serafini et al. 1994; Deiner et al. 1997; Yebra et al. 2003; Brankatschk and Dickson 2006). For spinal commissural neurons, immobilized netrin-1 was shown to mediate adhesion through DCC, generating forces necessary for commissural growth cones to extend and turn (Shekarabi et al. 2005; Moore et al. 2008; Moore et al. 2009). These studies support

the notion that a gradient of bound netrin-1 is formed in the embryonic spinal cord by the diffusion of netrin-1 from floor plate cells and subsequent association of the protein with cell membranes or ECM in the neural epithelium, and that this gradient is required to attract spinal commissural axons to the midline. The possibility that a gradient of netrin-1 would guide commissural axon in the embryonic spinal cord was first proposed shortly after the discovery of netrins, when a graded distribution of netrin mRNA was observed in the embryonic chick spinal cord (Kennedy et al. 1994; Serafini et al. 1996). Surprisingly, it took several years to directly visualize the distribution of netrin-1 protein in the chick and rodent spinal cords, ultimately revealing a graded distribution (Kennedy et al. 2006). Although netrin-1 has been shown to be essential for the majority of commissural axons to reach the ventral midline, the functional significance of the gradient *in vivo* has not been addressed. In chapter 5, we investigated the functional relevance of graded expression of the netrin-1 gene to spinal commissural axon guidance. We determined that genetically altering the amount of netrin-1 produced in the spinal cord modulates the formation of the ventral commissure. These findings support the conclusion that a graded distribution of netrin-1 is pertinent in directing the migration of spinal commissural axons to the midline, at least in early embryogenesis. Subsequent experiments will attempt to map the distribution of the gradients of netrin-1 at the different gene dosages and determine if genetically manipulating the distribution alters the trajectories taken by commissural axons.

CONCLUDING REMARKS

Netrin-1 is a key molecule directing neural development and adult neural function. Although the role of netrin-1 as a chemoattractant during development has been well-established, many of the specific signal transduction pathways and mechanisms of action underlying netrin function are still poorly understood. The findings in this thesis provide new insight into netrin-1 signalling by identifying new molecules involved in netrin-1 chemoattraction and re-evaluating the function of others. Additionally, our analysis of the consequences of genetically manipulating the level of netrin-1 expression in the embryonic

spinal cord provides insight into the function of a netrin-1 gradient to axon pathfinding. By advancing our knowledge of netrin function and axon guidance, these studies contribute to the broader understanding of the complexity of the nervous system and identify possible therapeutic strategies to promote axonal growth and regeneration following CNS injury or neurodegenerative diseases.

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