Signaling mechanisms underlying axon guidance downstream of the netrin-1 receptor DCC

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"…it is worth viewing basic science as a long-term investment that will yield completely unexpected dividends for humanity in the future. I believe that this progress underscores the importance of giving free rein to human inventiveness."

- Tony Pawson, 2008

DEDICATION

I dedicate this thesis to the memory of

Dr. Anthony “Tony” Pawson (1952-2013)

and

Dr. Alan Hall (1952-2015)

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My friends and extended family have been an enthusiastic audience and amazing cheerleaders over the years. I thank them for their continued interest in my

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LIST OF ABBREVIATIONS

BMP	bone morphogenetic factor
Boc	biregional cell adhesion molecule-related/down-regulated by oncogenes (Cdon) binding protein
C domain	central domain
Ca^{2+}	calcium
CAM	cell adhesion molecule
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
CNS	central nervous system
CRIB	Cdc42/Rac1 interactive binding
C-terminus	carboxy terminus
DCC	deleted in colorectal cancer
DLC1	deleted in liver cancer 1
DIV	days of culture *in vitro*
DRG	dorsal root ganglia
ECM	extracellular matrix
EGF	epidermal growth factor
Ena/VASP	enabled/ vasodilator-stimulated phosphoprotein
ER	endoplasmic reticulum
ERM	ezrin-radixin-moesin
ERMAD	ezrin-radixin-moesin association domain
ERK	extracellular signal-regulated kinase
F-actin	filamentous actin
FN	fibronectin
FRET	fluorescence resonance energy transfer
GAP	GTPase-activating protein
GDI	guanine nucleotide dissociation inhibitors
GEF	guanine exchange factor

GFP	green fluorescent protein
GPI	glycophosphatidylinositol
GST	glutathione S-transferase
Hh	hedgehog
ICD	intracellular domain
Ig	immunoglobulin
MAP	microtubule-associated proteins
mRNA	messenger RNA
NGF	nerve growth factor
Nrp	neuropilin
N-terminus	amino terminus
NTR	netrin-like module
N-WASP	neural Wiskott-Aldrich syndrome protein
P domain	peripheral domain
PAK	p21 activated protein
PC	point contact
PDGF	platelet-derived growth factor
pERM	phosphorylated ERM proteins
PIP_2	phosphatidylinositol 4,5-bisphosphate
PIP_3	phosphatidylinositol 3,4,5-triphosphate
PKC	protein kinase C
PITPα	phosphatidylinositol transfer protein-α
PNS	peripheral nervous system
RBD	Rho binding domain
RPTP	receptor protein tyrosine phosphatase
RGC	retinal ganglion cell
Rho	Ras homologous
ROCK	Rho kinase
ROI	region of interest
R-Ras	Related to Ras

RTK	Receptor tyrosine kinase
SEM	standard error of the mean
Sema3A	semaphorin 3A
SFK	Src family kinase
SH2	Src homology 2
SH3	Src homology 3
Shh	sonic hedgehog
Smo	smoothened
Sos	son of sevenless
TRITC	tetramethyl rhodamine isothiocyanate
T zone	transitional zone
UNC	uncoordinated
VEGF	vascular endothelial growth factor
WASP	Wiskott-Aldrich syndrome protein
WAVE	WASP-family verprolin-homologous protein

ABSTRACT

In order to reach the exceptional level of interconnectivity observed in the nervous system, newborn neurons must accomplish the feat of establishing appropriate synaptic connections as they navigate through the plethora of directional cues expressed in the extracellular environment. The distal tip of the axon, called the growth cone, expresses cell surface receptors that sense these extracellular cues and trigger intracellular signaling cascades that direct the growth of the axon. This thesis examines the molecular mechanisms that mediate attractive axon guidance downstream of the chemotropic cue netrin-1 and its receptor deleted in colorectal cancer (DCC).

DCC becomes phosphorylated following the binding of netrin-1 to its extracellular domain. Notably, the phosphorylation of the conserved tyrosine residue 1418 (Y1418) is essential for netrin-1 signal transduction in the vertebrate central nervous system. Here, the regulatory function of the Y1418 residue is explored in the context of netrin-1 signal transduction via the characterization of two novel protein interactions with DCC.

This thesis demonstrates that upon the binding of netrin-1, Y1418 phosphorylation mediates the recruitment to DCC of ezrin, a member of the actin-binding ezrin-radixin-moesin (ERM) protein family, and of p120RasGAP, a GTPase-activating protein (GAP) for proteins of the Ras subfamily of small GTPases. Both ezrin and p120RasGAP are shown to be required for netrin-1-dependent functions such as axon outgrowth and growth cone attraction. Furthermore, the discovery that the activity

of RhoA, a Rho subfamily GTPase, is required downstream of netrin-1 for the phosphorylation of the ERM proteins instigates the assessment of the spatiotemporal regulation of RhoA in response to netrin-1, which results in the detection of previously unreported netrin-1-induced RhoA activity. Taken together, the novel findings presented in this thesis improve our understanding of the signaling cascades that regulate netrin-1/DCC-mediated attractive axon guidance during the development of the nervous system.

RÉSUMÉ

L'interconnectivité exceptionnelle des réseaux neuronaux est acquise grâce aux neurones en développement qui doivent relever le défi d'établir des connexions synaptiques adéquates tout en naviguant dans un environnement extracellulaire dans lequel est exprimée une grande variété de molécules-guides. La région distale de l'axone, appelée le cône de croissance, exprime à sa surface des récepteurs qui engendrent des cascades de signalisation intracellulaire qui guide la croissance de l'axone. Cette thèse aborde les mécanismes moléculaires impliqués dans le guidage axonal en aval du facteur chémo-attractif nétrine-1 et de son récepteur « deleted in colorectal cancer » (DCC).

DCC est phosphorylé suite à la liaison de la nétrine-1 à son domaine extracellulaire. La phosphorylation de la tyrosine 1418 (Y1418) est notamment essentielle à la transduction de signaux en aval de nétrine-1 dans le système nerveux central des vertébrés. La caractérisation de deux nouvelles interactions avec DCC permet ici d'évaluer la fonction de la Y1418 dans la régulation de la signalisation intracellulaire induite par nétrine-1.

Cette thèse démontre que la phosphorylation de la Y1418 stimule l'interaction de DCC avec la protéine ezrine, membre de la famille de protéines ezrine-radixine-moesine (ERM) liant l'actine, et p120RasGAP, une « GTPase-activating protein » (GAP) inhibitrice des petites GTPases de la sous-famille Ras. Ezrine et p120RasGAP

sont requises pour la régulation des fonctions dépendantes de la nétrine-1 telles que la croissance axonale et l'attraction du cône de croissance. De plus, la découverte du rôle de la petite GTPase RhoA de la sous-famille Rho dans l'induction de la phosphorylation des protéines ERM a mené à l'évaluation de la régulation spatio-temporelle de RhoA en réponse à la nétrine-1 et à la détection inédite de l'activation de RhoA par nétrine-1. Finalement, les nouveaux mécanismes intracellulaires présentés dans cette thèse conduisent à une meilleure compréhension des cascades de signalisation qui régulent le guidage axonale médié par la nétrine-1 et son récepteur DCC pendant le développement du système nerveux central.

ORIGINAL SCHOLARSHIP AND CONTRIBUTIONS TO KNOWLEDGE

Original contributions (presented as Chapters 2, 3, and 4, respectively):

1. "The activation of ezrin-radixin-moesin proteins is regulated by netrin-1 through Src kinase and RhoA/Rho kinase activities and mediates netrin-1-induced axon outgrowth", Antoine-Bertrand, J., Ghogha, A., Luangrath, V., Bedford, F.K., and Lamarche-Vane, N., Molecular Biology of the Cell (2011) 22: 3734-3746

This study provides the first description of the regulation and function of ERM proteins during netrin-1/DCC signaling and demonstrates that ezrin, an ERM protein, is required for netrin-1-dependent axon outgrowth.

2. "Direct measurement of oscillatory RhoA activity in embryonic cortical neurons stimulated with the axon guidance cue netrin-1 using FRET", Antoine-Bertrand, J., Fu M., and Lamarche-Vane, N. *Edited and electronically published in Biology of the Cell on January 20, 2016 (doi: 10.1111/boc.201500077)*.

This study offers the first characterization of the spatiotemporal regulation of Rho GTPase activity downstream of netrin-1 and demonstrates that netrin-1 induces short-

term localized activation of RhoA in neuronal growth cones. This challenges the current notion that netrin-1 induces global inhibition of RhoA activity in neurons.

3. "p120RasGAP Contributes as a Scaffolding Protein to Regulate Netrin-1-Mediated Axon Outgrowth and Attraction in a GAP-dependent Manner", Antoine-Bertrand, J, Duquette P.M., Alchini R., Li X., Kennedy T.E., Fournier A.E., and Lamarche-Vane, N. *Edited and electronically published in the Journal of Biological Chemistry on December 28, 2015 (pii: jbc.M115.674846)*.

This study is the first report on the regulation and function of p120RasGAP during netrin-1/DCC signaling and demonstrates that p120RasGAP is required for netrin-1/DCC-dependent axon outgrowth and chemoattraction. It also provides the first direct evidence that Ras is activated downstream of netrin-1.

Published manuscripts not presented in this thesis:

4. "Spatial and temporal activation of the small GTPases RhoA and Rac1 by the netrin-1 receptor UNC5a during neurite outgrowth", Picard M., Petrie R.J., Antoine-Bertrand J., Saint-Cyr-Proulx E., Villemure J.F., Lamarche-Vane N. Cell Signalling (2009) 21(12):1961-73.

5. “Implication of rho GTPases in neurodegenerative diseases”, Antoine-Bertrand J., Villemure J.F., Lamarche-Vane N. Current Drug Targets (2011) 12(8):1202-15. (peer-reviewed review article)

CONTRIBUTION OF AUTHORS

CHAPTER 2: The activation of Ezrin-Radixin-Moesin (ERM) proteins is regulated by netrin-1 through Src kinase and RhoA/Rho kinase activities and mediates netrin-1-induced axon outgrowth

Judith Antoine-Bertrand: Designed, executed and analyzed all experiments. Assembled all the figures and co-wrote the manuscript.

Atefeh Ghogha: Designed, executed and analyzed the mass spectrometry experiment represented in Figure 2.1.

Vilayphone Luangrath: Generated the DCC (1-1120) construct in Figure 2.2.

Fiona K. Bedford: Contributed to the design of the cortical neuron experiments.

Nathalie Lamarche-Vane: Designed and analyzed all experiments. Co-wrote the manuscript.

CHAPTER 3: Direct measurement of oscillatory RhoA activity in embryonic cortical neurons stimulated with the axon guidance cue netrin-1 using FRET

Judith Antoine-Bertrand: Designed, executed and analyzed all experiments. Assembled all the figures and co-wrote the manuscript.

Min Fu: Designed and executed the live FRET imaging.

Nathalie Lamarche-Vane: Designed and analyzed all experiments. Co-wrote the manuscript.

CHAPTER 4: p120RasGAP Contributes as a Scaffolding Protein to Regulate Netrin-1-Mediated Axon Outgrowth and Attraction in a GAP-dependent Manner

Judith Antoine-Bertrand: Designed, executed and analyzed all experiments. Assembled all the figures and co-wrote the manuscript.

Philippe M. Duquette: Contributed to the execution of experiments for Figure 4.6 F and G and Figure 4.13.

Ricardo Alchini: Contributed to the design, execution and analysis of the Dunn chamber assay in Figure 4.8.

Xiaodong Li: Generated the DCC-PA construct in Figure 4.3.

Timothy E. Kennedy: Provided the recombinant netrin-1 VI-V in Figure 4.8.

Alyson E. Fournier: Contributed to the design and analysis of the Dunn chamber assay in Figure 4.8.

Nathalie Lamarche-Vane: Designed and analyzed all experiments. Co-wrote the manuscript.

Preface to Chapter 1

This chapter is a literature review of the signal transduction mechanisms that regulate axon guidance and outgrowth during the development of the central nervous system. The functions of the growth cone cytoskeleton and the regulation of growth cone dynamics by Rho GTPases and ERM proteins are described. The structure and function of axon guidance cues and their receptors are reviewed with an emphasis on signal transduction downstream of the axon guidance cue netrin-1 and its receptor Deleted in Colorectal Cancer (DCC). This first chapter aims to provide a summary of the current knowledge relevant to the original research articles presented in the following chapters of this thesis.

Chapter 1 – Introduction and Literature Review

1.1 Historical perspective

The establishment of proper synaptic connections is essential for the assembly of functional neuronal networks in invertebrates and vertebrates. At the turn of the 20th century, Santiago Ramón y Cajal marvelled at the complexity of the nervous system. His observations led to the postulation that axons innervate distant tissues by sensing molecular cues that are secreted by intermediate and final cellular targets (Ramón y Cajal, 1892). Ramón y Cajal's chemotaxis theory was challenged by his contemporaries Ross Granville Harrison and Paul Weiss who favoured a stereotropic guidance model in which the extracellular matrix (ECM) guides axons towards their targets by providing a "mechanical support for growing nerves" (Harrison, 1908; Harrison, 1910; Weiss, 1934). Weiss proposed that neuronal function is determined only after the establishment of a synaptic connection with a target tissue (Weiss, 1926; Weiss, 1941). For years, Weiss's "resonance principle" was the preferred model to explain axon guidance until Roger Sperry reinstated Ramón y Cajal's initial theory of chemotropism. Sperry demonstrated in fish and amphibians that severed retinal axons can regenerate their original connections, and suggested that stereotyped neuronal projections are ordered by the chemoaffinity of neurons for specific morphogenic gradients expressed by target cells (Sperry, 1963). Twenty years later, direct experimental evidence supporting

chemotropic axon guidance in the developing vertebrate peripheral and central nervous systems (PNS and CNS) was obtained in the laboratories of Alun Davies and Thomas Jessell, respectively (Lumsden and Davies, 1983; Tessier-Lavigne et al., 1988). Seminal work by Jessell and colleagues demonstrates that the commissural axons of sensory interneurons are attracted by a substance secreted by the neural tube in the embryonic rat spinal cord (Tessier-Lavigne et al., 1988). Netrin family proteins were later identified as the attractive factors (Kennedy et al., 1994). This discovery launched the “molecular age” of axon guidance research, which shifted the focus towards the identification and characterization of molecules implicated in neuronal signal transduction. To this day, the molecular mechanisms that regulate axon outgrowth and guidance continue to be decrypted and characterized.

1.2 Structure and function of the growth cone

Ramón y Cajal described the tip of an axon as a “conical protuberance” (Ramón y Cajal, 1890). This was the first published description of the growth cone, which he called “cône de croissance”. Remarkably, Ramón y Cajal deduced from the study of fixed tissues that the growth cone is a highly motile chemotactic structure. He described “a sort of club or ram, endowed with an exquisite chemical sensitivity, rapid amoeboid movements, and certain propelling force” (Ramón y Cajal et al., 1999). More than a century later, the molecular mechanisms that regulate the growth cone are still only partially understood and require further investigation.

1.2.1 The growth cone cytoskeleton

1.2.1.1 Actin filaments and microtubules in neurons

As denoted by Ramón y Cajal, growth cone morphology and motility are significant features of axon pathfinding. Although the tips of developing dendrites are considered to be growth cones, the biology of axonal growth cones has been more extensively characterized. The actin and microtubule cytoskeletons constitute the structural network that support morphological changes as the growth cone responds to environmental cues. The ends of filamentous (F)-actin and microtubules are functionally distinct due to head-to-tail subunit assembly that polarizes the polymers. Globular (G)-actin is added to the barbed ends of double-helical actin filaments and disassembled from the pointed ends (Pollard and Borisy, 2003). Actin filaments typically polymerize at the leading edge of motile cells with their barbed ends oriented outwards, and distal to the cell body in axons and dendrites (Chan et al., 2000; Small et al., 1978; Smith, 1988; Symons and Mitchison, 1991). Neuronal microtubules are hollow polymers composed of 13 protofilaments of α- and β-tubulin heterodimers (Gordon-Weeks, 2004; Howard and Hyman, 2003). Tubulin assembles at the plus end of microtubules and would dissociate from the minus end if it were not for microtubule-associated proteins (MAPs), which cap or stabilize microtubules in animal cells (de Forges et al., 2012; Jiang and Akhmanova, 2011; Stiess and Bradke, 2011). Microtubule plus ends grow distally in axons, but their

orientation is mixed in dendrites (Baas et al., 1988; Stepanova et al., 2003). Overall, the stereotyped distribution of actin filaments and microtubules in axons and dendrites establishes and maintains neuronal polarity (Neukirchen and Bradke, 2011; Stiess and Bradke, 2011).

1.2.1.2 Cytoskeletal organization of the growth cone

The axonal growth cone can be subdivided into two regions: the peripheral (P) and the central (C) domains (Geraldo and Gordon-Weeks, 2009; Lowery and Van Vactor, 2009; Vitriol and Zheng, 2012) (Figure 1.1A). The distal end of the axon expands into the thick C domain, which contains a dense array of bundled microtubules that transport organelles and vesicles. Some of these microtubules extend distally into a thin and motile area, the actin-rich P domain where the “leading edge” is punctuated by membrane protrusions, the filopodia and lamellipodia (Dent et al., 2011; Lewis and Bridgman, 1992). Filopodia are thin finger-like protrusions supported by F-actin bundles, whereas a meshwork of branched actin filaments constructs the sheet-like lamellipodia (Blanchoin et al., 2014). Between the P and C domains lies the transitional (T) zone (Figure 1.1A), the point of entry of exploratory microtubules into the P domain, which is characterized by the presence of contractile structures derived from the association of F-actin with the motor protein myosin II (Geraldo and Gordon-Weeks, 2009; Lowery and Van Vactor, 2009; Vitriol and Zheng, 2012).

The growth cone cytoskeleton is significantly reorganized during axon outgrowth. As it advances forward, the growth cone cycles between three stages: protrusion, engorgement and consolidation (Dent and Gertler, 2003; Goldberg and Burmeister, 1986; Lowery and Van Vactor, 2009). These stages, which were originally described by Goldberg and Burmeister, were in large part characterized by Paul Forscher, Daniel Suter and colleagues, who advantageously exploited the large size of the bag-cell growth cones of the *Aplysia* sea slug to resolve cytoskeletal dynamics using high resolution imaging (Goldberg and Burmeister, 1986; Suter and Miller, 2011).

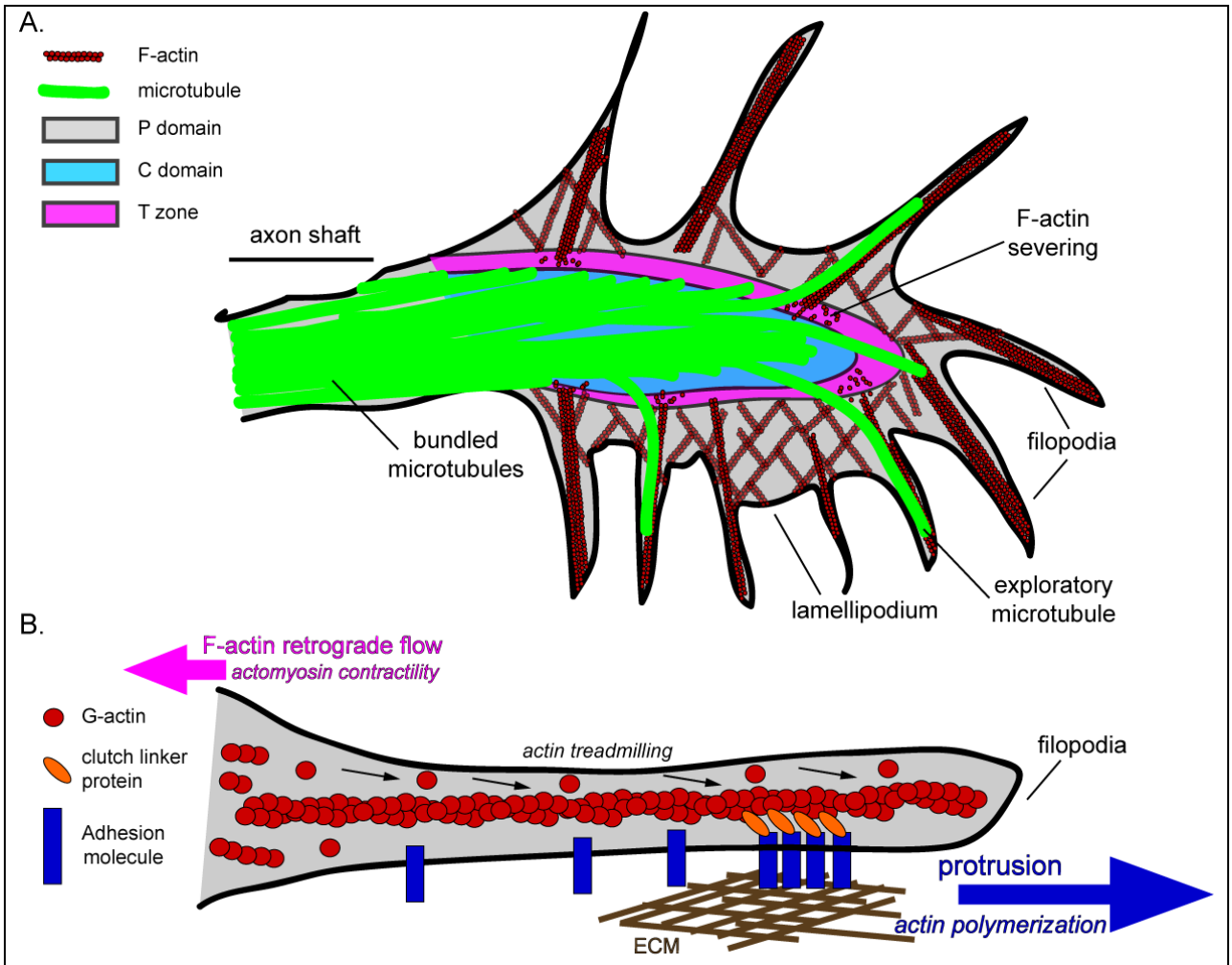

Figure 1.1 Structure of the growth cone cytoskeleton and molecular clutch

(A) The neuronal growth cone can be subdivided into the P domain, C domain and T zone. The P domain is actin-rich, whereas the C domain consists of bundled microtubules. In the T zone, the association between F-actin and myosin II generates actomyosin contractility. Adapted by permission from Macmillan Publishers Ltd on behalf of Cancer Research UK: Nature Reviews Molecular Cell Biology (Lowery and Van Vactor, 2009), copyright 2009. (B) In filopodia, the molecular clutch regulates growth cone advance. Actin polymerization induces membrane protrusion. Conversely,

actomyosin contractility produces F-actin retrograde flow and actin treadmilling by pulling bundles rearward and promoting F-actin severing, respectively. The molecular clutch couples F-actin with adhesion sites, creating traction that reduces retrograde flow and promotes filopodial protrusion. Adapted from Trends in Cell Biology (Giannone et al., 2009), Copyright 2009, with permission from Elsevier [OR APPLICABLE SOCIETY COPYRIGHT OWNER].

1.2.1.3 Protrusion

The protrusion stage is characterized by the rapid extension and retraction of filopodia and lamellipodia, a phenomenon that is powered by the balance between F-actin polymerization and retrograde flow (Craig et al., 2012; Dent et al., 2011; Lowery and Van Vactor, 2009; Mallavarapu and Mitchison, 1999; Medeiros et al., 2006; Yang et al., 2012). Actin polymerization is the force that pushes the membrane forward during cell migration and growth cone advance, but it also creates F-actin retrograde flow (Figure1.1B), a force that pushes the actin filaments and the associated membrane backwards (Blanchoin et al., 2014; Carlier and Pantaloni, 2007; Craig et al., 2012; Gomez and Letourneau, 2014; Medeiros et al., 2006; Pollard and Borisy, 2003). In the T zone, the motor myosin II contributes to F-actin retrograde flow by contracting and pulling actin filaments towards the C domain (Craig et al., 2012; Lin et al., 1996; Medeiros et al., 2006; Zhang et al., 2003). Actomyosin contractility also increases F-

actin severing behind the P domain, thus promoting actin treadmilling, a phenomenon during which F-actin is disassembled in the T-zone and G-actin is recycled for actin polymerization at the leading edge (Blanchoin et al., 2014; Carlier and Pantaloni, 2007; Craig et al., 2012; Medeiros et al., 2006; Pollard and Borisy, 2003; Van Goor et al., 2012; Yang et al., 2012).

1.2.1.4 Engorgement

The engorgement stage is highlighted by the invasion of microtubules into the P domain and by the emergence of vesicular trafficking (Dent and Gertler, 2003; Goldberg and Burmeister, 1986; Lowery and Van Vactor, 2009). The contraction of F-actin by myosin II in the T zone creates an array of condensed actin structures called actin arcs (Medeiros et al., 2006; Schaefer et al., 2008; Zhang et al., 2003). Actin arcs that run perpendicularly to the bundled microtubules of the C domain prevent microtubule advance into the P domain (Forscher and Smith, 1988; Lee and Suter, 2008; Schaefer et al., 2008) (Figure1.1A). As the growth cone advances, increased cell surface adhesion reduces F-actin retrograde flow and stabilizes membrane protrusions against retraction (Chan and Odde, 2008; Giannone et al., 2009; Lee and Suter, 2008; Letourneau, 1979; Letourneau and Shattuck, 1989; Lowery and Van Vactor, 2009; Schaefer et al., 2008; Shimada et al., 2008; Suter and Forscher, 2000; Toriyama et al., 2013). Bundled microtubules invade the space left behind the advancing P domain via

an actin-free corridor created by the disassembly of actin and the reorientation of actin arcs in the T zone (Schaefer et al., 2008). In parallel, unbundled microtubules that exhibit dynamic instability, which consists of alternating phases of rapid plus-end disassembly and shrinkage termed “catastrophe” and of “rescue” through plus-end assembly, extend into the P domain where they interact with F-actin and adhesion sites (Dent and Kalil, 2001; Lee and Suter, 2008; Schaefer et al., 2002; Schaefer et al., 2008; Suter et al., 1998; Suter et al., 2004; Wu et al., 2008) (Figure1.1A). Interactions with filopodial adhesion sites protect exploratory microtubules from dynamic instability and consequently, the polymers become more stable as cell surface adhesion increases (Gordon-Weeks, 2004; Kirschner and Mitchison, 1986; Lee and Suter, 2008; Schaefer et al., 2008).

1.2.1.5 Consolidation

Consolidation involves the contraction of the proximal part of the growth cone into a cylindrical axon shaft (Dent and Gertler, 2003; Goldberg and Burmeister, 1986; Lowery and Van Vactor, 2009). Microtubules are compressed in the wrist of the growth cone by longitudinal actin arcs and MAPs (Bielas et al., 2007; Burnette et al., 2008; Geraldo and Gordon-Weeks, 2009; Loudon et al., 2006; Lowery and Van Vactor, 2009; Schaefer et al., 2008). In addition, local inhibition of actin polymerization prevents the formation of membrane protrusions and axonal branches in the nascent axon shaft

(Bielas et al., 2007; Loudon et al., 2006; Lowery and Van Vactor, 2009; Mingorance-Le Meur and O'Connor, 2009).

In summary, axon elongation is achieved through the coordination of actin and microtubule dynamics in the growth cone and the iteration of the protrusion, engorgement and consolidation phases. The importance of F-actin-microtubule coordination is most apparent in the engorgement stage during which microtubule invasion and stabilization is regulated by F-actin in the T zone and P domain, respectively (Geraldo and Gordon-Weeks, 2009; Lowery and Van Vactor, 2009; Suter and Miller, 2011).

1.2.2 Chemotactic properties of the axonal growth cone

Chemotaxis is the ability of the cell to sense extracellular molecular gradients and to respond by polarizing and moving towards the source. The ECM proteins and cell adhesion molecules (CAM) influence axon guidance (Kiryushko et al., 2004; Mueller, 1999; Myers et al., 2011b; Tessier-Lavigne and Goodman, 1996). However, it is the graded expression of molecular cues that is truly instructive. Guidance cues released as gradients from a localized source can change the path of axon outgrowth by causing a growth cone to turn (de la Torre et al., 1997; Gundersen and Barrett, 1979, 1980; Song et al., 1998; Zheng et al., 1994). In fact, axon guidance cues expressed as

molecular gradients transmit positional and navigational information rather than simply delivering permissive or inhibitory signals to the growth cone (Song and Poo, 2001; Tessier-Lavigne and Goodman, 1996). Ultimately, the decision to turn towards or away from a given signal is determined by the intrinsic chemotactic properties of the growth cone (Lowery and Van Vactor, 2009; Mueller, 1999; Tojima, 2012).

Interestingly, the signaling pathways that regulate axon guidance are conserved in eukaryotic cells such as neutrophils, fibroblasts and the slime mold Dictyostelium discoideum (Mortimer et al., 2008; Van Haastert and Devreotes, 2004; von Philipsborn and Bastmeyer, 2007). Comparative studies of these cell migration models indicate that effective chemotaxis involves the integration of three distinct phenomena: 1) motility, 2) polarization and 3) directional sensing (Mortimer et al., 2008; Swaney et al., 2010; Van Haastert and Devreotes, 2004).

1.2.2.1 Motility

Motility is an ancient cellular process that is essential for many developmental and physiological functions, including axon guidance. The growth cone is the motile structure that drives chemotaxis during axon guidance. The regulation of the actin and microtubule cytoskeletons provides the structure and force required for growth cone motility and chemotaxis.

1.2.2.1.1 Filopodia

Attractive guidance cues promote the formation of membrane protrusions, causing the growth cone to turn on the side facing the cue, whereas repulsive guidance cues stimulate the disruption of protrusions, causing the growth cone to turn in the opposite direction (Dent et al., 2011; Gomez and Letourneau, 2014; Lowery and Van Vactor, 2009).

Filopodia have been more extensively studied than lamellipodia in the context of growth cone guidance. They are specialized domains that harbor cell surface receptors, which respond to axon guidance cues and ECM substrates (Davenport et al., 1993; Gallo, 2013; Gallo et al., 1997; Kerstein et al., 2013; Onishi et al., 2013; Shekarabi and Kennedy, 2002; Shim et al., 2013; Zheng et al., 1996). These receptors initiate the signaling cascades that mediate filopodial extension and retraction in response to permissive/attractive and inhibitory/repellent cues, respectively (Geraldo and Gordon-Weeks, 2009; Gupton and Gertler, 2007; Lowery and Van Vactor, 2009). Hence, filopodia are considered to be environmental sensors that initiate the regulation of growth cone motility during chemotaxis (Gallo, 2013; Heckman and Plummer, 2013; Mattila and Lappalainen, 2008).

1.2.2.1.2 F-actin and the molecular clutch

In motile cells, F-actin retrograde flow is regarded as a motor that idles when filopodia and lamellipodia are not stabilized by cell surface adhesions (Giannone et al., 2009; Lowery and Van Vactor, 2009; Mitchison and Kirschner, 1988). Mechanical resistance against the pulling force of F-actin retrograde flow is provided by a transient cell adhesion interface that links F-actin to the substratum and is called the molecular clutch (Figure 1.1B) (Chan and Odde, 2008; Giannone et al., 2009; Jay, 2000; Mitchison and Kirschner, 1988; Suter and Forscher, 2000).

When it is engaged, the molecular clutch generates the traction required for growth cone advance (Bridgman et al., 2001; Chan and Odde, 2008; Gomez and Letourneau, 2014; Heidemann et al., 1990; Koch et al., 2012; Oakley and Tosney, 1993). It bridges F-actin and the ECM via adhesion molecules such as integrins, L1 and *Aplysia* apCAM and this molecular linkage is transiently regulated by axon guidance cues during chemotaxis (Bard et al., 2008; Bechara et al., 2008; Carlstrom et al., 2011; Dequidt et al., 2007; Hines et al., 2010; Moore et al., 2012; Myers and Gomez, 2011; Schaefer et al., 2008; Shimada et al., 2008; Suter and Forscher, 2001; Toriyama et al., 2013; Woo and Gomez, 2006; Woo et al., 2009).

1.2.2.1.3 Coordination of microtubules with F-actin dynamics

The contribution of microtubules to growth cone motility was long considered to be structural rather than instructive partly because the dynamics of the tightly bundled

microtubules in the C domain were difficult to assess (Figure 1.1). Nonetheless, earlier studies show that microtubule rearrangements precede and influence growth cone turning, indicating that microtubules are directly involved in the polarization of growth cone motility (Challacombe et al., 1997; Lin and Forscher, 1995; Lin and Forscher, 1993; Rochlin et al., 1999; Sabry et al., 1991; Suter et al., 1998; Tanaka and Kirschner, 1991; Williamson et al., 1996). The presence of a small population of highly dynamic and tyrosinated exploratory microtubules in the P domain also indicates that microtubules play an active role in growth cone motility (Challacombe et al., 1996, 1997; Dent and Kalil, 2001; Mack et al., 2000; Rochlin et al., 1999; Sabry et al., 1991; Schaefer et al., 2002; Tanaka et al., 1995; Williamson et al., 1996). For instance, local alteration of microtubule dynamics in the P domain reveals that the destabilization of microtubules on one side of the growth cone is sufficient to initiate a turning response on the opposite side, and conversely, stabilization of microtubules on one side causes the growth cone to turn on that side (Buck and Zheng, 2002; Mack et al., 2000). Furthermore, F-actin dynamics are required for the induction of microtubule rearrangements during growth cone turning (Buck and Zheng, 2002; Challacombe et al., 1996; Gordon-Weeks, 2004; Lin and Forscher, 1993; Zhou et al., 2002).

Since microtubules have the ability to grow along F-actin bundles, the position of exploratory microtubules in the P domain is determined as much by F-actin retrograde flow than dynamic instability (Lee and Suter, 2008; Schaefer et al., 2002; Schaefer et

al., 2008; Suter et al., 1998; Zhou et al., 2002). As the growth cone turns, exploratory microtubules are “captured” by filopodial adhesion sites located on the turning side and become signaling platforms and/or signal carriers for key axon guidance molecules (Dent and Baas, 2014; Lee and Suter, 2008; Schaefer et al., 2008; Suter et al., 2004). Microtubule capture and stabilization is mediated by microtubule plus end tracking proteins (+TIPs), MAPs that are emerging as key players in the regulation of microtubule dynamics downstream of axon guidance cues (Dent et al., 2011; Geraldo and Gordon-Weeks, 2009; Gordon-Weeks, 2004; Liu and Dwyer, 2014).

In summary, the growth cone cytoskeleton regulates motility in response to axon guidance cues. While F-actin maintains growth cone motility by initiating membrane protrusion and adhesion, microtubules support the stabilization of filopodia and steer growth cone advance.

1.2.2.2 Directional sensing

The extracellular environment of growing axons constantly evolves in time and space. As they journey to reach their synaptic target, axons encounter a series of intermediate targets that express overlapping, and sometimes conflicting, molecular gradients. The integration and interpretation of complex navigational information is mediated by directional sensing, a phenomenon through which the growth cone

generates steep intracellular signaling gradients that spatially bias its response to guidance cue gradients (Mortimer et al., 2008; Swaney et al., 2010; von Philipsborn and Bastmeyer, 2007). Since growth cones encounter a plethora of extracellular cues and have the ability to respond to gradients as shallow as 0.1 % (Rosoff et al., 2004), mechanisms are in place to ensure that they accurately follow the navigational map laid out by axon guidance cues.

1.2.2.2.1 Receptor activation and trafficking

Whether the response to a given axon guidance cue is growth cone collapse, attraction or repulsion, the cue activates specific receptors at the surface of the growth cone and triggers signaling cascades that induce cytoskeletal rearrangements and alter growth cone motility (Bashaw and Klein, 2010; Dent et al., 2011; Huber et al., 2003; Mueller, 1999). Guidance cues that are expressed as molecular gradients induce asymmetric receptor activation on the side of the growth cone facing the gradient (Akiyama and Kamiguchi, 2015; Mortimer et al., 2008; Song and Poo, 2001; Tojima et al., 2011).

Since the binding of axon guidance cues to receptors is a stochastic process, a certain degree of receptor activation occurs across the growth cone, which generates noisy intracellular signals (Yuan et al., 2013). The noise can be reduced by clustering ligand-bound receptors at the plasma membrane in order to amplify the spatial bias

introduced by the graded distribution of axon guidance cues (Akiyama and Kamiguchi, 2015; Mortimer et al., 2008; Song and Poo, 2001; Swaney et al., 2010). The detergent resistant membranes (DRM), called lipid rafts, are involved in the compartmentalization and the polarization of signal transduction (Golub et al., 2004; Guirland and Zheng, 2007; Ibanez, 2004; Kamiguchi, 2006). They segregate axon guidance receptors and regulate signaling cascades in response to axon guidance cues (Bruckner et al., 1999; Guirland et al., 2004; Herincs et al., 2005; Petrie et al., 2009).

Aside from mediating receptor clustering at the cell surface, axon guidance cues can induce signaling feedback loops that regulate receptor endocytosis and exocytosis (Bashaw and Klein, 2010; Itofusa and Kamiguchi, 2011; O'Donnell et al., 2009). Notably, receptor trafficking mediates temporal gradient sensing via adaptation, a process through which sustained exposure to a given axon guidance cue will determine the sensitivity of growth cones to incoming molecular gradients (Ming et al., 2002; Piper et al., 2005). In cultured *Xenopus laevis* spinal and retinal growth cones, adaptation in response to axon guidance cues is characterized by growth cone desensitization mediated by fast receptor endocytosis, followed by slow protein synthesis-dependent resensitization (Ming et al., 2002; Piper et al., 2005). Growth cone adaptation allows neurons to modulate their responses based on their past exposures to axon guidance cues (Kaplan et al., 2014; Ming et al., 2002; Piper et al., 2005; Song and Poo, 2001; von Philipsborn and Bastmeyer, 2007).

1.2.2.2.2 Second messengers

Second messenger signaling is another important aspect of directional sensing. Several axon guidance cues induce an asymmetric increase in intracellular calcium (Ca^{2+}) in the growth cone (Akiyama and Kamiguchi, 2015; Gomez and Zheng, 2006; Henley and Poo, 2004; Tojima et al., 2011). This phenomenon is characterized by a higher concentration of intracellular Ca^{2+} on the side of the growth cone facing the source of the cue (Akiyama and Kamiguchi, 2015; Sutherland et al., 2014; Tojima et al., 2011). Experimental manipulation and quantification of intracellular Ca^{2+}, primarily conducted in *Xenopus*, have led to the conclusion that the amplitude of the intracellular Ca^{2+} gradients determines whether the growth cone turns towards or away from a cue (Gomez and Zheng, 2006; Henley and Poo, 2004; Sutherland et al., 2014; Tojima, 2012; Tojima et al., 2011). Negative cues, such as semaphorin 3A (Sema3A), generate low intracellular Ca^{2+} levels by mediating an influx of extracellular Ca^{2+} through the plasma membrane, whereas attractive cues like netrin-1 generate higher intracellular Ca^{2+} levels by combining the release of endoplasmic reticulum (ER) Ca^{2+} stores to extracellular Ca^{2+} influx (Akiyama and Kamiguchi, 2015; Bashaw and Klein, 2010; Sutherland et al., 2014; Tojima, 2012; Tojima et al., 2011).

Moreover, feedback regulation of intracellular Ca^{2+} refines and maintains the amplitude of Ca^{2+} gradients in response to axon guidance cues (Akiyama and

Kamiguchi, 2015; Tojima et al., 2011). For instance, the release of ER Ca^{2+} stores is regulated by the second messengers cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP). cAMP facilitates Ca^{2+} release from the ER and mediates growth cone attraction, whereas cGMP promotes repulsion by inhibiting Ca^{2+} release (Sutherland et al., 2014; Tojima, 2012; Tojima et al., 2011). Ultimately, the establishment of intracellular Ca^{2+} gradients influences growth cone steering via the asymmetric regulation of cytoskeletal dynamics, endocytosis and cell adhesion (Akiyama and Kamiguchi, 2015; Itofusa and Kamiguchi, 2011).

Of note, resting intracellular Ca^{2+} and cAMP/cGMP concentrations can change the polarity of the response to guidance cues (Bashaw and Klein, 2010; Ming et al., 1997; Nishiyama et al., 2003; Song et al., 1998; Song et al., 1997; Wen et al., 2004; Zheng, 2000). Although this influence may be cell-type specific, *in vitro* studies support the notion that the resting Ca^{2+} concentration of growth cones influences their ability to respond to guidance cues as they reach and move past intermediate targets *in vivo* (Kaplan et al., 2014; Mueller, 1999; Song and Poo, 2001; Tojima, 2012).

In summary, directional sensing enables the growth cone to respond with high fidelity to guidance cue gradients in time and space. Through this process, growth cones establish intracellular signaling gradients to adapt their responsiveness to axon guidance cues as they encounter them on their journey to reach their final target

1.2.2.3 Polarization

Polarization amplifies molecular asymmetry to the macroscopic level by integrating directional sensing and motility (Jilkine and Edelstein-Keshet, 2011). Unlike other chemotactic cells, the neuron is naturally polarized (Mortimer et al., 2008; von Philipsborn and Bastmeyer, 2007). The axonal growth cone is at the leading edge, the "front" of the cell, and moves almost independently from the trailing axon shaft, the "back" of the cell. The organization of the growth cone cytoskeleton into distinct morphological domains further establishes front-back polarity during axon outgrowth (Geraldo and Gordon-Weeks, 2009; Lowery and Van Vactor, 2009; Vitriol and Zheng, 2012) (Figure 1.1). Growth cone polarization can either be constitutive or evoked, and is supported by several cellular processes.

1.2.2.3.1 Constitutive front-back polarity

Neurite outgrowth necessitates the dramatic expansion of the plasma membrane, which is sustained by the long-range transport and delivery in axons and dendrites of lipids and other cargos (Bloom and Morgan, 2011; Pfenninger, 2009; Yap and Winckler, 2012). Consequently, polarized membrane trafficking regulates constitutive and directed axon outgrowth (Bloom and Morgan, 2011; Itofusa and Kamiguchi, 2011; Vitriol and Zheng, 2012). In the absence of external signals, membrane trafficking in the growth

cone assumes front-back asymmetry that involves exocytosis mediated by the vesicle-associated membrane protein VAMP7, a member of the Soluble NSF attachment protein receptor (SNARE) family of proteins (Itofusa and Kamiguchi, 2011; Martinez-Arca et al., 2000; Martinez-Arca et al., 2001; Meldolesi, 2011).

Polarized membrane trafficking is regarded as a “master regulator” of growth cone polarity since it spatially distributes the components of the growth cone machinery (Itofusa and Kamiguchi, 2011; Lasiecka and Winckler, 2011; Tojima et al., 2011; Vitriol and Zheng, 2012). For instance, growth cone motility is supported by the recycling of L1 and β1 integrin, CAMs associated with the molecular clutch, which are translocated to the C domain by F-actin retrograde flow, then removed from the surface by clathrin-mediated endocytosis, and transported back to the P domain where they are reinserted at the membrane by exocytosis (Dequidt et al., 2007; Eva et al., 2010; Itofusa and Kamiguchi, 2011; Kamiguchi and Lemmon, 2000; Kamiguchi et al., 1998; Kamiguchi and Yoshihara, 2001; Peretti et al., 2000; Tojima, 2012). The endosomes in which CAMs are recycled further contribute to front-back polarization by overseeing the anterograde transport of several other proteins that regulate the cytoskeleton and cell adhesion (Itofusa and Kamiguchi, 2011; Mortimer et al., 2008; Tojima et al., 2011).

1.2.2.3.2 Evoked left-right polarity

Conversely, axon guidance cues induce left-right asymmetry for adhesion and membrane trafficking, a phenomenon that involves VAMP2-dependent exocytosis (Itofusa and Kamiguchi, 2011; Myers et al., 2011b; Sutherland et al., 2014; Tojima, 2012; Tojima et al., 2011; Vitriol and Zheng, 2012). It is the graded expression of axon guidance cues that initially establishes left-right polarity by inducing asymmetric receptor activation (Akiyama and Kamiguchi, 2015; Mortimer et al., 2008; Song and Poo, 2001; Tojima et al., 2011). On one hand, repellent cues induce the loss of cell adhesion and endocytosis on the side of the growth cone facing cue (Gatlin et al., 2006; Guo et al., 2012; Hines et al., 2010; Kolpak et al., 2009; Tojima et al., 2010). On the other hand, attractive cues stimulate exocytosis and increase cell adhesion (Akiyama and Kamiguchi, 2010; Carlstrom et al., 2011; Cotrufo et al., 2011; Myers and Gomez, 2011; Tojima et al., 2007). Lipid rafts and receptor trafficking maintain and amplify left-right asymmetry by restricting the spatial distribution of key molecules within the growth cone (Bashaw and Klein, 2010; Guirland and Zheng, 2007; Itofusa and Kamiguchi, 2011; Kamiguchi, 2006; O'Donnell et al., 2009). Additionally, axon guidance cues induce the asymmetric synthesis of cytoskeletal regulators in the growth cone, which sustains left-right polarity (Jung et al., 2012; Shigeoka et al., 2013).

In the past years, Kamiguchi and colleagues have established an integrated model for growth cone chemotaxis (Figure 1.2). They propose that the graded expression of axon guidance cues generates intracellular counter-gradients that oppose

attractive and repulsive signals across the growth cone. For instance, an attractant may initiate attractive Ca^{2+}, cAMP and phosphatidylinositol 3,4,5-triphosphate (PIP_3) gradients on the side of the growth cone facing the cue, and simultaneously generate repulsive Ca^{2+}, cGMP and phosphatidylinositol 4,5-bisphosphate (PIP_2) signals on the opposite side (Akiyama and Kamiguchi, 2015; Itofusa and Kamiguchi, 2011; Tojima, 2012; Tojima et al., 2011). Though it is not clear whether it applies to all neuronal cell types in vertebrates, this prospective model proposes a credible representation of the regulation of growth cone chemotaxis by axon guidance cues.

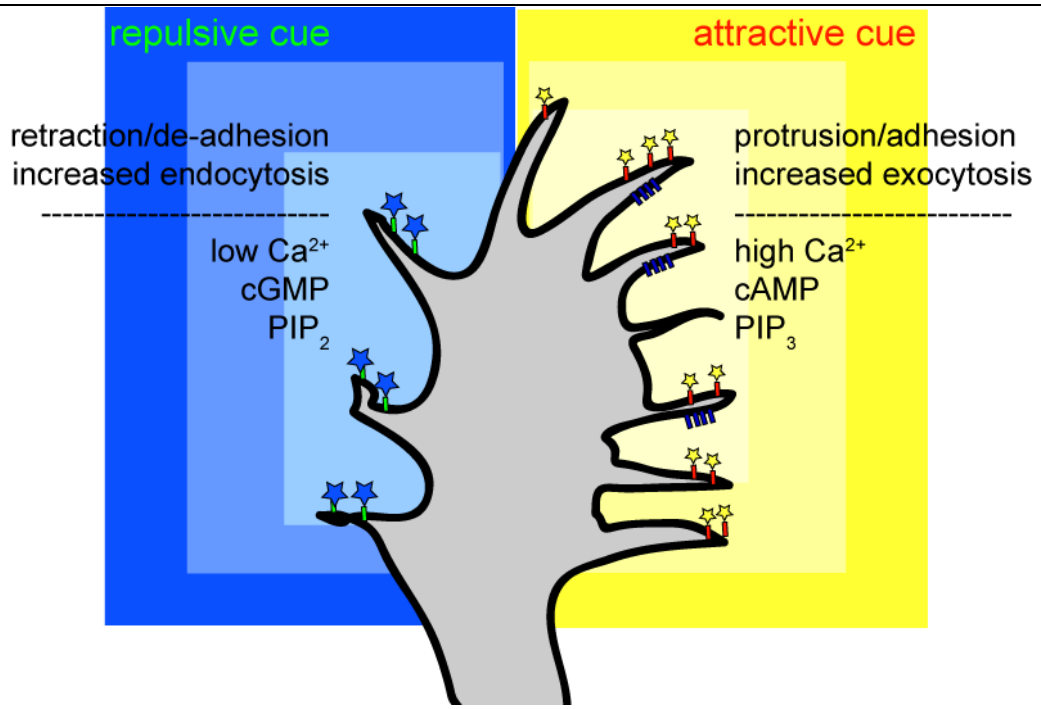

Figure 1.2 Regulation of neuronal growth cone chemotaxis

This model proposes that directional sensing generates second messenger gradients to mediate polarized growth cone motility in response to axon guidance cues (Akiyama and Kamiguchi, 2015).

1.3 Axon guidance signaling in the central nervous system

Axon guidance cues function over short- and long-range distances (Kolodkin and Tessier-Lavigne, 2011; Mueller, 1999; Tessier-Lavigne and Goodman, 1996). In the developing CNS, neurons form commissures that interconnect the left and right sides by projecting axons across the midline, an essential organizing center located in the neural tube (Castellani, 2013). The commissural neurons that extend axons down the dorsoventral axis of the spinal cord and across the ventral midline (Figure 1.3) inspired Ramón y Cajal more than a hundred years ago (Ramón y Cajal, 1890). In recent years, the study of spinal commissures has been instrumental in the identification and characterization of several axon guidance cues (Castellani, 2013; Evans and Bashaw, 2010; Tessier-Lavigne and Goodman, 1996). In short, the axons of these sensory interneurons are initially repelled from the dorsal roof plate by gradients of bone morphogenetic proteins (BMP) in the vertebrate spinal cord, and then, attracted to the ventral midline by netrin-1, sonic hedgehog (Shh) and vascular endothelial growth factor (VEGF), which are secreted by the floor plate (Augsburger et al., 1999; Butler and Dodd, 2003; Charron et al., 2003; Kennedy et al., 1994; Ruiz de Almodovar et al., 2011; Serafini et al., 1996). Once they cross the midline, axons are guided towards the brain by Wnt4, Shh and ephrins (Bourikas et al., 2005; Imondi and Kaprielian, 2001;

Lyuksyutova et al., 2003), and prevented from re-crossing by the chemorepulsion exerted by Slits and semaphorins (Long et al., 2004; Zou et al., 2000).

The journey of spinal commissural axons illustrates the complexity of axon pathfinding. Efficient axon guidance relies on the ability of growth cones to respond appropriately to the multiple guidance cue gradients they encounter. The molecules that direct spinal midline crossing are representative of the cues involved in axon guidance across the nervous system. The properties of the four classical families of guidance cues (Netrins, Slits, Ephrins, and Semaphorins) and those of non-conventional cues (Shh, BMP, Wnts, and VEGF) are summarized below.

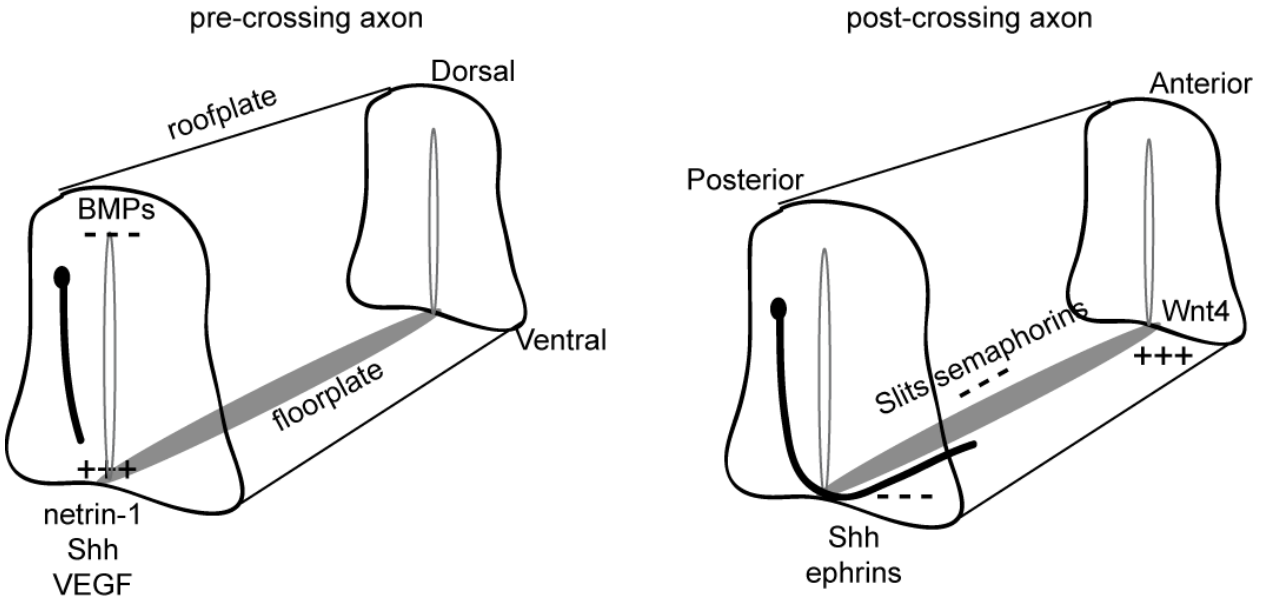

Figure 1.3 Regulation of midline crossing in the vertebrate spinal cord

The axons of pre-crossing commissural neurons are repelled away from the roof plate by BMPs and attracted to the floor plate by netrin-1, Shh and VEGF. Post-crossing

axons are attracted anteriorly by Wnt4 and repelled by Shh, ephrins, Slits and semaphorins.

1.3.1 Classical axon guidance cues and their receptors

1.3.1.1 Netrins

Members of the netrin family of proteins were first identified as factors expressed by the floor plate of the ventral midline, which guide commissural axon projections in the dorsoventral axis of Caenorhabditis elegans and vertebrates (Hedgecock et al., 1990; Ishii et al., 1992; Kennedy et al., 1994; Serafini et al., 1996; Serafini et al., 1994; Wadsworth et al., 1996). Netrins are phylogenetically conserved laminin-related proteins of approximately 75 kilodaltons (kDa) (Lai Wing Sun et al., 2011). Four secreted netrins have been identified in vertebrates: the closely related netrin-1, -2, and-3, and the more distantly related netrin-4 (Koch et al., 2000; Serafini et al., 1994; Wang et al., 1999; Yin et al., 2000). Two other members of the family, netrin G1 and G2, are attached to the plasma membrane by a glycophosphatidylinositol (GPI) anchor. (Nakashiba et al., 2000; Nakashiba et al., 2002). A seventh member of the family, netrin-5, was recently identified (Yamagishi et al., 2015). Netrins regulate diverse functions in the nervous system, which include axon guidance and branching, neuronal precursor migration, synaptogenesis and the development of oligodendrocytes (Lai Wing Sun et al., 2011; Moore et al., 2007; Yamagishi et al., 2015).

Netrin-1 has the ability to attract or repel axons in vertebrates due to the opposing functions of its receptor deleted in colorectal cancer (DCC) (Hong et al., 1999). Other netrin receptors include the DCC family receptor, neogenin, the C.elegans orthologues uncoordinated-5 (UNC5) A-D, Down syndrome cell adhesion molecule (DSCAM), Netrin G ligands and the amyloid precursor protein (Lai Wing Sun et al., 2011; Rama et al., 2012). Signal transduction downstream of netrin-1 and DCC has been extensively studied and is reviewed in the context of axon guidance signaling in Subchapter 1.5.

1.3.1.2 Slits

Slits were initially characterized as repellent factors in the midline of *Drosophila melanogaster* (Kidd et al., 1999; Rajagopalan et al., 2000; Simpson et al., 2000). They are large secreted glycoproteins that are structurally conserved across species (Brose et al., 1999; Rothberg et al., 1990). Vertebrate Slits (Slit1-3) are expressed in the embryonic ventral spinal cord where they prevent ipsilateral neurons from crossing the midline and contralateral neurons from re-crossing it (Brose et al., 1999; Holmes et al., 1998; Itoh et al., 1998; Long et al., 2004). Three Slit receptors, Robo1-3, are homologous to the *Drosophila* receptors and expressed in the CNS, including commissural axons (Kidd et al., 1999; Rajagopalan et al., 2000; Simpson et al., 2000; Yuan et al., 1999). Another receptor, Robo4, presents a more divergent molecular

structure and is also expressed in the CNS (Lee et al., 2001; Zheng et al., 2012). The intracellular domain of Robo receptors is not catalytically active, thus signal transduction is controlled by downstream signaling molecules (Chedotal, 2007). Robo1-3 are alternatively spliced to produce N-terminal variants (Ypsilanti and Chedotal, 2014; Ypsilanti et al., 2010). Of note, Robo1 has the ability to silence netrin-1 chemoattraction through the formation of a heterodimer with DCC, whereas Robo3 can suppress Slit-mediated Robo1 chemorepulsion and potentiate netrin-1-mediated chemoattraction (Chen et al., 2008; Jaworski et al., 2010; Sabatier et al., 2004; Stein and Tessier-Lavigne, 2001; Zelina et al., 2014). Besides their role in the establishment of spinal commissures, Slits serve as guidance cues in the brain, olfactory bulb and retina, and also regulate neuronal migration and axon branching (Ypsilanti and Chedotal, 2014; Ypsilanti et al., 2010).

1.3.1.3 Ephrins

Ephrins are the molecular cues that initially inspired Roger Sperry's chemoaffinity theory (Sperry, 1963). The graded expression of ephrins and their receptors is essential for the establishment of topographic axonal projections in the visual system (Feldheim and O'Leary, 2010). There are two classes of vertebrate ephrins: the five class A ephrins are GPI-linked and the three transmembrane class B ephrins have short cytoplasmic tails (Lisabeth et al., 2013). Ephrin-As and –Bs promiscuously bind to the

receptor tyrosine kinases (RTK) EphA and EphB, respectively (Gale et al., 1996). These receptors, nine EphA and five EphB receptors, constitute the largest of the RTK families (Lisabeth et al., 2013). Similarly to the function of Slits in axon guidance, the ephrin-Eph pairs promote the repulsion of ipsilateral and contralateral projections at the midline, but due to their attachment to the cell membrane, these activities are transduced over a short range (Egea and Klein, 2007; Lisabeth et al., 2013; Reber et al., 2007). Attachment to the cell membrane also imparts them with a unique feature: bi-directional signaling. Signal transduction from the ephrin-bound Eph receptors, termed “forward signaling”, is comparable to signaling downstream of other RTKs since it regulates the same network of proteins (Binns et al., 2000; Himanen et al., 2010; Lisabeth et al., 2013; Seiradake et al., 2010; Wagner et al., 2013; Wybenga-Groot et al., 2001; Zisch et al., 2000). In “reverse” signaling, Eph receptors serve as ligands for ephrins, which transmit cytoplasmic signals after being phosphorylated by Src family kinases (SFK) (Lisabeth et al., 2013; Xu and Henkemeyer, 2012). Signaling downstream of the ephrin-Eph pairs is influenced by various mechanisms, including alternative splicing and proteolytic cleavage (Klein, 2012; Lisabeth et al., 2013; Xu and Henkemeyer, 2012). These mechanisms, in addition to the promiscuous association of the ephrin-Eph pairs, contribute to the bifunctionality of ephrins, which have the ability to mediate either axon attraction or repulsion (Egea and Klein, 2007; Klein, 2012; Lisabeth et al., 2013; Reber et al., 2007).

1.3.1.4 Semaphorins

The first report on vertebrate semaphorins in the nervous system described the activity of a secreted factor, collapsin-1, that induces growth cone collapse in chick dorsal root ganglia (DRG) growth cones (Luo et al., 1993). Since then, the cue was renamed Sema3A and several more secreted, transmembrane and GPI-linked semaphorins have been identified. There are more than twenty vertebrate semaphorins, which are categorized in five classes (classes 3-7, except 5c) and share an extracellular "sema" domain with their invertebrate (classes 1, 2 and 5c) and viral (class V) counterparts (Semaphorin_Nomenclature_Committee, 1999; Yazdani and Terman, 2006). The sema domain, which comprises approximately 500 amino acids, mediates the dimerization of semaphorin molecules and receptors (Gherardi et al., 2004; Siebold and Jones, 2013). Plexins are the main receptors for semaphorins and are grouped in four classes (A-D) that include nine vertebrate family members (Tamagnone and Comoglio, 2000). They are transmembrane proteins that harbour an intracellular GTPase-activating protein (GAP) domain, which reduces the activity of the GTPases Ras and Rap in neurons (Oinuma et al., 2004a; Pascoe et al., 2015; Wang et al., 2012). Another family of semaphorin receptors, the Neuropilins (Nrp), are transmembrane proteins with a short cytoplasmic tail (Chen et al., 1998; He and Tessier-Lavigne, 1997; Kolodkin et al., 1997; Merte et al., 2010; Nakamura et al., 1998; Takahashi et al., 1999;

Tamagnone et al., 1999). Nrp1 and 2 form a holoreceptor complex with plexins, in which they are the obligate ligand-binding subunit of class 3 semaphorins (Fujisawa, 2004; Koncina et al., 2007; Parker et al., 2012). The CAMs L1 and β1 integrin also serve as co-receptors for semaphorin signaling in neurons (Bechara et al., 2008; Pasterkamp et al., 2003). Although they are commonly recognized as repellents, semaphorins mediate chemoattraction and chemorepulsion in the CNS (Fujisawa, 2004; Koncina et al., 2007). This bifunctionality is caused partly by the differential expression of multiple semaphorins and holoreceptor complexes throughout development (Jongbloets and Pasterkamp, 2014; Koncina et al., 2007).

1.3.2 Morphogens

Morphogens are secreted proteins expressed in long-range gradients that provide positional information and initiate transcriptional programs that specify cell fates. Several members of the Hedgehog (Hh), BMP and Wnt families of morphogens are recognized as axon guidance cues and direct the growth of axons via transcription-independent signaling pathways that differ from the canonical signaling cascades implicated in cell differentiation (Yam and Charron, 2013).

1.3.2.1 Sonic Hedgehog

Shh, a Hh family member, is secreted from the floor plate of the ventral midline in vertebrates (Figure 1.3), and establishes a gradient that determines the fate of several

classes of neurons along the dorsoventral axis (Gouti et al., 2015; Jessell, 2000; Le Dreau and Marti, 2012). Shh was revealed to regulate commissural axon guidance in the spinal cord by: 1) complementing netrin-1-dependent chemoattraction of pre-crossing axons to the ventral midline (Charron et al., 2003), and 2) guiding post-crossing axons along the longitudinal axis through chemorepulsion (Bourikas et al., 2005) (Figure 1.3). Similarly to classical guidance cues, the bidirectionality of Shh activity is determined by the cell-specific and time-dependent expression of receptors and signaling molecules (Yam and Charron, 2013). For instance, the non-canonical Shh receptor Boc [biregional cell adhesion molecule-related/down-regulated by oncogenes (Cdon) binding protein] mediates Shh-dependent attraction and repulsion of pre-crossing spinal commissural axons and ipsilateral retinal ganglion cell (RGC) projections, respectively (Fabre et al., 2010; Okada et al., 2006; Yam et al., 2009). Furthermore, Shh-dependent guidance of spinal contralateral projections requires a switch from Boc to the non-canonical receptor Hedgehog-interacting protein (Hhip) to promote repulsion in chick embryos (Bourikas et al., 2005), whereas in rodents, time-dependent expression of the 14-3-3 signaling proteins regulates the switch in the polarity of Shh-dependent guidance in the midline (Yam et al., 2012).

1.3.2.2 Bone morphogenic proteins

Bone morphogenic proteins (BMPs) are members of the Transforming growth factor β (TGF-β) superfamily that are secreted by the roof plate of the neural tube and specify dorsal cell types in vertebrates (Gouti et al., 2015; Le Dreau and Marti, 2012; Lee et al., 1998). The dorsoventral trajectory of pre-crossing spinal commissural axons is established by a combination of dorsal repulsion and ventral attraction. The BMP family members BMP7 and Growth differentiation factor 7 (GDF7) are secreted from the roof plate and form heterodimers that repel spinal commissural axons ventrally and away from the dorsal midline (Augsburger et al., 1999; Butler and Dodd, 2003). The canonical BMP receptor IB is required for dorsal repulsion of commissural neurons (Yamauchi et al., 2008).

1.3.2.3 Wnts

Wnts also induce cell fate specification in the neural tube (Gouti et al., 2015; Le Dreau and Marti, 2012; Muroyama et al., 2002). Wnt4 is expressed by the ventral midline in a decreasing anterior-to-posterior gradient that attracts post-crossing commissural axons anteriorly in the vertebrate spinal cord (Lyuksyutova et al., 2003). Conversely, Wnt1 and Wnt5a mediate axonal repulsion in the corticospinal tract and corpus callosum (Keeble et al., 2006; Liu et al., 2005). Furthermore, Wnts have a bifunctional role in anterior-posterior guidance of brainstem axons and in topographic mapping of the visual system (Fenstermaker et al., 2010; Schmitt et al., 2006). The

polarity of the response to Wnts is determined by the receptors Frizzled 3 (Fz) and receptor tyrosine kinase-related tyrosine kinase (Ryk), which mediate chemoattraction and chemorepulsion, respectively (Onishi et al., 2014; Yam and Charron, 2013).

1.3.2.4 Vascular endothelial growth factor

Although growth factors of the VEGF family are typically associated with the formation of vessels, they also hold many functions in the vertebrate nervous system, including neural migration and survival (Carmeliet and Ruiz de Almodovar, 2013; Mackenzie and Ruhrberg, 2012). The founding member, VEGF-A, is expressed by the floor plate of the spinal cord and mediates chemoattraction of pre-crossing commissural axons that is complementary to the attractive functions of netrin-1 and Shh (Ruiz de Almodovar et al., 2011). VEGF-A-mediated chemoattraction also guides contralateral RGC projections through the optic chiasm (Erskine et al., 2011). VEGF-A-evoked chemoattraction is mediated by two cell-specific VEGF receptors (VEGFR), VEGFR2/Flk1 and Nrp1, which are expressed by spinal commissural neurons and RGCs, respectively (Erskine et al., 2011; Ruiz de Almodovar et al., 2011).

1.4 Regulation of actin dynamics by Rho GTPases and ERM proteins

1.4.1 Rho GTPases: master regulators of F-actin dynamics

Although the classical axon guidance cues activate a wide range of cell surface receptors to mediate their functions, signal transduction downstream of these receptors converges to a common class of regulators, the Rho GTPases, among which Rac, Cdc42 and RhoA are considered to be master regulators of F-actin dynamics.

1.4.1.1 Rho GTPase structure and regulation

Ras homologous (Rho) family proteins are less than 25 kDa in size and are members of the Ras superfamily of small GTPases (Rojas et al., 2012). Only 20 mammalian proteins are considered to be part of the Rho subfamily of small GTPases (Hall, 2012; Vega and Ridley, 2007). The canonical Rho GTPases, RhoA, Rac1 and Cdc42, are the best characterized members of the family (Didsbury et al., 1989; Johnson and Pringle, 1990; Madaule and Axel, 1985). Their structure of Rho GTPases from the amino to carboxy terminus (N-, C-terminus) comprises a GTP/GDP binding domain, an effector binding domain, three other regions that mediate GTP/GDP binding, a hypervariable region, and a short amino acid sequence termed the CAAX box consisting of a cysteine, two aliphatic amino acids and another amino acid (Ridley, 2006).

The majority of Rho family members function as molecular switches that have the ability to convert guanosine triphosphate (GTP) into guanosine diphosphate (GDP) (Vetter and Wittinghofer, 2001). They cycle through GDP-bound and GTP-bound states,

which render them inactive and active, respectively. The GDP-GTP cycle is perpetuated by guanine nucleotide exchange factors (GEFs) and GAPs (Figure 1.4). GEFs activate GTPases by inducing the exchange of GDP for GTP, whereas GAPs inactivate them by stimulating the hydrolysis of GTP (Vetter and Wittinghofer, 2001). Rho GTPase activity is also regulated by guanine nucleotide dissociation inhibitors (GDI), which not only maintain GTPases in their inactive GDP-bound state, but also prevent interactions with cell membranes to control their subcellular localization by (Garcia-Mata et al., 2011). Cell membrane association is necessary for GTPase signal transduction and is mediated by the hypervariable region and the prenylation CAAX box (Boulter et al., 2012). Other processes regulate Rho GTPase activity and include phosphorylation, oxidation, ubiquitination and proteasome degradation (Boulter et al., 2012; Visvikis et al., 2010). The great diversity of their regulators (82 GEFs, 67 GAPs and 3 GDIs) highlights the essential role of Rho GTPases in the maintenance of cellular functions (Hall, 2012).

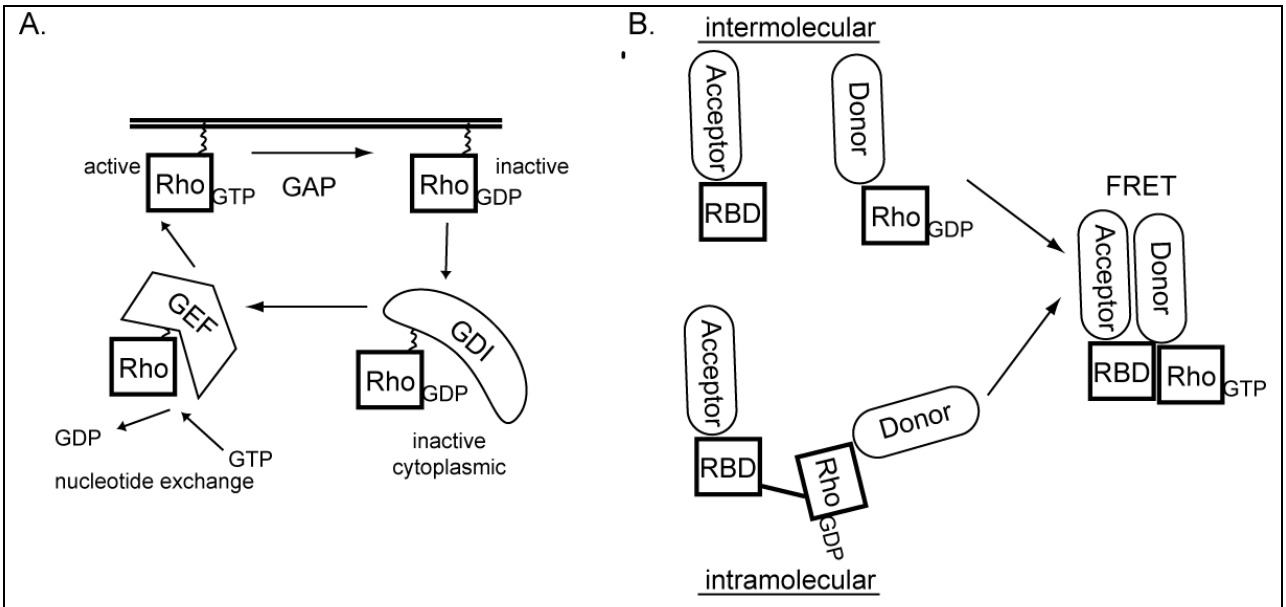

Figure 1.4 Regulation and detection of Rho GTPase activity

(A) Rho GTPases are molecular switches that cycle between an inactive GDP-bound and an active GTP-bound conformation. GEFs and GAPs mediate GTPase activation and inactivation, respectively. GDIs extract GTPases from membranes, rendering them inactive and cytosolic. (B) Inter- and intramolecular probes can be used to detect Rho GTPase activity in time and space.

1.4.1.2 Regulation of F-actin during cell migration

Rho GTPases are mostly regarded as master regulators of F-actin dynamics (Hall, 2012; Jaffe and Hall, 2005). The binding of GTP induces a conformational change in the switch I and II regions of the effector binding domain that enables the GTPase to interact with downstream molecules (Vetter and Wittinghofer, 2001). With over a hundred effectors identified thus far, Rho GTPases regulate an impressive number of

cellular processes, including cell polarity, endocytosis, morphogenesis and cytokinesis (Hall, 2012; Heasman and Ridley, 2008; Jaffe and Hall, 2005). The role of Rho GTPases as signaling molecules was first confirmed when their ability to regulate F-actin in response to extracellular cues was demonstrated in fibroblasts (Kozma et al., 1995; Ridley and Hall, 1992; Ridley et al., 1992). Then, the discovery that the three canonical Rho GTPases mediate the formation of focal adhesions in fibroblasts prompted the study of their regulation of F-actin dynamics during cell migration (Nobes and Hall, 1995; Ridley and Hall, 1992).

The classic model for cell motility is inspired by the migration of fibroblasts in a two-dimensional environment and describes a cycle that starts with the protrusion of the leading edge, followed by its adhesion to the substratum and the de-adhesion of the trailing edge, and ends with a contraction of the rear that causes the cell body to be pushed forward (Blanchoin et al., 2014; Pollard and Borisy, 2003; Raftopoulou and Hall, 2004). In this model, the spatial segregation of Rho GTPase activity polarizes the cell and regulates the protrusion-retraction cycle (Hanna and El-Sibai, 2013; Raftopoulou and Hall, 2004; Spiering and Hodgson, 2011).

In summary, Rac1 and Cdc42 promote actin polymerization and protrusions at the leading edge by stimulating the actin nucleation factor Arp2/3 through the activation of the Wiskott-Aldrich syndrome protein (WASP) family members, WASP-family verprolin-homologous protein (WAVE) and neural WASP (N-WASP), respectively (Chen

et al., 2010; Eden et al., 2002; Rohatgi et al., 1999). While Rac1 activity generates lamellipodia via the regulation of WAVE, Cdc42 activity induces the formation of filopodia via N-WASP and the formin mDia, an F-actin elongation factor (Yang and Svitkina, 2011). Though both GTPases have the ability to regulate actin polymerization and focal adhesions via their common effector p21 activated kinase (PAK), Rac1 activity is primarily associated with the regulation of nascent adhesions, whereas Cdc42 establishes the direction of cell migration by regulating cell polarity (Hanna and El-Sibai, 2013; Raftopoulou and Hall, 2004).

RhoA activity is typically associated with the trailing edge of migrating cells where Rho kinase (ROCK) promotes contractility by activating myosin light chain (Amano et al., 1996; Kawano et al., 1999; Kimura et al., 1996). However, observations suggesting that RhoA promotes actin polymerization at the leading edge via the bundling activity of mDia challenged the paradigm dictating that RhoA activity exclusively regulates contractility at the rear of the cell (Kurokawa and Matsuda, 2005; Lawson and Burridge, 2014; Watanabe et al., 1997). Other findings also contributed to the emergence of the notion that the subcellular localization and the timing of activation could determine the function of Rho GTPases. For example, the ability of RhoA, Rac1 and Cdc42 to stabilize actin filaments through the inhibition of the F-actin severing protein cofilin yields cytoskeletal rearrangements that are unique to each GTPase (Edwards et al., 1999; Maekawa et al., 1999; Sumi et al., 2001). At the time of their discovery, these novel

mechanisms called attention to the need to develop new experimental approaches that can resolve the spatio-temporal regulation of Rho GTPases.

1.4.1.3 Imaging Rho GTPase activity in time and space using FRET

Fluorescence Resonance Energy Transfer (FRET) is the non-radiative transfer of excitation energy from a donor fluorophore to an acceptor fluorophore with an absorption spectrum that overlaps with the emission spectrum of the donor (Herman et al., 2001). FRET can be detected either by exciting the donor and detecting the light emitted by the acceptor or by comparing the fluorescence lifetime of the donor in the presence and absence of the acceptor (Herman et al., 2001). Since FRET is only effective when the distance between the donor and acceptor molecules is between 1 and 10 nanometers (nm), the phenomenon can be exploited to study protein-protein interactions and conformational changes (Herman et al., 2001). In recent years, the combination of FRET with optical microscopy has produced a method that surpasses the limitations of optical resolution and biochemical assays by quantitatively measuring and localizing protein activities and interactions in living cells (Aoki et al., 2013; Herman et al., 2001). Notably, FRET imaging in individual cells resolves the small subcellular pools of activated Rho GTPases that are not detectable in pull-down activation assays, which biochemically measure the average GTPase activity among a collection of cells (Pertz, 2010; Spiering and Hodgson, 2011). As a result, FRET imaging has been

instrumental in the study of the spatio-temporal dynamics of GTPases during various cellular processes, including the regulation of Rac1, Cdc42 and RhoA during cell migration (Kiyokawa et al., 2011; Pertz, 2010).

The biosensors that are used to detect Rho GTPase activity are designed to be Rho GTPase activity reporters. As such, the occurrence of FRET is determined by the interaction of the GTPase with the GTPase binding domain of its effector, the Cdc42/Rac1 interactive binding (CRIB) domain or the Rho binding domain (RBD) (Pertz, 2010). The FRET probes typically consist of the Rho GTPase and the associated effector CRIB domain or RBD expressed as individual acceptor and donor fluorescent fusion proteins, respectively, or as a fusion protein that harbours both fluorophores (Figure 1.4). The use of such biosensors confirmed that Rac1, Cdc42 and RhoA are activated at the leading edge of live migrating cells (Itoh et al., 2002; Kraynov et al., 2000; Kurokawa and Matsuda, 2005; Pertz et al., 2006). FRET imaging not only detected the presence of active RhoA at the leading edge, but the cross-correlation of the resulting Rho GTPase activity maps with the displacement of the leading edge also revealed that RhoA activation is synchronous with the onset of protrusions and precedes Rac1 and Cdc42 activation (Machacek et al., 2009).

This new information led to the proposal of a new model in which RhoA activity initiates the formation of protrusions that are stabilized at the leading edge by the subsequent activation of Rac1 and Cdc42 (Lawson and Burridge, 2014; Machacek et

al., 2009; Spiering and Hodgson, 2011). The resolving power of FRET imaging improved our understanding of the role of Rho GTPases during cell migration and confirmed that Rho GTPase activity is coordinated in time and space rather than strictly segregated. Though it was initially observed in the context of cell migration, the coordination of Rho GTPase activities likely regulates other cellular processes that involve morphological changes.

1.4.1.4 Regulation of Rho GTPases by axon guidance cues

Rho GTPase signaling is implicated in several aspects of the development of the nervous system, including dendritic spine morphogenesis, myelination, neuronal migration, proliferation and survival (Antoine-Bertrand et al., 2011b; Duquette and Lamarche-Vane, 2014; Govek et al., 2005; Hall and Lalli, 2010; Stankiewicz and Linseman, 2014). Since the regulation of cytoskeletal rearrangements determines the direction in which an axon grows (Subchapter 1.2), it is not surprising that axon guidance signaling converges to the canonical Rho GTPases. According to a longstanding dogma, Rac1 and Cd42 are positively regulated by attractive guidance cues and promote axon outgrowth, whereas RhoA is activated by repulsive cues, promotes growth cone collapse and inhibits axon outgrowth (Govek et al., 2005; Hall and Lalli, 2010). However, in recent years, the regulation of Rho GTPases by axon

guidance cues was shown to be more complex than the initial model (Bashaw and Klein, 2010; Robichaux and Cowan, 2014).

In the context of netrin-1 signaling, Rho GTPase activation in neurons is best characterized downstream of the DCC receptor, which activates Rac1 and Cd42 (Briancon-Marjollet et al., 2008; Li et al., 2002b; Shekarabi and Kennedy, 2002; Shekarabi et al., 2005), and inhibits RhoA activity (Moore et al., 2008a). While regulators for Cdc42 and RhoA have yet to be identified, the GEFs Trio and DOCK180 activate Rac1 and promote netrin-1- dependent axon guidance (Briancon-Marjollet et al., 2008; DeGeer et al., 2013; Li et al., 2008). In accordance with the paradigm, netrin-1/DCC-evoked axon outgrowth and guidance are positively regulated by Rac1 and Cdc42 (Briancon-Marjollet et al., 2008; DeGeer et al., 2013), and inhibited by RhoA (Moore et al., 2008a). Yet, FRET imaging reveals that RhoA is activated in nascent neurites when DCC is expressed in neuroblastoma cells (Picard et al., 2009), indicating that the regulation of RhoA may be time- and space-dependent downstream of DCC. This mechanistic feature of netrin-1/DCC signaling is discussed in Subchapter 5.3.

Signal transduction downstream of the repellent ligands Slits and their Robo receptors also challenges the traditional model for the regulation of Rho GTPases as Slit2/Robo1 signaling activates both Rac1 and RhoA, and inhibits Cdc42 activity in cultured non-neuronal cells (Fan et al., 2003; Wong et al., 2001). Slit2 activates RhoA and inhibits Cdc42 to repel migrating neurons (Guan et al., 2007; Wong et al., 2001), a

process that requires the Cdc42-specific GAP activity of Slit-Robo GAP 1 (SrGAP1) (Wong et al., 2001). Of note, Slit-mediated axon repulsion in *Drosophila* requires the functional contribution of the Rac/Ras GEF Son of Sevenless (Sos) and the Rac/Cdc42 GAP Vilse/CrossGAP, both of which regulate Rac downstream of Robo (Hu et al., 2005; Lundstrom et al., 2004; Yang and Bashaw, 2006). In line with these findings, vertebrate Slit2 and Robo1 promote the recruitment of Sos to the plasma membrane of non-neuronal cells and induce membrane ruffling, a hallmark of Rac1 activity (Yang and Bashaw, 2006). This suggests that Slits also activate Rac1 via Sos to mediate repulsion in vertebrates.

The GEF ephexin1 maintains the equilibrium between Rac1, Cdc42 and RhoA activities in the absence of ligand, and upon EphA4 activation via ephrin forward signaling, ephexin1 shifts the balance toward RhoA activation, causing growth cone collapse in RGCs (Sahin et al., 2005; Shamah et al., 2001). In addition, the RacGAP α2-chimaerin is activated downstream of EphA4 and inhibits Rac1 activity (Iwasato et al., 2007; Shi et al., 2007; Wegmeyer et al., 2007). Paradoxically, ephrin-mediated growth cone collapse and axon guidance require the expression of Vav family GEFs, which mediate ephrin-Eph endocytosis, a process that favours cell detachment and requires Rac1 activity (Cowan et al., 2005; Jurney et al., 2002; Marston et al., 2003). In parallel, Eph-ephrin reverse signaling activates Rac1 and Cdc42, and requires the RacGEF activity of DOCK180 and the kinase activity of the Rac/Cdc42 effector PAK to mediate

repulsive hippocampal axon pruning (Xu and Henkemeyer, 2009). Thus, ephrin signaling also features unconventional Rho GTPase regulation as Rac1 activity is solicited to promote axon repulsion instead of attraction.

Similarly, semaphorins differentially regulate Rho GTPases to mediate growth cone collapse and repulsion. RhoA and Rac1 activities are required for semaphorin-induced growth cone collapse in DRG and hippocampal neurons (Jin and Strittmatter, 1997; Kuhn et al., 1999; Swiercz et al., 2002; Vastrik et al., 1999). RhoA is transiently inactivated by p190RhoGAP/ARHGAP35 and activated by the GEFs PDZ-RhoGEF and LARG in response to Sema4D/Plexin-B1 signaling in non-neuronal cells (Barberis et al., 2005; Swiercz et al., 2002). Furthermore, Rac1 is activated by the GEF FARP2 downstream of Sema3A and the Nrp1/Plexin-A1 holoreceptor complex in DRG neurons (Toyofuku et al., 2005). It was suggested that active Rac1 is sequestered by Plexins in *Drosophila* (Hu et al., 2001; Vikis et al., 2002). Though this mechanism remains to be confirmed in vertebrates, one plausible hypothesis is that Rac1 activity mediates receptor endocytosis downstream of semaphorins to promote growth cone collapse (Jurney et al., 2002). Of note, FARP2 is also required for the recruitment of the Rho family GTPase Rnd1 to Plexin-A1 and for Rnd1-dependent R-Ras inactivation (Oinuma et al., 2004a; Oinuma et al., 2004b; Toyofuku et al., 2005).

Overall, Rho GTPase regulation downstream of axon guidance cues diverges from the established paradigm. Functional assays in dissociated neurons and the

detection of GTPase activity *in vitro* have been essential to forge our current understanding of the regulation of Rho GTPases in the context of axon guidance. Yet, several questions regarding the coordination and timing of Rho GTPase activities remain since these techniques cannot resolve the spatio-temporal regulation of Rho GTPases *in vivo*.

1.4.2 ERM proteins: bridging the plasma membrane and the actin cytoskeleton

Ezrin-radixin-moesin (ERM) proteins play a significant role in the establishment of specialized apical domains and regulate several cellular processes through their ability to bridge the plasma membrane and the actin cytoskeleton.

1.4.2.1 Structure and regulation of the ERM protein family

ERM proteins are closely related proteins that link membrane-associated proteins to the cell cortex, a thin layer of F-actin that lines the plasma membrane (Blanchoin et al., 2014; McClatchey, 2014). These proteins of approximately 80 kDa are part of the Band 4.1 or FERM (four-point-one-ezrin-radixin-moesin) protein superfamily, which includes the prototypic erythrocyte protein Band 4.1, the cytoskeletal protein talin and the signaling protein focal adhesion kinase (FAK) (Bosanquet et al., 2014; Bretscher et al., 2002). Mammalian ezrin, radixin and moesin share more than 70% amino acid identity and have a high degree of structural conservation (Fievet et al.,

2007). The ERM protein family includes a fourth member, the neurofibromatosis type 2 (NF2) tumour suppressor protein, merlin. Although merlin is structurally related, its regulation and functions diverge from the properties of the three other family members (Bretscher et al., 2002; Cooper and Giancotti, 2014; McClatchey and Fehon, 2009), and will not be presented here.

The function of ERM proteins is largely attributed to their molecular structure, which comprises an N-terminal FERM domain, a central α-helical domain and a C-terminal domain that harbours a 30 aa F-actin-binding region (Algrain et al., 1993; Turunen et al., 1994). The FERM domain contains approximately 300 residues and interacts with membranes in three different ways: it binds directly to membrane PIP_2 or the cytoplasmic tail of membrane proteins, or indirectly via scaffold proteins (Bretscher et al., 2002; McClatchey, 2014). Additionally, the FERM domain binds with high affinity to a region consisting of approximately 100 aa residues in the C-terminus of ERM proteins (Gary and Bretscher, 1995). The interaction between the two domains, also called N-ERMAD and C-ERMAD (amino- and carboxy-ERM association domains), determines the three-dimensional conformation of ERM proteins.

ERM proteins alternate between a so-called dormant cytoplasmic state and an active membrane-bound state. A significant amount of ezrin exists as a soluble cytoplasmic monomer in which the N- and C-ERMAD interact, consequently masking the F-actin binding region and keeping the protein dormant (Berryman et al., 1995;

Bretscher et al., 1995; Gary and Bretscher, 1995). Besides enabling self-association, head-to-tail interactions produce homo- and heterotypic ERM protein dimers and oligomers that are detectable *in vivo* (Andreoli et al., 1994; Berryman et al., 1995; Bretscher et al., 1995; Gary and Bretscher, 1993). However the biological significance of ERM protein oligomers remains to be determined. ERM protein activation involves the transition from the closed dormant state to an open conformation, a process during which the C-terminus is released from the FERM domain and becomes free to interact with F-actin. This structural conversion involves two steps: 1) the binding of membrane PIP_2 by the FERM domain, and 2) the phosphorylation of a conserved C-terminal threonine (Thr567, Thr564 and Thr558 in ezrin, radixin and moesin, respectively) (Fievet et al., 2007; Fievet et al., 2004; Yonemura et al., 2002).

The three-dimensional structures of ERM proteins obtained by crystallography and electron microscopy corroborate the N-/C-ERMAD conformational masking model and provide information on the structural impact of lipid-binding and phosphorylation during ERM protein activation (Ben-Aissa et al., 2012; Edwards and Keep, 2001; Hamada et al., 2000; Ishikawa et al., 2001; Pearson et al., 2000). Three regions in the clover-leaf-shaped FERM domain have been shown to bind to lipids (Barret et al., 2000; Ben-Aissa et al., 2012; Hamada et al., 2000). These sites mediate the association of the FERM domain with membranes and membrane-associated proteins, and also regulate the interaction of ERM proteins with F-actin by destabilizing the N-/C-ERMAD

association (Barret et al., 2000; Braunger et al., 2014; Chen et al., 2015; Jayasundar et al., 2012). The second phase of ERM protein activation, the phosphorylation of the conserved C-terminal threonine residue, mediates the association of ERM proteins with the cytoskeleton and induces their translocation from the cytosol to actin-rich membrane extensions such as lamellipodia, filopodia and microvilli (Gautreau et al., 2000; Hayashi et al., 1999; Matsui et al., 1999; Nakamura et al., 2000; Oshiro et al., 1998; Shaw et al., 1998). The addition of the negative charge in the C-terminus likely weakens the affinity of the FERM domain for the C-terminus and is considered to be a secondary activation step that stabilizes ERM proteins in their open F-actin-binding conformation (Fievet et al., 2007; Maniti et al., 2013; Matsui et al., 1998; Pearson et al., 2000). Several kinases, including protein kinase C (PKC) family members and ROCK, can phosphorylate the conserved threonine residue in a tissue and cell-dependent manner (Clucas and Valderrama, 2014). Of note, the phosphorylation of ezrin on tyrosine residues (Y145, 353 and 477) also regulates its function (Clucas and Valderrama, 2014). However, the impact of tyrosine phosphorylation on the structure or the function of ERM proteins remains to be determined. Overall, the significant number of kinases that phosphorylate ERM proteins indicates that ezrin, radixin and moesin are not simply scaffolds that bridge the plasma membrane and F-actin, but are also signaling molecules.

1.4.2.2 General functions of ERM proteins

ERM proteins are widely expressed across cell types and tissues (Sato et al., 1992). They are presumed to be paralogues and functionally redundant due to their highly conserved structure and activation mechanism. While moesin knockout mice do not display any abnormalities (Doi et al., 1999), the ablation of ezrin or radixin expression causes overt phenotypes, mainly the loss of specialized, actin-rich, apical membrane domains. Adult radixin-deficient mice develop deafness and mild liver injury due to the degeneration of cochlear stereocilia and the lack of hepatic microvilli, respectively (Kikuchi et al., 2002; Kitajiri et al., 2004). The loss of ezrin expression results in microvilli and villi malformations in the retina and intestinal epithelium, respectively (Bonilha et al., 1999; Bonilha et al., 2006; Saotome et al., 2004). Overall, the cell-specific phenotypes in mice that express only two of the three proteins indicate that ERM proteins can compensate for each other in a tissue-specific manner (Fehon et al., 2010; Fievet et al., 2007).

ERM proteins are indeed co-expressed in many tissues and cell types, yet each protein possesses unique expression patterns and functions (Doi et al., 1999; Fehon et al., 2010; Maniti et al., 2013; Sato et al., 1992). Ezrin, the first ERM protein identified, is mainly expressed in epithelial and mesothelial cells, and localizes in the apical membrane of these cells in actin-rich structures like microvilli, filopodia and membrane ruffles (Berryman et al., 1993; Bonilha et al., 1999; Bretscher, 1983; Franck et al., 1993; Saotome et al., 2004; Sato et al., 1992). Radixin is highly expressed in hepatocytes

where it is found in microvilli and is the only ERM protein that is expressed at adherens junctions (Amieva et al., 1994; Tsukita and Hieda, 1989). Moesin is also found in actin-rich membrane domains located at the cell surface, but its expression is less widespread than ezrin and mostly detectable in endothelial cells and lymphoid cells (Amieva and Furthmayr, 1995; Berryman et al., 1993; Franck et al., 1993; Lankes and Furthmayr, 1991; Sato et al., 1992).

Generally, ERM proteins regulate the mechanical properties of the cell cortex and contribute to the establishment of cell polarity in vertebrates. They define primordial apical surfaces in developing embryos, regulate cortical rigidity and spindle orientation during mitosis and meiosis, and are required for lumen morphogenesis in tissues such as the intestinal epithelium (Fehon et al., 2010; McClatchey, 2014). Another important aspect of ERM protein function is the regulation of transmembrane receptors. ERM proteins can regulate the cell surface distribution of membrane receptors by linking them to cortical actin and restricting their localization to actin-rich domains (Bretscher et al., 2002; Fehon et al., 2010; McClatchey and Fehon, 2009). They also interact with many cytosolic signaling proteins such as protein kinases and Rho GTPase regulators, and regulate cellular processes, including lymphocyte activation, cell migration and cell adhesion (Arpin et al., 2011; Bretscher et al., 2002; Clucas and Valderrama, 2014; Fehon et al., 2010; McClatchey and Fehon, 2009; Parameswaran and Gupta, 2013; Viswanatha et al., 2013).

1.4.2.3 ERM proteins in axon outgrowth and guidance

ERM proteins are expressed in several regions of the developing and adult CNS, including the spinal cord, hippocampus and cortex (Gimeno et al., 2004; Gronholm et al., 2005; Johnson et al., 2002; Paglini et al., 1998; Sato et al., 1992). They localize in axonal projections in the spinal cord and are enriched in actin-rich growth cone structures like lamellipodia, filopodia and radial striations (Birgbauer et al., 1991; DiTella et al., 1994; Gonzalez-Agosti and Solomon, 1996; Goslin et al., 1989; Paglini et al., 1998). They are also expressed in dendritic filopodia and the distal processes of glial cells (Derouiche and Frotscher, 2001; Furutani et al., 2007; Gatto et al., 2003; Gronholm et al., 2005).

Growing evidence suggests that ERM proteins contribute to the regulation of axon outgrowth and guidance. On one hand, the suppression of radixin and moesin expression in hippocampal and DRG neurons reduces growth cone motility by altering filopodial and lamellipodial dynamics (Castelo and Jay, 1999; Paglini et al., 1998). On the other hand, radixin accumulates in the peripheral domain of growth cones upon nerve growth factor (NGF)-mediated or electrical stimulation in sympathetic neurons, and ezrin activity is required for cortical axon guidance (Gonzalez-Agosti and Solomon, 1996; Mintz et al., 2008). ERM protein activity is also required for cortical axon outgrowth and branching in response to injury (Haas et al., 2004, 2007; Mintz et al.,

2008; Ruan et al., 2008). Interestingly, ERM proteins are activated by key signaling molecules that are regulated by axon guidance cues, mainly Rho GTPases. In non-neuronal cells, RhoA and Cdc42 induce the phosphorylation of the conserved threonine residue via their effectors ROCK and myotonic dystrophy kinase-related Cdc42-binding kinase (MRCK), respectively (Clucas and Valderrama, 2014; Nakamura et al., 2000). In neurons, ERM protein activation is required for the induction of filopodial protrusions and neurite outgrowth downstream of the extracellular cues glutamate and NGF, respectively (Jeon et al., 2010; Kim et al., 2010). Glutamate mediates ERM protein phosphorylation via RhoA, while NGF induces phosphorylation via Rac1 (Jeon et al., 2010; Kim et al., 2010). In contrast, ERM proteins are transiently dephosphorylated downstream of the repulsive guidance cue Sema3A (Gallo, 2008; Jeon et al., 2010; Kim et al., 2010; Mintz et al., 2008). The inhibition of ERM protein activity is required for the internalization of the Sema3A receptors L1 and Nrp1, a process known to be required for Sema3A-mediated growth cone collapse (Castellani et al., 2004; Fournier et al., 2000; Mintz et al., 2008; Schlatter et al., 2008).

In summary, ERM protein activity is differentially regulated in order to mediate opposing guidance responses: ERM protein activation promotes positive axon outgrowth and guidance, while inactivation inhibits outgrowth and promotes growth cone collapse and repulsion. Moreover, the enrichment of ERM proteins in the actin-rich P domain of growth cones indicates that these proteins regulate F-actin dynamics. Yet,

the molecular mechanisms through which ERM proteins regulate axon outgrowth and guidance are still largely unknown.

1.5 Netrin-1/DCC signal transduction in the vertebrate CNS

Netrins function as chemotropic guidance cues for axons and migrating cells. They transduce their diverse functions through several families of cell surface receptors that control distinct signaling cascades. Signal transduction downstream of the prototypic secreted netrin, netrin-1, and its receptor DCC mediates the attraction of growing axons, a process that is essential for the development of the nervous system.

1.5.1 Structure and function of netrin-1 and its receptors

1.5.1.1 Netrin-1

The netrins are members of the laminin superfamily of proteins and consist of approximately 600 amino acids. The receptor-binding N-terminal sequences of netrins share homology with the N-terminal VI and V domains of laminins, a positively charged globular domain and a domain made up of three EGF repeats, respectively (Lai Wing Sun et al., 2011; Rajasekharan and Kennedy, 2009). While the C-terminal GPI linker of netrin G1 and G2 tethers the proteins to the plasma membrane, the C-domain or netrin-like module (NTR) of the secreted netrins (netrin-1 to -4), is enriched in basic amino

acids and binds heparin, which enables netrins to decorate the ECM (Lai Wing Sun et al., 2011; Rajasekharan and Kennedy, 2009).

Netrin-1 regulates tissue morphogenesis within and outside the nervous system by controlling processes such as cell adhesion, migration and proliferation (Bradford et al., 2009; Cirulli and Yebra, 2007). It is expressed in the developing and adult nervous systems where it mediates the development and maturation of oligodendrocytes, neuronal precursor migration, and several stages of axogenesis, including synaptogenesis and axon guidance (Lai Wing Sun et al., 2011). For instance, netrin-1 functions at a short-range in the mature nervous system to mediate cell-cell contacts between neurons at synapses, as well as between oligodendrocytes and axons at paranodal junctions (Goldman et al., 2013; Jarjour et al., 2008; Manitt et al., 2001) Additionally, the developmental defects observed in mice deficient for netrin-1 expression indicate that netrin-1 is also required for the long-range attractive guidance of axonal projections across the midline in the embryonic brain and spinal cord (Bin et al., 2015; Serafini et al., 1996; Yung et al., 2015). Furthermore, netrin-1-mediated chemoattraction guides other axon tracts within the developing CNS, including thalamic, cortical, hippocampal and RGC projections (Barallobre et al., 2000; Braisted et al., 2000; Deiner et al., 1997; Metin et al., 1997; Richards et al., 1997). It has also been proposed that netrin-1 serves as a chemorepellent for motor neurons in the developing PNS (Colamarino and Tessier-Lavigne, 1995; Dillon et al., 2005; Dillon et al., 2007).

However, the hypomorphic nature of the loss-of-function mutation originally generated to characterize netrin-1 functions in mice has warranted the re-evaluation of the functions previously attributed to netrin-1 in the developing nervous system (Bin et al., 2015; Serafini et al., 1996; Yung et al., 2015).

In light of the phenotypic analysis of newly generated *netrin-1* null mouse lines, the formerly demonstrated role of netrin-1 as a cell survival factor is proposed to be restricted to specific cells within the nervous system since the complete loss of netrin-1 expression does not cause cell death in the spinal cord, DRGs or spinal accessory nerve (Bin et al., 2015; Furne et al., 2008; Llambi et al., 2001; Mehlen and Guenebeaud, 2010; Mehlen et al., 1998; Yung et al., 2015). Also in opposition with what was previously reported, the complete loss of netrin-1 expression indicates that netrin-1 does not function as a major repulsive cue for trochlear motor axons in the PNS (Colamarino and Tessier-Lavigne, 1995; Yung et al., 2015). Finally, the characterization of the *netrin-1* null mice confirms that spinal midline crossing is primarily mediated by netrin-1 and suggests that the guidance cues Shh and VEGF only play minor roles in commissural axon attraction in the spinal cord (Bin et al., 2015; Yung et al., 2015).

1.5.1.2 Netrin-1 receptors

DCC and neogenin are members of the DCC receptor family. DCC was first identified in humans as a candidate tumour suppressor, encoded within a region of

chromosome 18q that is deleted in colorectal cancers (Fearon and Vogelstein, 1990). The ectodomain of DCC comprises four immunoglobulin (Ig)-like domains and six fibronectin (FN) type III domains, and shares a high degree of homology with the ectodomain of neogenin (Cho et al., 1994; Vielmetter et al., 1994). The interaction between netrin-1 and its receptors DCC and neogenin engages an interface that includes the FN4, FN5 and FN6 domains (Finci et al., 2015; Finci et al., 2014; Geisbrecht et al., 2003; Kruger et al., 2004; Xu et al., 2014). The cytoplasmic tail of DCC is catalytically inactive and comprises three highly conserved domains (P1, P2, and P3) that are involved in receptor dimerization and signal transduction (Hong et al., 1999; Lai Wing Sun et al., 2011). For instance, the P3 domain mediates the formation of DCC homodimers in response to netrin-1 (Stein et al., 2001).

The interaction of DCC with netrin-1 regulates cell-cell adhesion at paranodal junctions and synapses (Goldman et al., 2013; Horn et al., 2013; Jarjour et al., 2008), whereas DCC mediates chemoattraction in response to netrin-1 (Keino-Masu et al., 1996). Several axon tracts in the spinal cord and the brain express DCC and the loss of DCC expression in mice causes defects in the formation of spinal and cerebral commissures that are comparable to those observed in netrin-1-deficient mice (Bin et al., 2015; Fazeli et al., 1997; Keino-Masu et al., 1996; Serafini et al., 1996; Yung et al., 2015). Moreover, mutations in the *dcc* locus are associated with mirror movement disorders in humans and mice, a reported consequence of midline crossing defects

(Finger et al., 2002; Srour et al., 2010). In contrast, neogenin functions as a receptor for the repulsive guidance molecule (RGM), but its netrin receptor functions are generally associated to cell adhesion rather than to axon guidance (Lai Wing Sun et al., 2011; Rajagopalan et al., 2004). Yet, the recent revision of the phenotypes of *dcc* and *netrin-1* mutant mice suggests that netrin-1-dependent spinal commissural axon guidance, which until then was thought to be regulated exclusively by DCC, also requires neogenin (Fazeli et al., 1997; Xu et al., 2014). Of note, Down syndrome cell adhesion molecule (DSCAM), another netrin-1 receptor of the Ig superfamily, is also expressed on spinal commissural axons, but the depletion of the protein does not induce defects in spinal or cerebral commissures (Andrews et al., 2008; Ly et al., 2008; Palmesino et al., 2012).

UNC5 A-D receptors are widely expressed across the nervous system including in neurons that express DCC (Ackerman et al., 1997; Engelkamp, 2002; Keino-Masu et al., 1996; Leonardo et al., 1997). The ectodomain of the UNC5 receptors is composed of two Ig domains that bind to netrin-1 and two thrombospondin type I domains (Ackerman et al., 1997; Engelkamp, 2002; Geisbrecht et al., 2003; Leonardo et al., 1997). The cytoplasmic tail of UNC5 receptors consists of a ZU-5 domain, a DCC-binding (DB) motif and a death domain (Hong et al., 1999; Tong et al., 2001). The heterodimerization of UNC5 and DCC via their respective DB motif and P1 domain

causes a shift of the response to netrin-1 from chemoattraction to repulsion that is transduced by the intracellular domain (ICD) of UNC5 (Hong et al., 1999).

Netrin-1 also binds to α6β4 and α3β1 integrins, but the interaction involves the NTR module rather than the N-terminus of netrin-1 (Yebra et al., 2003). Although the role of this interaction is well characterized in the context of cell adhesion and migration outside the nervous system (Nikolopoulos and Giancotti, 2005; Yebra et al., 2003), its functional significance in the nervous system remains to be determined.

1.5.2 DCC receptor signaling in the developing vertebrate CNS

1.5.2.1 DCC receptor signaling cascades

Following netrin-1 binding, several signaling proteins are activated and recruited to DCC (Figure 1.5). The functional significance of protein complex assembly was experimentally demonstrated in the context of netrin-1-dependent axon outgrowth and chemoattraction in vertebrate embryos *in vivo*, dissociated primary neurons or tissue explants. This process has led to the establishment of a model for netrin-1/DCC signal transduction in neurons.

1.5.2.1.1 Activation of DCC

The binding of netrin-1 induces the dimerization of DCC via the P3 domain, as well as the phosphorylation of tyrosine residues on the receptor (Meriane et al., 2004;

Ren et al., 2004; Stein et al., 2001). Similarly to ligand-dependent RTK activation, which also requires receptor dimerization and tyrosine phosphorylation (Schlessinger, 2014), these events contribute to the activation of DCC and the initiation of netrin-1/DCC signaling. DCC also becomes phosphorylated on serine and threonine residues in response to netrin-1 (Meriane et al., 2004). However, to this day, functional relevance in the context of axon outgrowth and guidance has only been demonstrated for the tyrosine phosphorylation of DCC.

In brief, tyrosine kinases Src and FAK are constitutively bound to DCC via the P3 domain (Li et al., 2004; Ren et al., 2004). The binding of netrin-1 stimulates the activation and the recruitment of additional Src and FAK proteins to DCC (Li et al., 2004; Liu et al., 2004; Ren et al., 2004). The tyrosine kinase Fyn is also activated and recruited to DCC in response to netrin-1, and along with Src, Fyn initiates the phosphorylation of the receptor on tyrosine residues (Li et al., 2004; Liu et al., 2004; Meriane et al., 2004). Fyn also phosphorylates the RacGEF Trio and mediates the activation of Rac1 in response to netrin-1 (DeGeer et al., 2013; Meriane et al., 2004).

1.5.2.1.2 Regulation of Rho GTPases and the cytoskeleton

Netrin-1/DCC signaling induces growth cone expansion and protrusion by increasing actin polymerization and F-actin content within the growth cone (Lebrand et al., 2004; Marsick et al., 2010; Shekarabi et al., 2005). The regulation of Rho GTPases

downstream of netrin-1/DCC signaling likely mediates the changes in growth cone dynamics. In fact, two RacGEFs, Trio and DOCK180, are recruited to DCC in response to netrin-1 and both contribute to the mediation of netrin-1-dependent axon outgrowth and chemoattraction (Briancon-Marjollet et al., 2008; DeGeer et al., 2013; Li et al., 2008). The loss of Trio expression abolishes netrin-1-dependent Rac1 activation in neurons, suggesting that Rac1 is primarily activated by Trio downstream of netrin-1 (Briancon-Marjollet et al., 2008). Of note, the molecular chaperone heat shock cognate protein 70 regulates the localization of Trio and its ability to activate Rac1 in response to netrin-1 (DeGeer et al., 2015). Moreover, the adapter protein Nck-1, which is constitutively bound to DCC, mediates the recruitment and activation of an effector of Rac1 and Cdc42, the serine/threonine kinase PAK1, which also interacts with Trio (Briancon-Marjollet et al., 2008; Li et al., 2002a; Shekarabi et al., 2005). Concurrent with the positive regulation of Rac1 and Cdc42 activity, RhoA activity is inhibited downstream of netrin-1/DCC signaling to further promote axon outgrowth and guidance (Moore et al., 2008a).

Besides PAK1, several proteins that promote F-actin polymerization are solicited downstream of netrin-1 and DCC. Enabled/ vasodilator-stimulated phosphoprotein (ENA/VASP), the Cdc42 effector N-WASP, and the unconventional motor myosin X promote filopodia formation in response to netrin-1, and netrin-1-dependent increases in growth cone protrusions and F-actin correlate with cofilin activation (Lebrand et al.,

2004; Marsick et al., 2010; Shekarabi and Kennedy, 2002; Shekarabi et al., 2005; Zhu et al., 2007). Additionally, netrin-1 mediates chemoattraction by modulating microtubule dynamics through the recruitment to DCC of the β-tubulin isoform TUBB3 and the regulation of the microtubule-binding activity of MAP1B (Del Rio et al., 2004; Qu et al., 2013).

1.5.2.1.3 Regulation of second messengers

Other receptor proximal signaling events include the regulation of second messengers. Netrin-1 induces the activation of phosphatidylinositol transfer protein-α (PITPα) and the hydrolysis of PIP_2 by phospholipase Cγ (PLCγ), a process that generates the second messengers diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3) (Ming et al., 1999; Xie et al., 2005; Xie et al., 2006). Furthermore, the regulation of intracellular Ca^{2+} and cAMP concentrations represents an intriguing aspect of netrin-1/DCC signal transduction. An increase in the levels of intracellular Ca^{2+} and cAMP as well as the activity of the cAMP-dependent protein kinase A (PKA) are required for netrin-1-dependent chemoattraction in *Xenopus* spinal neurons (Hong et al., 2000; Ming et al., 1997; Shim et al., 2005; Wang and Poo, 2005). In contrast, netrin-1 does not alter cAMP concentration or PKA activity in rat DRG neurons or spinal commissural neurons (Bouchard et al., 2004; Moore and Kennedy, 2006; Moore et al., 2008b). Yet, the frequency of intracellular Ca^{2+} fluctuations, or Ca^{2+} transients, is elevated following

netrin-1 stimulation in *Xenopus* spinal neurons and rat hippocampal neurons (Nicol et al., 2011; Ros et al., 2015). In light of these conflicting reports, the requirement for Ca^{2+}/cAMP signaling downstream of netrin-1 and DCC appears to be cell-specific and should be assessed further.

1.5.2.1.4 Regulation of ERK activity and protein synthesis

Netrin-1/DCC signaling also regulates axon guidance through the stimulation of local protein synthesis in growth cones (Campbell and Holt, 2001; Leung et al., 2006; Tcherkezian et al., 2010; Welshhans and Bassell, 2011). For instance, netrin-1-dependent growth cone attraction requires a local increase in β-actin messenger RNA (mRNA) translation (Leung et al., 2006; Welshhans and Bassell, 2011). In the spinal cord, DCC constitutively interacts with the translation machinery, including ribosomal subunits and initiation factors of the eIF protein family (Tcherkezian et al., 2010). Upon binding of netrin-1, the extracellular signal-regulated kinases (ERK) 1 and 2 are activated and recruited to DCC via the P1 domain (Campbell and Holt, 2003; Forcet et al., 2002; Ma et al., 2010). Netrin-1-stimulated ERK1/2 activity promotes transcription and protein synthesis by activating proteins such as the transcription factor Elk-1 and the eukaryotic initiation factor (eIF4E), respectively (Campbell and Holt, 2003; Forcet et al., 2002). Assays performed in non-neuronal cells have provided some insight into the mechanisms that regulate netrin-1-dependent protein synthesis. These assays have

demonstrated that the P1 domain interacts with the ribosomal protein L5 and that netrin-1 promotes the dissociation of translational components from DCC (Tcherkezian et al., 2010). Furthermore, the P1 domain is required for DCC-induced protein translation *in vitro* (Tcherkezian et al., 2010). This suggests that netrin-1 promotes local protein synthesis in growth cones by releasing the translation machinery from DCC-mediated inhibition.

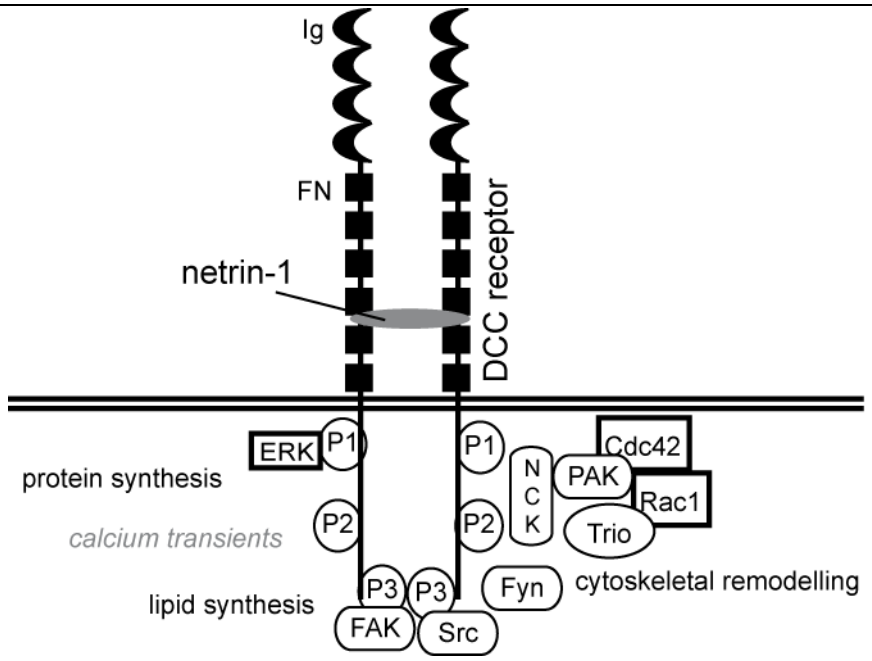

Figure 1.5 Chemoattractive netrin-1 signal transduction in vertebrates

Netrin-1 binding triggers the activation and the recruitment of several proteins to DCC. The signaling cascades initiated by the activation of DCC induce protein and lipid synthesis, as well as cytoskeletal remodelling to promote chemoattraction. The generation of calcium transients (in gray) downstream of netrin-1 may be cell specific.

1.5.2.2 Regulation of DCC signaling

Netrin-1/DCC signal transduction is modulated by different molecular mechanisms in vertebrates. For instance, proteins expressed in the extracellular environment have been shown to regulate the response of neurons to netrin-1. The ECM component heparin can bind to both netrin-1 and DCC and the cell-autonomous expression of heparan sulfate, which is related to heparin and also found in the ECM, is essential for netrin-1/DCC-mediated axon guidance in the spinal cord (Bennett et al., 1997; Matsumoto et al., 2007; Serafini et al., 1994). Conversely, the basement membrane protein laminin-1 converts netrin-1-dependent chemoattraction to repulsion in *Xenopus* spinal neurons (Hopker et al., 1999). Furthermore, netrin-1/DCC signaling is modulated by several intracellular mechanisms.

1.5.2.2.1 Co-receptors

Several DCC co-receptors are known to regulate netrin-1-mediated signal transduction. The heterodimerization of UNC5 and DCC mediates netrin-1-dependent chemorepulsion in *Xenopus* spinal neurons (Hong et al., 1999), whereas protein interacting with C-kinase-1 (PICK1) and PKC mediate the internalization of UNC5 via endocytosis, resulting in the conversion of netrin-1-mediated chemorepulsion to chemoattraction in rat hippocampal neurons (Bartoe et al., 2006; Williams et al., 2003).

Moreover, the Slit receptor Robo1 forms a complex with DCC that silences netrin-1-dependent chemoattraction in *Xenopus* spinal neurons (Stein and Tessier-Lavigne, 2001). In contrast, Robo3 and the amyloid precursor protein each serve as DCC co-receptors that potentiate netrin-1-dependent chemoattraction in commissural neurons (Beamish and Kennedy, 2015; Rama et al., 2012; Zelina et al., 2014). The adenosine A2b receptor was also identified as a DCC co-receptor, but its activation is not required for netrin-1-mediated chemoattraction in the spinal cord (Corset et al., 2000; Stein et al., 2001).

1.5.2.2.2 mRNA and protein processing

The regulation of the surface expression of DCC also has an effect on the response to netrin-1. Alternative splicing produces short and long DCC isoforms that bear different distances between the netrin-1-binding FN4 and FN5 repeats in the ectodomain (Xu et al., 2014). It has been proposed that the distance between the FN repeats modulates netrin-1-dependent signal transduction by regulating the three-dimensional architecture of the netrin-1/DCC complex as well as the multimerization of DCC (Finci et al., 2015; Finci et al., 2014; Xu et al., 2014). In parallel, two distinct mechanisms that involve the removal of DCC from the cell surface likely contribute to the loss of responsiveness to netrin-1, a mechanism that is essential as axons reach their target. The intramembranous cleavage of the DCC ectodomain by presenilin is

required for the proper guidance of motor and commissural neurons in the spinal cord, and the subsequent cytoplasmic cleavage by gamma-secretase produces a DCC ICD fragment that may serve as a transcriptional coactivator in vertebrates (Bai et al., 2011; Neuhaus-Follini and Bashaw, 2015; Taniguchi et al., 2003). Additionally, netrin-1-dependent ubiquitination and proteasome degradation of DCC downregulates DCC surface expression in cortical neurons (Kim et al., 2005).

1.5.2.2.3 Receptor trafficking

Another important aspect of the regulation of netrin-1/DCC signaling is receptor trafficking. Lipid rafts regulate the distribution of netrin-1 and DCC at the cell surface and are required for netrin-1/DCC signal transduction (Guirland et al., 2004; Herincs et al., 2005; Petrie et al., 2009). In *Xenopus*, spinal neuron chemoattraction is modulated by phases of desensitization and resensitization to netrin-1, which are believed to be essential for long-range chemotaxis (Ming et al., 2002). This phenomenon called adaptation is mediated by the fast endocytosis (desensitization) and the slower protein-synthesis (resensitization) of DCC (Ming et al., 2002; Piper et al., 2005). In mammals, DCC surface expression is increased by netrin-1, as well as by the activation of PKA and the inhibition of RhoA, which incidentally potentiate netrin-1-dependent axon outgrowth and chemoattraction in spinal commissural neurons (Bouchard et al., 2004; Moore et al., 2008a; Moore and Kennedy, 2006). Moreover, netrin-1 induces a transient

decrease of DCC surface expression in growth cones relative to axon shafts in cortical neurons (DeGeer et al., 2013). This shift in the distribution of surface DCC is regulated by Trio, an essential regulator of netrin-1 chemoattraction, and could modulate the response to netrin-1 in a way that is comparable to adaptation in *Xenopus* (Briancon-Marjollet et al., 2008; DeGeer et al., 2013).

1.6 Concluding remarks

Altogether, the regulation of axon outgrowth and guidance in the developing nervous system implicates several signaling pathways that must be coordinated. The cytoskeletal organization and chemotactic properties of the growth cone define the context in which axon guidance cues must establish neuronal networks. Each guidance cue yields specificity to the response of growing axons through the mediation of distinct signaling cascades.

Signal transduction downstream of netrin-1 and DCC constitutes a representative example of the complexity of axon guidance signaling. Netrin-1/DCC-mediated signaling induces axon outgrowth and chemoattraction and these responses are modulated by diverse molecular mechanisms that involve a plethora of proteins. Yet, after twenty years of research, several questions remain regarding the function of these signaling mechanisms and could be answered through the identification of additional proteins that regulate netrin-1/DCC signaling.

Rationale and objectives

This thesis examines the molecular mechanisms that mediate signal transduction downstream of the guidance cue netrin-1 and its receptor DCC. Netrin-1/DCC signaling promotes axon guidance in the developing nervous system, but also serves diverse developmental functions outside the nervous system (Lai Wing Sun et al., 2011). The studies presented here were motivated by the discovery that the phosphorylation of the conserved tyrosine residue 1418 (Y1418 in *Rattus norvegicus*) of DCC was essential for netrin-1-dependent signal transduction in neurons (Li et al., 2004; Meriane et al., 2004). Here, the molecular mechanisms by which Y1418 phosphorylation regulates netrin-1/DCC signaling are explored via the characterization of two novel protein interactions with DCC. The spatiotemporal regulation of RhoA activity in response to netrin-1 is also explored.

In an effort to elucidate the complex signaling cascades that mediate axon guidance downstream of netrin-1 and DCC in rat embryonic cortical neurons, the specific objectives of the thesis are as follows:

Objective 1 (Chapter 2): Characterize the functional interaction between DCC and the ERM protein ezrin downstream of netrin-1

The objective was established following the identification by mass spectrometry of ezrin as a protein that interacts with DCC upon the phosphorylation of Y1418. In

order to complete this objective, the regulation and function of ERM proteins are evaluated in response to netrin-1/DCC signaling in cortical neurons.

Objective 2 (Chapter 3): Demonstrate that netrin-1 signaling induces the transient activation of RhoA

The data obtained during the completion of objective 1 suggests that netrin-1 induces the activation of RhoA in cortical neurons. This conclusion differs from previous studies that report that netrin-1 does not activate RhoA, but rather inhibits its activity (Li et al., 2002b; Moore et al., 2008a). To address the discrepancy, the spatiotemporal regulation of RhoA in response to netrin-1 is assessed using FRET imaging in live neurons.

Objective 3 (Chapter 4): Characterize the functional interaction between p120RasGAP and DCC downstream of netrin-1

The discovery that the N-terminus of p120RasGAP interacts with DCC upon the phosphorylation of Y1418 not only raises questions concerning the function of p120RasGAP, but also regarding the regulation and the role of Ras activity during netrin-1-dependent axon guidance. To answer these questions, the molecular mechanisms that regulate the interaction between p120RasGAP and DCC are

examined and the function of p120RasGAP is assessed during netrin-1-dependent axon outgrowth and chemoattraction.

Preface to Chapter 2

Post-translational modifications such as phosphorylation create binding sites that mediate dynamic protein-protein interactions (Seet et al., 2006). The phosphorylation of the conserved Y1418 residue of DCC is essential for netrin-1 signal transduction in the central vertebrate nervous system (Li et al., 2004; Meriane et al., 2004).

The rationale of the study presented in Chapter 2 is to characterize the function of proteins that interact with DCC following the netrin-1-dependent phosphorylation of Y1418 in rat embryonic cortical neurons. Mass spectrometry has proven to be a valuable tool in the search for novel protein-protein interactions and was used here to search for rat embryonic brain proteins that interact with DCC upon the phosphorylation of Y1418. Several potential phospho-dependent interactions were identified using this method, but the interaction between DCC and the ERM protein ezrin is of particular interest because the regulation of actin-binding proteins is essential to axon outgrowth and guidance (Dent et al., 2011; Gomez and Letourneau, 2014; Vitriol and Zheng, 2012). This chapter describes the molecular mechanisms that mediate the recruitment of ezrin to DCC as well as the activation of ERM proteins downstream of netrin-1 in cortical neurons. The significance of those mechanisms is highlighted by the discovery that ezrin activity is required for netrin-1-dependent axon outgrowth. At the time of its publication in Molecular Biology of the Cell in 2011, this study was the first report on the regulation and the function of ERM proteins downstream of netrin-1 and DCC.

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Chapter 2 - The activation of Ezrin-Radixin-Moesin (ERM) proteins is regulated by netrin-1 through Src kinase and RhoA/Rho kinase activities and mediates netrin-1-induced axon outgrowth

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2.1 Abstract

The receptor Deleted in Colorectal Cancer (DCC) mediates the attractive response of axons to the guidance cue netrin-1 during development. Upon netrin-1 stimulation, DCC is phosphorylated and induces the assembly of signaling complexes within the growth cone, leading to activation of cytoskeleton regulators, namely the GTPases Rac1 and Cdc42. The molecular mechanisms that link netrin-1/DCC to the actin machinery remain unclear. In this study we seek to demonstrate that actin-binding proteins, ezrin-radixin-moesin (ERM), are effectors of netrin-1/DCC signaling in embryonic cortical neurons. We show that ezrin associates with DCC in a netrin-1-dependent manner. We demonstrate that netrin-1/DCC induce ERM phosphorylation

and activation, and that the phosphorylation of DCC is required in that context. Moreover, Src kinases and RhoA/Rho kinase activities mediate netrin-1-induced ERM phosphorylation in neurons. We also observed that phosphorylated ERM proteins accumulate in growth cone filopodia where they colocalize with DCC upon netrin-1 stimulation. Finally, we show that loss of ezrin expression in cortical neurons significantly decreased axon outgrowth induced by netrin-1. Together, our findings demonstrate that netrin-1 induces the formation of an activated ERM/DCC complex in growth cone filopodia, which is required for netrin-1–dependent cortical axon outgrowth.

2.2 Introduction

During the development of the central nervous system (CNS), extracellular cues guide axons to the appropriate cellular target. At the budding periphery of axons, the neuronal growth cone integrates attractive and repulsive sensory cues and translates them into the appropriate response (Guan and Rao, 2003; Lowery and Van Vactor, 2009). The GTPases Rac1, Cdc42 and RhoA, members of the Rho family of small GTPases, are major intracellular signaling molecules regulated downstream of most, if not all, axon guidance cues, including the netrins, ephrins, semaphorins and slits (Govek et al., 2005; Huber et al., 2003; O'Donnell et al., 2009). In the context of axon outgrowth and guidance, Rho GTPases drive the cytoskeletal rearrangements that mediate processes such as membrane protrusion or retraction, axonal shaft

consolidation and receptor endocytosis (Hall and Lalli, 2010; Lowery and Van Vactor, 2009).

Netrins are conserved bifunctional axon guidance molecules that can either attract or repel growing axons depending on the nature of the neuron (Rajasekharan and Kennedy, 2009). In vertebrates, netrin-1 attracts and promotes the growth of a wide variety of neuronal cell types, including cortical and spinal commissural neurons (Kennedy *et al.*, 1994; Metin *et al.*, 1997; Richards *et al.*, 1997). The receptor Deleted in Colorectal Cancer (DCC) mediates the attractive responses induced by netrin-1 and is expressed in the spinal cord and the forebrain (Keino-Masu *et al.*, 1996; Shu *et al.*, 2000). A deficiency in either netrin-1 or DCC expression prevents the formation of spinal and cerebral commissures (Fazeli et al., 1997; Serafini et al., 1996). In recent years, the signaling cascade regulated by netrin-1 and DCC has been extensively studied. Netrin-1 induces the phosphorylation of DCC on tyrosine, serine and threonine residues (Meriane *et al.*, 2004). Src kinase activity and netrin-1-dependent phosphorylation of the C-terminal tyrosine residue 1418 (Y1418) of DCC are essential to netrin-1-mediated axon outgrowth and attraction (Li *et al.*, 2004; Meriane *et al.*, 2004; Ren *et al.*, 2008). Ultimately, netrin-1/DCC signal transduction leads to Rac1 and Cdc42 activation (Li et al., 2002b; Shekarabi and Kennedy, 2002), while RhoA activity is inhibited (Moore et al., 2008a). Although the F-actin regulators N-WASP and ADF/cofilin have been implicated in netrin-1 signaling (Marsick et al., 2010; Shekarabi et al., 2005), the regulation of

actin-binding proteins in the context of netrin-1/DCC signaling is still poorly understood. The ezrin-radixin-moesin (ERM) proteins are conserved molecules that bridge the plasma membrane with the actin cytoskeleton. They share a similar domain structure that is characterized by a N-terminal FERM (Four-point one, ezrin, radixin, moesin) domain, an α-helical linker region, and a C-terminal F-actin-binding domain (Fehon *et al.*, 2010). In the cytoplasm, the phosphorylation of a conserved C-terminal threonine residue is a crucial step in the activation of ERM proteins (Fievet et al., 2004; Gary and Bretscher, 1995; Gautreau et al., 2000). Upon phosphorylation, activated ERM proteins translocate from the cytoplasm to the plasma membrane where their FERM domain binds to membrane-associated proteins such as cell-surface receptors (McClatchey and Fehon, 2009). Through their affinity for F-actin and their recruitment to the cell cortex, ERM proteins create an interface between transmembrane receptors and the cortical actin network (Charrin and Alcover, 2006; Fehon et al., 2010; Niggli and Rossy, 2008). ERM proteins, including the brain-specific homolog merlin, are expressed during the development of the CNS (spinal cord and brain) in mouse and rat embryos (Kikuchi et al., 2002; Paglini et al., 1998; Ramesh, 2004; Saotome et al., 2004). In primary embryonic neurons, ERM protein activity has been associated with growth cone dynamics, neurite outgrowth and branching (Cheng et al., 2005; Haas et al., 2007; Paglini et al., 1998)

Here, we demonstrate that ERM proteins are effectors of netrin-1/DCC signaling in embryonic cortical neurons. We show that ezrin interacts with DCC in a netrin-1-dependent manner. In response to netrin-1/DCC stimulation, ERM proteins are phosphorylated and activated in cortical neurons. Furthermore, we show that Src kinases and RhoA/Rho kinase activities are required for netrin-1 to mediate ERM activation. Additionally, netrin-1 induces the accumulation of phosphorylated ERM proteins (pERM) in growth cone filopodia where they colocalize with the receptor DCC. Finally, impairing ERM activity inhibits DCC-mediated neurite outgrowth in mouse N1E-115 neuroblastoma cells and loss of ezrin expression blocks netrin-1-induced axon outgrowth in primary cortical neurons. Altogether, these results show that netrin-1 induces the formation of a pERM-DCC complex in growth cone filopodia, and that this complex is required for netrin-1-dependent cortical axon outgrowth.

2.3 Materials and methods

Plasmids

The plasmids pRK5, pRK5-DCC, pRK5-DCC-Y1418F and pEGFP were previously described (Li et al., 2002b; Meriane et al., 2004). pRK5-DCC (1-1120) was previously described (Tcherkezian *et al.*, 2010). For ezrin constructs, mammalian expression plasmids (pCB6-ezrin-VSVG, pCB6-ezrinT567D-VSVG, pCB6-ezrinT567A-VSVG, pCB6-ezrinΔ29-VSVG) and the plasmids encoding glutathione *S*-transferase (GST), GST-ezrin and GST-ezrinNT were kindly provided by Dr M. Arpin (Institut Curie, Paris,

France) and previously described (Algrain et al., 1993; D'Angelo et al., 2007; Gautreau et al., 2000; Gautreau et al., 1999).

Cell culture and transfection

Cell culture was maintained in a humidified incubator at 37ºC with 5% CO_2. HEK293 cells and N1E-115 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Wisent Bioproducts) supplemented with 10% fetal bovine serum and antibiotics. N1E-115 cells were plated on laminin (25 µg/mL, BD Biosciences)-treated dishes. Cells were transfected overnight with pRK5, pRK5-DCC, pRK5-DCC (11120), pCB6-ezrin-VSVG, pCB6-ezrinΔ29-VSVG or pCB6-ezrinT567D-VSVG using polyethylenimine (PEI, PolyScience) prepared according to the manufacturer's instructions. Briefly, HEK293 cells and N1E-115 cells were plated in 100 mm-dishes and transfected when they reached 70-80% of confluency. cDNA constructs (5 µg) were incubated with PEI (30 µg) in 1 mL of DMEM for 15 minutes. The transfection mixes were then added to cells with 9 mL of fresh supplemented DMEM. For the neurite outgrowth assay, N1E-115 cells were plated in 35 mm-dishes at a cell density of 1.25 x 10^6 on laminin-treated coverslips. The next day, cells were transfected with pRK5, pRK5-DCC, pCB6-ezrin-VSVG, pCB6-ezrinT567D-VSVG, pCB6-ezrinT567A-VSVG, or pCB6-ezrinΔ29-VSVG using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions.

Primary cortical neuron culture, transfection and electroporation

Cortical neurons from E18 rat embryos were dissociated mechanically and plated on poly-D-lysine (0.1 mg/mL; Sigma)-treated dishes. Neurons were cultured overnight in attachment medium: Minimal Essential Medium (MEM, Invitrogen) supplemented with 1 mM sodium pyruvate (Invitrogen), 0.6% D-glucose (Sigma) and 10% horse serum. The medium was replaced the next day with maintenance medium: Neurobasal-A medium (Invitrogen) supplemented with 2% B27 (Invitrogen) and 1% L-glutamine (Invitrogen). For transfections, dissociated neurons were plated on poly-L-lysine (0.1 mg/mL, Sigma)-treated coverslips at a cell density of 75,000 cells/well in 24-well dishes. Neurons were cultured 6 hours in attachment medium. The medium was then replaced overnight with maintenance medium. Neurons were transfected at 1DIV for 24 hours with pEGFP, pCB6-ezrin-VSVG or pCB6-ezrinΔ29-VSVG using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. Neurons were treated for the indicated times with the following reagents: purified myc-netrin-1 (500 ng/mL); glutamate (50 µM) was generously provided by Dr D. Bowie (McGill University, Canada); function-blocking anti-DCC (AF5) antibody (5 µg/mL for 24h; Calbiochem), ImmunoPure mouse immunoglobulin G (IgGs) (5 µg/mL for 24h; Pierce), PP2 (10 µM overnight; Calbiochem), toxin B (1 ng/mL for 24h) previously described (Li et al., 2002b), C3 transferase (1 µg/mL for 6h; Cytoskeleton, Inc), NSC23766 (100 µM for 2h; Calbiochem), Y27632 (20µM for 6h; Calbiochem) DMSO (Fisher), and glycerol (Fisher).

Recombinant chick netrin-1 was produced and purified as previously described (Serafini *et al.*, 1994). The Amaxa Rat Neuron Nucleofector Kit (Lonza) was used to introduce 300nM of synthetic ezrin siRNA (ON-TARGETplus SMARTpool, Thermo Scientific), Cdc42 siRNA (Silencer Select; Ambion, Applied Biosystems) or Negative Control siRNA (Silencer #1; Ambion, Applied Biosystems) with or without 2ug of pmaxGFP Vector (Lonza) in cortical neurons as per the manufacturer's instruction.

Antibodies

The following antibodies were used for immunoblotting and immunofluorescence at the indicated concentrations: mouse monoclonal anti-DCC, clone G97-449 (BD Biosciences Inc), 1:4000 (immunoblot, IB) and 1:500 (immunofluorescence, IF); mouse monoclonal (AF5) anti-DCC (Calbiochem), 1:1000 (IB); mouse anti-actin (Sigma), 1:2000 (IB); rabbit polyclonal anti-ezrin (kindly provided by Dr M. Arpin) (Algrain *et al.*, 1993), 1:7000 (IB) and 1:500 (IF); mouse monoclonal anti-VSVG (Sigma), 1:40000 (IB) and 1:300 (IF); rabbit polyclonal anti-pERM [Ezrin (Thr567)/Radixin (Thr564)/Moesin (Thr 558)] (Cell Signaling Technology), 1:1000 (IB) and 1:300 (IF); rabbit polyclonal anti-Cdc42 (Cell Signaling), 1:500 (IB); donkey anti-mouse-alexa488 (Molecular Probes), 1:1000 (IF); goat anti-rabbit-TRITC (Sigma), 1:500 (IF).

Affinity purification and mass spectrometry

An affinity column was prepared using a phosphopeptide corresponding to amino acids 1409 to 1423 of rat DCC (Small Scale Peptide Synthesis, W.M. Keck Facility, Yale University, USA), phosphorylated on tyrosine 1418 (KPTEDPASVpYEQDDL) coupled to Affigel (Bio-Rad) according to the manufacturer's protocol. An unphosphorylated peptide and Affigel beads were used as negative controls. Protein lysates from E13 rat brains were loaded on each column and proteins bound to the affinity columns were eluted using a gradient of sodium chloride by Fast Protein Liquid Chromatography. The eluted proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and proteins were detected using the Coomassie blue gel staining method. An 85 kDa band that was only present in the phosphopeptide affinity purification was cut and sent to be identified by tandem mass-spectrometry. Protein identification was made with the Mascot software (Matrix Science).

GST pull-down

Recombinant GST proteins were purified as described (Gautreau *et al.*, 1999). Transfected HEK293 cells were lysed in 1% Triton X-100 lysis buffer (25 mM Tris-HCl pH7.5, 1% Triton X-100, 10 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM Na_2VO_4, 20 mM NaF, 1X Complete protease inhibitor cocktail from Roche). Lysates were precleared with 30 µL of glutathione-agarose beads (Sigma) for 2h at 4ºC and then incubated with 10 µg of either GST, GST-ezrin, or GST-ezrinNT fusion proteins coupled to glutathione-

agarose beads for 3h at 4ºC. Beads were washed three times in ice-cold lysis buffer and boiled in SDS sample buffer. Total cell lysates and GST pull-down-associated proteins were resolved by SDS-PAGE and transferred onto nitrocellulose membrane. Membranes were stained with Ponceau S (Sigma), immunoblotted with antibodies against DCC, and visualized using enhanced chemiluminescence (ECL) (PerkinElmer).

Reverse transcription polymerase chain reaction (RT-PCR)

mRNA was extracted from purified total RNA obtained from homogenized cortices or dissociated cortical neurons in culture (1, 2 and 3DIV) using the QIAshredder homogenizer and RNeasy Mini kits (QIAGEN) according to the manufacturer's protocol. First strand cDNA was synthesized using M-MLV reverse transcriptase (Invitrogen). The Expand High Fidelity PCR system (Roche) was used to amplify cDNA fragments (sizes are indicated) using the following forward and reverse primers: ezrin, GGAGGTTCGAAAGGAGAACC and ACCCAGACTTGTGCATTTCC, 233 bp; moesin, TGGTCCAGGAAGACTTGGAG and TGCACACGCTCATTCTTCTC, 205 bp; radixin, CTCCATGCTGAGAACGTCAA and CCTCGGGTTCTGCTAGTGAG, 242 bp; merlin, TGCTATGCCTCAGTCCACAG and TGCTGAGGTGTCAAGTCCTG, 248 bp; GAPDH, AGACAGCCGCATCTTCTTGT and CTTGCCGTGGGTAGAGTCAT, 207 bp. The primers were annealed at 55ºC, and 30 cycles were carried out.

Immunoprecipitations and Immunoblotting

Dissociated cortical neurons (2DIV) and transfected N1E-115 cells were lysed in RIPA lysis buffer (150 mM NaCl, 50 mM Hepes pH7.5, 1% NP-40, 10 mM EDTA pH8.0, 0.5% Sodium Deoxycholate, 0.1% SDS). Prior to cell lysis, cortical neurons were incubated with netrin-1 or control Neurobasal –A media. For immunoprecipitation, 1 mg of protein lysates were incubated with 20 µL of Protein G Sepharose (GE Healthcare) and 1.5 µg of anti-DCC antibodies (Calbiochem) or mouse IgGs for 3h at 4ºC. Beads were washed three times in ice-cold lysis buffer and boiled in SDS sample buffer. Immunoprecipitated proteins and total cell lysates were resolved by SDS-PAGE and transferred to nitrocellulose membrane for immunoblotting with the appropriate antibodies and visualized by ECL.

Quantitative densitometry of ERM phosphorylation and of Cdc42 and ezrin downregulation

Following treatment with the indicated reagents, treated and untreated dissociated cortical neurons (2DIV) were lysed in RIPA buffer following incubation with netrin-1 or control media. Protein lysates were resolved by SDS-PAGE and transferred to nitrocellulose for immunoblotting with anti-pERM, anti-ezrin, anti-Cdc42 and anti-actin antibodies. Quantitative densitometry for pERM, ezrin, Cdc42 and actin bands was measured using the Quantity One software (Bio-Rad). The ratio of the densitometry of

pERM over that of total ezrin, of Cdc42 over that of actin and of ezrin over that of actin was then calculated. Student's unpaired t-test was used for statistical analysis, and the data were presented as the mean of the pERM/ezrin, Cdc42/actin or ezrin/actin ratios ±SEM.

Immunofluorescence and microscopy

Transfected N1E-115 cells and dissociated cortical neurons (2DIV) were fixed with 3.7% formaldehyde in phosphate buffered saline (PBS) for 15 min, quenched in 0.1 M glycine for 5 min, permeabilized in 0.25% Triton X-100 for 5 min, and blocked with 0.2% or 10% bovine serum albumin (BSA) for 30 min, respectively. N1E-115 cells and cortical neurons were immunostained with primary (1h) and secondary antibodies (45 min) in 0.2% or 3% BSA, respectively. A 5-min wash in PBS was done between each step. For the pERM and DCC co-immunostaining in dissociated cortical neurons (2DIV), the trichloroacetic acid (TCA) fixation and staining methods described previously were used (Hayashi *et al.*, 1999). Phalloidin-TRITC (1:5000, Sigma) was used to visualize actin in N1E-115 cells. Glass coverslips were mounted with Prolong (Molecular Probes). Cells were examined with an Olympus IX81 motorized inverted microscope using the 40X U PLAN Fluorite and 60X U PLAN S-APO oil objective lenses. Images were recorded with a CoolSnap 4K (Photometrics) and analysed with the Metamorph software (Molecular Devices).

Quantification of colocalization using Pearson's correlation coefficient and quantification of the accumulation of phosphorylated ERM proteins (pERM) in growth cones

Dissociated cortical neurons were incubated with netrin-1 or control media at 2DIV, and immunofluorescence was performed. Images of the pERM and DCC co-immunostaining were acquired with the 60X objective lens and deconvoluted using the AutoQuant X2 software (MediaCybernetics). The Pearson's correlation coefficient (*r*) was then calculated for each set of images using the Metamorph software. More than 50 growth cones per condition were analysed in least 3 independent experiments. Student's unpaired t-test was used for statistical analysis, and the data were presented as mean *r* ±SEM. The accumulation of pERM in growth cones was determined by analysing more than 75 neurons in 3 independent experiments. Student's unpaired t-test was used for statistical analysis, and the data were presented as mean percentage ±SEM.

Axon outgrowth assay

Two hours after transfection or 24h after electroporation, netrin-1 (250 ng/mL), glutamate (50 µM) or control media was added to dissociated cortical neurons at 1DIV. Immunofluorescence was performed 24h after transfection. Images were acquired with the 40X objective lens and the length of each axon was measured using Metamorph.

Axon outgrowth was then represented as the percentage of axons that are longer than 35 or 50 μm after treatment with netrin-1 or glutamate over the percentage observed with control media for each transfection. More than 100 axons were counted per condition, and at least 3 independent experiments were performed. Student's unpaired t-test was used for statistical analysis, and the data were presented as mean percentage ±SEM.

2.4 Results

Netrin-1 regulates the interaction of ezrin with DCC in embryonic cortical neurons

We have previously demonstrated that DCC is phosphorylated by the Src kinase Fyn on Y1418 and that this phosphorylation is required to mediate Rac1 activation and neurite outgrowth (Meriane *et al.*, 2004). To identify proteins that interact with phosphorylated Y1418, we incubated embryonic day 13 (E13) rat brain protein lysates with Affigel beads coupled to a 15-amino acid DCC peptide phosphorylated on Y1418 or to an unphosphorylated control peptide (Figure 2.1A). The bound proteins were resolved by SDS-PAGE and stained with Coomassie blue (Figure 2.1A). Tandem mass spectrometry (MS/MS) analysis performed on one band of approximately 85 kDa (asterisk in Figure 2.1A), which was enriched after incubation with the DCC phosphopeptide, yielded 4 peptides corresponding to the ERM proteins, ezrin, radixin and moesin. The domain architecture of ezrin is characterized by the N-terminal FERM domain, the α-helical linker region, and the C-terminus that harbors the actin-binding

domain (Figure 2.1B). Since ERM proteins associate with proteins found at the plasma membrane via their FERM domain (Fehon *et al.*, 2010), we first determined whether the FERM domain of ezrin (ezrinNT) could interact with DCC *in vitro* by GST pull-down assays. Indeed, the FERM domain of ezrin expressed as a glutathione S-transferase (GST) fusion protein was found to interact with DCC overexpressed in HEK293 cells (Figure 2.1C). To further characterize the interaction, DCC was co-expressed with the constitutively active mutant protein ezrinT567D (ezrinTD) in N1E-115 cells, and a mouse monoclonal antibody against the extracellular domain of DCC was used to immunoprecipitate DCC from protein lysates. As shown in Figure 2.2A, VSVG-tagged ezrinT567D was able to co-immunoprecipitate with DCC. When ezrin was co-expressed with DCC in HEK293 cells stimulated with netrin-1, we observed that ezrin was able to interact with DCC in a netrin-1-dependent manner (Figure 2.2B). However, DCC lacking the intracellular domain (DCC (1-1120)) or inactive ezrin lacking the F-actin binding domain (ezrinΔ29) abolished the ezrin-DCC interaction (Figure 2.2B). These results confirm that the intracellular domain of DCC and the F-actin-binding domain of ezrin (including T567) are required to promote netrin-1-induced recruitment of ezrin to DCC in HEK293 cells. We next determined whether endogenous ezrin and DCC associate in embryonic cortical neurons. We first confirmed by RT-PCR that E18 cortical neurons cultured for 0 to 3 days *in vitro* (DIV) express ezrin mRNA, and found that all 3 ERM mRNAs as well as their brain-specific homolog merlin were expressed (Figure 2.2C).

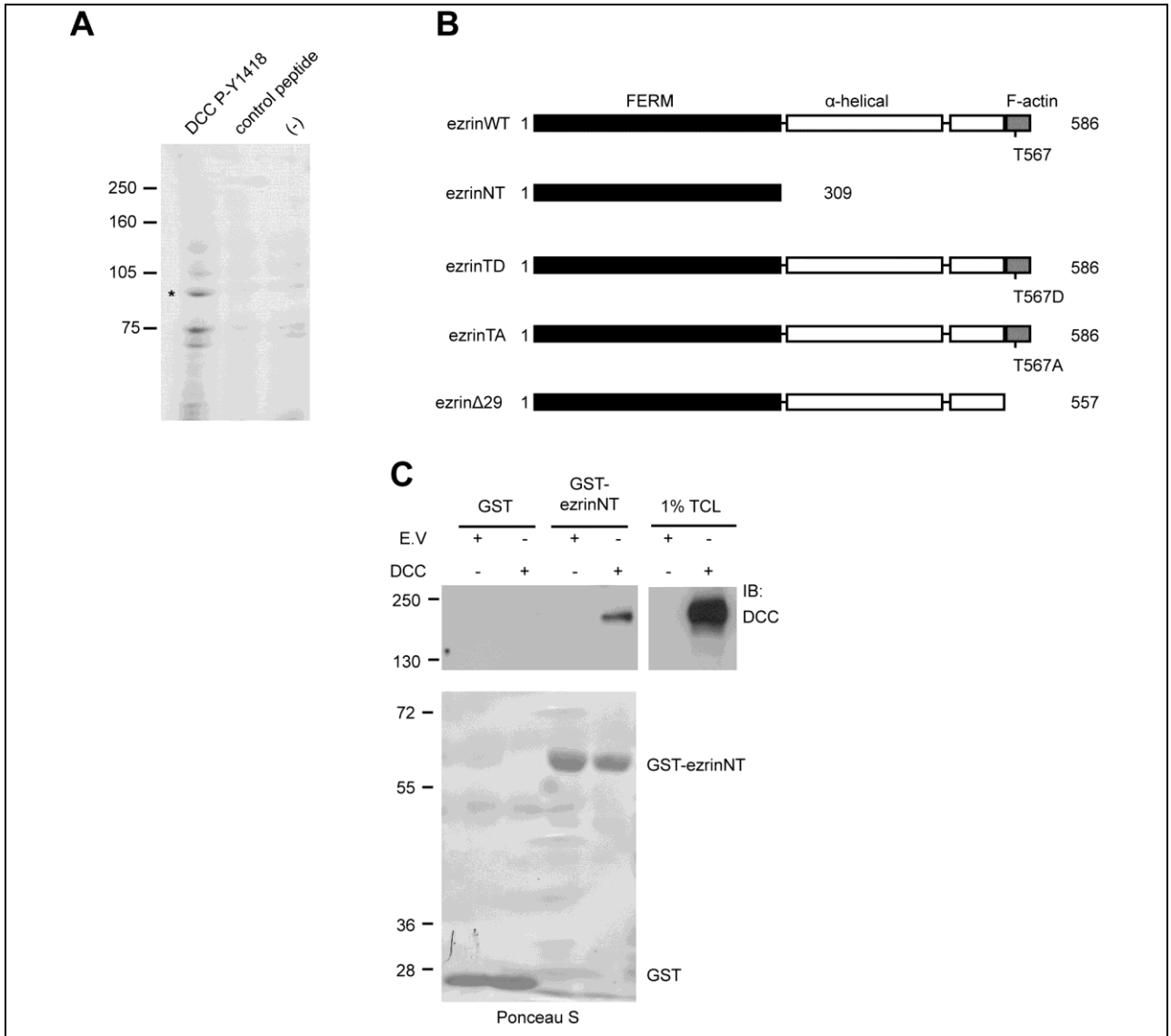


Figure 2.1 Ezrin interacts with DCC

(A) E13 rat brain protein lysates were incubated with Affigel-DCC-P-Y1418 phosphopeptide, Affigel-DCC unphosphorylated peptide (control) or Affigel beads (-). Bound proteins were resolved by SDS-PAGE and stained with Coomassie blue. Peptides matching the ERM protein sequences of ezrin, radixin and moesin were identified by MS/MS analysis of the band at approximately 85kDa represented by the asterisk (*). (B) The domain architecture of wild-type ezrin (ezrinWT) and ezrin mutant

proteins. EzrinWT consists of the N-terminal FERM domain, the α-helical linker region and the C-terminal F-actin-binding domain. (C) Lysates of HEK293 cells transfected with pRK5 or pRK5-DCC were incubated with GST or GST-ezrinNT fusion proteins coupled to glutathione-agarose beads (n=4). GST pull-down-associated proteins and 1% of total cell lysates (TCL) were resolved by SDS-PAGE and immunoblotted (IB) with antibodies against DCC. Purified GST proteins were stained with Ponceau S prior to immunoblotting (lower panel).

The protein expression of DCC and ezrin was also confirmed by immunoblot (Figure 2.2D). In rat E18 cortical neurons at 2DIV, netrin-1 transiently stimulated the interaction of ezrin and DCC (Figure 2.2E). Co-immunoprecipitation of ezrin with DCC was observed after 5 minutes of netrin-1 stimulation and was significantly reduced after a 20 minute-stimulation (Figure 2.2 E and F). Only 0.26% of the total amount of DCC expressed in HEK293 cells was found to interact with GST-ezrinNT (Figure 2.1C), whereas the interaction of ezrin with DCC was increased by 6 folds after stimulation of cortical neurons with netrin-1 (Figure 2.2F). Together, these results show that ezrin interacts with DCC via its FERM domain, that netrin-1 positively regulates the assembly of ezrin-DCC protein complexes in embryonic cortical neurons, and that this interaction is weak in the absence of netrin-1.

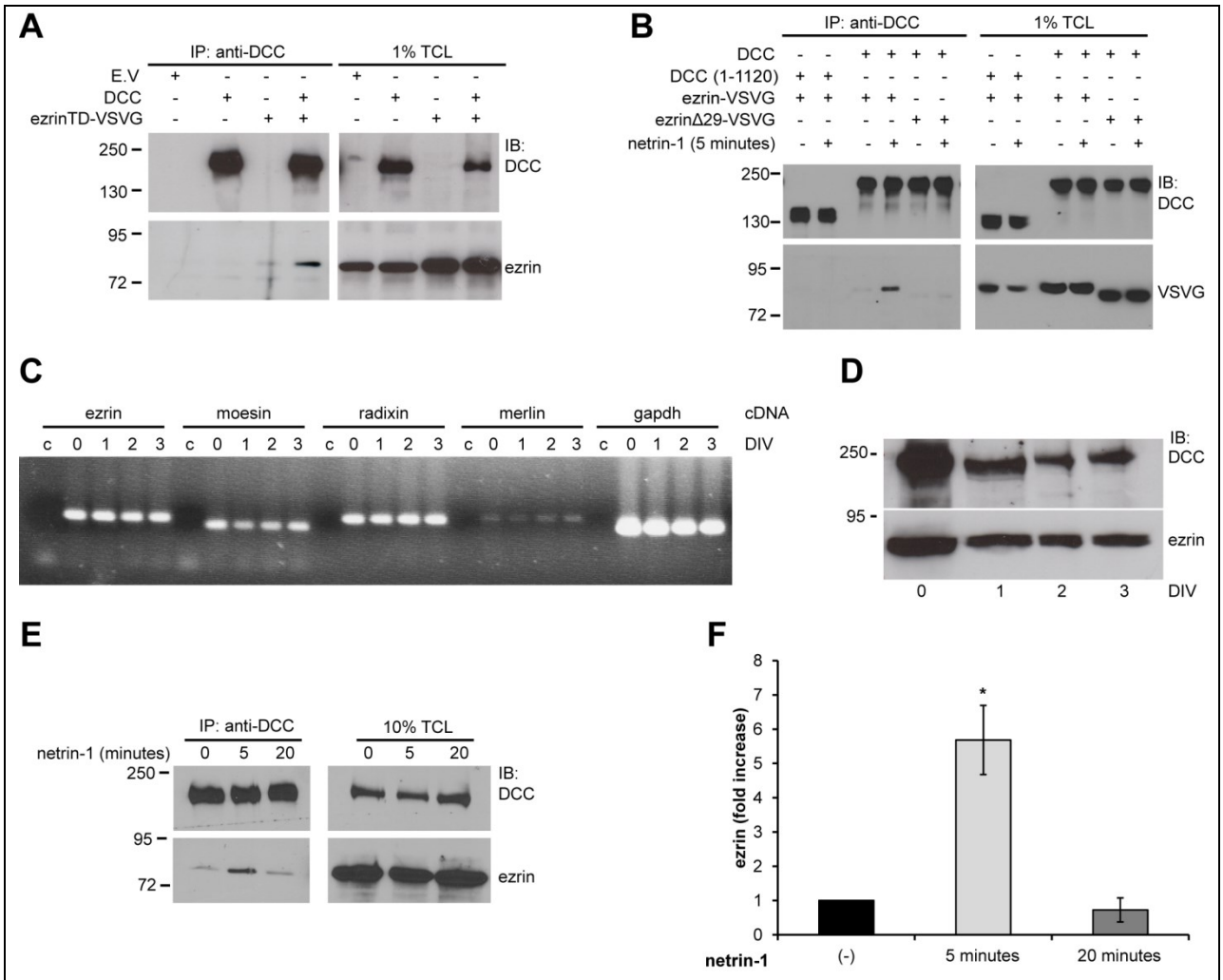


Figure 2.2 Netrin-1 regulates the interaction between ezrin and DCC

Protein lysates of N1E-115 cells (A) and HEK293 cells (B) transfected with empty vector (E.V.), pRK5-DCC, pRK5-DCC (1-1120), VSVG-tagged ezrinTD, ezrin wild-type, or ezrinΔ29 as indicated or (E) lysates of embryonic rat cortical neurons (E18, 2DIV) stimulated with netrin-1 for various periods of time were submitted to immunoprecipitation (IP) using anti-DCC antibodies. Immunoprecipitated proteins and 1% (A, B) or 10% (E) of total cell lysates (TCL) were resolved by SDS-PAGE followed by immunoblotting (IB) using anti-DCC, -ezrin or -VSVG antibodies. mRNA (C) or

proteins (D) were extracted from E18 cortices (0DIV) or cultured cortical neurons (1, 2, and 3 DIV). (C) RT-PCR amplification of cDNA from ezrin (233bp), moesin (205bp), radixin (242bp), merlin (248bp) and gapdh (207bp). Control reactions (c) were performed with water instead of cDNA for each primer pair. (D) Protein lysates of cortical neurons were resolved by SDS-PAGE and immunoblotted with antibodies against DCC or ezrin. (F) Quantitative densitometry of the amount of ezrin immunoprecipitated with DCC in (E) represented as the fold increase relative to unstimulated neurons (n=3). The increase in the amount of ezrin immunoprecipitated is significant after 5 minutes of netrin-1 stimulation (*$p<0.01$).

Netrin-1/DCC induces ERM phosphorylation in embryonic cortical neurons

Next, we wanted to assess whether netrin-1 and DCC regulate the activation of ERM proteins in neurons. The activation state of ERM proteins in cell lysates was monitored by immunoblotting against phosphorylated ERM proteins (pERM) using a phosphospecific ERM antibody that recognizes the phosphorylated form of the conserved C-terminal threonine residue in all three ERM proteins (Figure 2.1B). In embryonic cortical neurons stimulated with netrin-1 for different periods of time, we found that netrin-1 stimulation increased pERM levels after 5 minutes of stimulation (Figure 2.3A). There was a significant activation peak in ERM phosphorylation between 5 and 10 minutes of stimulation with pERM/ezrin ratios of 1.94±0.43 and 1.67±0.09,

respectively (Figure 2.3B). Past 10 minutes of netrin-1 stimulation, ERM phosphorylation decreased but appeared to be maintained. To confirm that DCC mediates the regulation of ERM proteins by netrin-1, dissociated embryonic cortical neurons were incubated with a function-blocking DCC antibody or with mouse immunoglobulin G (IgGs) as a negative control prior to netrin-1 stimulation. Blocking the function of DCC resulted in the inhibition of netrin-1-mediated ERM phosphorylation in cortical neurons (Figure 2.3 C and D), thus confirming that DCC is required for netrin-1 to mediate ERM phosphorylation.

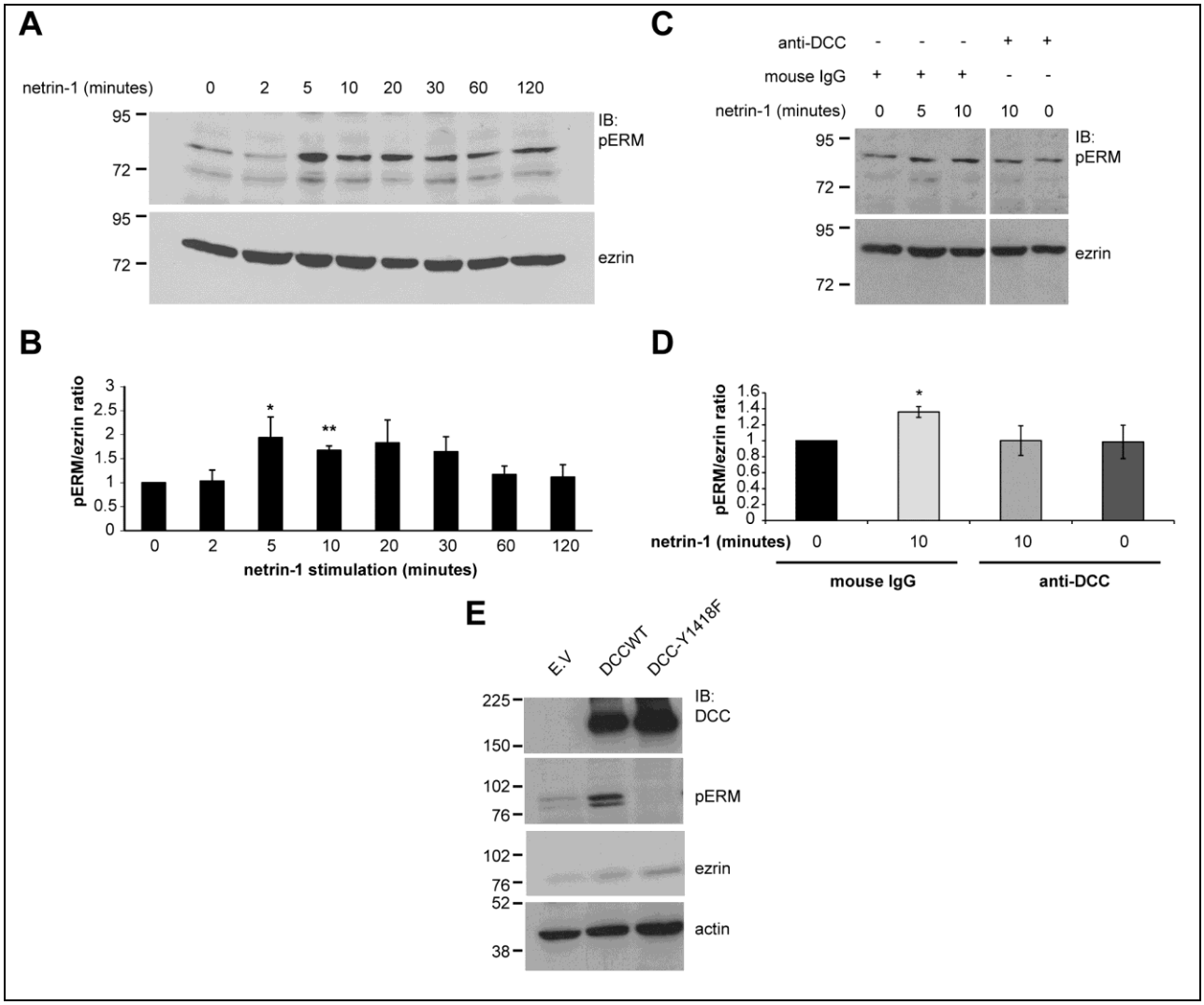


Figure 2.2 Netrin-1 induces the phosphorylation of ERM proteins through DCC in embryonic cortical neurons

(A) Protein lysates of embryonic rat cortical neurons (E18, 2DIV) stimulated with netrin-1 for various periods of time or (E) protein lysates of NIE-115 cells transfected with empty vector (E.V.), pRK5-DCC, or pRK5-DCC-Y1418F were resolved by SDS-PAGE followed by immunoblotting using anti-DCC, -pERM, -ezrin, or –actin antibodies. (B) Quantitative densitometry of (A) is represented as the ratio of pERM over total ezrin and corresponds to the average of at least three independent experiments. The increase in pERM levels after 5 and 10 minutes of netrin-1 stimulation was significant (*$p<0.05$; **$p<0.005$). (C) The experiment was as described in (A), except that function-blocking DCC antibodies, were added before netrin-1 stimulation. Mouse IgGs were used as a negative control. (D) Quantitative densitometry of (C) is represented as the ratio of pERM over total ezrin and corresponds to the average of at least three independent experiments. The increase in pERM after 10 minutes of netrin-1 stimulation is significant (*$p<0.05$). Error bars represent the standard error of the mean (SEM). (E) The upper pERM band represents ezrin and radixin and the lower band is moesin.

The phosphorylation of DCC on Y1418 has previously been shown to be important for netrin-1/DCC signaling (Li *et al.*, 2004; Meriane *et al.*, 2004; Ren *et al.*, 2008). In addition, we found that a DCC-Y1418 phosphopeptide was able to recruit

ERM proteins in embryonic rat brain protein lysates (Figure 2.1A). Thus, we examined whether the phosphorylation state of DCC-Y1418 influences pERM levels in N1E-115 cells, which endogenously express netrin-1 (Li et al., 2002b). We found that the expression of wild-type DCC in these cells increased significantly the phosphorylation of ERM proteins, whereas the expression of the phospho-deficient mutant DCC-Y1418F led to a reduction in ERM phosphorylation below the basal pERM levels observed in control cells transfected with empty vector (Figure 2.3E). Taken together, these results suggest that netrin-1 induces DCC-dependent ERM phosphorylation in cortical neurons and that the phosphorylation of DCC on Y1418 is required for netrin-1-mediated activation of ERM proteins.

Src kinases and RhoA/Rho kinase activities are required for netrin-1/DCC to mediate ERM activation

Axon outgrowth and guidance mediated by netrin-1/DCC signaling requires the function of Src family kinases (Li *et al.*, 2004; Liu *et al.*, 2004; Meriane *et al.*, 2004; Ren *et al.*, 2008). To determine whether Src kinases also regulate ERM phosphorylation in response to netrin-1, embryonic cortical neurons were treated with netrin-1 in the presence of the Src kinase inhibitor PP2 or DMSO as a control. Under these conditions, netrin-1 could no longer induce ERM phosphorylation in PP2-treated cortical neurons (Figure 2.4 A and B).

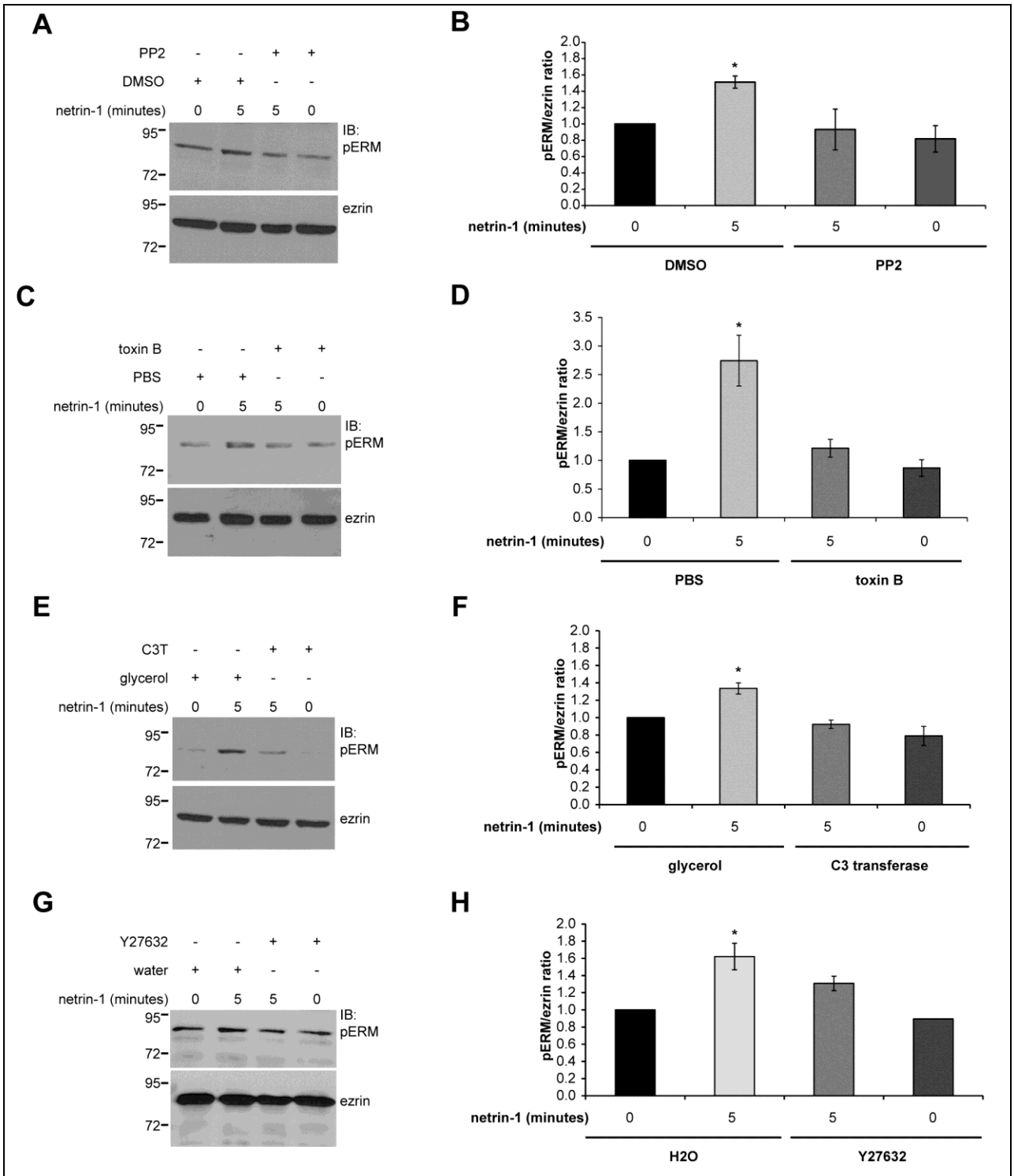


Figure 2.3 Src family kinases and RhoA/Rho kinase mediate ERM protein phosphorylation in cortical neurons stimulated with netrin-1

Embryonic rat cortical neurons (E18, 2DIV) were incubated with netrin-1 for the indicated times following treatment with either: (A and B) Src kinase inhibitor PP2 or DMSO as a negative control, (C and D) Rho GTPase inhibitor toxin B or PBS as a negative control, (E and F) RhoA inhibitor C3 Transferase (C3T) or glycerol as a negative control or (G and H) Rho kinase inhibitor Y27632 or water (H2O) as a negative control. Protein lysates were resolved by SDS-PAGE and immunoblotted with anti-pERM or -ezrin antibodies. Quantitative densitometry is represented as the ratio of pERM over total ezrin and corresponds to the average of at least three independent experiments. The increase in pERM after 5 minutes of netrin-1 stimulation was significant (*$p<0.05$), while there was no significant increase after the PP2, toxin B, C3 transferase or Y27632 treatment compared to unstimulated neurons (0 minutes). Error bars represent SEM.

Rho GTPases promote ERM phosphorylation in neuronal and non-neuronal cells (Haas et al., 2007; Jeon et al., 2010; Kim et al., 2010; Nakamura et al., 2000). In addition, netrin-1 has been found to regulate Rho GTPase activities in the mammalian brain and spinal cord (Briancon-Marjollet et al., 2008; Li et al., 2002b; Moore et al., 2008a). The requirement for Rho GTPase activity in netrin-1-mediated ERM phoshorylation was first evaluated by inhibiting the activity of RhoA, Rac1 and Cdc42 with toxin B in cortical neurons (Figure 2.4C). The toxin B treatment significantly

reduced the ability of netrin-1 to induce ERM phosphorylation (pERM/ezrin ratio = 1.21±0.15) compared to the pERM levels observed with the control PBS treatment (pERM/ezrin ratio = 2.74±0.44) (Figure 2.4 C and D). Toxin B treatment did not influence ERM phosphorylation in unstimulated cortical neurons, thus confirming that the inhibitory effect observed with toxin B is specific to netrin-1-induced ERM phosphorylation. We next determined the specific contribution of RhoA, Rac1, and Cdc42 in the context of ERM phosphorylation by inhibiting RhoA or Rac1 activities with C3 transferase (C3T) and the NSC23766 compound, respectively, or by downregulation of Cdc42 expression with synthetic siRNA targeting Cdc42. The inhibition of Rac1 by NSC23766 treatment or depletion of Cdc42 did not inhibit netrin-1-dependent ERM phosphorylation in cortical neurons (Supplemental Figure 2.1). On the other hand, the inhibition of RhoA by C3 transferase treatment was sufficient to block ERM phosphorylation induced by netrin-1 without altering the basal level of ERM phosphorylation in unstimulated cortical neurons (Figure 2.4 E and F). Furthermore, the inhibition of Rho kinase by Y27632 treatment also interfered with the ability of netrin-1 to induce ERM phosphorylation (Figure 2.4 G and H). Therefore, these data demonstrate that the activities of Src kinases and RhoA/Rho kinase are solicited downstream of netrin-1 and DCC to mediate ERM phosphorylation.

Netrin-1 induces the accumulation of pERM and their colocalization with DCC in neuronal growth cones

We next examined the localization of activated ERM proteins in netrin-1-stimulated cortical neurons by immunostaining neurons with antibodies specific for pERM. In unstimulated cortical neurons, pERM were mostly localized in the filopodia emanating from neuronal cell bodies and practically absent from axons or growth cones (Figure 2.5A). After 5 and 20 minutes of netrin-1 stimulation, the pERM immunostaining was clearly increased (Figure 2.5A), which is reminiscent of the increase in pERM levels observed in cortical neuron lysates (Figure 2.3B). Netrin-1 induced a significant accumulation of pERM in growth cones compared to unstimulated neurons (Figure 2.5A), whereas no significant difference was observed for the ezrin immunostaining (Supplemental Figure 2.2A). When images of the pERM and DCC immunostainings were merged, colocalization between pERM and DCC was observed in growth cones after 5 and 20 minutes of stimulation with netrin-1 and not in unstimulated neurons (Figure 2.5 A and B). Moreover, netrin-1-induced pERM-DCC colocalization appeared to be restricted to the tips of the neuronal growth cones, in filopodia (Figure 2.5B). However, the level of pERM-DCC colocalization induced by netrin-1 was variable after 20 minutes of stimulation (Figure 2.5B, a and b), an observation that was confirmed when Pearson's correlation was used to quantify pERM-DCC colocalization exclusively in growth cones. The Pearson's correlation coefficient (r) is considered significant when

its value is between 0.5 and 1. Thus, we found that pERM-DCC colocalization was only significant after 5 minutes of netrin-1 stimulation (r=0.55±0.03) compared with 20 minutes of stimulation (r=0.44±0.13) or with unstimulated growth cones (r=0.38±0.03) (Figure 2.5C). Interestingly, inhibition of RhoA and Rho kinase by toxin B, C3 transferase or Y27632 also abolished the accumulation of pERM in the growth cones (Figure 2.6 and Supplemental Figure 2.3). Therefore, these results are in good agreement with the observation that netrin-1 transiently induces the interaction of ezrin with DCC in cortical neurons (Figure 2.2E), and demonstrate that netrin-1 induces the accumulation of pERM in growth cone filopodia where they interact with DCC, in a RhoA/Rho kinase-dependent manner.

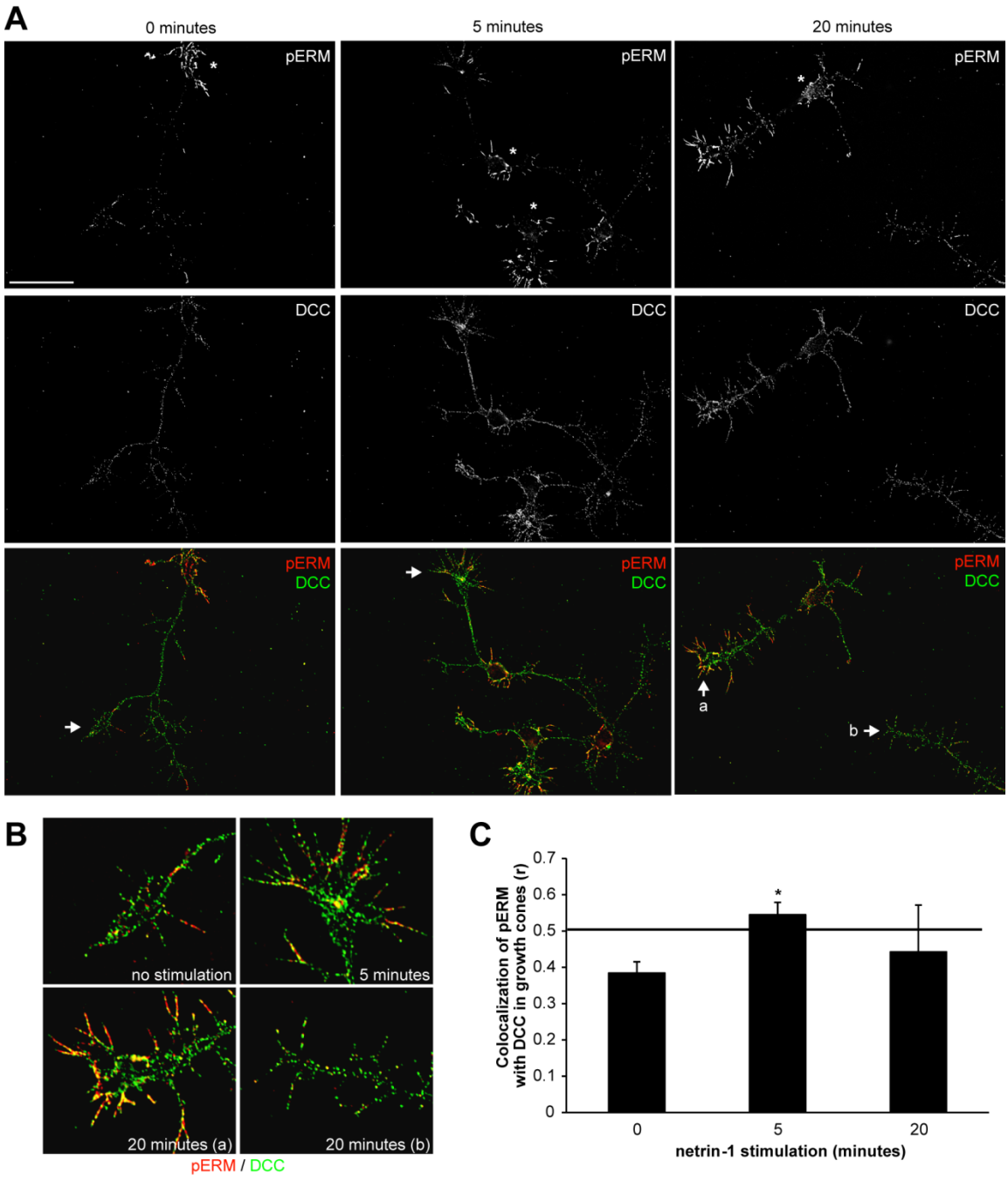


Figure 2.4 Netrin-1 induces the accumulation of phosphorylated ERM proteins and their colocalization with DCC in growth cone filopodia

(A) Embryonic rat cortical neurons (E18, 2DIV) were incubated with netrin-1 for the indicated times, fixed and immunostained with antibodies against DCC (green) and

pERM (red). Cell bodies are represented by asterisks (*). Scale bar, 5 μm. (B) Magnification of regions designated by arrows in (A). Growth cones after 20 minutes of netrin-1 stimulation: a) with pERM-DCC colocalization, b) with no significant pERM-DCC colocalization. (C) Quantification of pERM-DCC colocalization in growth cones using Pearson's correlation coefficient (*r*); *p<0.05. The minimal *r*-value for significant pERM-DCC colocalization is 0.5 (horizontal line). Error bars represent SEM.

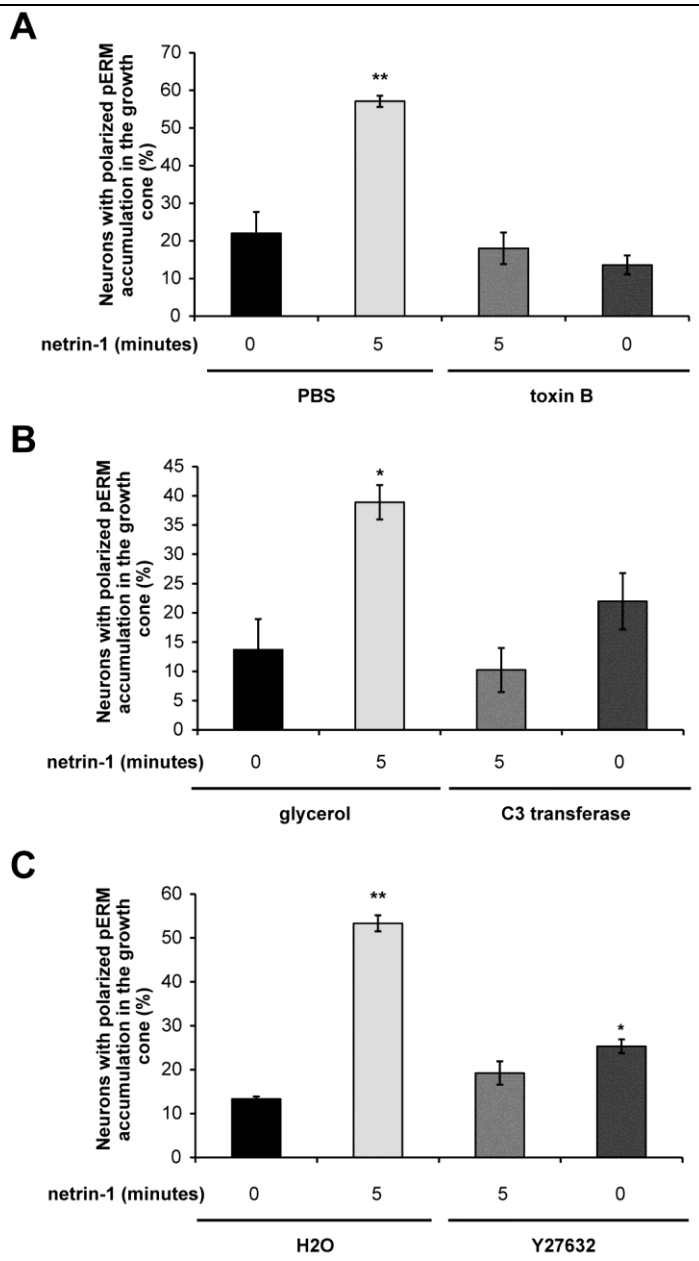


Figure 2.5 RhoA/Rho kinase are required to mediate the accumulation of phosphorylated ERM proteins in growth cones upon netrin-1 stimulation

Embryonic rat cortical neurons (E18, 2DIV) were incubated with netrin-1 for the indicated times following treatment with either: (A) Rho GTPase inhibitor toxin B or PBS as a negative control, (B) RhoA inhibitor C3 Transferase (C3T) or glycerol as a negative

control or (C) Rho kinase inhibitor Y27632 or water (H2O) as a negative control. Neurons were fixed and immunostained with anti-pERM and –DCC antibodies (Supplemental Figure 2.3). The quantification of the accumulation of phosphorylated ERM proteins (pERM) in growth cones corresponds to 3 independent experiments. Student's unpaired t-test was used for statistical analysis, and the data were presented as mean percentage ±SEM (*$p<0.05$ and **$p<0.005$).

Impairing ERM activity inhibits netrin-1-mediated axon outgrowth

The expression of DCC in N1E-115 cells induces neurite outgrowth (Figure 2.7 A and C) and was previously shown to occur in a netrin-1-dependent manner (Li et al., 2002b). To assess whether ezrin also induces neurite outgrowth in these cells, various ezrin protein mutants were expressed (Figure 2.7B). The expression of wild-type ezrin (ezrinWT) or constitutively active ezrinT567D (ezrinTD) induced a significant increase in neurite outgrowth compared with the empty vector control (Figure 2.7 B and C). Although the overexpression of ezrinWT or ezrinTD promoted neurite outgrowth in N1E-115 cells, the morphology of these neurites was different from those observed with DCC overexpression (Figure 2.7A). EzrinWT overexpression was associated with shorter neurites, and cells expressing ezrinTD exhibited more neuritic branching (Figure 2.7B). However, expression of ezrinT567A (ezrinTA), which keeps ezrin in its inactive conformation, or ezrinΔ29 in which the F-actin binding domain is deleted (Figure 2.1B)

did not induce neurite outgrowth in N1E-115 cells (Figure 2.7 B and C). The impact of ERM activity in DCC-mediated neurite outgrowth was then assessed by co-expressing DCC and ezrin protein mutants in neuroblastoma cells. The expression of the inactive protein mutants, ezrinTA or ezrinΔ29, significantly inhibited DCC-induced neurite outgrowth (Figure 2.7C). However, the percentage of transfected cells exhibiting neurite outgrowth was not altered when ezrinWT or ezrinTD were co-expressed with DCC (Figure 2.7C). Therefore, we conclude that ezrin is able to promote the extension of neurites in N1E-115 cells and that its activity is required for DCC-dependent neurite outgrowth.

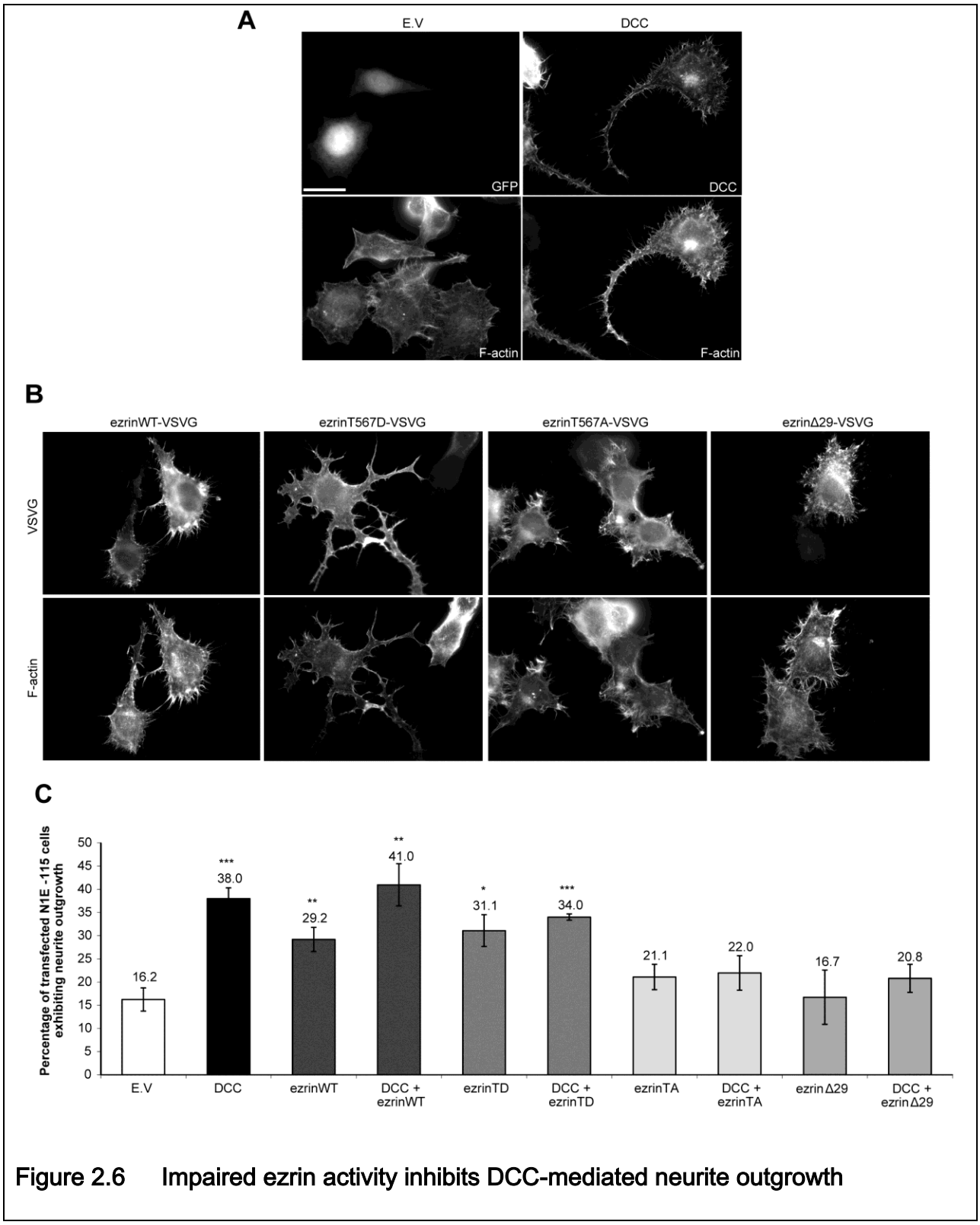


Figure 2.6 Impaired ezrin activity inhibits DCC-mediated neurite outgrowth

(A and B) N1E-115 cells were transfected with either pEGFP (E.V), pRK5-DCC, pCB6-ezrin-VSVG (ezrinWT), pCB6-ezrinT567D-VSVG (ezrinTD), pCB6-ezrinT567A-VSVG (ezrinTA) or pCB6-ezrinΔ29-VSVG (ezrinΔ29), fixed and immunostained with antibodies against DCC or VSVG. F-actin was labelled with phalloidin-TRITC (tetramethyl rhodamine isothiocyanate). Scale bar, 5 μm. (C) The percentage of transfected N1E-115 cells with neurites longer than their cell body was measured 24h post-transfection. The values correspond to the average of at least three independent experiments in which at least 150 transfected cells were counted. Error bars represent SEM (*p<0.05, **p<0.01 and ***p<0.001).

Since netrin-1 induces axon outgrowth in cortical neurons (Briancon-Marjollet et al., 2008; Liu et al., 2004), we examined the impact of ERM proteins on netrin-1-induced axon outgrowth in embryonic cortical neurons. Dominant negative ezrinΔ29 (EzΔ29) (D'Angelo *et al.*, 2007), wild-type ezrin (EzWT) or a green fluorescent protein (GFP) control were expressed in neurons stimulated with netrin-1 for 24 hours. Axon outgrowth was quantified as the percentage of transfected neurons that exhibit an axon longer than 35 μm over that of the control medium incubation (Figure 2.8). Netrin-1 did induce significant axon outgrowth in cortical neurons expressing GFP (Figure 2.8 A and B). However, it failed to promote axon outgrowth in neurons expressing ezrinΔ29, whereas the expression of ezrinWT did not affect the ability of netrin-1 to induce axon extension

(Figure 2.8A and B). Additionally, downregulation of ezrin expression by electroporation of synthetic siRNA targeting ezrin was sufficient to abolish the ability of netrin-1 to induce axon outgrowth (Figure 2.8 C and D). We have previously reported that glutamate stimulation induces axon outgrowth in embryonic cortical neurons (Briancon-Marjollet *et al.*, 2008). Similarly, we observed that glutamate was capable of inducing axon outgrowth, but the expression of ezrinΔ29 significantly impaired its ability to promote axon outgrowth in cortical neurons (Figure 2.8B). Collectively, these results reveal an important role for ezrin in mediating the effects of netrin-1/DCC signaling on axon extension. The implication of ERM proteins in glutamate-induced axon outgrowth also suggests that ERM proteins play a more general role in axon outgrowth that consists of coupling surface receptors to their downstream functions in response to extracellular cues.

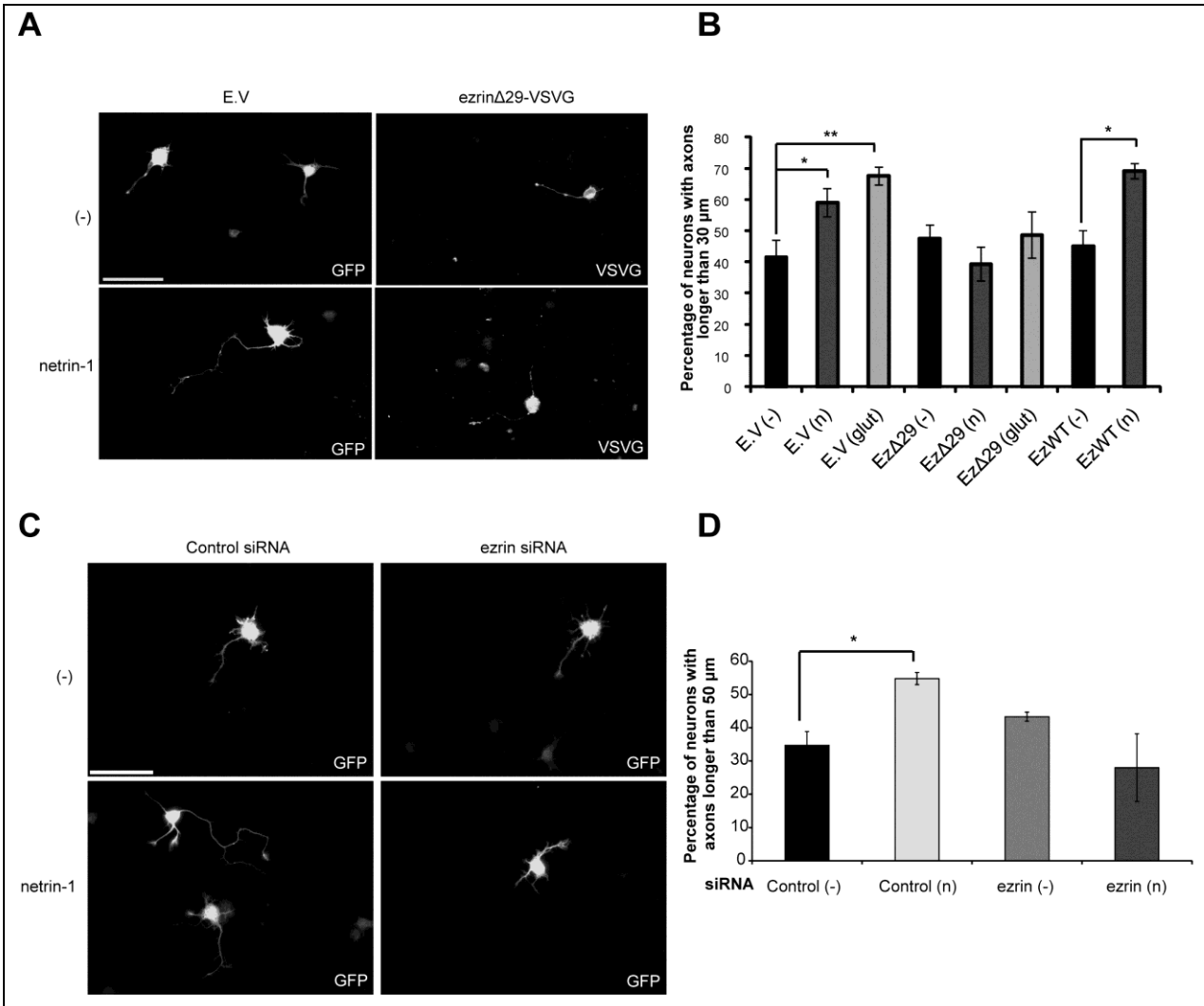


Figure 2.7 Dominant negative ezrin and ezrin downregulation inhibit netrin-1-mediated axon outgrowth

(A and B) Embryonic rat cortical neurons (E18) were transfected at 1DIV with pEGFP (empty vector, E.V), pCB6-ezrin-VSVG (ezWT) or pCB6-ezrinΔ29-VSVG (ezΔ29) and incubated 24 hours with netrin-1 or control medium. Neurons were fixed at 2DIV and immunostained with anti-VSVG antibodies. (C and D) Embryonic rat cortical neurons (E18) were electroporated at 0DIV with pmaxGFP and Negative Control siRNA or with pmaxGFP and ezrin siRNA to downregulate ezrin (Supplemental

Figure 2.2 B and C) and incubated 24 hours with netrin-1 or control medium. Quantification of axon outgrowth in transfected cells following a 24 hours-incubation with control medium (-), netrin-1 (n) or glutamate (glut) is represented as the percentage of transfected cortical neurons with axons longer than 35 µm (B) or 50 µm (D) in length. The values correspond to the average of at least three independent experiments. Error bars represent SEM (*p<0.05, **p<0.001). Scale bar, 50 µm.

2.5 Discussion

In this study, we provide evidence that the axon guidance cue netrin-1 transiently induces ERM phosphorylation and promotes the interaction of ezrin with DCC in embryonic cortical neurons. We show that netrin-1 induces the accumulation of pERM in filopodial protrusions and colocalization with DCC at the extremities of neuronal growth cones. Moreover, our findings demonstrate that ezrin is required for netrin-1 to induce axon outgrowth in primary cortical neurons. The interaction between DCC and ezrin has previously been described *in vitro* and in colonic cancer cells (Martin et al., 2006a; Tcherkezian et al., 2010). The intracellular domain (ICD) of DCC harbors a juxtamembrane ERM-binding region and its deletion prevented *in vitro* recruitment of FERM domains (ezrin and merlin) (Martin et al., 2006a). We also confirmed that the cytoplasmic tail of DCC is required to mediate the interaction DCC-ezrin in response to netrin-1 stimulation. Our proteomic data also reveals that the phosphorylation of the tyrosine residue 1418 positively regulates the interaction of ERM proteins with a

phosphopeptide corresponding to the C-terminus of DCC *in vitro*. This is in good agreement with the finding that the C-terminus of DCC mediates the interaction with the FERM-containing protein Myosin X (Zhu *et al.*, 2007). Thus, both the juxtamembrane region and the C-terminus of DCC are implicated in the recruitment of ERM proteins to DCC and our results suggest that phosphorylation of Y1418 is a regulatory step that promotes ERM-DCC complex formation in response to netrin-1 stimulation.

It is well established that the intramolecular interaction between the FERM domain and the C-terminus of ERM proteins inhibits their function (Berryman *et al.*, 1995; Pearson *et al.*, 2000). The affinity of the FERM domain for phosphatidylinositol 4,5 bisphosphate (PIP2) recruits ERM proteins to the plasma membrane (Barret *et al.*, 2000). Subsequently, phosphorylation of their conserved C-terminal threonine residue releases the FERM and the actin-binding domains (Fievet et al., 2004; Matsui et al., 1998). The sequential activation of ERM proteins enables them to interact with both the plasma membrane and the actin cytoskeleton. Recent findings have shown that the extracellular cues Sema3A, NGF and glutamate regulate ERM phosphorylation in neuronal cells (Jeon et al., 2010; Kim et al., 2010; Mintz et al., 2008). Here, we demonstrate that netrin-1 increases ERM phosphorylation in a DCC-dependent manner in primary cortical neurons. Of note, the formation of the ezrin-DCC protein complex and ERM phosphorylation occur in parallel and peak after 5 minutes of netrin-1 stimulation. Unlike the ezrin-DCC interaction that was not detected past 5 minutes of netrin-1

stimulation, ERM phosphorylation decreases but appears to be maintained. This suggests that netrin-1 regulates the interaction in time and space by restricting the pool of ezrin that can interact with DCC. We also show that Src kinase activity is required for netrin-1-induced ERM phosphorylation in cortical neurons. Src family kinases mediate tyrosine phosphorylation of DCC in response to netrin-1, which is essential for promoting netrin-1-dependent functions, such as axon outgrowth and guidance in vertebrates (Li *et al.*, 2004; Meriane *et al.*, 2004; Ren *et al.*, 2008). Furthermore, the expression of a phospho-deficient mutant DCC-Y1418F has been shown to inhibit Rac1 activation in COS-7 cells, neurite outgrowth in N1E-115 cells, and axon turning of *Xenopus* commissural neurons (Li *et al.*, 2004; Meriane *et al.*, 2004; Ren *et al.*, 2008). We provide further evidence of the significance of Y1418 phosphorylation in netrin-1 signaling by demonstrating that DCC expression increases ERM phosphorylation in N1E-115 cells, while DCC-Y1418F inhibits it. Taken together, these results reveal that Src kinase-dependent tyrosine phosphorylation of DCC mediates ERM activation in cortical neurons stimulated with netrin-1.

We found that inhibiting RhoA, Rac1 and Cdc42 with toxin B blocks netrin-1-dependent ERM phosphorylation in cortical neurons. Rho GTPases are regulated by netrin-1 and DCC in neuronal cells (Li et al., 2002b; Moore et al., 2008a; Picard et al., 2009; Shekarabi and Kennedy, 2002). In addition, they have also been implicated in ERM activation in neuronal cells. Indeed, RhoA and Rho-kinase promote ERM

phosphorylation in cortical and hippocampal neurons (Haas *et al.*, 2007; Kim *et al.*, 2010). A recent study also implicates the positive regulation of Akt-dependent moesin phosphorylation by Rac1 in PC12 cells (Jeon *et al.*, 2010). Although it has been reported that active Cdc42 promotes pERM accumulation in fibroblasts (Nakamura *et al.*, 2000), its role in ERM activation in neurons has yet to be defined. Our data reveal that inhibiting both Rac1 and Cdc42 does not decrease netrin-1-induced ERM phosphorylation, while RhoA and Rho kinase activities are required for netrin-1 to increase ERM phosphorylation in cortical neurons. Interestingly, *Moore et al. (2008)* have previously reported that netrin-1 induces whole-cell inhibition of RhoA activity in spinal commissural neurons and that prolonged RhoA inhibition promotes DCC-dependent axon outgrowth induced by netrin-1. Yet we find that RhoA/Rho kinase activity mediates the initial and short-lived peak in netrin-1-dependent ERM phosphorylation. Moreover, we have previously shown that DCC is able to locally activate RhoA in neurites using Fluorescence Resonance Energy Transfer (FRET) intermolecular probes (Picard *et al.*, 2009), suggesting that a spatial and temporal regulation of RhoA would be a required step for netrin-1/DCC to mediate ERM activation during axon extension. Without contradicting previous reports, our study offers new insight into the regulation of RhoA activity by netrin-1. We propose that RhoA signaling is regulated by netrin-1 in at least two different ways: 1) short-term local

activation to mediate ERM phosphorylation, and 2) long-term global inhibition to promote axon outgrowth.

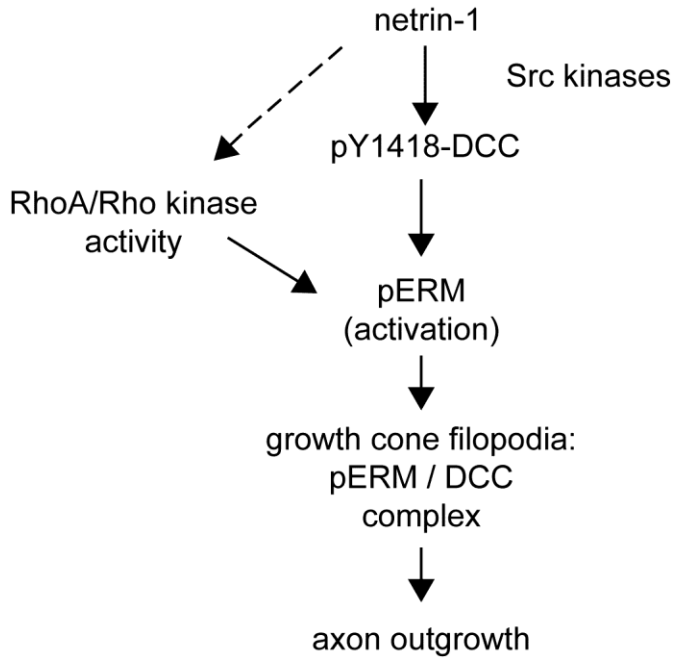
Once they are phosphorylated, activated ERM proteins have been shown to localize to plasma membrane protrusions such as microvilli in non-neuronal cells (Yonemura *et al.*, 2002) and growth cone filopodia in neurons where they are asymmetrically distributed (Haas *et al.*, 2004, 2007; Mintz *et al.*, 2008). Consistent with this, we found that netrin-1 stimulation of cortical neurons leads to the accumulation of pERM in growth cone filopodia where they colocalize with DCC, in a RhoA/Rho kinase-dependent manner. This suggests that activated ERM proteins localize to growth cones to mediate netrin-1-dependent functions. Both netrin-1 and Sema3A regulate the endocytosis of their respective receptors, DCC and it co-receptor Npn1, in growth cones (Piper *et al.*, 2005). In parallel, ERM protein activity was reported to mediate Sema3A-induced growth cone collapse by regulating the internalization of Npn1 and L1 (Mintz *et al.*, 2008). This highlights the interesting possibility that ERM proteins may regulate the internalization of DCC in response to netrin-1.

We show that the activity of ERM proteins is required for axon outgrowth induced by netrin-1 and DCC. In a separate study, Howe *et al.* have found that DCC forms a complex with ezrin and protein kinase A (PKA). Interestingly, they observed that silencing the expression of ezrin in rat hippocampal neurons impairs axon turning in response to netrin-1. Furthermore, downregulation of ERM expression in IMR-32 cells

blocks netrin-1-induced PKA activation and phosphorylation of its cytoskeletal targets VASP/MENA (Howe *et al.*, submitted). Given that ERM proteins are actin-binding proteins, loss of ERM activity in hippocampal and dorsal root ganglia neurons reduces filopodial dynamics in growth cones (Gallo, 2008; Paglini et al., 1998). Consequently, the inhibition of protrusive activity due to the loss of ERM expression severely impairs neurite outgrowth and axonal extension in hippocampal neurons (Paglini *et al.*, 1998). Filopodial protrusions are also positively regulated by netrin-1 in cortical neurons, while the axon guidance cue Sema3A has an inhibitory effect (Dent *et al.*, 2004). These data and those presented in our study support burgeoning evidence that ERM activation in neuronal growth cones promotes axon outgrowth by regulating filopodial dynamics. The transmembrane receptors L1, CHL1 and Fas have also been reported to mediate neurite branching through ERM proteins (Cheng *et al.*, 2005; Ruan *et al.*, 2008; Schlatter *et al.*, 2008). We found that ERM protein activity is also required for glutamate-induced axon outgrowth in cortical neurons, which confirms that the role of ERM proteins in axon outgrowth is not exclusive to netrin-1. Here, we propose a model in which netrin-1 promotes the phosphorylation of DCC and ERM proteins in a Src kinase and RhoA/Rho kinase-dependent manner to positively regulate their interaction in growth cone filopodia leading to axon outgrowth (Figure 2.9). In conclusion, this study contributes to our current knowledge of netrin-1/DCC signal transduction by providing

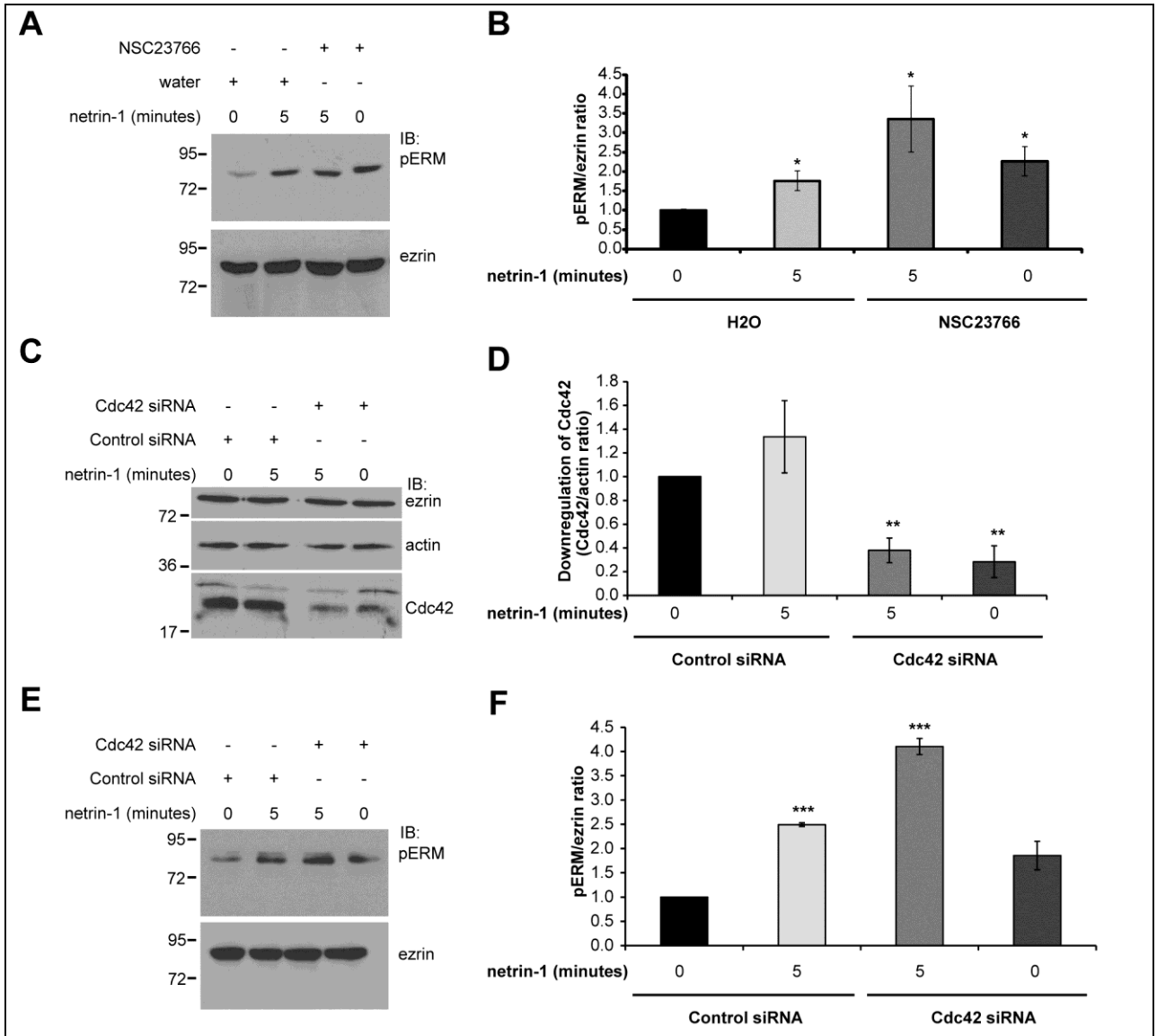
novel insight into the regulation and the function of actin cytoskeleton regulators in the context of axon outgrowth.

Figure 2.8 Regulation and function of the ERM-DCC complex downstream of netrin-1 in cortical neurons



Netrin-1 promotes tyrosine phosphorylation of DCC via Src kinases. The signaling cascade downstream of netrin-1 and its phosphorylated receptor promotes the phosphorylation of ERM protein in concert with RhoA and its effector Rho kinase. The mechanism by which netrin-1 activates RhoA has yet to be determined (dashed arrow). Upon their phosphorylation, activated ERM proteins (pERM) accumulate in growth cone filopodia where they form a complex with DCC. The phosphorylation of the receptor on Y1418 positively regulates ERM protein activation thus promoting ERM-DCC complex assembly. Ultimately, the activity of ERM proteins promotes axon outgrowth downstream of netrin-1, likely through their interaction with both DCC and the actin cytoskeleton in growth cone filopodia.

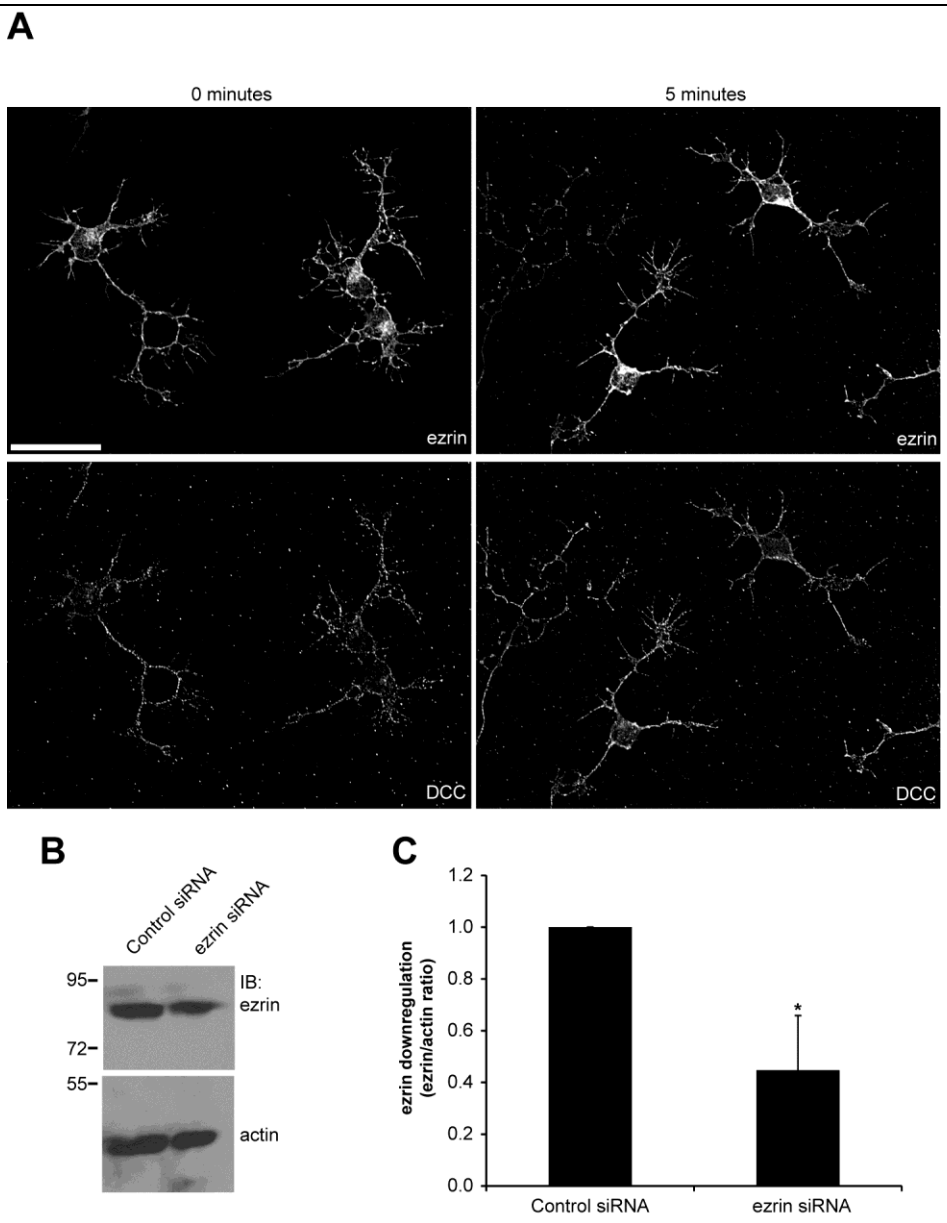
2.6 Supplemental information



Supplemental Figure 2.1 Netrin-1-dependent ERM phosphorylation is not blocked by Rac1 inhibition or Cdc42 downregulation

Embryonic rat cortical neurons (E18, 2DIV) were incubated with netrin-1 for the indicated times following treatment with either: (A and B) Rac1 inhibitor NSC23766 or water as a negative control or (C-F) Cdc42 siRNA or Control Negative siRNA

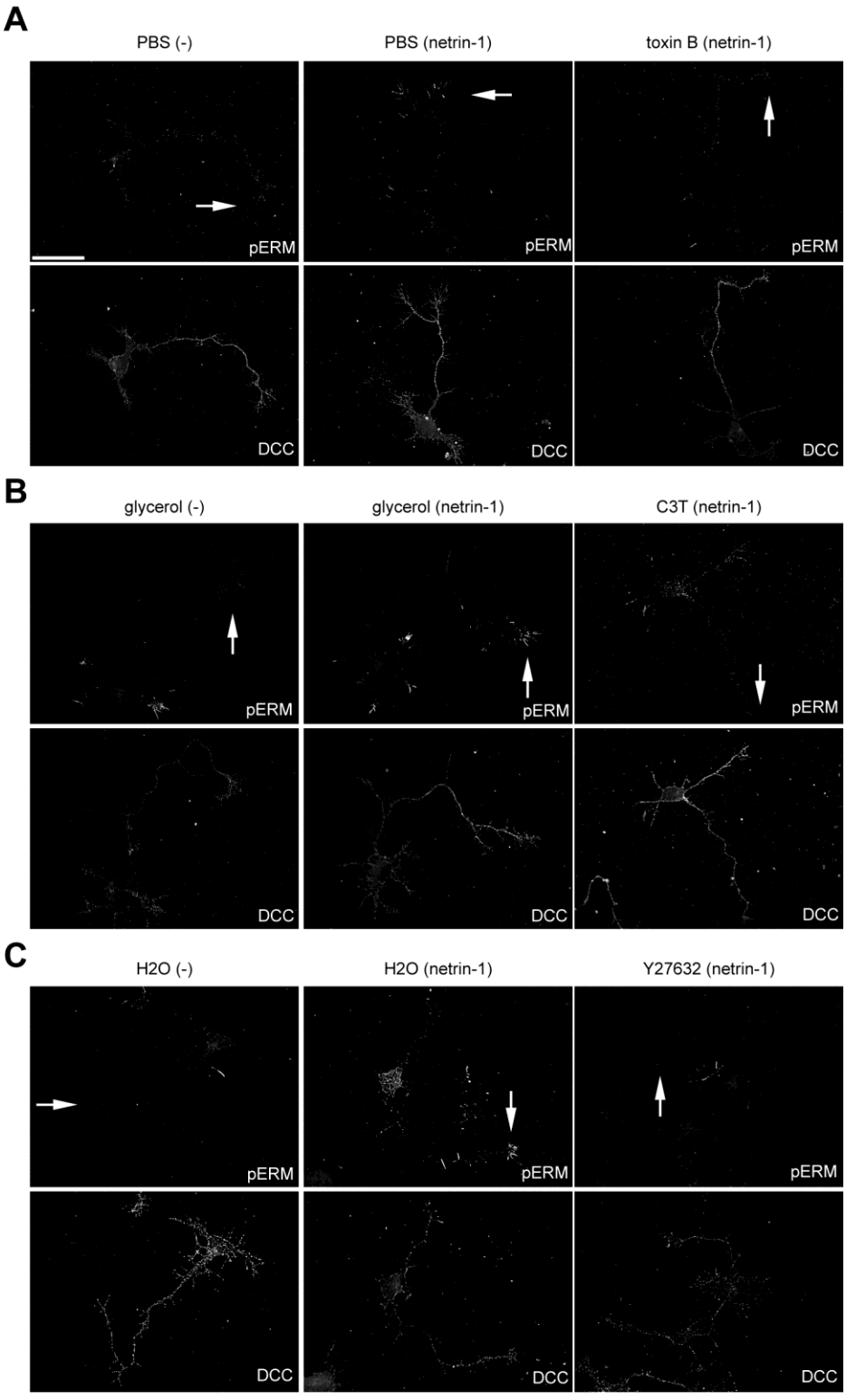
electroporation. Protein lysates were resolved by SDS-PAGE and immunoblotted with anti-pERM, –ezrin, -actin, or –Cdc42 antibodies. (D) Quantitative densitometry of Cdc42 downregulation is represented as the ratio of Cdc42 over actin. Cdc42 expression is downregulated by at least 62%. (F) Quantitative densitometry is represented as the ratio of pERM over total ezrin and corresponds to the average of at least three independent experiments. Error bars represent SEM (*$p<0.05$, **$p<0.01$, ***$p<0.005$).



Supplemental Figure 2.2 Subcellular localization and downregulation of ezrin in cortical neurons

(A) Embryonic rat cortical neurons (E18, 2DIV) were incubated with netrin-1 for 0 or 5 minutes, fixed and immunostained with antibodies against DCC and ezrin. Scale bar, 5 μm. Total ezrin proteins, unlike phosphorylated ERM proteins, do not accumulate in growth cones following netrin-1 stimulation. (B) Embryonic rat cortical neurons were

electroporated with Negative Control or ezrin siRNA and protein lysates were resolved by SDS-PAGE and immunoblotted with anti–ezrin and –actin antibodies. (C) Quantitative densitometry of ezrin donregulation in (B) is represented as the ratio of ezrin over actin. Ezrin expression is downregulated by 55%. Error bars represent SEM (*$p<0.05$).



Supplemental Figure 2.3 Toxin B, C3 transferase and Y27632 inhibit netrin-1-induced accumulation of phosphorylated ERM proteins in growth cones

Embryonic rat cortical neurons (E18, 2DIV) were incubated with netrin-1 for 0 or 5 minutes following treatment with either: (A) Rho GTPase inhibitor toxin B or PBS as a negative control, (B) RhoA inhibitor C3 Transferase (C3T) or glycerol as a negative control or (C) Rho kinase inhibitor Y27632 or water (H2O) as a negative control. Neurons were fixed and immunostained with anti-pERM and –DCC antibodies. The quantification of the accumulation of phosphorylated ERM proteins (pERM) in growth cones (arrows) is represented in Figure 2.6. Scale bar, 5 μm.

Preface to Chapter 3

The use of FRET imaging in live cells has provided tremendous insight into the dynamic regulation of Rho GTPase activation in migrating cells. The detection of RhoA activity in nascent membrane protrusions challenges the existing paradigm regarding the inhibitory effects of RhoA on protrusive activity (Pertz, 2010; Spiering and Hodgson, 2011). Similarly, FRET imaging in live neurons indicates that RhoA positively regulates growth cone motility (Fritz et al., 2013; Nakamura et al., 2005; Picard et al., 2009). However, the effects of axon guidance cues on RhoA activity have not been examined in growth cones using FRET imaging.

In light of the findings of Chapter 2, which suggest that RhoA is transiently activated downstream of netrin-1 and mediates ERM protein activation, the main objective of the next chapter is to confirm that netrin-1 activates RhoA since previous studies attest to the contrary (Li et al., 2002b; Moore et al., 2008a). The discrepancy is successfully addressed as netrin-1 is shown to mediate short-term region-specific activation of RhoA in rat embryonic cortical neurons. The use of FRET imaging was essential to this discovery since the subtle effects of netrin-1 on RhoA activity likely renders netrin-1-dependent RhoA activation undetectable in biochemical assays such as those previously used (Li et al., 2002b; Moore et al., 2008a). Rather than disproving former conclusions, Chapter 3 offers a discussion regarding the spatiotemporal

regulation of RhoA downstream of netrin-1 in neurons. An edited version of this chapter was accepted for publication in 2016 by Biology of the Cell.

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Chapter 3 – Direct measurement of oscillatory RhoA activity in embryonic cortical neurons stimulated with the axon guidance cue netrin-1 using FRET

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4.1 Abstract

Background information: Rho GTPases play an essential role during the development of the nervous system. They induce cytoskeletal rearrangements that are critical for the regulation of axon outgrowth and guidance. It is generally accepted that Rac1 and Cdc42 are positive regulators of axon outgrowth and guidance, while RhoA is a negative regulator. However, spatiotemporal control of their activity can modify the function of Rho GTPases during axonal morphogenesis. Signaling downstream of the axon guidance cue netrin-1 and its receptor Deleted in Colorectal Cancer (DCC)

triggers the activation of Rac1 and the inhibition of RhoA to promote axon outgrowth. However, our previous work also suggests that netrin-1/DCC signaling can activate RhoA in a time- and region-specific manner.

Results: Here, we visualized RhoA activation in response to netrin-1 in live embryonic cortical neurons using Fluorescence Resonance Energy Transfer (FRET). RhoA activity oscillated in unstimulated neurons and netrin-1 increased the amplitude of the oscillations in growth cones after 5 minutes of stimulation. Within this period of time, netrin-1 transiently increased RhoA activity and modulated the pattern of RhoA oscillations. We found that the timing of netrin-1-induced RhoA activation was different in whole neurons, cell bodies and growth cones.

Conclusions: We conclude that netrin-1 modulates the spatiotemporal activation of RhoA in embryonic cortical neurons.

Significance: This study demonstrates for the first time the short-term localized activation of RhoA in neuronal growth cones by the axon guidance cue netrin-1.

4.2 Introduction

During the development of the nervous system, the establishment of the neuronal circuitry is achieved by the integration of numerous intracellular signals induced by axon guidance cues (Bashaw and Klein, 2010). This signal integration takes place within the neuronal growth cone located at the tip of the growing axon where guidance cues induce dramatic cytoskeletal rearrangements, mediating growth cone attraction or

repulsion (Dent et al., 2011). Rho GTPases are important regulators of the actin and microtubule cytoskeletons and as such they are signaling targets for axon guidance cues (Antoine-Bertrand et al., 2011b; Hall and Lalli, 2010; O'Donnell et al., 2009). Rho GTPases are molecular switches that cycle between two conformational states, active/GTP-bound and inactive/GDP-bound. The current paradigm for the regulation of Rho GTPases during axon outgrowth and guidance specifies that Rac1 and Cdc42 promote attractive axonal growth while RhoA mediates neurite retraction and growth cone collapse (Gonzalez-Billault et al., 2012; Kozma et al., 1997; Thies and Davenport, 2003). During axon extension, the growth cone advances by cycling through the following three morphological phases: protrusion of actin filaments in the peripheral domain (P-domain), engorgement of the microtubules from the central domain (C-domain) into the newly formed protrusions, and consolidation of the growth cone into the axon shaft (Dent et al., 2011). Growth cone collapse and neurite retraction are mediated by an increase in RhoA activity, which causes the loss of polymerized actin (F-actin) and increases actomyosin contractility in the growth cones (Aizawa et al., 2001; Amano et al., 1998; Gallo, 2006; Murray et al., 2010; Zhang et al., 2003; Zhou and Cohan, 2001). Moreover, pharmacological inactivation of RhoA or Rho kinase (ROCK) promotes axon extension (Da Silva et al., 2003; Hirose et al., 1998; Jalink et al., 1994). Thus, the general consensus is that RhoA activity is inhibited during axon outgrowth and guidance (Hall and Lalli, 2010).

Netrin-1 and its receptor Deleted in Colorectal Cancer (DCC) have been shown to inhibit RhoA activity in commissural neurons (Moore et al., 2008a). However, we reported in a previous study using Fluorescence Resonance Energy Transfer (FRET) that RhoA is activated in DCC-expressing N1E-115 neuroblastoma cells that exhibited newly formed neurites (Picard et al., 2009). Since N1E-115 cells endogenously express netrin-1, we hypothesized that RhoA activation could be required for certain aspects of netrin-1/DCC-dependent axon outgrowth such as axon consolidation (Burnette et al., 2008; Loudon et al., 2006; Picard et al., 2009). Furthermore, we recently demonstrated that ezrin-radixin-moesin (ERM) proteins are required for netrin-1/DCC-mediated axon outgrowth and that their activation is mediated by RhoA/ROCK activities in response to netrin-1 (Antoine-Bertrand et al., 2011a). This study provided additional evidence that RhoA is activated by netrin-1/DCC signaling in cortical neurons (Antoine-Bertrand et al., 2011a). These seemingly conflicting reports highlight the importance of localization and timing for the regulation of RhoA activation during netrin-1/DCC-dependent signal transduction. We propose that RhoA signaling is regulated in at least two ways in response to netrin-1: short-term local activation in the growth cone (Antoine-Bertrand et al., 2011a), and long-term global inhibition (Moore et al., 2008a). However, transient local activation of RhoA by netrin-1 still remains to be demonstrated.

A number of studies have demonstrated that RhoA activity positively regulates growth cone dynamics, including axon branching and regeneration (Ahnert-Hilger et al.,

2004; Arakawa et al., 2003; Burnette et al., 2008; Fritz et al., 2013; Jin and Strittmatter, 1997; Kuhn et al., 1999; Loudon et al., 2006; Nakamura et al., 2005; Ohnami et al., 2008; Sebok et al., 1999). Thus, RhoA activity can support growth cone maintenance and axon outgrowth based on the cellular context. Here, we show early and transient activation of RhoA in response to netrin-1 stimulation in dissociated rat embryonic cortical neurons by FRET, which enabled the detection of subtle localized changes in RhoA activation. RhoA activity was measured over time by FRET in live whole neurons, as well as in specific neuronal regions such as the cell bodies and the growth cones. We detected RhoA activity oscillations in unstimulated cortical neurons. Netrin-1 stimulation modulated the pattern of RhoA oscillations and transiently increased RhoA activity, but the onset and duration of the increase varied in whole neurons, cell bodies and growth cones. Furthermore, the amplitude of RhoA oscillations was increased within 5 minutes of netrin-1 stimulation specifically in growth cones. Altogether, this study demonstrates that netrin-1 stimulation induces transient and local activation of RhoA in cortical neurons.

4.3 Materials and Methods

DNA constructs

YFP-RBD, CFP-RhoA, CFP-RhoAN19 and CFP-RhoAL63 were generated as described previously (Picard et al., 2009).

Cell culture and transfection

Cell culture was maintained in a humidified incubator at 37°C with 5% CO_2. Cortical neurons from E18 rat embryos were dissociated mechanically. 100 000 neurons were plated on 24-well glass bottom dishes (MaTek Corp, Ashland, MA) treated with poly-L-lysine (0.1 mg/ml; Sigma-Aldrich). Neurons were cultured in DMEM containing 10% FBS, which was replaced 4h later with Neurobasal-A medium supplemented with 2% B27 and 1% L-glutamine (Invitrogen). 24 hours later, Lipofectamine 2000 (Invitrogen) was used according to the manufacturer's instructions (DNA:Lipofectamine ratio of 1:2) to transfect 62.25 ng YFP-RBD with or without 125 ng CFP-RhoA, 62.25 ng CFP-RhoAN19 or 62.25 ng CFP-RhoAL63 per well. Neurons were then processed for live imaging.

Live cell imaging and Fluorescence Resonance Energy Transfer (FRET) microscopy

After 2 days *in vitro* (DIV), neurons plated on glass bottom dishes were imaged 12h post-transfection after the media was replaced with phenol free Neurobasal medium (Invitrogen) supplemented with 2% B27, 1% L-glutamine and 5mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES). For stimulations, neurons were treated with 500 ng/ml of recombinant netrin-1 produced and purified as previously described (Kennedy et al., 2006; Serafini et al., 1994), or with phosphate buffered saline (PBS) as a control. To compare the activity of RhoA, RhoAN19 and RhoAL63, neurons

were examined using an Olympus IX81 motorized inverted microscope (60× U PLAN S-APO oil objective lens) equipped with a chamber to maintain a temperature of 37 °C and a 5% CO_2 atmosphere. The fluorescent images were captured with the Semrock Multi Band CFP/YFP-2X2-OMF filter set for FRET and CoolSnap 4K camera (Photometrics). The exposure time for 2x2 binning was 20 ms. To determine RhoA activity in netrin-1-stimulated neurons, neurons were imaged at 37 °C and 5% CO_2 atmosphere with a Zeiss LSM780 confocal microscope (63×/1.40 oil Plan-Apochromat objective lens) for 10 minutes every 20s. The 458 nm and 514 nm argon lasers set at 2% intensity were used for excitation, and the GaAsP detectors were used for detection of the CFP channel (Ex. CFP/Em. CFP), YFP channel (Ex. YFP/Em. YFP) and FRET channel (Ex.CFP/ Em. YFP).

Image analysis

Images were analyzed with the MetaMorph software (Molecular Devices). The corrected FRET/CFP images for single and timelapse acquisitions were generated with the MetaMorph sensitized emission FRET dialog using the following formula: corrected FRET= raw FRET – (A x Acceptor) – (B x Donor). Acceptor, Donor and raw FRET images were obtained with the YFP, CFP and FRET acquisition settings, respectively. The fluorescence intensities of CFP, YFP and raw FRET images from neurons transfected with YFP-RBD, CFP-RhoA, CFP-RhoAN19 or CFP-RhoAL63 were used to

determine coefficients A (YFP correction factor: $FRET^{YFP}/YFP^{YFP}$) and B (CFP correction factor: $FRET^{CFP}/CFP^{CFP}$). Coefficient A was 0.19; coefficient B was 0.82 for CFP-RhoA and CFP-RhoAN19, and 0.80 for CFP-RhoAL63. Net FRET efficiency (nFRET) was determined by measuring the fluorescence intensity of the corrected FRET/CFP ratio images. An intensity value of 200 represents a FRET efficiency of 20%. For single acquisitions, nFRET efficiency was represented as the fold increase relative to the nFRET efficiency of CFP-RhoA. For timelapse acquisitions, maximum projections of CFP time series were used to define regions of interests (ROI: whole cell, cell body, growth cone, background) prior to fluorescence intensity measurements. For each cell time series, the timepoints for which the CFP fluorescence intensity was not within the standard deviation measured for all the acquired timepoints were considered to be out of focus and discarded. Normalized FRET efficiency ratios (rFRET) for each ROI were obtained by dividing the nFRET efficiency of the timepoints acquired between -40 s and 600 s by the highest nFRET efficiency measurement within the time series. For each ROI, the average rFRET between -40 s and 360 s and for each timepoint was measured.

CFP, YFP and FRET (FRET/CFP) images were generated from background subtracted single and timelapse acquisitions, respectively.

Amplitude and statistical measurements

The rFRET of each timepoint was substracted by the rFRET of the following timepoint to calculate the average rFRET peak-to-peak amplitude between -40 s and 360 s. Student's unpaired t-test was used for statistical analysis. The data are presented as the mean ± the standard error of the mean (SEM).

4.4 Results

Validation of the FRET intermolecular probes to study RhoA activation in cortical neurons

To examine RhoA activity in embryonic rat cortical neurons, we measured RhoA FRET efficiency using intermolecular CFP/YFP probes (Figure 3.1A). The FRET probes used in this study were previously validated in N1E-115 neuroblastoma cells and were the following: YFP-Rho binding domain (RBD) of Rhotekin as the acceptor, and CFP-RhoA, -RhoAN19 and -RhoAL63 as the donors (Picard et al., 2009). The constitutively active CFP-RhoAL63 and dominant negative CFP-RhoAN19 donor probes were used to validate the FRET measurements in cortical neurons. We transfected the CFP/YFP pairs in embryonic cortical neurons and measured the net FRET efficiency (nFRET) 12 hours post-transfection (Figure 3.1B), as described in Materials and Methods. The expression of CFP-RhoAL63 with YFP-RBD produced a 2-fold increase in nFRET compared to CFP-RhoA ($p=0.04$) (Figure 3.1C). As expected, the expression of dominant negative CFP-RhoAN19 in cortical neurons did not produce a significant increase in nFRET compared to CFP-RhoA (Figure 3.1C). These nFRET

measurements were comparable to those previously reported with the same FRET probes expressed in N1E-115 cells (Picard et al., 2009). Therefore, we conclude that RhoA activity can be accurately detected by FRET using the CFP-RhoA/YFP-RBD intermolecular probes expressed in embryonic cortical neurons.

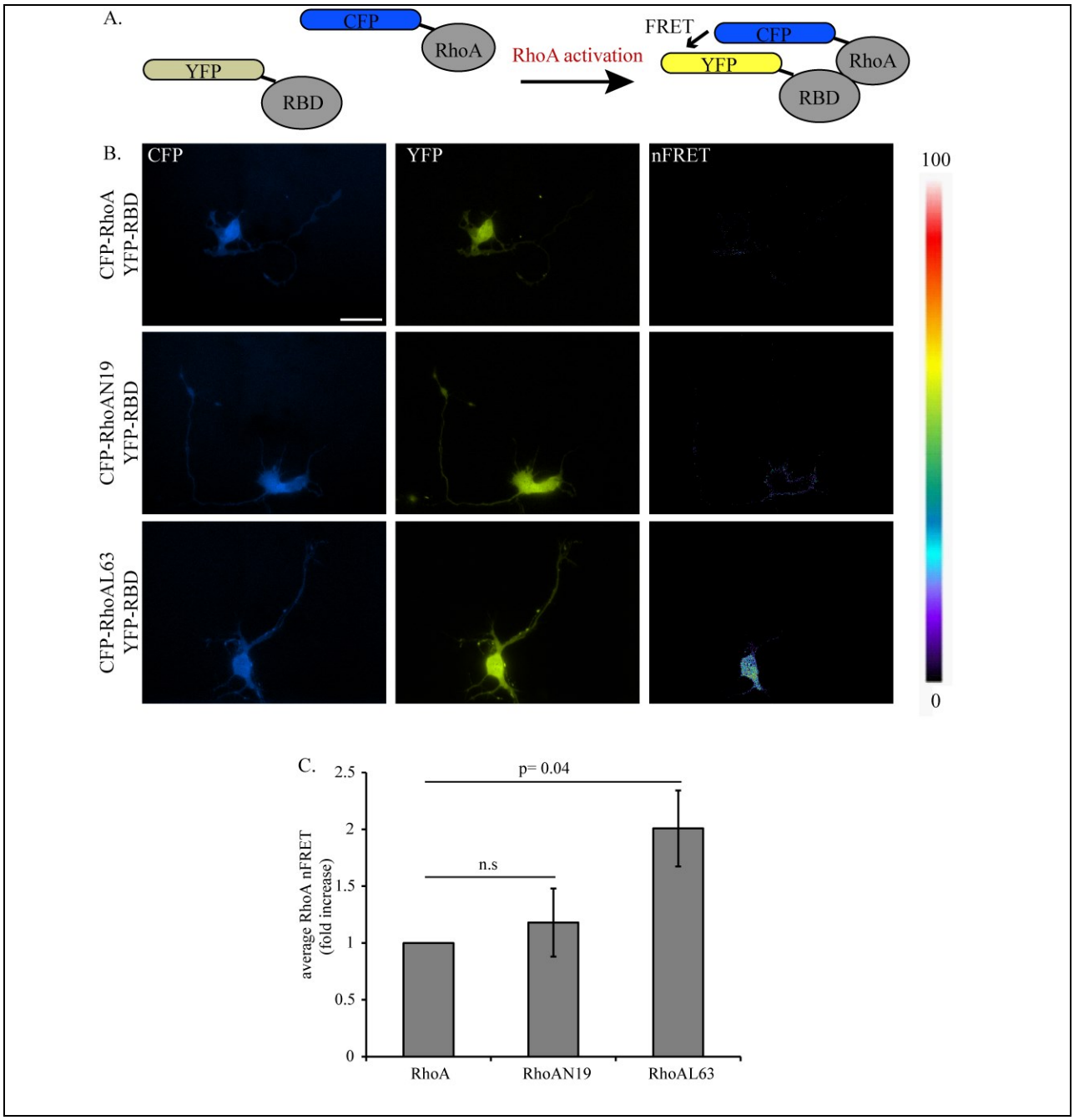

Figure 3.1 Constitutively active RhoA increases FRET efficiency in embryonic cortical neurons

(A) Schematic structure of the CFP-YFP intermolecular probes used to measure RhoA activity. FRET is measured when CFP-RhoA is activated and interacts with the effector domain YFP- RBD. (B) Representative CFP, YFP and FRET images of E18 rat cortical neurons expressing YFP-RBD with CFP-RhoA, CFP-RhoAN19 or CFP-RhoAL63 (number of neurons= 47, 39, 32, respectively) after 2 days *in vitro*. RhoA activity is displayed as net FRET efficiency (nFRET). nFRET was calculated from the fluorescence intensity of FRET/CFP ratio images (nFRET images). The nFRET intensity scale (0-100%) is represented on the right. Scale bar represents 20 µm. (C) RhoA activity is represented as the fold increase relative to average CFP-RhoA nFRET (±SEM). Student's unpaired t-test was used for statistical analysis over three independent experiments (n.s: non-significant).

Region-specific RhoA activity oscillations in embryonic cortical neurons

To determine RhoA activity in embryonic cortical neurons over a period of time, embryonic rat cortical neurons were transfected with the CFP-RhoA/YFP-RBD pair and imaged before and after being treated with 500ng/ml netrin-1 or PBS as a control. The onset of the treatment was set at 0 s. CFP images of unstimulated and netrin-1-treated neurons at time 0 s were used to define the regions of interest (ROI) (Figure 3.2A).

Since our previous work suggested that RhoA is activated within 5 minutes of stimulation with netrin-1 (Antoine-Bertrand et al., 2011a), we measured the nFRET efficiency between -40 s and 360 s (over 6 minutes). During this period of time, we observed that RhoA nFRET efficiency oscillated in unstimulated neurons, as represented in Figure 3.2B. However, the average RhoA nFRET efficiencies of 43%, 44% and 33%, were not statistically different between -40 s and 360 s in whole cells, cell bodies and growth cones, respectively (Figure 3.2C). The timing of the oscillations in nFRET efficiency was different between acquisitions. Notably, the nFRET efficiencies varied between acquisitions for each timepoint. These variations prevented the use of statistical analysis to compare nFRET efficiencies between conditions and between cell regions. To overcome this technical issue, RhoA nFRET measurements for each time series were divided by the highest nFRET efficiency value within the time series, providing normalized RhoA nFRET ratios (rFRET) that were used to assess RhoA activity between -40 and 360 s (Figure 3.3).

In unstimulated neurons, whole cell and growth cone rFRET continuously oscillated across average values of 0.930±0.002 (±SEM) and 0.91±0.05, respectively (Figure 3.3A, B and G). Cell body rFRET oscillated across an average value of 0.860±0.013, which was significantly lower than whole cell rFRET (Figure 3.3C and G). The oscillation range of RhoA activity was evaluated by measuring the average peak-to-peak amplitude for RhoA rFRET timepoints between -40 s to 360 s. The small

oscillations observed in whole cell RhoA rFRET (Figure 3.3A) were translated into an average rFRET amplitude of 0.022 (Figure 3.4A). In comparison, RhoA rFRET oscillations in growth cones and cell bodies (Figure 3.3B and C) corresponded to larger rFRET amplitudes of 0.033 and 0.060, respectively (Figure 3.4A). The RhoA rFRET oscillation range was significantly higher in cell bodies and growth cones than in whole cells by 2.9- and 1.5-fold, respectively (Figure 3.4B). Together, these results demonstrate that basal RhoA activity oscillates in unstimulated embryonic cortical neurons. The amplitude of RhoA activity oscillations is greater in cell bodies and growth cones than in whole neurons, suggesting that RhoA activity is regulated differently in specific neuronal regions.

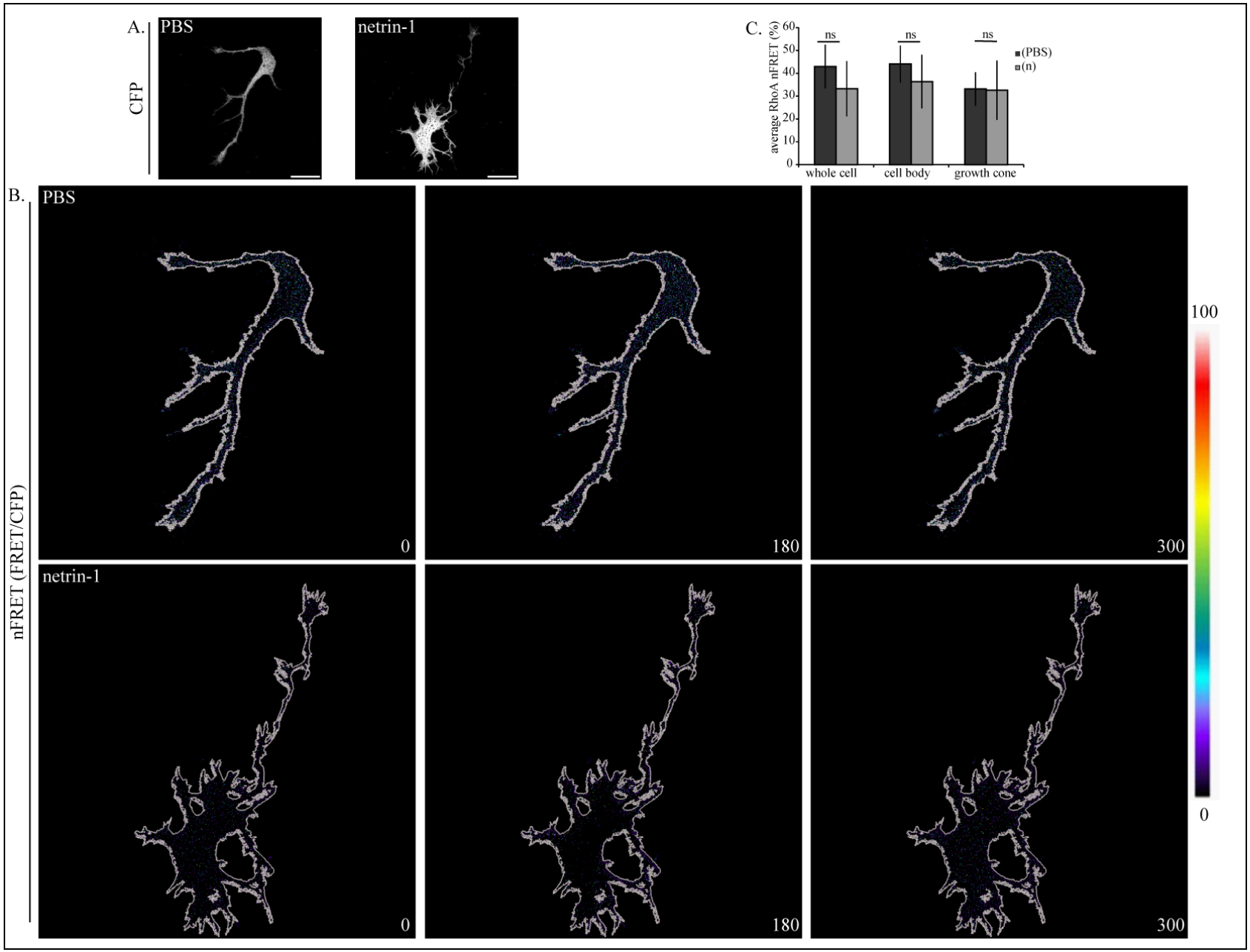
A.
PBS
netrin-1
CFP
C.
average RhoA nFRET (%)
60
50
40
30
20
10
0
ns
ns
ns
(PBS)
(n)
whole cell
cell body
growth cone
B.
nFRET (FRET/CFP)
PBS
netrin-1
0
180
300
0
180
300
100
0

Figure 3.2 RhoA activity oscillates in embryonic cortical neurons

Live E18 rat cortical neurons expressing CFP-RhoA and YFP-RBD were imaged at 2 days *in vitro* in the presence of 500 ng/ml netrin-1 or PBS as a control. (A) Representative CFP images were acquired at 0 s, when PBS or netrin-1 was added. Scale bars represent 20 µm. (B) RhoA activity is displayed as net FRET efficiency (nFRET). RhoA nFRET was calculated from the fluorescence intensity of FRET/CFP ratio images. RhoA nFRET timelapse images were obtained from FRET/CFP ratio images acquired at 0 s, 180 s, and 300 s. The cells are outlined in gray. The nFRET intensity scale (0-100%) is represented on the right. (C) RhoA activity is represented as the average net FRET efficiency (nFRET; ±SEM) in percentage (%). Average RhoA nFRET in whole cell, cell body and growth cone were calculated between -40 s to 360 s (PBS= 3 cells, netrin-1= 9-12 cells). Student's unpaired t-test was used for statistical analysis over three independent experiments (n.s: non-significant).

Region-specific effects of netrin-1 on RhoA activity oscillations in embryonic cortical neurons

We then assessed the effects of netrin-1 on RhoA activity in embryonic cortical neurons. Similarly to unstimulated neurons, RhoA nFRET efficiency oscillated in neurons imaged in the presence of netrin-1 (Figure 3.2B). The average RhoA nFRET efficiencies of 33%, 36% and 33% were not statistically different between -40 s and 360

s in whole cells, cell bodies and growth cones, respectively, or compared to untimulated neurons left (Figure 3.2C). RhoA rFRET in whole cells, cell bodies and growth cones oscillated across average values of 0.95±0.01, 0.93±0.03 and 0.90±0.02, respectively (Figure 3.3D-G). The average RhoA rFRET between -40s and 360 s was not significantly different in netrin-1-treated neurons compared to neurons left unstimulated (Figure 3.3G). However, netrin-1 stimulation significantly reduced the average RhoA rFRET in growth cones but not in cell bodies, compared to whole neurons (Figure 3.3G). In addition, netrin-1 modulated the pattern of RhoA rFRET oscillations in stimulated neurons (Figure 3.3D-F) compared to neurons left unstimulated (Figure 3.3A-C). RhoA rFRET measurements in whole cells revealed that RhoA activity remained above average levels from 20 s to 185 s after the addition of netrin-1 (compare Figure 3.3D with Figure 3.3A). RhoA rFRET oscillations in cell bodies were altered after the addition of netrin-1 from 0 s to 360 s (compare Figure 3.3F with Figure 3.3C). RhoA activity in cell bodies initially decreased between 0 s and 90 s, then increased and remained above average levels from 100 s to 330 s (Figure 3.3F). RhoA rFRET in growth cones continued to oscillate after the addition of netrin-1 (compare Figure 3.3E with Figure 3.3B), though RhoA activity remained above average levels from 120 s to 240 s (Figure 3.3E). Furthermore, the oscillation range of RhoA activity in netrin-1-stimulated growth cones was significantly higher than in growth cones left unstimulated (Figure 3.4A). The corresponding rFRET amplitude in growth cones was 0.07, which

was 2-fold greater than in whole cells after netrin-1 stimulation (Figure 3.4A and B). Netrin-1 stimulation did not have a significant effect on the amplitude of RhoA rFRET oscillations in whole cells or cell bodies (Figure 3.4A and B). Taken together, these results demonstrate that netrin-1 regulates RhoA activity in embryonic cortical neurons in a region-specific manner.

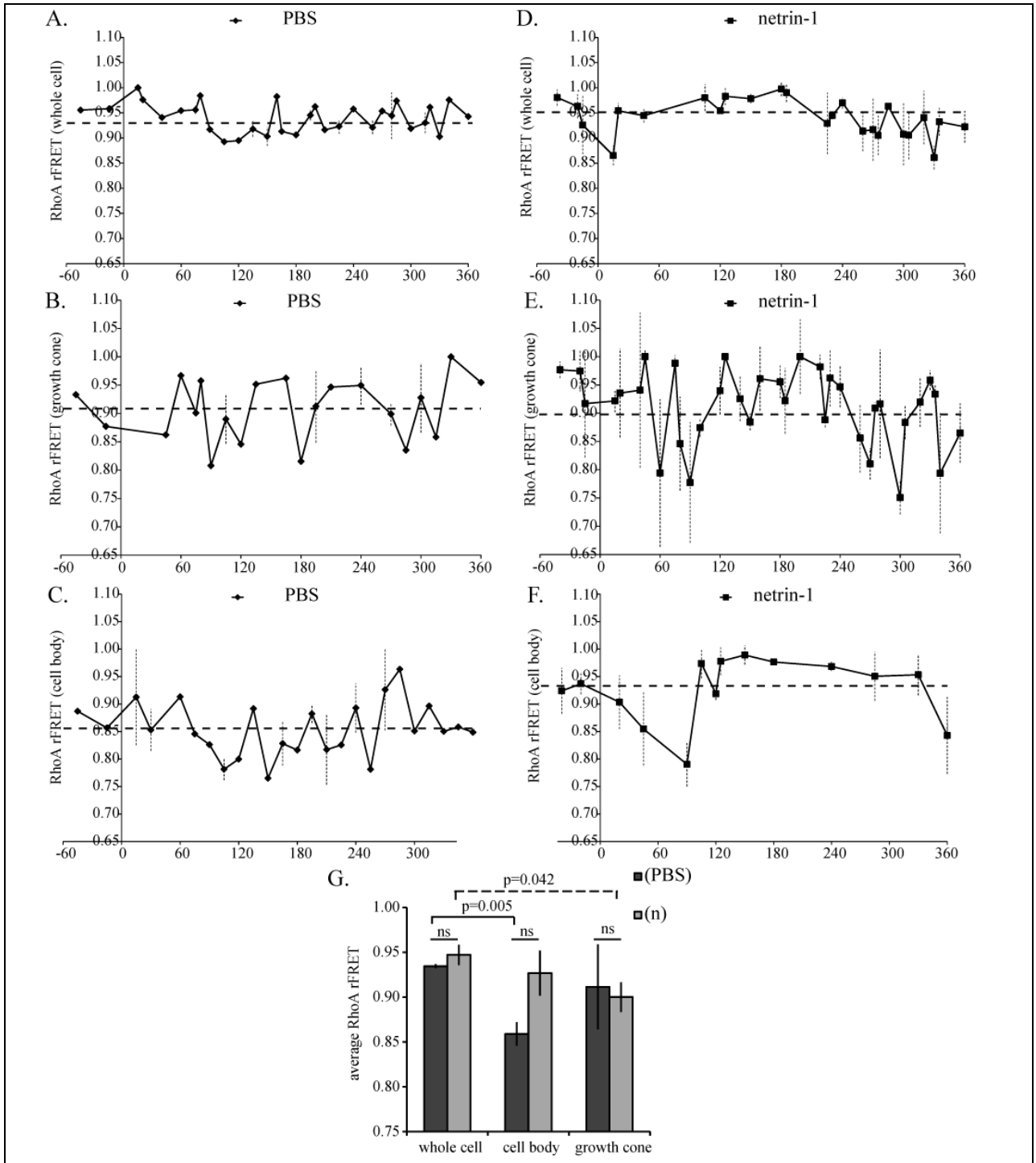

Figure 3.3. Netrin-1 modulates RhoA activity in embryonic cortical neurons

Live E18 rat cortical neurons expressing CFP-RhoA and YFP-RBD were imaged at 2 days *in vitro* in the presence of PBS as a control (A-C) or 500 ng/ml netrin-1(n) (D-F).

(A-F) RhoA activity is represented as the average normalized net FRET efficiency (rFRET; ±SEM). Average RhoA rFRET in the whole cell (A,D), growth cone (B, E) and cell body (C, F) were calculated for each timepoint from the fluorescence intensity of FRET/CFP ratio images acquired every <20 s between -40 s and 360 s (PBS= 3 cells, netrin-1= 9-12 cells). (G) Average RhoA rFRET in whole cell, cell body and growth cone between -40 s and 360 s were calculated and displayed as dashed lines in A-F. Student's unpaired t-test was used for statistical analysis over three independent experiments (n.s: non-significant).

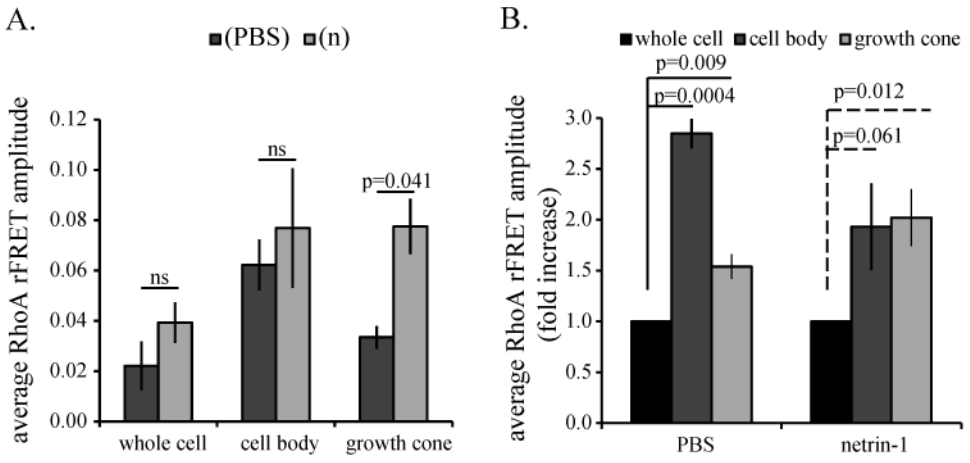

Figure 3.4 Netrin-1 regulates RhoA activity in a region-specific manner in embryonic cortical neurons

Live E18 rat cortical neurons expressing CFP-RhoA and YFP-RBD were imaged at 2 days *in vitro* in the presence of 500 ng/ml netrin-1 (n) or PBS as a control. (A) The oscillation range of RhoA activity in whole cell, cell body and growth cone was

evaluated by measuring the average peak-to-peak amplitude of normalized net FRET efficiency (rFRET), which is represented in Fig. 3, from -40 s to 360 s. (B) Average RhoA rFRET peak-to-peak amplitude is represented as the fold increase relative to whole cell RhoA rFRET peak-to-peak amplitude (±SEM) in unstimulated or netrin-1-treated neurons. Student's unpaired t-test was used for statistical analysis over three independent experiments (PBS= 3 cells, netrin-1= 9-12 cells; n.s: non-significant).

4.5 Discussion

In this study, we demonstrate using FRET imaging that netrin-1 mediates early onset, short-term RhoA activity in embryonic cortical neurons. Previously, we had indirectly shown that netrin-1/DCC signaling activates RhoA by demonstrating that RhoA/ROCK activities are required to activate ERM proteins within 5 minutes of netrin-1 stimulation in embryonic cortical neurons (Antoine-Bertrand et al., 2011a). Since netrin-1 activated ERM proteins mostly in growth cone filopodia and within a short time frame (Antoine-Bertrand et al., 2011a), the rationale was to measure RhoA activation using FRET imaging, a method that has been successful to measure spatiotemporal Rho GTPase activation in migrating non-neuronal cells and neurons (Kiyokawa et al., 2011; Pertz, 2010). Moreover, biochemical approaches that measure whole cell GTPase activity such as effector pull-down assays and G-Lisa did not detect RhoA activation in response to netrin-1/DCC signaling (Li et al., 2002b; Moore et al., 2008a).

RhoA activity oscillated in unstimulated cortical neurons and was modulated by short exposure to netrin-1. These oscillations are representative of Rho GTPase cycling between GDP- and GTP-bound states, a process thought to be important for the exploratory behaviour of growth cones and the rapid detection of extracellular cues (Das and Verde, 2013; Meinhardt, 1999; Sakumura et al., 2005). GTPase cycling is regulated by guanine exchange factors (GEFs) and GTPase-activating proteins (GAPs). Rho GTPase cycling in time and space is tightly linked to morphological dynamics in spontaneously migrating fibroblasts (Machacek et al., 2009). Here, we found that RhoA activity oscillations in untreated neurons have greater amplitude in cell bodies and growth cones than in the whole neurons. Moreover, netrin-1 significantly increased the amplitude of RhoA activity oscillations specifically in the growth cone. One interesting theory, which has been proposed for migrating cells, suggests that spatiotemporal activation of Rho GTPases results from the combined activity of several Rho GTPase pools that have distinct regulators and effectors within specific cellular locations and different functions (Pertz, 2010). Thus, our results suggest that at least two RhoA pools are differentially regulated in the cell bodies and growth cones of cortical neurons.

We also found that within 5 minutes of stimulation, netrin-1 transiently increased RhoA activity above average levels and netrin-1 modulated the pattern of RhoA oscillations in whole neurons. The onset and duration of the increase in netrin-1-dependent RhoA activity was different in each region of the neuron analyzed. The

increase in RhoA activity detected in whole cortical neurons lasted 3 minutes (180 s) after the addition of netrin-1. In cell bodies, RhoA activity initially decreased in the presence of netrin-1 followed by an increase that remained elevated for almost 6 minutes (330 s). Furthermore, RhoA activity was increased between 2 and 4 minutes in growth cones following the addition of netrin-1. This result confirms that RhoA is locally activated by netrin-1 in cortical growth cones, and supports our previous findings showing that RhoA activity mediates ERM activation in growth cone filopodia necessary for netrin-1/DCC-dependent axon outgrowth (Antoine-Bertrand et al., 2011a). Overall, these results lead to the establishment of a novel model for the spatiotemporal regulation of RhoA activity by netrin-1 and its implication in cortical axon outgrowth. In this model, late onset long-term global RhoA inhibition by netrin-1/DCC signaling is preceded by early onset short-term local activation in the growth cone, most likely as a result of the subcellular distribution of specific RhoA GAPs, GEFs and/or effectors in cortical neurons (Pertz, 2010).

Netrin-1 regulates filopodial dynamics in growth cones through the reorganization of the actin cytoskeleton and redistribution of actin-binding proteins, including ERM proteins (Antoine-Bertrand et al., 2011a; Dent et al., 2004; Lebrand et al., 2004; Marsick et al., 2010; Shekarabi et al., 2005; Welshhans and Bassell, 2011). ERM proteins are F-actin-binding proteins that regulate protein interactions and signal transduction at the interface between cortical actin and the plasma membrane (Fehon et al., 2010; Neisch

and Fehon, 2011). In the growth cones, they are considered to be an integral part of the molecular clutch that provides traction and counteracts F-actin retrograde flow to promote growth cone protrusions (Dent et al., 2011; Giannone et al., 2009; Lowery and Van Vactor, 2009; Marsick et al., 2012b). F-actin retrograde flow is required for growth cone motility (Van Goor et al., 2012). Notably, disrupting ERM protein activity increases F-actin retrograde flow, which impairs the formation of filopodial protrusions in neuronal growth cones (Castelo and Jay, 1999; Gallo, 2008; Marsick et al., 2012b; Paglini et al., 1998; Teuliere et al., 2011). The actin-depolymerizing-factor (ADF)/cofilin and the motor protein myosin II also regulate F-actin retrograde flow downstream of RhoA/ROCK activities (Aizawa et al., 2001; Dent et al., 2011; Sumi et al., 1999). Activated ERM proteins and activated ADF/cofilin accumulate in neuronal growth cones to promote filopodial protrusions within 5 minutes of stimulation with attractive cues such as netrin-1 (Antoine-Bertrand et al., 2011a; Marsick et al., 2010; Marsick et al., 2012b). ERM protein activation is mediated in part by RhoA/ROCK activities to promote axon attraction, outgrowth and branching (Antoine-Bertrand et al., 2011a; Cheng et al., 2005; Fievet et al., 2004; Gary and Bretscher, 1995; Gautreau et al., 2000; Haas et al., 2007; Jeon et al., 2002; Kim et al., 2010; Marsick et al., 2012b; Ruan et al., 2008; Teuliere et al., 2011). Conversely, RhoA/ROCK activities reduce actin dynamics and impair growth cone protrusions through the inhibition of cofilin in response to repulsive axon guidance cues (Aizawa et al., 2001; Geneste et al., 2002; Marsick et al., 2012a; Sumi et al.,

2001). Therefore, the localization and timing of RhoA activation in growth cones must be tightly controlled in order to mediate the concurrent activation of ERM proteins and cofilin in response to netrin-1. Moreover, FRET imaging revealed in N1E-115 neuroblastoma cells and dorsal root ganglion (DRG) neurons that RhoA is more active in the P-domain, where filopodia form, than in the C-domain (Fritz et al., 2013; Nakamura et al., 2005). Interestingly, RhoA activity is maintained in the P-domain during growth cone retraction induced by lysophosphatidic acid (Nakamura et al., 2005; Zhang et al., 2003). In this context, the structure of the P-domain, mainly filopodia, remains unaffected and growth cone spreading is maintained, even though the P-domain is pulled rearward due to increased contractility in the C-domain and axon shaft (Nakamura et al., 2005; Zhang et al., 2003). Therefore, these studies demonstrate that RhoA activity can also contribute to the maintenance of growth cone filopodia. Similarly, an elegant correlative study of protrusion-retraction cycles in spontaneously migrating fibroblasts by Danuser and colleagues demonstrated that increased RhoA activity is temporally and spatially coupled to protrusions (Machacek et al., 2009). Since our work shows that RhoA is activated early and transiently by netrin-1 in a region-specific manner in neurons, we propose that netrin-1/DCC signaling transiently activates RhoA in the P-domain of cortical growth cones to establish traction via ERM proteins and promote filopodial protrusions. Spatiotemporal regulation of Rho GTPases is likely mediated by the differential distribution of GEFs, GAPs and effectors in the cell (Pertz,

2010). The identification of these regulators and effectors downstream of specific extracellular cues is certainly the next step on the way to understanding how Rho GTPase activities are coordinated to regulate growth cone dynamics in neurons and migration in non-neuronal cells.

Preface to Chapter 4

Members of the Ras subfamily of GTPases are essential regulators of cellular function and integrate signals from various cellular compartments, including signaling from plasma membrane receptors (Prior and Hancock, 2012). It has been generally assumed that Ras activity mediates ERK activation downstream of netrin-1 and DCC (Campbell and Holt, 2003; Forcet et al., 2002). Yet, the regulation and the role of Ras activity have not been investigated in the context of netrin-1/DCC signaling.

The rationale for the study presented in Chapter 4 is to characterize the function of proteins that interact with DCC via a phosphotyrosine-binding Src homology 2 (SH2) domain since Y1418 phosphorylation is required for netrin-1 signaling in the vertebrate nervous system (Li et al., 2004; Meriane et al., 2004). Among the potential interactions uncovered, the discovery that the N-terminal SH2 domain of p120RasGAP binds to DCC prompted the examination of the role of Ras activity downstream of netrin-1 in the nervous system. This chapter describes the functional interaction between p120RasGAP and DCC during netrin-1 signal transduction in rat embryonic cortical neurons. The study represents the first report of netrin-1-dependent Ras activation and proposes a mechanism for the role of p120RasGAP during netrin-1-mediated axon outgrowth and chemoattraction that includes Ras-dependent and Ras-independent functions. An edited version of this chapter was accepted for publication in 2016 by the Journal of Biological Chemistry.

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Chapter 4 – p120RasGAP Contributes as a Scaffolding Protein to Regulate Netrin-1-Mediated Axon Outgrowth and Attraction in a GAP-dependent Manner

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4.1 Abstract

The receptor Deleted in Colorectal Cancer (DCC) mediates attraction of growing axons to netrin-1 during brain development. In response to netrin-1 stimulation, DCC becomes a signaling platform to recruit proteins that promote axon outgrowth and guidance. The Ras GTPase-activating-protein (GAP), p120RasGAP, inhibits Ras activity and mediates neurite retraction and growth cone collapse in response to

repulsive guidance cues. Here, we show an interaction between p120RasGAP and DCC that positively regulates netrin-1-mediated axon outgrowth and guidance in embryonic cortical neurons. The N-terminus of p120RasGAP, consisting of two SH2 domains and one SH3 domain, interacts with DCC through two phosphorylated tyrosine residues (Y1361 and Y1418) and a class II PxxPxR(/K) motif. In response to netrin-1, p120RasGAP is recruited to DCC and forms a multiprotein complex with focal adhesion kinase (FAK) and extracellular signal-regulated kinase (ERK) in a Src family kinase-dependent manner. Netrin-1 induces Ras activation in cortical neurons and Ras/ERK activities are aberrantly elevated in p120RasGAP-deficient neurons. The expression of p120RasGAP N-terminus is sufficient to restore netrin-1-dependent axon outgrowth in p120RasGAP-deficient neurons. Thus, we provide a novel mechanism that exploits both the scaffolding properties of the N-terminus and the GAP activity of p120RasGAP to tightly regulate netrin-1/DCC-dependent axon outgrowth and guidance.

4.2 Introduction

Netrin-1 is one of the many extracellular cues that guide axons to their target during the development of the central nervous system (CNS) (Bashaw and Klein, 2010; Lykissas et al., 2007; Sanchez-Camacho and Bovolenta, 2009). It has the ability to attract or repel axons via several transmembrane receptors (Lai Wing Sun et al., 2011). The DCC receptor is expressed in the spinal cord and the forebrain of vertebrates and

mediates netrin-1-dependent attraction of neurons (Keino-Masu et al., 1996; Shu et al., 2000). Deficiencies in netrin-1 or DCC expression result in the loss of cerebral and spinal commissures (Fazeli et al., 1997; Serafini et al., 1996). In humans, genetic variations within the *dcc* locus have been linked to neurological disorders such as congenital mirror movement (Depienne et al., 2011; Srour et al., 2010), schizophrenia (Grant et al., 2012) and Parkinson's disease (Kim et al., 2011). Upon netrin-1 stimulation, DCC becomes phosphorylated on threonine, serine and tyrosine residues (Meriane et al., 2004). Phosphorylation at the C-terminal tyrosine residue 1418 (Y1418) of DCC by Src family kinases is essential for netrin-1 to mediate axon outgrowth and guidance in vertebrates (Antoine-Bertrand et al., 2011a; Li et al., 2004; Meriane et al., 2004; Ren et al., 2008). The significance of Y1418 phosphorylation in the netrin-1/DCC signaling pathway is highlighted by its position within the P3 motif (amino acids 1392 to 1445 in *R. norvegicus*), a highly conserved region of the DCC C-terminal tail that regulates the recruitment of several proteins including focal adhesion kinase (FAK), Src, Fyn, ezrin and Myosin X (Antoine-Bertrand et al., 2011a; Hirano et al., 2011; Li et al., 2004; Liu et al., 2004; Meriane et al., 2004; Ren et al., 2008; Ren et al., 2004; Wei et al., 2011; Zhu et al., 2007).

The neuronal growth cone is found at the distal periphery of an extending axon where the signals from guidance cues are integrated. The signaling cascades initiated by the receptors expressed on the surface of the growth cone produce a coordinated

cellular response by regulating cytoskeletal rearrangements (Dent et al., 2011; Vitriol and Zheng, 2012). Rho GTPases are important mediators of the classic axon guidance cues netrins, slits, ephrins and semaphorins during cytoskeletal reorganization in growth cones (Bashaw and Klein, 2010; Hall and Lalli, 2010). Netrin-1/DCC signal transduction activates Rac1 in neurons, whereas RhoA is inhibited (Briancon-Marjollet et al., 2008; Li et al., 2002b; Moore et al., 2008a; Shekarabi and Kennedy, 2002). Ras GTPases are also regulated by ephrins, semaphorins and neurotrophins during neuronal development, but their role in netrin-1/DCC signaling has not been explored (Hall and Lalli, 2010; Skaper, 2012). The extracellular-regulated kinase (ERK) is activated downstream of netrin-1 and DCC and required for netrin-1-dependent axon outgrowth and guidance (Campbell and Holt, 2003; Forcet et al., 2002; Ming et al., 2002), but it remains unclear whether Ras mediates ERK activation downstream of netrin-1 and DCC. Until now, the Ras GTPase-activating-protein (GAP), p120RasGAP, was considered only to be an inhibitor of axon outgrowth and guidance due to the activity of its C-terminal RasGAP domain (Elowe et al., 2001; Endo and Yamashita, 2009; Hancock et al., 2014). In addition to its C-terminal GAP domain, the N-terminus of p120RasGAP, comprising one SH3 and 2 SH2 domains, interacts with a wide variety of proteins to regulate cell survival, proliferation and migration (King et al., 2013; Pamonsinlapatham et al., 2009). Here, we identified p120RasGAP in a Src homology 2 (SH2) domain screen for proteins that interact with the phosphorylated Y1418 residue of

DCC. Our results provide evidence for the dual function of p120RasGAP as a GAP and a scaffolding protein to promote netrin-1/DCC signaling. We show that p120RasGAP is required to regulate netrin-1-mediated ERK and FAK activation, axon outgrowth and guidance in neurons. p120RasGAP forms a Src-family-kinase-dependent signaling complex with DCC in response to netrin-1. The N-terminus of p120RasGAP interacts with DCC and is required for netrin-1-mediated axon outgrowth. Moreover, p120RasGAP is essential for the attractive response of axons to netrin-1 in cortical neurons. Together, this novel mechanism adds another layer to the intricacy of the multiple and essential signaling pathways regulated by netrin-1 and DCC during axon extension and attraction.

4.3 Materials and Methods

Antibodies and reagents

The following antibodies were purchased: anti-GST, anti-RasGAP B4F8 and anti-DCC A-20 (Santa Cruz Biotechnology); anti-phosphotyrosine (pY) 4G10, anti-tubulin and anti-DCC AF5 (Millipore); anti-DCC G92-13 (BD Biosciences Pharmingen); anti-FAK (BS Biosciences); anti-phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) and anti-p44/42 MAPK (Erk1/2) (Cell Signaling Technology); anti-FAK [pY861] and anti-FAK [pY397] (Life Technologies Novex); anti-active Ras (NewEast Biosciences); anti-ezrin provided by M. Arpin (Algrain et al., 1993); anti-DCC [pY1418] (polyclonal antibodies raised in

rabbit against the peptide KPTEDPASVpYEQDDL (DCC-pY1418)); anti-mouse Alexa 488, anti-mouse Cy3 and anti-rabbit Cy3, anti-rabbit Alexa 555 (Life Technologies Molecular Probes); anti-goat Immunoglobulin G (IgG) Cy3 (Sigma). The following reagents were used: recombinant chick netrin-1 and netrin-1 VI-V were produced and purified as previously described (Kennedy et al., 2006; Serafini et al., 1994), glutamate provided by D. Bowie, PP2 (Calbiochem), DMSO (Thermo Scientific), NSC23766 (Calbiochem).

Plasmids, sequence alignment and siRNAs

The plasmids pRK5, pRK5-DCC, pRK5-DCC-Y1418F, pRK5-DCC-Y1361F, pRK5-DCC 1-1327 and pRK5-DCC 1-1421 were previously described (Li et al., 2002b; Meriane et al., 2004; Tcherkezian et al., 2010). pRK5-DCC-P1336A/P1339A was generated by site-directed mutagenesis (Stratagene). The pCDNA3-GAP and pCDNA3-GAP-N (human) constructs were provided by T. Pawson (Kulkarni et al., 2000). The plasmids encoding GST and GST- human p120RasGAP (N-SH2, SH3, C-SH2 and SH2-SH3-SH2) were provided by L. Larose. pmaxGFP was purchased from Lonza. The amino acid sequence alignment of DCC orthologs was generated with the BioEdit software Clustal W plugin. The following siRNAs were purchased: Silencer Negative Control No.1 siRNA (Life Technologies Ambion) and previously described 5'-

GCAGGGAAATCTGGAAGCTACCTTA-3'p120RasGAP siRNA (Dharmacon) (Endo and Yamashita, 2009).

Purification of GST fusion proteins

Production of GST and GST-p120RasGAP proteins was induced with 0.5 mM isopropylthiogalactopyranoside (IPTG) for 2 h at 37°C. Bacteria pellets were resuspended in BME-Phosphate buffer (1:1000 beta-mercaptoethanol (BME), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1mM ethylenediaminetetraacetic acid (EDTA) in PBS), supplemented with 0.5 mg/ml lysozyme and incubated for 30 min at 4 °C. Resuspended pellets were frozen in an ethanol/dry ice bath, thawed in warm water, sonicated and incubated with 1% Triton X-100 for 10 min at 4 °C. Protein lysates obtained after centrifugation were incubated with glutathione-agarose beads (Sigma) for 2 h at 4°C. The beads were washed three times in BME-Phosphate buffer and stored at 4°C or proteins were eluted with 5 mM glutathione buffer in 50 mM Tris-HCl pH 8.0 and concentrated with a Nanosep 10K Omega column (PALL) and stored at 4°C. Purity and concentration were determined by Coomassie Blue-stained SDS-PAGE.

Peptides, SH2 domain array and dot blot assays

The following peptides were purchased from Keck MS & Proteomics Resource (USA): biotinylated TEDPASVpYEQDDLSE (DCC-pY1418). The SH2 Domain-Based RTK

Profiling Kit (Signosis) was used according to the manufacturer's instructions with some modifications. Streptavidin-HRP was directly added to samples of 50 or 100 nM of DCC-pY1418 without any primary antibodies. Absorbance was read spectrophotometrically at 450 nm. Absorbance fold increase was calculated by normalizing the absorbance of each condition with the GST control absorbance. For the dot blots, 20 µg DCC-Y1418 and 300 µg BSA were spotted onto nitrocellulose membranes. Membranes were incubated with freshly purified GST or GST-p120RasGAP proteins overnight (O/N) at 4°C. The membranes were immunoblotted with anti-GST, anti-DCC-pY1418 antibodies.

Cell culture, transfection and electroporation

Cell culture was maintained in a humidified incubator at 37°C with 5% CO^2. HEK293 cells and COS-7 cells were cultured in DMEM (Wisent Bioproducts) supplemented with 10% fetal bovine serum (FBS) and antibiotics. Cells were transfected overnight using polyethylenimine (PEI; PolySciences, PA) according to the manufacturer's instructions (Antoine-Bertrand et al., 2011a; DeGeer et al., 2013). Cortical neurons from E18 rat embryos (Charles River) were dissociated mechanically and plated on dishes treated with poly-D-lysine (0.1 mg/ml; Sigma-Aldrich) or glass coverslips treated with poly-L-lysine (0.1 mg/ml; Sigma-Aldrich). Neurons were cultured in 10% FBS DMEM for 4h and the medium was replaced by Neurobasal-A medium supplemented with 2% B27 and 1% L-glutamine (Invitrogen) (Antoine-Bertrand et al., 2011a). The Amaxa Rat Neuron

Nucleofector Kit (Lonza) was used as per the manufacturer's instructions to electroporate siRNAs and plasmids. Neurons were treated with the following reagents: purified recombinant netrin-1 or netrin-1 VI-V (200 or 500 ng/ml), glutamate (50 µM), PP2 (10 µM), NSC23766 (100 µM), DMSO or water.

GST pull-downs

Transfected HEK293 and COS-7 cells were lysed in 1% Triton X-100 lysis buffer as previously described (Briancon-Marjollet et al., 2008). Protein lysates (1mg) were pre-cleared with 30 µl of glutathione–agarose beads (Sigma Aldrich) for 2 h at 4°C and incubated with 10 or 20 µg of fresh GST or GST-p120RasGAP proteins coupled to glutathione–agarose beads for 3 h at 4°C. Beads were washed three times in ice-cold lysis buffer and boiled in SDS sample buffer.

Immunoprecipitation

Cortical neurons (2DIV) lysates were prepared as previously described (Antoine-Bertrand et al., 2011a). Protein lysates (1mg) were incubated with 1.5 µg of anti-DCC antibodies (Millipore) or 4ug of anti-p120RasGAP with protein G Sepharose beads (GE Healthcare) for 3 h at 4°C.

Immunoblotting and quantitative densitometry

Proteins were resolved by SDS–PAGE and transferred onto nitrocellulose membrane. Membranes were stained with Ponceau S (Sigma-Aldrich), immunoblotted with the indicated antibodies, and visualized using enhanced chemiluminescence (Millipore). Optical density was measured using the Quantity One software (Bio-Rad). The following optical density ratios were calculated: co-immunoprecipitated DCC and p120RasGAP over immunoprecipitated p120RasGAP; phosphotyrosines over p120RasGAP, DCC-pY1418 over DCC, p120RasGAP over ezrin, FAK-pY861 over tubulin, and pERK over ERK or tubulin. Optical density fold change was calculated by normalizing the ratio of each condition with the control ratio.

Rac1 and Ras G-LISA assays

Cortical neuron (2DIV) lysates were prepared and processed as per the manufacturer's instructions (Cytoskeleton). Absorbance was read spectrophotometrically at 492 nm. Optical density fold increase was calculated by normalizing each condition with the control's optical density.

Immunofluorescence

Cortical neurons (2DIV) were fixed 30 min with 3.7% formaldehyde in 20% sucrose PBS at 37°C, quenched 5 min in 0.1 M glycine at room temperature, permeabilized 5 min in 0.25% Triton X-100, and blocked 30 min with 3% BSA. Primary and secondary

antibodies were incubated in 0.3% BSA. A 15 min fixation with 10% trichloroacetic acid in water was used for phosphospecific antibodies (Hayashi et al., 1999). Neurons were examined with the following: Olympus IX81 motorized inverted microscope (40× U PLAN Fluorite and 60× U PLAN S-APO oil objective lenses) with a CoolSnap 4K camera (Photometrics); Zeiss LSM780 confocal microscope (63×/1.40 oil Plan-Apochromat) with 488 nm argon and 561 nm DPSS lasers and a GaAsP detector. Pearson's correlation coefficient and fluorescence intensity quantification were measured with the Metamorph software.

Axon outgrowth and Dunn chamber assays

Axon length of GFP-expressing cortical neurons (2DIV) was measured with Metamorph. Cortical neurons (2DIV) were plated on coverslips used for Dunn chamber assembly as previously described (Yam et al., 2009). Gradients were generated with purified netrin-1 VI-V (200 ng/ml) or buffer containing PBS in the outer well. Cell images were acquired every 3–4 min for at least 90 min on a temperature controlled stage. Neurites of at least 10 μm in length were tracked in GFP-expressing neurons. The final position of the growth cone was used to determine the angle turned over 90 min relative to the gradient position. Measurements are presented in rose histograms in bins of 10° with the length of each segment representing the frequency of measurements in percent. Percentage

distribution of turned angles, average turned angle and average displacement are also represented.

Statistical analysis

Statistical analysis was performed with GraphPad Prism 6. The data are presented as the mean ± the standard error of the mean (SEM).

4.4 Results

The N-terminal SH2 domain of p120RasGAP interacts with DCC via the phosphorylated Y1418 residue in vitro

To identify SH2-containing proteins that bind to the phosphorylated Y1418 (pY1418) residue of DCC, we screened an SH2 domain array using as bait a 15-amino acid (a.a.) synthetic DCC peptide comprising pY1418 (DCC-pY1418). Amongst an array of 46 SH2 domains, the N-terminal SH2 (N-SH2) domain of p120RasGAP (Figure 4.1A) bound to the DCC-pY1418 peptide as revealed by a colorimetric enzyme-linked immunosorbent assay (ELISA). The N-SH2 domain of p120RasGAP displayed a 1.24- and 1.36-fold increase in absorbance relative to the GST control with 50 and 100 nM of DCC-pY1418 peptide, respectively (Figure 4.1B).

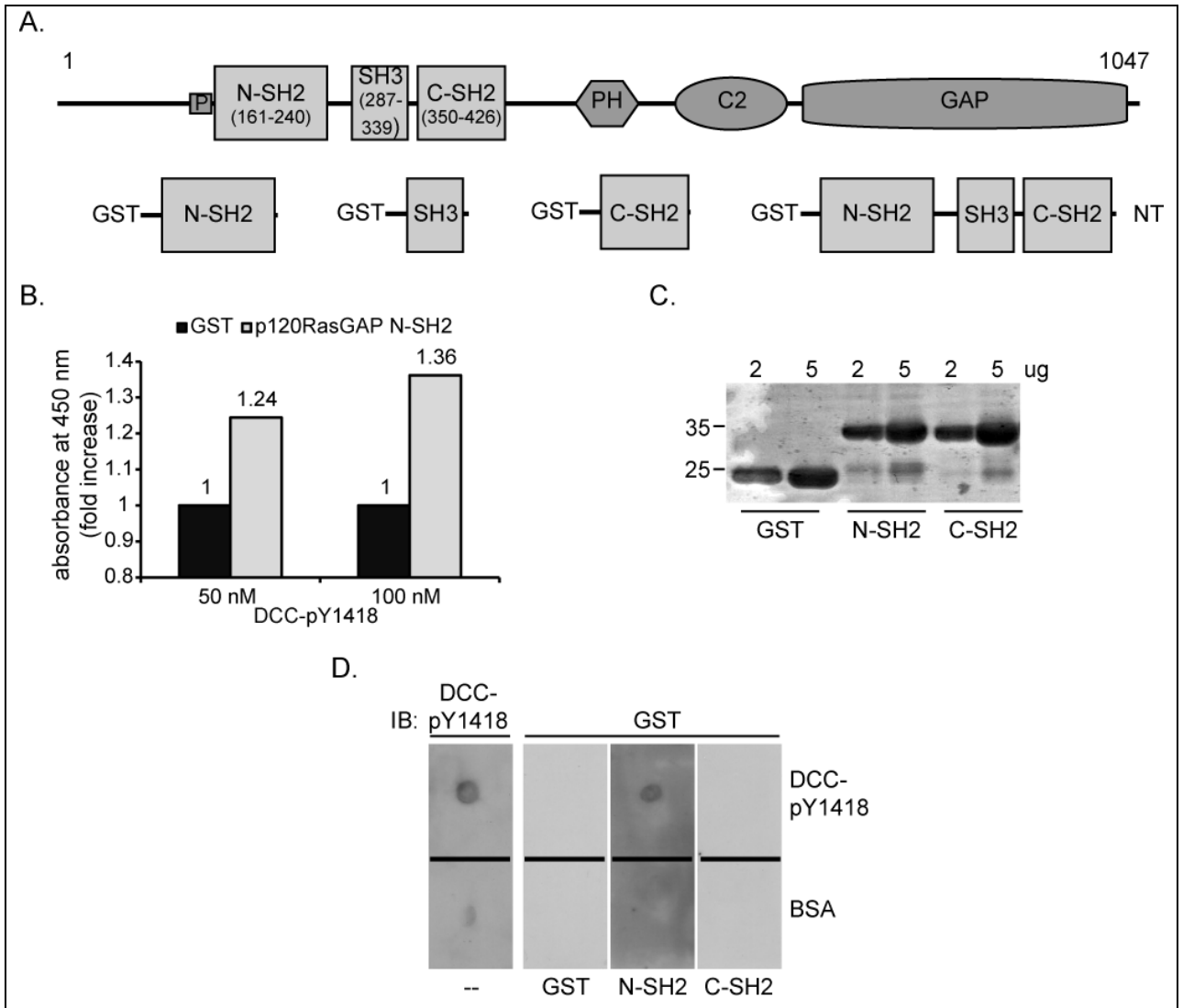


Figure 4.1 The N-terminal SH2 domain of p120RasGAP interacts *in vitro* with DCC via phosphorylated tyrosine Y1418

(A) p120RasGAP contains a proline-rich region (P), N- and C-terminal Src Homology 2 (N-SH2, C-SH2), Src Homology 3 (SH3), pleckstrin homology (PH), calcium-dependent phospholipid-binding (C2) and GTPase-activating protein (GAP) domains. GST-p120RasGAP fusion proteins N-SH2, SH3, C-SH2, and SH2-SH3-SH2 (NT) were used to perform all GST pull-down assays and dot blots. (B) A synthetic DCC peptide

containing phosphorylated Y1418 (DCC-pY1418) was used as bait to screen a SH2 domain array by ELISA. Binding of p120RasGAP N-SH2 with 50 and 100 nM of DCC-pY1418 peptide is represented as the fold increase in absorbance relative to the absorbance obtained with a GST control. (C) 2 and 5 ug of purified GST, GST-p120RasGAP N-SH2 and C-SH2 were resolved by SDS-PAGE and the proteins stained with Coomassie Blue. (D) The DCC-pY1418 peptide was spotted onto nitrocellulose membranes with BSA as a control, and each membrane was incubated with either purified GST, GST-p120RasGAP N-SH2 or C-SH2 (100ng/ml) followed by immunoblotting with anti-GST antibodies. One membrane was immunoblotted with phosphospecific anti-DCC-pY1418 (DCC-pY1418) antibodies (IB: immunoblot).

To validate the interaction between the N-SH2 domain of p120RasGAP and DCC-pY1418, purified GST fusion proteins of the individual N-SH2 and C-terminal SH2 (C-SH2) domains of p120RasGAP (Figure 4.1 A and C) were tested for their ability to bind to immobilized DCC-pY1418 peptide in a dot blot assay (Figure 4.1 D). The N-SH2 domain was the sole domain capable of interacting with DCC-pY1418 and did not bind to a control spot of BSA (Figure 4.1D). GST control protein did not bind to the DCC peptide or to BSA (Figure 4.1 D), and tyrosine phosphorylation of DCC-pY1418 was confirmed with a phosphospecific antibody raised against DCC-pY1418 (Figure 4.1D).

Thus, we conclude that the N-SH2 domain of p120RasGAP interacts directly with DCC via pY1418.

Src family kinase activity regulates the assembly of a DCC multiprotein signaling complex in embryonic cortical neurons stimulated with netrin-1

Next, we examined the interaction of p120RasGAP and DCC in dissociated embryonic day 18 (E18) rat cortical neurons, which are a good cellular model to investigate netrin-1/DCC-induced signaling pathways in the context of axon outgrowth and guidance (Antoine-Bertrand et al., 2011a; Briancon-Marjollet et al., 2008; DeGeer et al., 2013; Metin et al., 1997; Richards et al., 1997; Shu et al., 2000). DCC and p120RasGAP co-immunoprecipitated and the interaction peaked after 10 minutes of stimulation with netrin-1 (Figure 4.2A and B). In addition, phosphorylation of DCC on Y1418 was observed after 10 and 30 minutes of netrin-1 stimulation in embryonic cortical neurons using the phosphospecific anti-DCC-pY1418 antibody (Figure 4.2C and D). p120RasGAP was also tyrosine phosphorylated in response to netrin-1 (Figure 4.2E and F). Since the Src family tyrosine kinases regulate netrin-1/DCC-mediated intracellular signaling (Antoine-Bertrand et al., 2011a; DeGeer et al., 2013; Li et al., 2004; Meriane et al., 2004), we sought to test their role in regulating the interaction between p120RasGAP and DCC. Src family kinase inhibition with the PP2 inhibitor reduced netrin-1-dependent tyrosine phosphorylation of p120RasGAP and the

interaction between p120RasGAP and DCC (Figure 4.2E-G). Furthermore, PP2 abolished the recruitment of activated FAK (FAK-pY397) and ERK (pERK) to p120RasGAP in response to netrin-1 stimulation (Figure 4.2E). We validated the efficacy of the PP2 inhibitor by demonstrating a decrease in netrin-dependent phosphorylation of FAK on Y861 as described previously (Liu et al., 2004) (Figure 4.2E). Taken together, these results show that the assembly of a DCC-p120RasGAP-pFAK-pERK protein complex induced by netrin-1 requires the activity of Src family kinases.

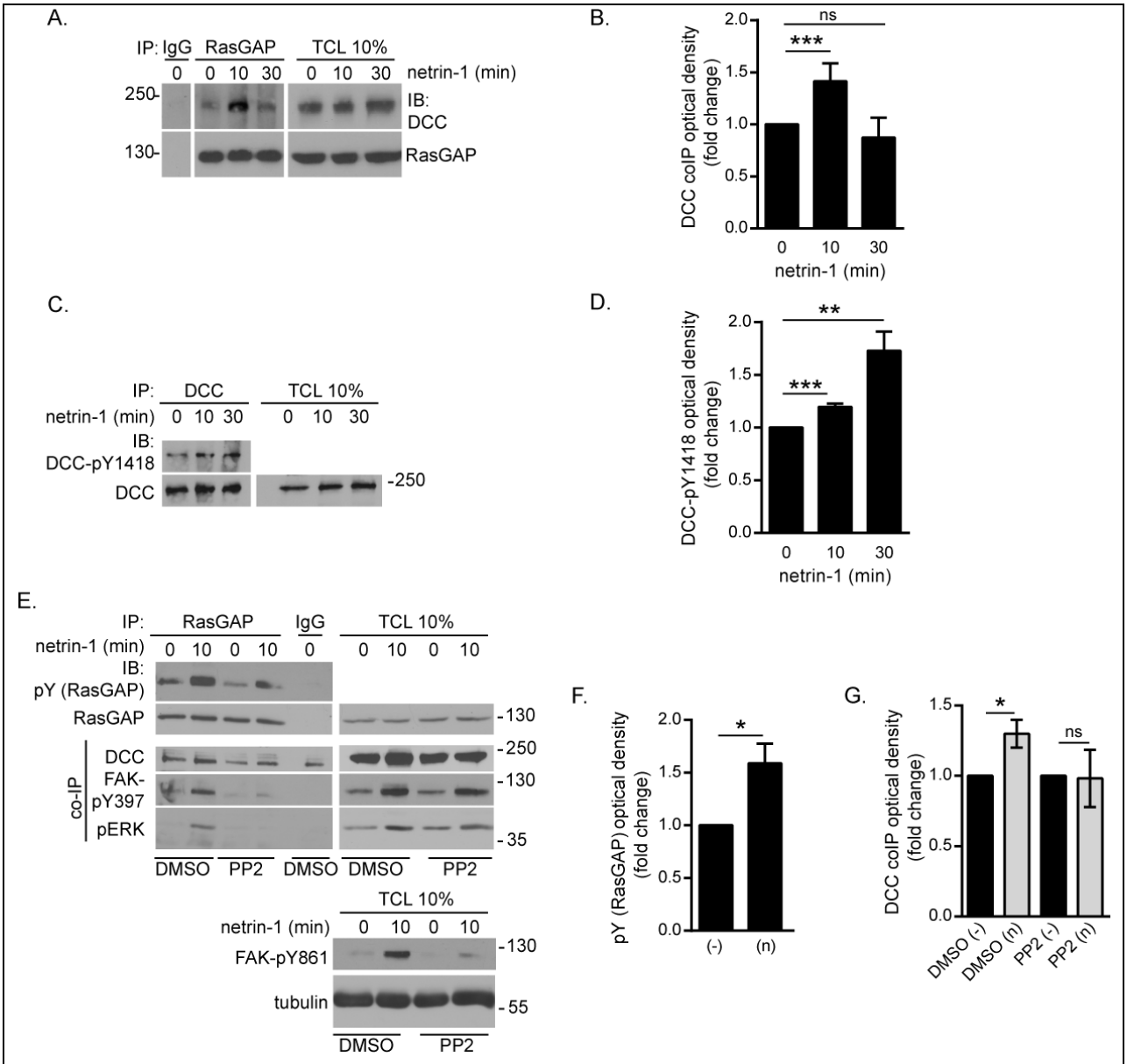


Figure 4.2 Src family kinase activity regulates the assembly of a DCC multiprotein signaling complex in embryonic cortical neurons stimulated with netrin-1

E18 embryonic rat cortical neurons were stimulated with netrin-1 (500 ng/ml) for the indicated times after being cultured 2 days *in vitro* (2DIV). (A) p120RasGAP was immunoprecipitated (IP) from cell lysates with anti-p120RasGAP antibodies or mouse immunoglobulin G (IgG) as control. IP proteins and total cell lysates (TCL) were

resolved by SDS-PAGE and immunoblotted (IB) with antibodies against the indicated proteins. (B) Quantitative densitometry (±SEM) of DCC co-IP with p120RasGAP is represented as the fold change relative to 0 min of netrin-1 stimulation for at least three independent experiments. Unpaired Student's t test: ***p<0.005. (C) DCC was immunoprecipitated (IP) from cell lysates with anti-DCC antibodies followed by immunoblotting (IB) with phosphospecific anti-DCC-pY1418 antibodies (DCC-pY1418). (D) Quantitative densitometry (±SEM) of DCC-pY1418 is represented as the fold change relative to 0 min of netrin-1 stimulation for at least three independent experiments. Unpaired Student's t test: **p<0.01, ***p<0.005. (E) Neurons were treated for 1h with the Src family kinase inhibitor PP2 or DMSO as a control prior to stimulation with netrin-1. p120RasGAP was IP from cell lysates, resolved by SDS-PAGE and immunoblotted (IB) with antibodies against the indicated proteins (pY (RasGAP): anti-phosphotyrosine antibodies). Quantitative densitometry (±SEM) of pY (RasGAP) (F) and DCC co-IP with p120RasGAP (G) is represented for at least three independent experiments (-, unstimulated; n, netrin-1). Unpaired Student's t test: *p<0.05.

Cooperative interactions of p120RasGAP SH2 domains with DCC and FAK

To further investigate the molecular interactions between p120RasGAP and DCC, GST-p120RasGAP proteins were incubated with protein lysates from HEK293 cells that overexpressed either wild-type DCC or DCC mutant proteins (Figure 4.3 A

and B). DCC proteins did not bind to the GST protein control (Figure 4.3C). p120RasGAP N-SH2 interacted with DCC, DCC-Y1361F, and with DCC 1-1421 lacking the P3 region (Figure 4.3 A and D). It failed to interact with DCC 1-1327 truncated before the P2 region indicating that the region preceding the P3 domain, and including P2, is important for binding (Figure 4.3 A and D). Surprisingly, DCC-Y1418F was still able to interact with p120RasGAP N-SH2 (Figure 4.3 D). Since FAK interacts with both DCC and p120RasGAP (Hecker et al., 2004; Ren et al., 2004; Serpente et al., 1996), we suspected that DCC-Y1418F might be indirectly pulled-down by p120RasGAP N-SH2 via an interaction with FAK. The phosphorylation of Y397 on FAK has been shown to mediate its interaction with the N-SH2 domain of p120RasGAP (Hecker et al., 2004). Indeed, FAK-pY397 specifically interacted with the N-SH2 domain of p120RasGAP (Supplemental Figure 4.1). GST-N-SH2 pulled-down FAK-pY397 along with DCC and DCC mutant proteins (Figure 4.3D), suggesting that DCC-Y1418F interacted indirectly with GST-N-SH2 through binding to FAK-pY397. Furthermore, the expression of either DCC 1-1421 or DCC 1-1327 significantly abrogated phosphorylation of FAK on Y397 in total cell lysates, confirming that the amino acid region 1422-1445 of DCC is important for the phosphorylation of FAK on Y397 in HEK293 cells (Figure 4.3B). Consequently, the expression of DCC 1-1421 or DCC 1-1327 impaired or completely abolished the interaction of FAK-pY397 with GST-N-SH2, respectively (Figure 4.3D).

To compensate for their weak affinity for phosphorylated tyrosine residues, it has been proposed that the 2 SH2 domains of p120RasGAP simultaneously bind to two adjacent tyrosine residues on binding partners such as p190RhoGAP, EphB2, epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) receptors (Holland et al., 1997; Hu and Settleman, 1997; Kazlauskas et al., 1990; Margolis et al., 1990). The intracellular domain of DCC comprises 4 conserved tyrosine residues, including Y1418 (Meriane et al., 2004). Following the identification of Y1418 as a direct binding site for p120RasGAP N-SH2 but not C-SH2 (Figure 4.1D), we selected Y1361 as a candidate binding site for the C-SH2 since it is the closest to Y1418 in the intracellular domain of DCC (Figure 4.3A). DCC, DCC-Y1418F, and DCC 1-1421 but not DCC 1-1327 nor DCC-Y1361F interacted with GST-C-SH2 (Figure 4.3E), demonstrating that p120RasGAP C-SH2 interacted with DCC via the phosphorylated Y1361 residue. FAK-pY397 was also pulled-down indirectly with p120RasGAP C-SH2 via DCC or DCC-Y1418F interaction, but not with DCC 1-1327, DCC-Y1361F, or DCC 1-1421 (Figure 4.3E). Finally, the SH2-SH3-SH2 domains of p120RasGAP interacted strongly with FAK-pY397 and DCC, DCC-Y1418F, -Y1361F, and 1-1421, but not with DCC 1-1327 (Figure 4.3F). Altogether, these data demonstrate that cooperative binding of the N- and C-SH2 domains of p120RasGAP promote an interaction with DCC via phosphorylated Y1418 and Y1361, respectively (Figure 4.3G).

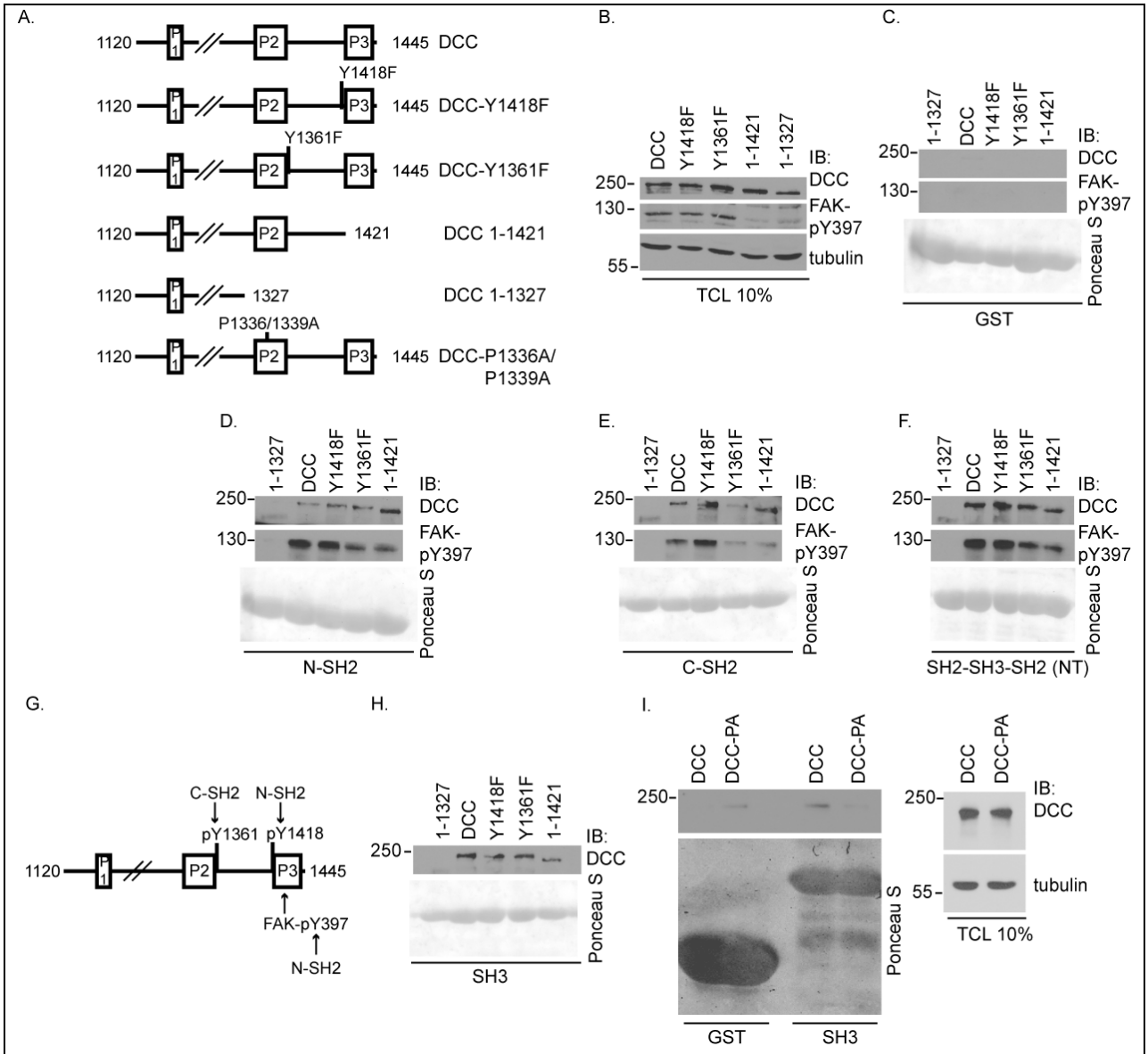


Figure 4.3 The N-terminus of p120RasGAP forms three independent interactions with DCC

(A) The intracellular domain of rat DCC (1120-1445 a.a.) contains three conserved regions (P1, P2, P3). The conserved tyrosine residue in phosphodeficient mutants DCC-Y1418F and DCC-Y1361F was substituted for a phenylalanine residue. Truncation mutants DCC 1-1421 and 1-1327 are truncated before the P3 or the P2, respectively. In

DCC-P1336A/P1339A, the PxxP motif was mutated by replacing the two proline residues for alanine residues. (B) DCC, DCC-Y1418F, DCC-Y1361F, DCC 1-1421 and DCC 1-1327 were expressed in HEK293 cells. Proteins from cell lysates were pulled-down using purified GST control protein (C) or GST-p120RasGAP N-SH2 (D), C-SH2 (E), SH2-SH3-SH2 (NT) (F), or SH3 (H). Associated proteins and total cell lysates (TCL) were resolved by SDS-PAGE and immunoblotted (IB) with antibodies against DCC, FAK-pY397 and tubulin. GST fusion proteins were stained with Ponceau S. (G) The N-SH2 and C-SH2 domains of p120RasGAP interact with phosphorylated (p) Y1418 and Y1361, respectively. The N-SH2 also indirectly interacts with DCC via FAK-pY397. (I) DCC or DCC-PA (DCC-P1336A/P1339A) were expressed in COS-7 cells. Proteins were pulled-down with GST or GST-SH3. Blots are representative of more than 3 experiments.

The SH3 domain of p120RasGAP interacts with DCC via a PxxP motif

We then examined whether the SH3 domain of p120RasGAP could also interact with DCC. Indeed, GST-p120RasGAP-SH3 interacted with DCC and all DCC mutant proteins, except DCC 1-1327 (Figure 4.3H). Since GST-p120RasGAP-SH3 interacted with DCC between a.a. 1328 and 1421, we searched for and identified 7 PxxP motifs in that region that could serve as a binding site. Thus far, the SH3 domain of p120RasGAP is considered to be atypical since its structure diverges from conventional

SH3 domains (Ross et al., 2007; Yang et al., 1994) and its interaction with G3BP1 or Aurora kinase has been reported to occur via non-PxxP motifs (Gigoux et al., 2002; Kennedy et al., 2001). Nevertheless, it has been proposed that the last proline-rich region of p200RhoGAP mediates its interaction with p120RasGAP SH3 (Shang et al., 2007). A closer analysis of the p200RhoGAP proline-rich region revealed a class II PxxPxR(/K) motif (PSLPQK) that could interact with p120RasGAP SH3. Only one of the 7 PxxP motifs that we have identified in DCC, ^{1336}PTHPLR^{1341}, represents a class II motif and is conserved in vertebrate DCC orthologs (Supplemental Figure 4.2). As shown in Figure 4.3I, p120RasGAP SH3 interacted with DCC but not with DCC-PA (P1336A/P1339A) in which two prolines have been replaced by alanine residues (Figure 4.3 A and I). These results demonstrate that the ^{1336}PTHPLR^{1341} motif in the P2 region of DCC mediates the interaction with the SH3 domain of p120RasGAP.

Netrin-1 promotes the recruitment of p120RasGAP to DCC in cortical growth cones

To assess the function of p120RasGAP in netrin-1/DCC-mediated signaling in cortical neurons, we first evaluated its localization relative to DCC by immunostaining cortical neurons following netrin-1 stimulation. Visualization by confocal microscopy revealed that p120RasGAP and DCC were both expressed in the cell bodies, axons, and growth cones of cortical neurons (Figure 4.4A). Netrin-1 increased the fluorescence intensity of p120RasGAP and DCC in the axons and growth cones (Figure 4.4A).

Quantification of Pearson's correlation coefficient revealed that the correlation between p120RasGAP and DCC fluorescence was significantly increased in growth cones after 10 minutes of stimulation with netrin-1 (Figure 4.4B). In whole cells, the correlation coefficients in unstimulated and netrin-1-stimulated neurons were 0.25 and 0.27, respectively. In growth cones, the correlation coefficient in unstimulated neurons was 0.16, and increased significantly to 0.24 with netrin-1 stimulation ($p=0.028$) (Figure 4.4B). These results indicate that netrin-1 promotes the recruitment of p120RasGAP to DCC in growth cones.

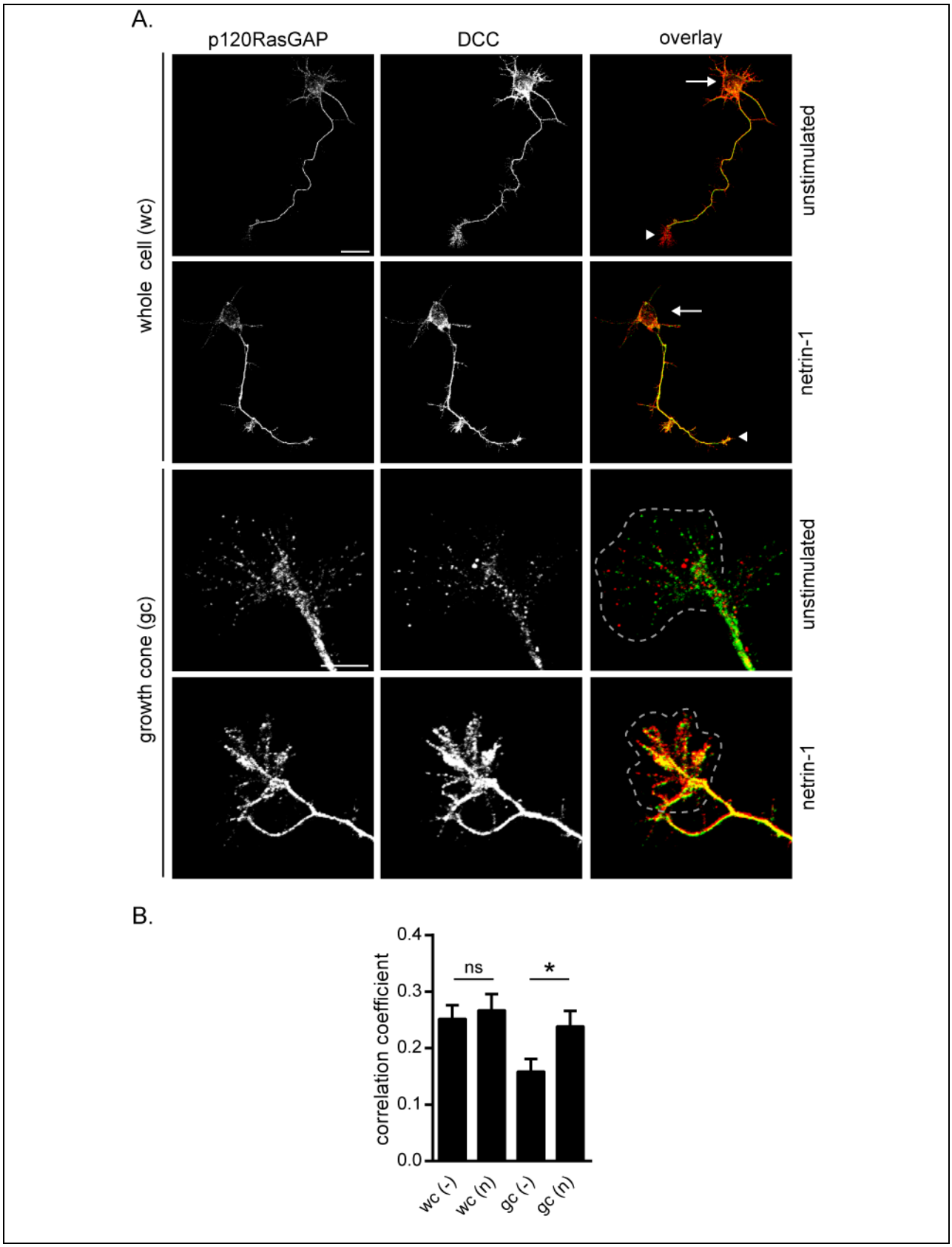

A.
p120RasGAP
DCC
overlay
whole cell (wc)
growth cone (gc)
unstimulated
netrin-1
unstimulated
netrin-1
B.
correlation coefficient
0.4
0.3
0.2
0.1
0.0
ns
*
wc (-)
wc (n)
gc (-)
gc (n)

Figure 4.4 p120RasGAP is recruited to DCC in cortical growth cones stimulated with netrin-1

(A) E18 embryonic rat cortical neurons (2DIV) were incubated with 500 ng/ml netrin-1 (n) or left unstimulated (-) for 10 minutes and immunostained with antibodies against DCC and p120RasGAP. Arrows indicate cell bodies and arrowheads indicate growth cones. The gray dashed outlines represent the growth cones. Scale bars=50 μm (wc) and 20 μm (gc). (B) The correlation between DCC and p120RasGAP fluorescence in (A) was measured using Pearson's correlation coefficient (±SEM) in whole cells (wc) and growth cones (gc) in three independent experiments (number of neurons = 65, 51, 54, 40 from left to right). Unpaired Student's *t* test: ns, not significant; *p=0.028.

p120RasGAP regulates ERK and FAK activation in netrin-1-stimulated embryonic cortical neurons

Next, we explored the role of p120RasGAP in netrin-1-mediated ERK and FAK activation in embryonic cortical neurons. Endogenous p120RasGAP expression was downregulated in E18 rat cortical neurons by electroporating synthetic siRNA targeting the 5' end of p120RasGAP mRNA (RASA) (34), which led to a significant decrease of p120RasGAP expression compared to control siRNA (Figure 4.5 A and B). In cell lysates from cortical neurons stimulated with netrin-1, downregulation of p120RasGAP had no effect on netrin-1-dependent phosphorylation of ERK and FAK (Supplemental

Figure 4.3). Given that ERK and FAK phosphorylation are receptor-proximal events, we reasoned that phosphorylation may occur in discrete cellular compartments that would not be efficiently detected in whole cell lysates. To overcome this limitation, we chose to monitor ERK and FAK activity by immunofluorescence and confocal microscopy (Figure 4.5 C and F). We quantified the average fluorescence intensity of pERK and FAK-pY397 at the plasma membrane of cortical neurons (Figure 4.5 D, E, G and H). After 10 minutes of netrin-1 stimulation, cortical neurons electroporated with control siRNA displayed a significant increase by 1.66- and 1.91-fold in pERK fluorescence intensity in whole cells and growth cones, respectively (Fig 5 D and E). In p120RasGAP-depleted neurons, pERK was significantly increased by 1.35- and 1.53-fold in whole cells and growth cones, respectively, and the addition of netrin-1 did not further increase its activity (Fig 5 D and E). FAK-pY397 was also aberrantly increased by 1.20-fold in whole p120RasGAP-deficient neurons (Figure 4.5G). After 10 minutes of netrin-1 stimulation, FAK-pY397 fluorescence intensity specifically and significantly increased by 1.52-fold in the growth cones of cortical neurons electroporated with control siRNA, and netrin-1 did not further increase the levels of FAK-pY397 in p120RasGAP-deficient neurons (Figure 4.5H). No change in total ERK or FAK fluorescence intensity was detected in p120RasGAP-deficient or netrin-1-stimulated neurons (Supplemental Figure 4.4). Overall, p120RasGAP depletion in cortical neurons caused aberrant activation of both ERK and FAK in whole cell and neuronal growth cone and prevented ERK and FAK

phosphorylation induced by netrin-1. Therefore, these results reveal that p120RasGAP is necessary for the proper regulation of ERK and FAK activities in netrin-1-stimulated cortical neurons.

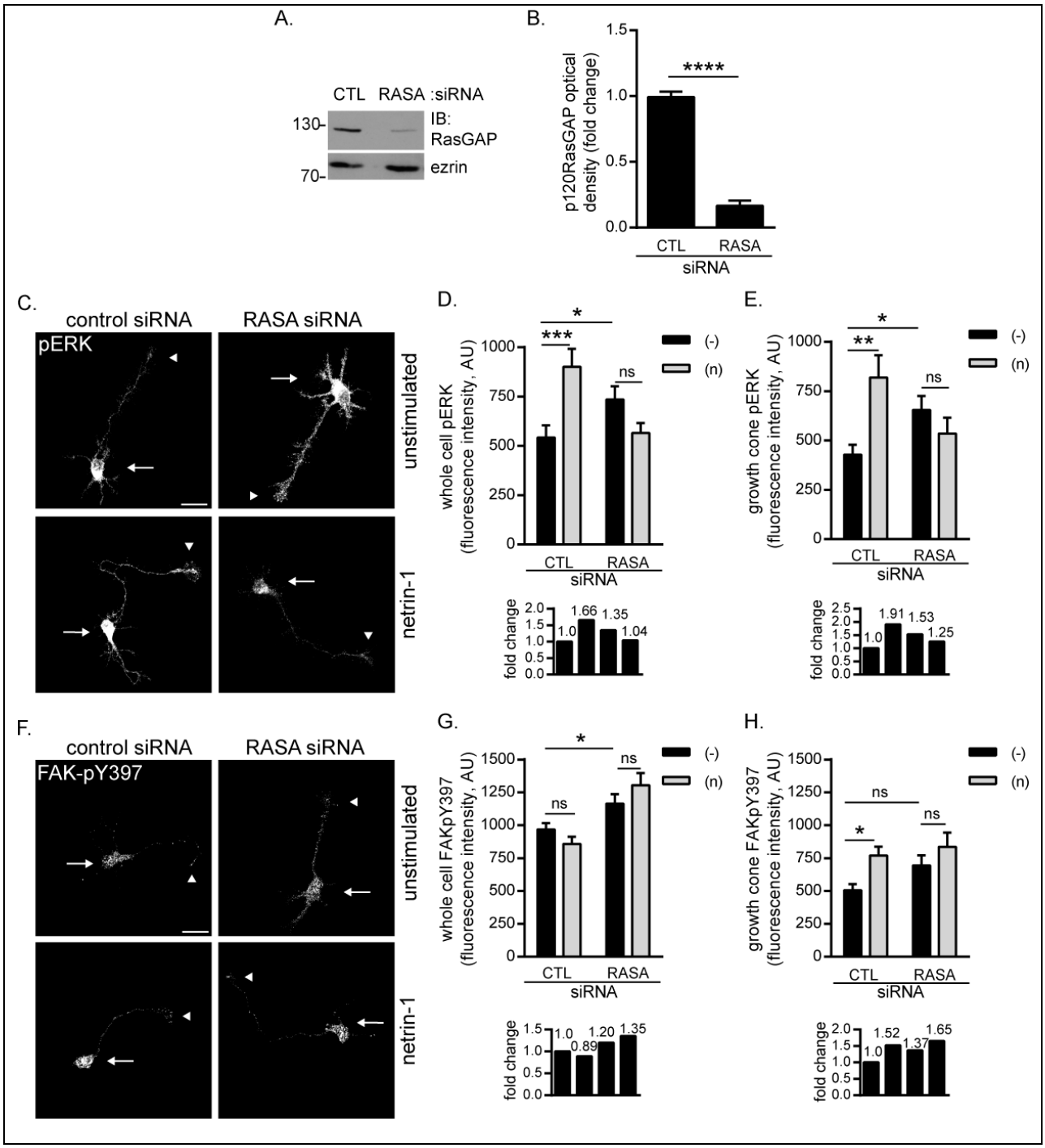


Figure 4.5 p120RasGAP regulates ERK and FAK activation in embryonic cortical neurons stimulated with netrin-1

E18 embryonic rat cortical neurons (2DIV) were incubated with 500 ng/ml netrin-1 (n) or left unstimulated (-) for 10 minutes. Control (CTL) or p120RasGAP (RASA) siRNA was electroporated in neurons at 0DIV with pGFP as a transfection reporter plasmid. (A) Total cell lysates were resolved by SDS-PAGE and immunoblotted (IB) with antibodies against p120RasGAP and ezrin as a loading control. (B) Quantitative densitometry of (A) is represented as the fold change (±SEM) relative to control siRNA measured in ten independent experiments. Unpaired Student's *t* test: ****p<0.0001. (C-H) The neurons were immunostained with antibodies against pERK (C) and FAK-pY397 (F). Arrows indicate cell bodies and arrowheads indicate growth cones. Scale bars=50 µm. pERK (D, E) and FAK-pY397 (G, H) fluorescence intensity (arbitrary units, AU; ±SEM) of GFP-expressing neurons in (C) and (F), respectively, and the fold change relative to unstimulated control neurons were measured in whole cells (number of neurons: pERK=35, 32, 36, 40 and FAK-pY397=64, 69, 65, 56) and growth cones (number of neurons: pERK=30, 27, 35, 28 and FAK-pY397=54, 60, 59, 50) in three independent experiments. Two-way ANOVA, Fisher's least significant difference (LSD) posttest: ns, not significant; *p<0.05, **p<0.005, ***p<0.001.

Netrin-1 activates Ras in embryonic cortical neurons and p120RasGAP is required to maintain basal Ras activity in neurons

Although the activation of ERK in response to netrin-1 has been previously reported (Campbell and Holt, 2003; Forcet et al., 2002; Ming et al., 2002), the activation of Ras in response to netrin-1 has not yet been demonstrated. To address this issue, we assessed the activation of Ras in response to netrin-1 in cortical neuron lysates by measuring Ras-GTP in a G-LISA assay. E18 rat cortical neurons were stimulated for 0 to 30 minutes with netrin-1 before cell lysates were harvested. Ras-GTP levels were significantly increased after 30 minutes of netrin-1 stimulation by 1.53-fold (Figure 4.6A). Consistent with previous reports, we also detected Rac1 activation in response to netrin-1, which occurred as early as 10 minutes following stimulation (Briancon-Marjollet et al., 2008) (Figure 4.6B). FAK and ERK phosphorylation were induced between 5 and 10 minutes of stimulation and maintained until 30 minutes of netrin-1 stimulation (Figure 4.6 C, D and E). We conclude that the onset and duration of Rac1, FAK and ERK activation are concurrent and precede Ras activation. These results led us to postulate that Rac1 may regulate ERK activity prior to Ras activation in response to netrin-1 stimulation (Aslan et al., 2013; Eblen et al., 2002; Jiang et al., 2000; Wang et al., 2013). To test this hypothesis, we monitored netrin-1-dependent ERK activation in the presence of the Rac1 inhibitor NSC23766. The inhibition of Rac1 impeded ERK

activation after 10 minutes of netrin-1 stimulation (Figure 4.6 F and G), suggesting that Rac1 does indeed promote early-onset ERK activation downstream of netrin-1.

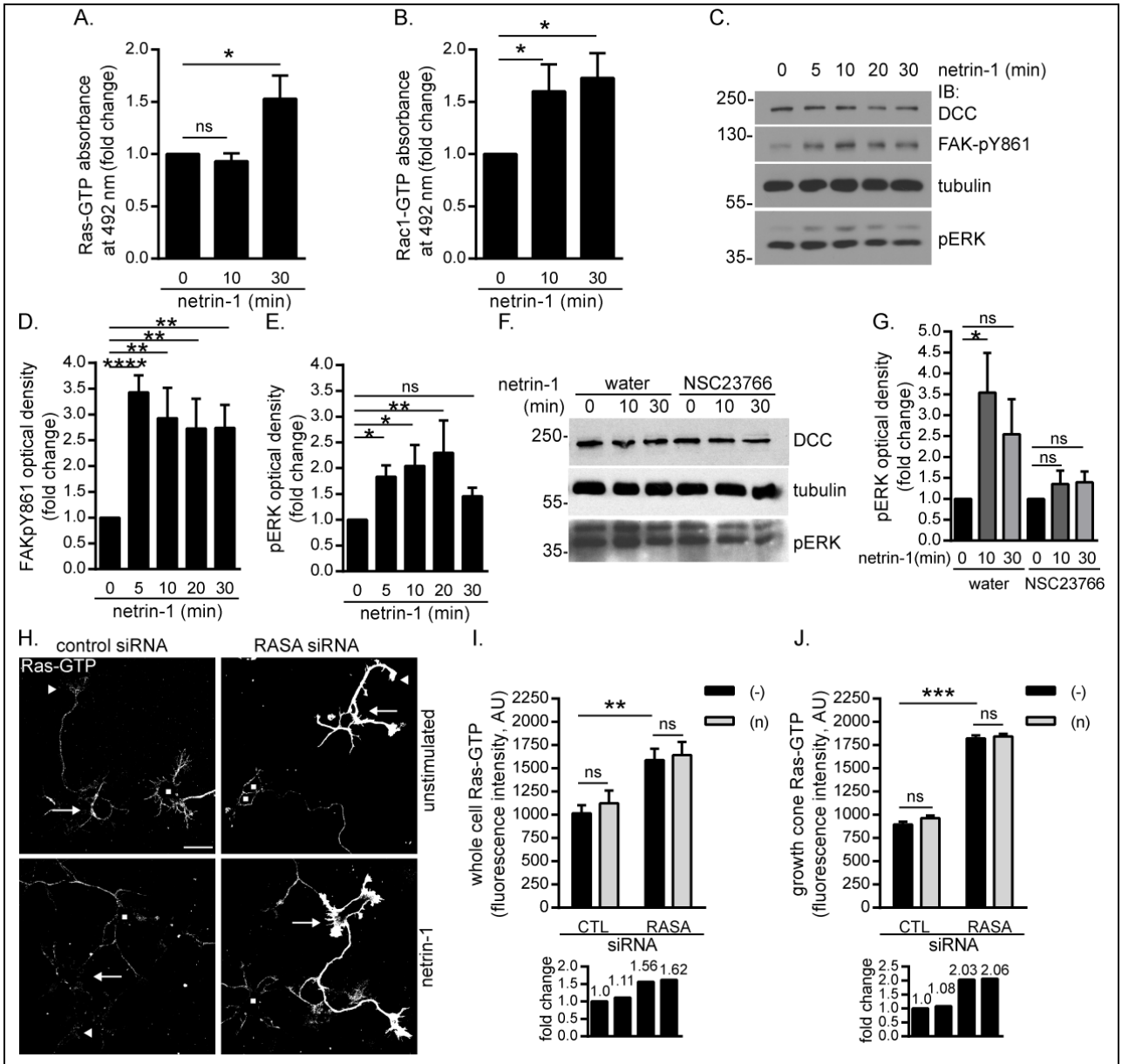


Figure 4.6 Netrin-1 activates Ras in embryonic cortical neurons and p120RasGAP is required to maintain basal Ras activity in neurons

E18 embryonic rat cortical neurons (2DIV) were stimulated with netrin-1 (500 ng/ml) for the indicated times. Ras-GTP and Rac1-GTP levels from total cell lysates were measured using G-LISA colorimetric assays. The levels of Ras-GTP (A) and Rac1-GTP (B) in each lysate were evaluated by measuring the absorbance at 492 nm which is represented as the fold change (±SEM) relative to the unstimulated lysate (0 min netrin-1) in at least three independent experiments. One-way ANOVA, Fisher's LSD posttest: ns, not significant; *p<0.05, **p<0.005, ****p<0.0005. (C) Total cell lysates were resolved by SDS-PAGE and immunoblotted (IB) with antibodies against the indicated proteins. (D, E) Quantitative densitometry (±SEM) of phosphorylated FAK (FAK-Y861) and ERK (pERK) is represented as the fold increase relative to 0 min of netrin-1 stimulation for at least three independent experiments. (F) Neurons were treated for 2h with the Rac1 inhibitor NSC23766 or water as a control prior to stimulation with netrin-1. Total cell lysates were resolved by SDS-PAGE and immunoblotted (IB) with antibodies against the indicated proteins. (G) Quantitative densitometry (±SEM) of phosphorylated ERK (pERK) is represented as the fold increase relative to 0 min of netrin-1 stimulation for six independent experiments. One-way ANOVA, Fisher's LSD posttest: ns, not significant; *p<0.05, **p<0.005, ****p<0.0005. (H) Control (CTL) or p120RasGAP (RASA) siRNA was electroporated in neurons at 0DIV with pGFP as a transfection reporter plasmid and were incubated with 500 ng/ml netrin-1 (n) or left unstimulated (-) for 30 minutes. Neurons were immunostained with anti-Ras-GTP antibodies. Arrows

indicate cell bodies and arrowheads indicate growth cones of GFP-expressing neurons. Squares represent untransfected neurons. Scale bar=50 μm. (I, J) Ras-GTP fluorescence intensity (arbitrary units, AU; ±SEM) of GFP-expressing neurons in (H) and the fold change relative to unstimulated control neurons for whole cells and growth cones (number of neurons=31, 29, 28, 32, 27, 29) was measured in three independent experiments. Two-way ANOVA, Fisher's LSD posttest: ns, not significant; **p<0.005, ***p<0.001.

To determine the role of p120RasGAP in Ras activation in neurons, we evaluated Ras-GTP in p120RasGAP-deficient cortical neurons by immunofluorescence using anti-Ras-GTP antibodies and confocal microscopy (Figure 4.6H). In cortical neurons electroporated with control siRNA, anti-Ras-GTP antibodies did not reveal any significant change in fluorescence intensity after 30 minutes of netrin-1 stimulation in whole cells or growth cones (Figure 4.6 I and J). This discrepancy with the Ras G-LISA assay data suggests that fluorescence intensity using the anti-Ras-GTP antibodies was not sufficient to detect local changes in Ras activity in neurons. Nonetheless, in p120RasGAP-deficient cortical neurons, Ras-GTP levels showed a 1.56- and 2.03-fold increase in whole cells and growth cones, respectively, which was not further elevated by netrin-1 stimulation (Figure 4.6 I and J). Therefore, these results demonstrate that

netrin-1 leads to Ras activation in cortical neurons and that p120RasGAP is required to maintain low levels of Ras-GTP in neurons.

The SH2-SH3-SH2 domains of p120RasGAP mediate netrin-1-dependent cortical axon outgrowth

We then explored the role of p120RasGAP in netrin-1-induced axon outgrowth in cortical neurons (Antoine-Bertrand et al., 2011a; Briancon-Marjollet et al., 2008). p120RasGAP siRNA was electroporated together with GFP cDNA as a reporter to visualize the neurons. We measured the average axon length of GFP-expressing neurons after 24 hours of incubation with netrin-1. Netrin-1 significantly increased the average axon length compared to unstimulated control neurons (Figure 4.7 A and B), as previously described (Antoine-Bertrand et al., 2011a; Briancon-Marjollet et al., 2008; DeGeer et al., 2013). Depletion of p120RasGAP resulted in significantly longer axons (103.8 μm, $p<0.0001$) (Figure 4.7 A and B). Surprisingly, netrin-1 significantly decreased the length of p120RasGAP-deficient neurons (88.6 μm, $p=0.0051$) (Figure 4.7 A and B). To assess the specificity of the p120RasGAP effects on axon extension, we analyzed the glutamate response in p120RasGAP-deficient neurons. Consistent with previous studies, glutamate stimulation increases axon outgrowth in cortical neurons and the effect was independent of p120RasGAP (Antoine-Bertrand et al., 2011a; Briancon-Marjollet et al., 2008) (Figure 4.7C). Thus, the decrease in outgrowth in p120RasGAP-

deficient neurons was specific to netrin-1, suggesting that p120RasGAP is required for netrin-1 to positively regulate axon extension.

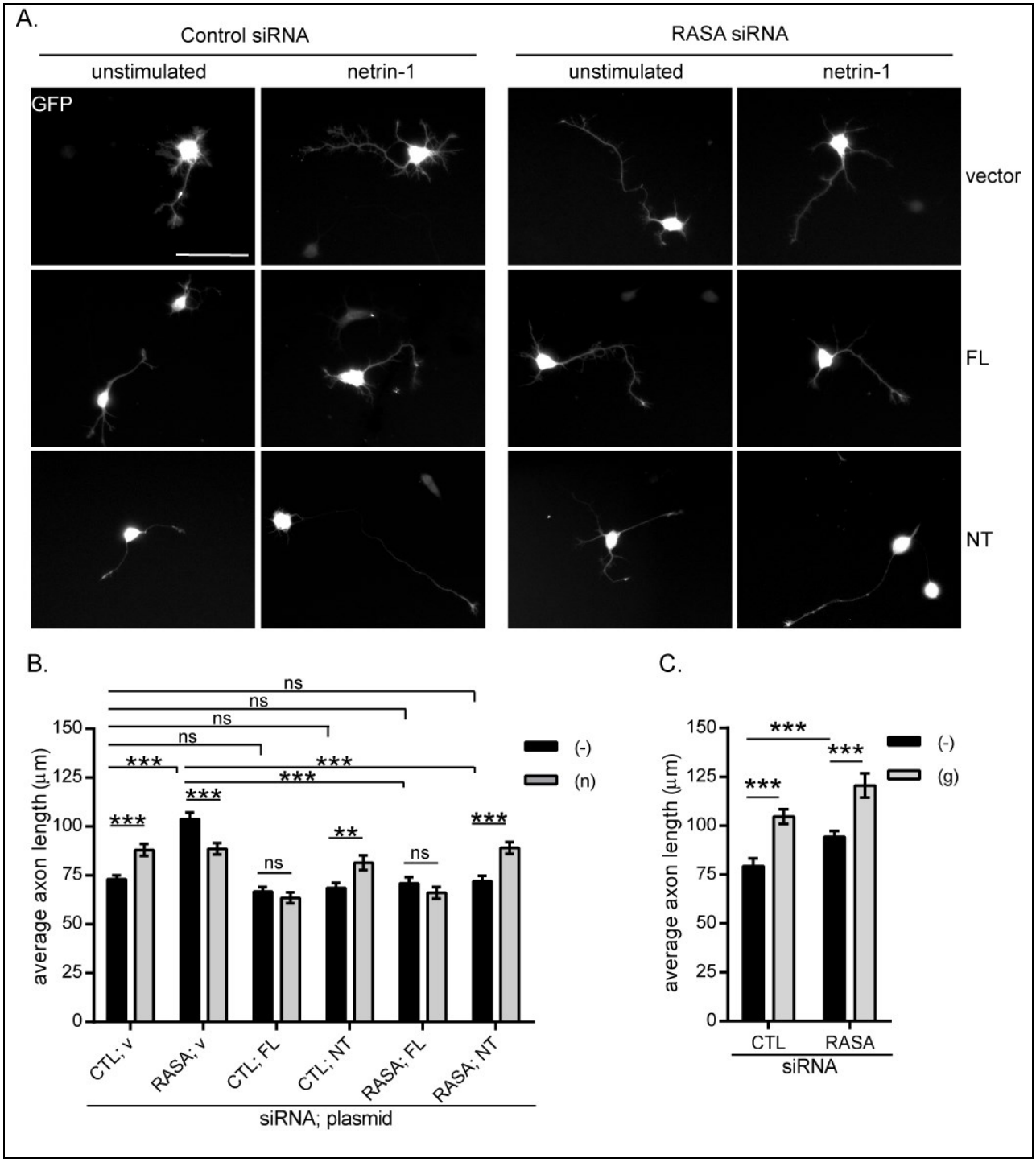


Figure 4.7 The N-terminus of p120RasGAP mediates netrin-1-dependent cortical axon outgrowth

Control (CTL) or p120RasGAP (RASA) siRNA was electroporated in E18 embryonic rat cortical neurons at 0DIV with pGFP as a transfection reporter plasmid. Neurons at 1DIV were incubated with 200 ng/ml netrin-1 (n), 50 μM glutamate (g) or left unstimulated (-) for 24 hours and axon outgrowth was assessed in GFP-expressing neurons. (A) Control vector (v), full-length (FL) or N-terminal (NT) p120RasGAP were co-expressed with control or p120RasGAP siRNA and pGFP in cortical neurons. Scale bar=50 μm. (B, C) Axon outgrowth was measured and expressed as the average axon length (μm; ±SEM) in at least three independent experiments (number of neurons: in (B) 381, 224, 272, 191, 228, 180, 222, 165, 146, 162, 179, 182 and in (C) 299, 210, 381, 190). Two-way ANOVA, Fisher's LSD posttest: ns, not significant; *$p<0.05$, **$p<0.005$, ***$p<0.001$.

To determine the domains within p120RasGAP responsible for the response to netrin-1, we expressed siRNA-resistant human full-length (FL) or the NT domain of p120RasGAP in control or p120RasGAP-deficient neurons (Figure 4.7 A and B, and Supplemental Figure 4.5). p120RasGAP-FL overexpression in control neurons inhibited netrin-1-induced axon outgrowth (Figure 4.7 A and B). In contrast, netrin-1 was able to significantly stimulate axon outgrowth of control neurons expressing p120RasGAP-NT

suggesting that the GAP domain blocks netrin-1 responsiveness (Figure 4.7 A and B). In p120RasGAP-deficient neurons, introduction of p120RasGAP-FL restored the axon length to a length comparable to unstimulated control neurons validating the specificity of the siRNA (Figure 4.7 A and B). Similar to control neurons overexpressing p120RasGAP-FL, these neurons did not respond to netrin-1 stimulation (Figure 4.7 A and B). Furthermore, p120RasGAP-NT expression in p120RasGAP-depleted neurons was able to restore netrin-1-dependent axon outgrowth (Figure 4.7 A and B). Thus, the positive regulation of netrin-1-dependent axon outgrowth by p120RasGAP is mediated by the N-terminal scaffolding SH2-SH3-SH2 domains while overexpressed GAP activity blocks netrin-1 signaling.

p120RasGAP is required for netrin-1-dependent attraction of embryonic cortical neurons

To determine whether p120RasGAP regulates netrin-1-dependent chemoattraction, we evaluated the impact of p120RasGAP depletion on cortical growth cone turning in response to a netrin-1 gradient using a Dunn chamber turning assay (Kent et al., 2010; Yam et al., 2012; Yam et al., 2009). The growth cones of cortical neurons electroporated with control siRNA randomly turned with no particular preference for any direction when exposed to a control phosphate-buffered saline (PBS) gradient (Figure 4.8A), but were attracted to a netrin-1 gradient (Figure 4.8B). The

introduction of p120RasGAP siRNA inhibited the attractive response to netrin-1 and growth cones reverted to turning randomly (Figure 4.8 C and D). 64% of the control growth cones were attracted to the netrin-1 gradient, whereas the percentage that turned towards the netrin-1 gradient in p120RasGAP-deficient neurons (46%) was similar to the percentage that turned towards PBS in control neurons (43%) (Figure 4.8E). In fact, the turned angle of growth cones in response to netrin-1 (9.22° ± 4.03º: mean angle turned ± standard error of the mean (SEM)) was significantly reduced when p120RasGAP was depleted (-4.39° ± 3.86º) (Figure 4.8F). p120RasGAP downregulation did not have an effect on displacement rates during the time the growth cones were imaged (Figure 4.8G). These results demonstrate that p120RasGAP is required for netrin-1-dependent chemoattraction.

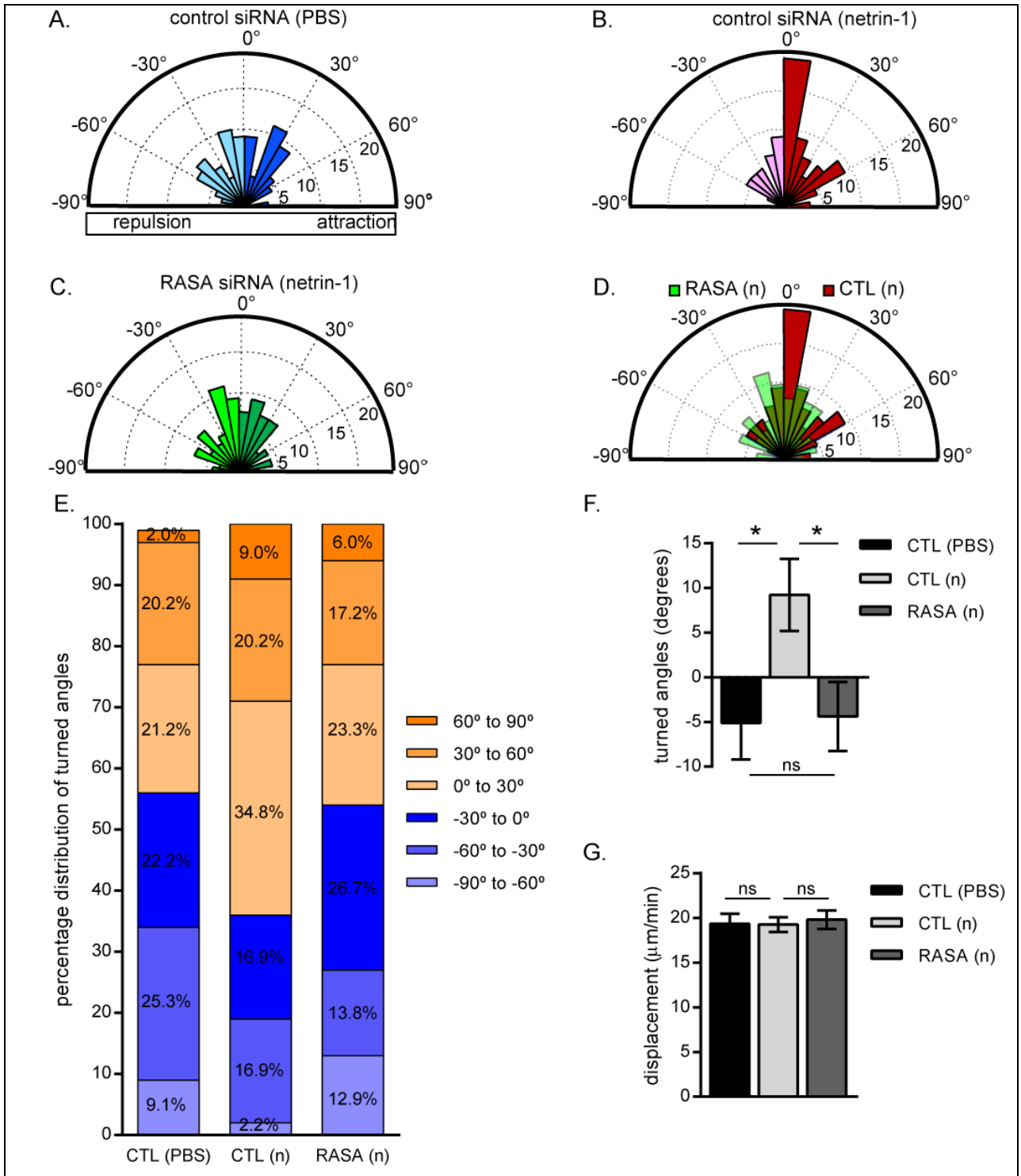


Figure 4.8 p120RasGAP is required for netrin-1-dependent attraction

Control (CTL) or p120RasGAP (RASA) siRNA was electroporated with pGFP as a transfection reporter plasmid in E18 embryonic rat cortical neurons at 0 DIV. At 2 DIV,

the neurons were exposed to a control PBS or a 200 ng/ml netrin-1 VI-V (n) gradient for 90 minutes. (A-C) Rose histograms represent the distribution of turned angles of cortical growth cones when exposed to a control PBS (A) or a netrin-1 gradient (B, C). Responses of individual neurons were clustered in 10° bins and the percentage of total neurons per bin is represented by the radius of each segment (number of neurons=99, 89, 116, respectively). (D) The overlay of the rose histograms presented in (B) and (C) is illustrated to compare the response to netrin-1 of control and p120RasGAP-deficient neurons. (E) The bar graph represents the turned angles percentage distribution of cortical growth cones in panels (A-C). (F) The mean turned angle (±SEM) toward the gradient was measured in degrees for each condition. (G) The mean displacement (±SEM) for a 90-minute netrin-1 treatment was calculated. One-way ANOVA, Fisher's LSD posttest: ns, not significant; *p< 0.05.

4.5 Discussion

In this study, we report a novel interaction between p120RasGAP and the netrin-1 receptor DCC in embryonic cortical neurons. We demonstrate that p120RasGAP is essential for netrin-1 to efficiently mediate axon outgrowth and attraction of embryonic cortical neurons. We find that p120RasGAP plays two roles in netrin-1/DCC signaling: it serves as a scaffolding protein and it regulates Ras/ERK activities in a time-dependent manner. To our knowledge, this study is the first one to demonstrate the positive role of

p120RasGAP activity during axon outgrowth and guidance and to show that netrin-1 activates Ras in primary neurons.

Although ERK activity was reported to be involved in netrin-1-mediated axon outgrowth and guidance (Campbell and Holt, 2003; Forcet et al., 2002), the mechanism by which ERK is activated by netrin-1 remained to be elucidated (Campbell and Holt, 2003; Forcet et al., 2002; Ming et al., 2002). The canonical pathway to ERK activation begins with the activation of Ras GTPase and the sequential activation of downstream kinases Raf, MEK and ERK (McKay and Morrison, 2007). Ras activity positively regulates many aspects of neuronal development, including axon initiation, elongation and guidance (Hall and Lalli, 2010), whilst the axon guidance cues of the Ephrin and Semaphorin protein families induce neurite retraction and growth cone collapse in part due to their inhibition of Ras activity (Dail et al., 2006; Elowe et al., 2001; Oinuma et al., 2004a). Here, we demonstrate that netrin-1 increases significantly the levels of Ras-GTP in cortical neurons after 30 minutes of stimulation (Figure 4.6A). However, netrin-1-mediated rapid and sustained ERK activation occurs much earlier than Ras activation (Figure 4.6 C and E), indicating that early activation of ERK is independent of Ras activity in cortical neurons. An alternative route to ERK activation is mediated by Rac1 and its effector p21-activated kinase (PAK), which bypass Ras and directly activate the Raf/MEK/ERK cascade (Aslan et al., 2013; Eblen et al., 2002; Jiang et al., 2000; Wang et al., 2013). Indeed, both Rac1 and ERK activation were observed from 5 to 10

minutes of netrin-1 stimulation and sustained until 30 minutes of treatment in cortical neurons (Figure 4.6 B, C and E). Furthermore, we also demonstrated that Rac1 activity was required for the early-onset ERK activation downstream of netrin-1 (Figure 4.6 F and G). In light of these observations, we propose that ERK activation by netrin-1 is initially mediated by Rac1/PAK signaling, and later mediated by Ras activation in order to sustain long-term ERK activation in cortical neurons (Figure 4.9). The delay in netrin-1-mediated Ras activation also suggests that Ras activity is tightly regulated before and after netrin-1 stimulation. As a GAP, p120RasGAP stimulates the GTPase activity of Ras to inactivate the GTPase (Gideon et al., 1992; Marshall et al., 1989; Vogel et al., 1988). In the absence of netrin-1 treatment, p120RasGAP-deficient cortical neurons exhibit an aberrant increase in Ras/ERK activities (Figure 4.5 C, D and E and Figure 4.6 H, I and J), showing that p120RasGAP acts as a negative regulator of Ras activity in unstimulated conditions (Figure 4.9). Netrin-1 does not further increase Ras/ERK activities in these neurons, indicating that p120RasGAP is required for netrin-1 signaling.

Ras is eventually activated by netrin-1, which implies that netrin-1 inhibits the GAP activity of p120RasGAP. *Endo et al.* have reported that p120RasGAP is sequestered and inhibited by the neogenin receptor via an interaction with FAK-pY397 (Endo and Yamashita, 2009). The repulsive guidance cue, RGMa, releases p120RasGAP from that interaction to mediate the inhibition of Ras activity (Endo and

Yamashita, 2009). Other studies have shown that the GAP activity of p120RasGAP is inhibited when its N-terminus interacts with p190RhoGAP, FAK, p200RhoGAP or SOCS-3 (Cacalano et al., 2001; Hecker et al., 2004; Moran et al., 1991; Shang et al., 2007). Tyrosine phosphorylation of these proteins, except for p200RhoGAP, mediated the interaction (Cacalano et al., 2001; Hecker et al., 2004; Moran et al., 1991). Here, we find that netrin-1-induced Ras activation is preceded by p120RasGAP tyrosine phosphorylation and the formation of a DCC-p120RasGAP protein complex in cortical neurons (Figure 4.2E and F and Figure 4.6A). The N-SH2 and C-SH2 domains recognize and bind to the phosphorylated tyrosine residues Y1418 and Y1361 of DCC, respectively, which are both conserved amongst vertebrate DCC orthologs (Figure 4.3 D and E, and Supplemental Figure 4.2). The phosphorylation of FAK-Y397 mediates interactions with both the N-SH2 domain of p120RasGAP and DCC (Hecker et al., 2004; Ren et al., 2004). Additionally, the SH3 domain of p120RasGAP interacts with the ^{1336}PTHPLR^{1341} motif of DCC (Figure 4.3I). All these interactions occur within or close to the P2 and P3 regions of DCC (Figure 4.3G), suggesting that these domains sequester and inactivate p120RasGAP activity. Moreover, earlier studies from Pawson and colleagues suggested that p120RasGAP regulates the level and duration of PDGF-dependent Ras/ERK activation in fibroblasts (Kulkarni et al., 2000; van der Geer et al., 1997). Conversely, the absence of p120RasGAP recruitment to the EphB2 receptor delayed and reduced ephrinB1-dependent ERK inhibition in neuroblastoma cells (Elowe

et al., 2001). Extracellular factors such as PDGF, EGF, and fibroblast growth factor (FGF) have been reported to induce tyrosine phosphorylation of p120RasGAP and activation of Ras (Ellis et al., 1990; Kaplan et al., 1990; Liu and Pawson, 1991; Molloy et al., 1989; Moran et al., 1991; Woodcock and Hughes, 2004). In this study, we demonstrate that tyrosine phosphorylation of p120RasGAP is concurrent with the interaction of DCC, FAK, and ERK in response to netrin-1 (Figure 4.2E). Based on these results, we propose that netrin-1 mediates the tyrosine phosphorylation and sequestration of p120RasGAP to inhibit its GAP activity and promote the activation of Ras (Figure 4.9). Thus, we conclude that p120RasGAP regulates the timing of netrin-1-dependent Ras activation and that this regulation is essential for netrin-1-dependent functions in cortical neurons.

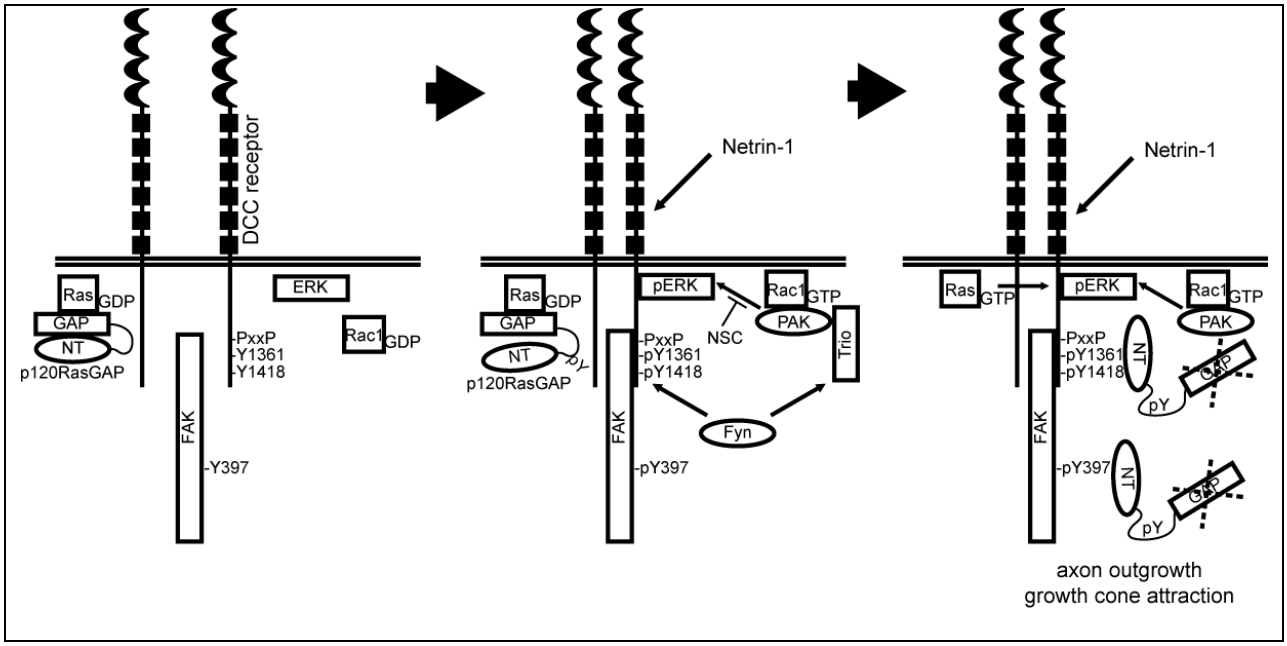


Figure 4.9 Proposed model for the role of p120RasGAP in netrin-1/DCC-mediated axon outgrowth and guidance

In the absence of ligand, the GAP domain of p120RasGAP maintains Ras inactive (Ras-GDP). ERK and Rac1 (Rac1-GDP) are also inactive. Within 5 to 10 minutes of netrin-1 stimulation, DCC-Y1418/Y1361, the guanine exchange factor (GEF) Trio and p120RasGAP are phosphorylated by Src family kinases, including Fyn (Briancon-Marjollet et al., 2008; DeGeer et al., 2013). Rac1 is activated (Rac1-GTP) by Trio and activates ERK (pERK). Activated FAK (pY397) and ERK are then recruited to DCC. After the onset of netrin-1 stimulation, p120RasGAP is sequestered by DCC via the direct interaction of the N-terminal SH2-SH3-SH2 domains (NT) of p120RasGAP with DCC or an indirect interaction with FAK. Then, p120RasGAP serves as a scaffold to form multiprotein complexes essential to netrin-1/DCC signaling. Through these interactions and tyrosine phosphorylation, the GAP activity of p120RasGAP would be blocked and no longer inhibits Ras. Activated Ras (Ras-GTP) can then mediate sustained ERK activation in response to netrin-1. In conclusion, the N-terminal and GAP domains of p120RasGAP positively regulate netrin-1/DCC-dependent axon outgrowth and growth cone attraction.

This novel role as a positive modulator of the attractive function of netrin-1 is in marked contrast with previous reports that describe p120RasGAP as a negative

regulator of axon outgrowth and guidance in primary neurons (Dail et al., 2006; Elowe et al., 2001; Endo and Yamashita, 2009; Hancock et al., 2014; Oinuma et al., 2004a). We further substantiate the evidence for a positive role of p120RasGAP by demonstrating that the N-terminus of p120RasGAP is required to mediate axon outgrowth in response to netrin-1. In addition to the PH, C2 and C-terminal GAP domains, the N-terminus of p120RasGAP, comprising one SH3 and 2 SH2 domains, interacts with various proteins to regulate cell survival, proliferation and migration (King et al., 2013; Pamonsinlapatham et al., 2009). In cortical neurons, the depletion of p120RasGAP expression leads to a significant increase in axon length of unstimulated neurons and the inhibition of netrin-1-induced axon outgrowth (Figure 4.7A and B). In p120RasGAP-deficient neurons, re-expression of full-length or the N-terminal SH2-SH3-SH2 domains of p120RasGAP is sufficient to restore the axon length to one that is comparable to unstimulated control neurons. However, netrin-1-induced axon outgrowth is only restored by the expression of the N-terminal SH2-SH3-SH2 domains of p120RasGAP in these neurons. These results suggest that overexpression of p120RasGAP-FL inhibits netrin-1-mediated outgrowth due to its GAP activity which represses axon extension as reported by previous studies (Dail et al., 2006; Elowe et al., 2001; Endo and Yamashita, 2009; Hancock et al., 2014; Oinuma et al., 2004a). Thus, the N-terminus of p120RasGAP is sufficient to regulate netrin-1-mediated axon outgrowth independently of the GAP domain. Among its GAP-independent functions, it has been demonstrated

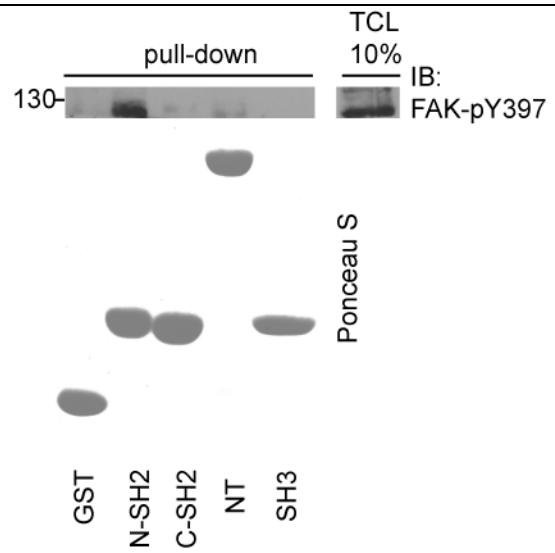
that the N-terminus of p120RasGAP can also serve as a Ras effector and a RhoA inhibitor. Firstly, the expression of the N-terminus increases Ras and ERK activation in fibroblasts (Elowe et al., 2001), and promotes Ras-dependent differentiation of PC12 cells *(Leblanc et al., 1998; Nakata and Watanabe, 1996).* These studies and others suggest that the N-terminus of p120RasGAP forms interactions that promote Ras signaling (Gideon et al., 1992; Tocque et al., 1997; Yatani et al., 1990). Secondly, cell migration is severely impaired in p120RasGAP-deficient mouse fibroblasts due in part to cell polarity defects and the lack of focal adhesion turnover at the leading edge of cells (Kulkarni et al., 2000). Both defects are rescued by the expression of p120RasGAP-NT (Kulkarni et al., 2000). Focal adhesion turnover at the leading edge of migrating fibroblasts is regulated by a protein complex consisting of p120RasGAP, FAK and p190RhoGAP, which inhibits RhoA activation and promotes cell migration (Kulkarni et al., 2000; McGlade et al., 1993; Tomar et al., 2009). Src family kinase activity regulates the interaction of the SH2 domains of p120RasGAP with FAK and p190RhoGAP to promote their recruitment to the plasma membrane and the cytoskeleton (Hu and Settleman, 1997; Kulkarni et al., 2000; Moran et al., 1991). In our study, the recruitment of p120RasGAP to DCC in response to netrin-1 indicates that p120RasGAP regulates specific functions in growth cones, the “leading edge of neurons” (Figure 4.4). We show that netrin-1 promotes the interaction of p120RasGAP with DCC, FAK and ERK, and that similarly to the regulation of the p120RasGAP-FAK-p190RhoGAP complex, these

interactions are dependent on Src family kinase activity (Figure 4.2E). Other proteins involved in netrin-1/DCC signaling such as NCK, Src and ezrin also interact with p120RasGAP (Antoine-Bertrand et al., 2011a; Ger et al., 2011; Li et al., 2004; Li et al., 2002a; Park et al., 1992; Viswanatha et al., 2013). Furthermore, tyrosine phosphorylation of p120RasGAP is also regulated by Src family kinase activity (Figure 4.2E), and could induce conformational changes that favor complex formation. With two SH2 and one SH3 domain, the N-terminus of p120RasGAP has the ability to engage in a wide range of protein interactions and to form multiple and diverse multiprotein complexes. Thus, we conclude that netrin-1 activates the scaffolding function of the N-terminus of p120RasGAP via Src family kinase activity to regulate netrin-1/DCC signaling in neuronal growth cones and promote netrin-1-mediated axon outgrowth.

In conclusion, the depletion of p120RasGAP expression severely impairs netrin-1/DCC signaling. Netrin-1 cannot stimulate ERK and FAK activities, axon outgrowth or growth cone turning in p120RasGAP-deficient cortical neurons. In fact, netrin-1 decreases axon outgrowth in these neurons. It will be of great interest to address whether this reduction in axon outgrowth results from the loss of p190RhoGAP regulation in p120RasGAP-deficient cortical neurons. We propose that p120RasGAP not only regulates the timing of netrin-1-dependent Ras activation but that it is also a molecular clutch and scaffold that primes and engages DCC in attractive netrin-1 signaling by recruiting key regulators (Figure 4.9). Until now, the functions of the GAP domain had been studied

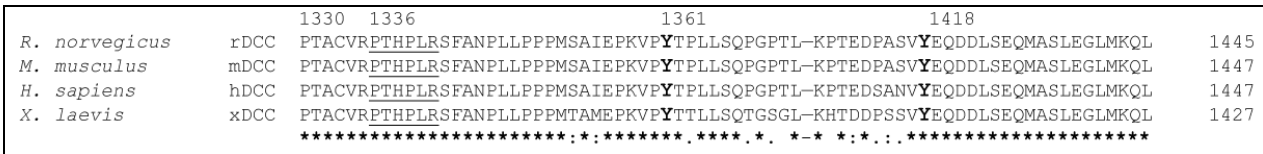
separately from the functions of the N-terminus. Studies that examine how the molecular interactions of p120RasGAP are integrated to initiate a unified cellular response will certainly be more successful at deciphering the intricacies of p120RasGAP functions in the future. The significance of these findings is not limited to the mechanisms of axon growth and guidance since Ras/p120RasGAP activities and netrin-1/DCC signaling are implicated in the regulation of vascular development and the progression of cancer (King et al., 2013; Larrieu-Lahargue et al., 2012; Mehlen and Guenebeaud, 2010; Pamonsinlapatham et al., 2009). Notably, the autosomal dominant disorder capillary malformation-arteriovenous malformation (CM-AVM) is caused by heterozygous mutations in the p120RasGAP locus, *RASA1* (Bayrak-Toydemir and Stevenson, 2014; Eerola et al., 2003; Revencu et al., 2013; Revencu et al., 2008). The study of netrin-1 signal transduction outside the nervous system will undoubtedly add to our understanding of the physiological functions of p120RasGAP.

4.6 Supplemental information



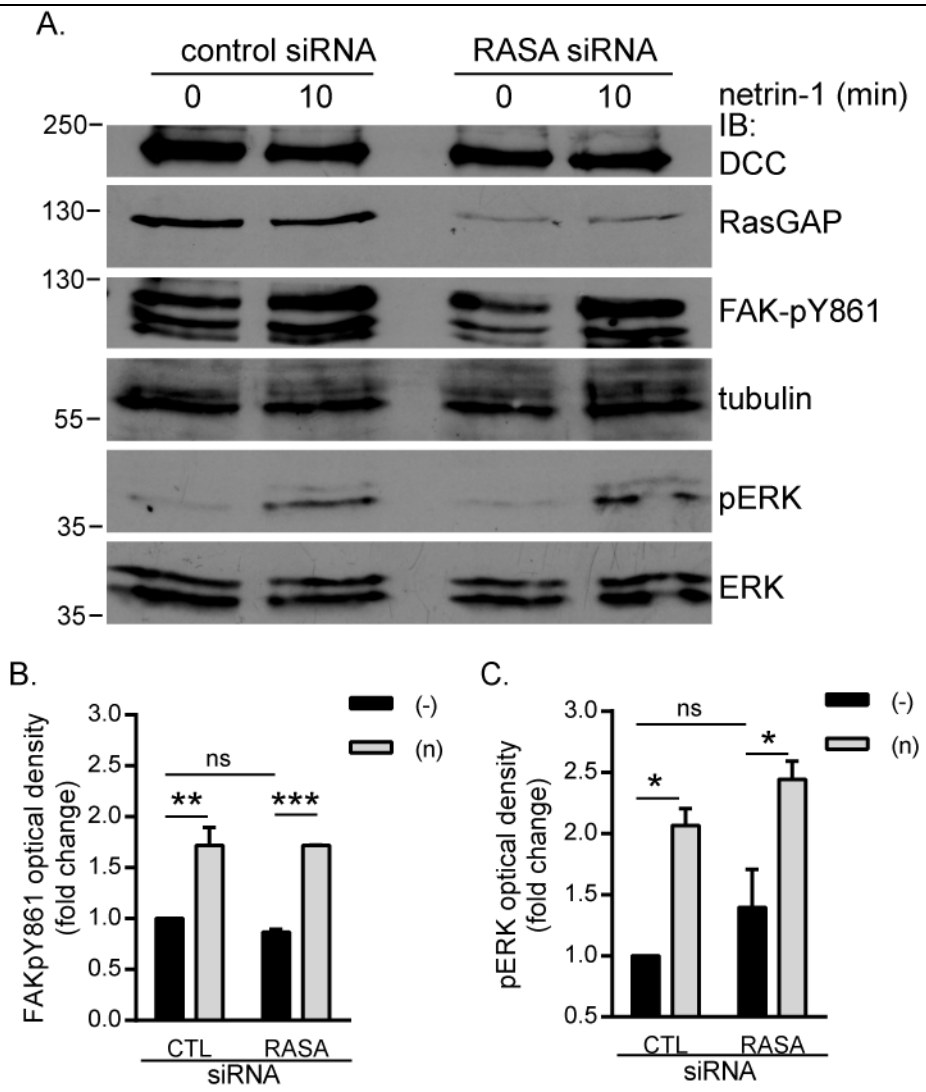
Supplemental Figure 4.1 The N-SH2 domain of p120RasGAP interacts with FAK-pY397

Proteins from HEK293 cell lysates were pulled-down using purified GST-p120RasGAP N-SH2, C-SH2, SH3, SH2-SH3-SH2 (NT) or GST as control. Associated proteins (pull-down) and total cell lysates (TCL) were resolved by SDS-PAGE and immunoblotted (IB) with antibodies against FAK-pY397. GST fusion proteins were stained with Ponceau S.



Supplemental Figure 4.2 The P1336/P1339 PxxP motif and tyrosines Y1361 and Y1418 are conserved in vertebrate DCC orthologs

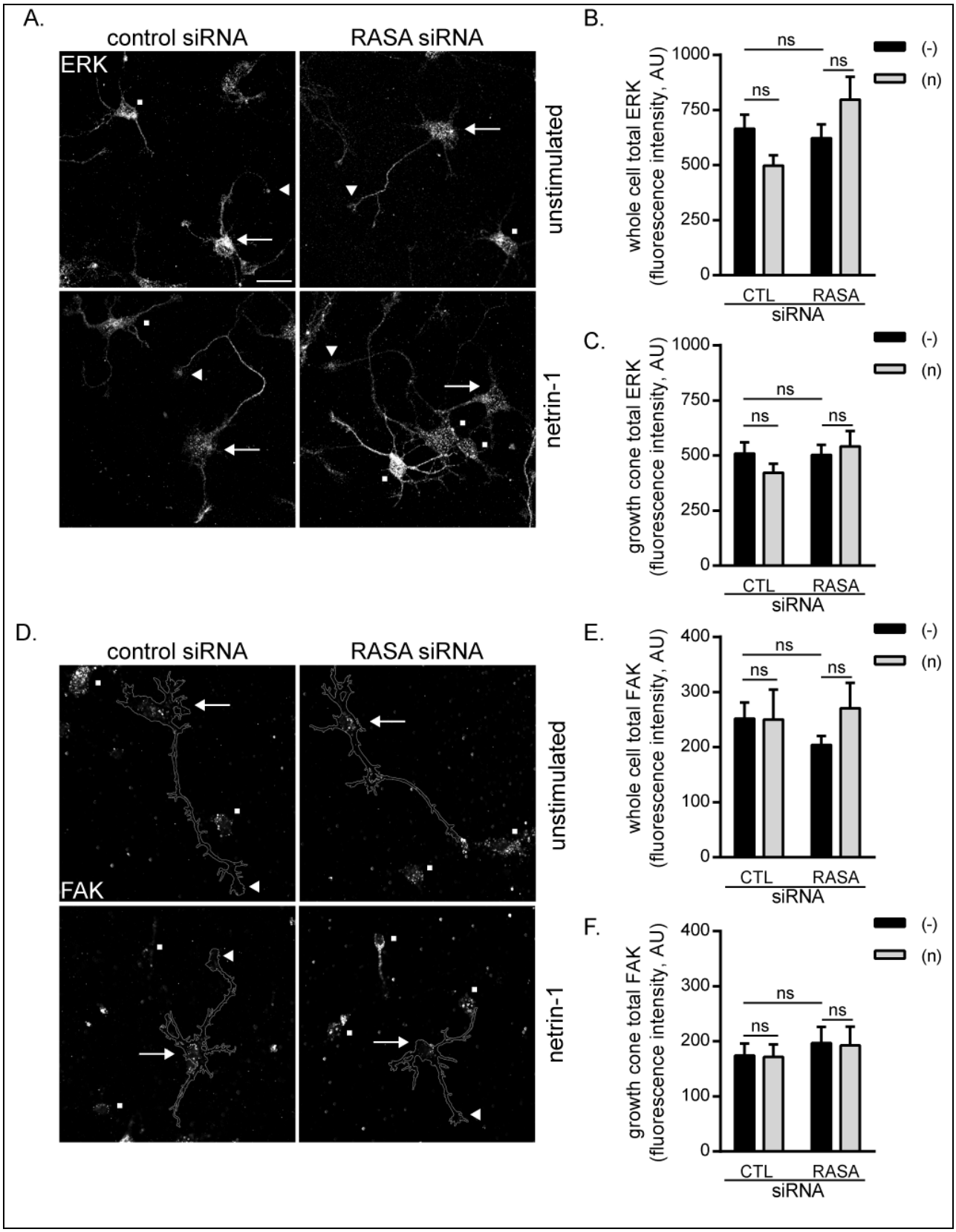
Amino acid sequence alignment of DCC proteins from different vertebrate species was performed with Clustal W software. The conserved P1336/P1339 PxxP motif is underlined and the conserved tyrosine residues are in bold.



Supplemental Figure 4.3 p120RasGAP downregulation does not alter netrin-1-dependent ERK and FAK activation in cortical neuron lysates

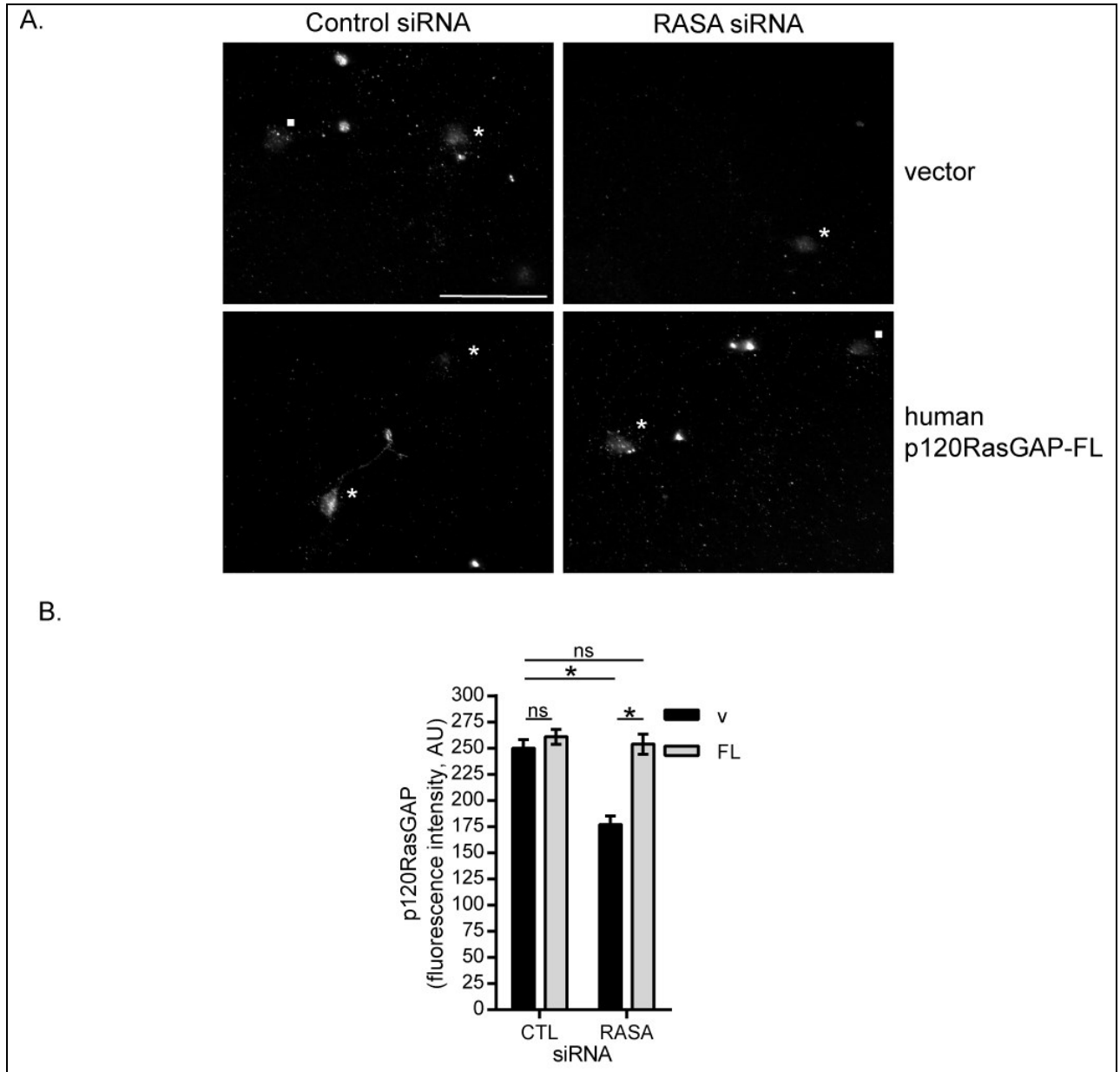
Control (CTL) or p120RasGAP (RASA) siRNA was electroporated in E18 embryonic rat cortical neurons at 0 DIV with pGFP as a transfection reporter. The neurons were incubated at 2DIV with netrin-1 (500ng/ml) for 10 minutes (n) or left unstimulated (-). (A) Total cell lysates were resolved by SDS-PAGE and immunoblotted (IB) with antibodies against the indicated proteins. (B, C) Quantitative densitometry of (A) is represented as

the fold change (±SEM) relative to unstimulated control neurons for FAK-pY861 (B) and pERK (C) from at least three independent experiments. Two-way ANOVA, Tukey's posttest: *p<0.05, **p<0.005, ***p<0.001.


A.
control siRNA
RASA siRNA
ERK
unstimulated
netrin-1
B.
whole cell total ERK (fluorescence intensity, AU)
1000
750
500
250
0
ns
ns
ns
(-)
(n)
CTL
RASA
siRNA
C.
growth cone total ERK (fluorescence intensity, AU)
1000
750
500
250
0
ns
ns
ns
(-)
(n)
CTL
RASA
siRNA
D.
control siRNA
RASA siRNA
FAK
unstimulated
netrin-1
E.
whole cell total FAK (fluorescence intensity, AU)
400
300
200
100
0
ns
ns
ns
(-)
(n)
CTL
RASA
siRNA
F.
growth cone total FAK (fluorescence intensity, AU)
400
300
200
100
0
ns
ns
ns
(-)
(n)
CTL
RASA
siRNA

Supplemental Figure 4.4 RasGAP downregulation does not alter total ERK and FAK expression in embryonic cortical neurons

E18 embryonic rat cortical neurons (2DIV) were incubated with 500 ng/ml netrin-1 (n) or left unstimulated (-) for 10 minutes. Control (CTL) or p120RasGAP (RASA) siRNA was electroporated in neurons at 0DIV with pGFP as a transfection reporter plasmid. The neurons were immunostained with antibodies against ERK (A) or FAK (D). Arrows and arrowheads indicate the cell bodies and growth cones of GFP-expressing neurons (gray outlines in D), respectively. Squares indicate untransfected neurons. Scale bars=50 μm. Total ERK (B, C) and total FAK (E, F) fluorescence intensity (arbitrary units, AU; ±SEM) of GFP-expressing neurons in (A) and (D), respectively was measured in whole cells (number of neurons: ERK=41, 33, 38, 38 and FAK=24, 19, 23, 21) and growth cones (number of neurons: ERK=41, 33, 38, 38 and FAK=23, 19, 23, 21) in three independent experiments. Two-way ANOVA, Tukey's posttest: ns, not significant.



Supplemental Figure 4.5 Human p120 RasGAP is expressed in p120RasGAP-deficient rat embryonic cortical neurons

Control (CTL) or p120RasGAP (RASA) siRNA was electroporated in E18 embryonic rat cortical neurons at 0 DIV with control vector or full-length (FL) human p120RasGAP with pGFP as a transfection reporter plasmid. (A) The cortical neurons from Figure 4.6A

were immunostained with anti-p120RasGAP antibodies. Asterisks indicate GFP-expressing neurons and squares indicate untransfected neurons. Scale bar=50 μm. (B) p120RasGAP fluorescence intensity (arbitrary units, AU; ±SEM) of GFP-expressing neurons in (A) was measured (n=38, 37, 45, 24). Two-way ANOVA, Tukey's posttest: * $p<0.0001$.

Chapter 5 – General Discussion

5.1 Major findings

The work presented in this thesis was undertaken with the aim of acquiring a greater understanding of the molecular mechanisms that regulate axon outgrowth and chemoattraction downstream of the axon guidance cue netrin-1 and its receptor DCC. This venture led to the identification of two proteins, ezrin and p120RasGAP, that interact with DCC upon the phosphorylation of the conserved Y1418 residue and mediate netrin-1/DCC-dependent functions, and to the discovery that netrin-1 signaling transiently activates RhoA. Each study used rat embryonic cortical neurons as an experimental model.

Chapter 2 reveals that the activity of the ERM protein ezrin is required for netrin-1/DCC-mediated axon outgrowth, and that netrin-1 transiently induces the recruitment of ezrin to DCC as well as the activation of ERM proteins. Notably, netrin-1-dependent ERM protein activation is prominent in growth cone filopodia, and mediated by SFK and RhoA/ROCK activities. The requirement for RhoA activity in ERM protein activation constitutes the first indication that RhoA may be activated downstream of netrin-1.

Chapter 3 then confirms that netrin-1 regulates RhoA activity in time and space. FRET imaging reveals that RhoA activity levels constantly oscillate in live neurons and that netrin-1 transiently modulates oscillations by increasing RhoA activity in a region-specific manner. The study provides the first assessment of the spatiotemporal regulation of Rho GTPase activity downstream of netrin-1.

Lastly, Chapter 4 demonstrates that p120RasGAP is required for netrin-1/DCC-dependent axon outgrowth and chemoattraction. p120RasGAP regulates basal Ras activity in neurons and its recruitment to DCC is mediated by SFKs in response to netrin-1. The study provides the first direct evidence that Ras is activated downstream of netrin-1, and offers new insight on the scaffolding functions of p120RasGAP as well as its role as an effector of netrin-1/DCC signaling.

Overall, these original findings contribute to the advancement of knowledge by providing the first reports of the involvement of ERM proteins and p120RasGAP in netrin-1/DCC signaling, and the first demonstration that RhoA and Ras are activated downstream of netrin-1.

5.2 ERM proteins serve as scaffold and signaling proteins downstream of netrin-1

As the sensory apparatus of the neuron, the growth cone must constantly probe and adapt to the environment. Directional motility is achieved in part via the regulation of protrusion, cell adhesion and traction in the P domain. Subchapter 1.2 describes how these actin-based processes influence the protrusion-retraction cycle of growth cone filopodia. The functions of ERM proteins in netrin-1/DCC signaling, including their role in the regulation of growth cone motility, are discussed here.

5.2.1 Netrin-1 engages the molecular clutch

Filopodia are transient microdomains that serve as environmental sensors and mechanical units for cell motility. Much of our knowledge on the regulation of filopodial dynamics during axon guidance is owed to the study of apCAM-mediated contact guidance in the large growth cones of *Aplysia* neurons. The molecular clutch concept was validated by measuring the forces generated by F-actin dynamics upon the formation of adhesion sites between growth cone filopodia and beads coated with apCAM ligands (Craig et al., 2012; Suter and Miller, 2011). Similarly to apCAM, the vertebrate adhesion molecules, N-cadherin, L1, and β1 integrin, assemble transient point contact (PC) adhesions, which couple F-actin to the ECM and resemble the focal adhesions found in fibroblasts (Bard et al., 2008; Chan and Odde, 2008; Dequidt et al., 2007; Giannone et al., 2009; Myers et al., 2011b; Shimada et al., 2008).

PC adhesions promote growth cone advance by engaging the molecular clutch and their assembly has been shown to be regulated by several axon guidance cues. For instance, the repellent cues myelin-associated glycoprotein and Sema3A destabilize adhesion sites, whereas attractive cues BDNF and netrin-1 positively regulate them (Bechara et al., 2008; Carlstrom et al., 2011; Dequidt et al., 2007; Hines et al., 2010; Myers and Gomez, 2011; Toriyama et al., 2013; Woo and Gomez, 2006). Two different models have been proposed to describe netrin-1 signal-force transduction via the molecular clutch (Figure 5.1). One model suggests that netrin-1 signaling engages the molecular clutch by regulating the assembly of the shootin1-cortactin protein complex,

which couples L1-mediated substrate adhesions to F-actin retrograde flow (Kubo et al., 2015; Shimada et al., 2008; Toriyama et al., 2013). The other model is based on evidence demonstrating that the association of netrin-1 with the ECM mediates growth cone expansion, axon outgrowth and chemoattraction (Moore et al., 2009; Moore et al., 2012; Serafini et al., 1994). The traction force generated by the interaction of DCC with substrate-attached netrin-1 is proposed to mechanically activate FAK, resulting in FAK-mediated stabilization of PC adhesions and increased growth cone traction (Moore et al., 2012). Another possibility is that other netrin-1 receptors that interact with FAK, such as integrins, participate in the establishment of the molecular clutch and also contribute to FAK-dependent mechanotransduction in response to netrin-1 (Armendariz et al., 2014; Myers et al., 2011a; Yebra et al., 2003). However, at least two thirds of the traction generated by substrate-absorbed netrin-1 is mediated by DCC *in vitro*, indicating that DCC is the primary adhesion molecule that engages the molecular clutch in neurons downstream of netrin-1 (Figure 5.1).

Several proteins that interact with DCC also associate with F-actin and could serve as linker proteins in DCC-mediated PC adhesions. Potential linker proteins include N-WASP and the FERM domain-bearing proteins FAK, myosin X and ezrin (Martin et al., 2006b; Ren et al., 2004; Shekarabi et al., 2005; Zhu et al., 2007). ERM proteins such as ezrin are enriched in the P domain and regulate growth cone motility, axon outgrowth and guidance. Although the molecular mechanisms through which ERM

proteins promote these processes are not well understood, the conclusions drawn from several studies converge towards a general consensus regarding function of ERM proteins in growth cone filopodia. For instance, the inhibition of ERM protein activity increases F-actin retrograde flow in growth cones (Castelo and Jay, 1999; Gallo, 2008; Marsick et al., 2012b; Paglini et al., 1998; Teuliere et al., 2011). Additionally, ezrin interacts with the clutch protein L1 via its juxtamembrane region and another region that overlaps with the binding site of AP2, a clathrin adapter protein that regulates L1 endocytosis (Dickson et al., 2002; Sakurai et al., 2008). Ezrin likely increases growth cone traction by coupling L1 to F-actin retrograde flow and preventing AP2-mediated L1 endocytosis (Giannone et al., 2009; Sakurai et al., 2008).

In this thesis, Chapter 2 reveals that netrin-1 activates ERM proteins in cortical neurons, particularly in growth cone filopodia (Antoine-Bertrand et al., 2011a). Since the publication of these results, ERM protein activity was shown to mediate the regulation of growth cone traction in response to axon guidance cues. ERM proteins are required in the P domain to decrease F-actin retrograde flow and increase filopodial L1 and β1–integrin expression in response to NGF (Marsick et al., 2012b). In contrast, the inactivation of ERM protein activity in response to the repulsive cues Sema3A, ephrinA2 and Slit3 correlates with L1 endocytosis and reduced peripheral F-actin in growth cones (Marsick et al., 2012a; Mintz et al., 2008). Thus, ERM proteins may also regulate growth cone traction downstream of netrin-1.

In fact, all three ERM proteins were shown to interact with DCC in the embryonic rat brain, but only ezrin has been reported to contribute to netrin-1-mediated axon outgrowth in neurons (Antoine-Bertrand et al., 2011a; Deming et al., 2015). Based on its recognized role as a molecular clutch linker protein and the findings of Sheetz and colleagues (Giannone et al., 2009; Moore et al., 2012), ezrin could contribute to netrin-1 signal-force transduction by coupling L1 and DCC to F-actin retrograde flow (Figure 5.1). This exciting hypothesis could be evaluated by measuring the impact of the ezrin-L1 and ezrin-DCC protein complex formation on F-actin retrograde flow and growth cone traction downstream of netrin-1.

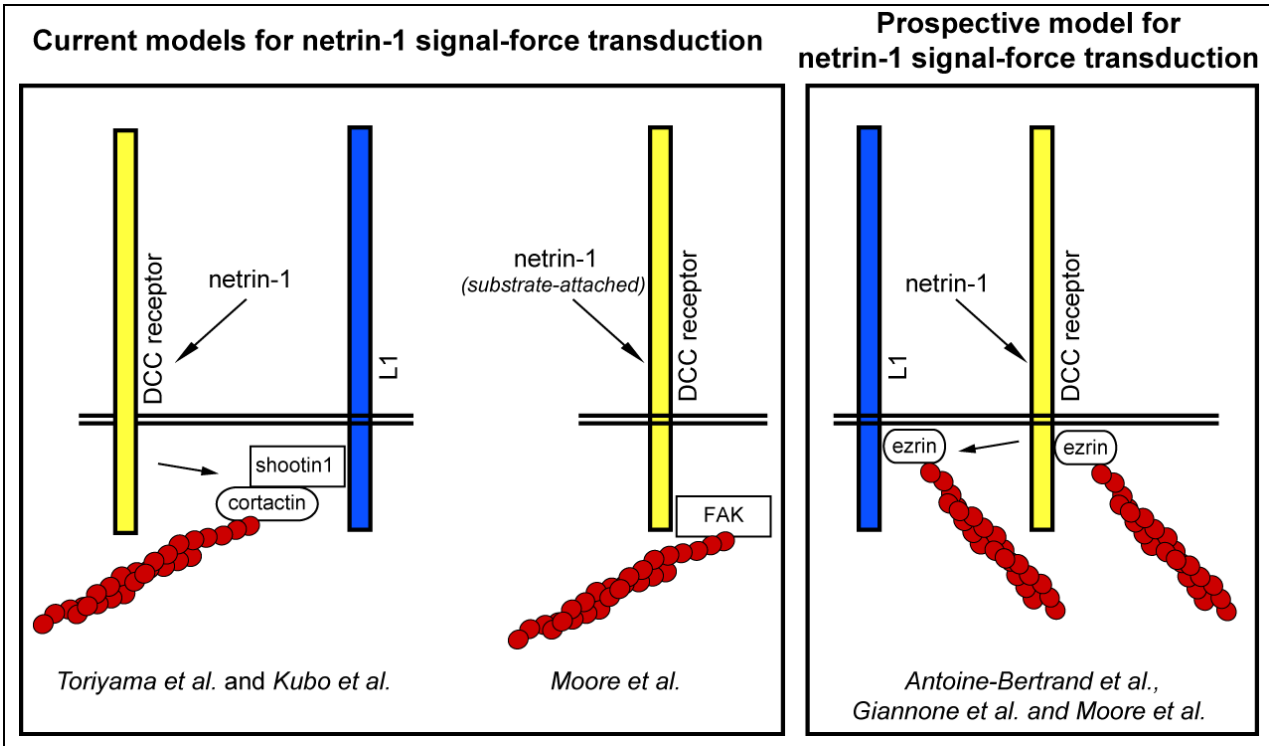


Figure 5.1 Current and prospective models for netrin-1 signal-force transduction

Netrin-1/DCC signaling engages the molecular clutch by promoting the assembly of the shootin1-cortactin protein complex, which couples L1 to F-actin retrograde flow (Kubo et al., 2015; Toriyama et al., 2013). Substrate-attached netrin-1 mechanically activates FAK, stabilizes substrate adhesions via DCC, and increases growth cone traction (Moore et al., 2009; Moore et al., 2012). Finally, a new model emerges from the findings of Chapter 2. The activation of ezrin by netrin-1 may engage the molecular clutch by mediating the interaction of ezrin with L1 and DCC to couple the receptors to F-actin retrograde flow (Antoine-Bertrand et al., 2011a; Giannone et al., 2009; Moore et al., 2012).

5.2.2 ERM protein signaling beyond the molecular clutch

Beyond their role as cytoskeletal linker proteins, ERM protein functions are surprisingly broad and are likely to contribute to several aspect of netrin-1 signal transduction downstream of DCC. For instance, a recent study has revealed that radixin couples PKA activity to DCC in the rat CNS and that this function is required to mediate netrin-1-dependent chemoattraction in human neuroblastoma cells (Deming et al., 2015). These findings are in agreement with other reports stating that PKA activity is required for netrin-1-mediated chemoattraction in *Xenopus* spinal neurons (Hong et al., 2000; Ming et al., 1997).

Ezrin also regulates Rho GTPase activity in epithelial cells and neurons. In epithelial cells, ezrin inhibits RhoA and activates Rac1 and Cdc42 through its regulation

of the RhoA GAP Gmip and the RhoGEFs PLEKHG6 and Dbl, respectively (D'Angelo et al., 2007; Hatzoglou et al., 2007; Prag et al., 2007; Pujuguet et al., 2003; Ruan et al., 2008). Hippocampal neurons that are deficient for ezrin expression exhibit increased RhoA activity, which confirms that ezrin inhibits RhoA activity in these neurons (Matsumoto et al., 2014). Based on these findings, the regulation of Rho GTPases by ezrin appears to parallel Rho GTPase regulation during netrin-1/DCC signaling, suggesting that ezrin contributes to Rho GTPase signaling downstream of netrin-1 and DCC (Antoine-Bertrand et al., 2011b; Lai Wing Sun et al., 2011). Additionally, ERM proteins regulate the subcellular localization of the RhoGEFs DOCK180 and Dbl (Grimsley et al., 2006; Prag et al., 2007; Vanni et al., 2004). Thus, ERM proteins may further regulate the function of Rho GTPases downstream of netrin-1 by determining the localization of regulators such as the RacGEFs DOCK180 and the Dbl family GEF Trio, which are proven contributors of netrin-1/DCC signaling during axon guidance (Briancon-Marjollet et al., 2008; Li et al., 2008).

In parallel to the regulation of Rho GTPases, ezrin interacts with the RasGEF Sos and is required for the activation of Ras in response to RTK signaling (Geissler et al., 2013; Sperka et al., 2011). Unlike the interaction with the GEF Dbl, the impact of the interaction between ERM proteins and Sos is not limited to the control of the subcellular of Sos; the interaction with ERM proteins also stimulates the GEF activity of Sos towards Ras (Geissler et al., 2013; Sperka et al., 2011; Takahashi et al., 1998).

Moreover, the discovery that p120RasGAP preferentially interacts with the closed conformation of ezrin in epithelial cells supports a model in which inactive ezrin mediates Ras inhibition via p120RasGAP and active ezrin mediates Ras activation via Sos (Geissler et al., 2013; Sperka et al., 2011; Viswanatha et al., 2013). Since ERM proteins are activated downstream of netrin-1 (Antoine-Bertrand et al., 2011a), the netrin-1-dependent activation of Ras reported in Chapter 4 could be the result of ezrin activation, which may displace p120RasGAP in favour of Sos downstream of netrin-1.

In summary, FAK, ezrin and the shootin1-cortactin protein complex have the ability to couple L1 and DCC to F-actin retrograde flow in response to netrin-1 (Figure 5.1). Molecular clutch engagement via these protein complexes contributes to netrin-1-dependent axon outgrowth and chemoattraction. Additionally, the interactions between ERM proteins and GTPase regulators indicate that ERM proteins also serve as adapter proteins for GTPase signaling and suggest that ERM proteins regulate the activity of Rho and Ras GTPases during netrin-1-mediated axon outgrowth and chemoattraction.

5.3 Netrin-1 regulates Rho and Ras GTPase activities in time and space

Small GTPases function within signaling modules that define their cellular functions. Their regulators, GEFs, GAPs, and GDIs, determine where and when GTPases become activated (Bos et al., 2007; Cherfils and Zeghouf, 2013). Chapters 2, 3, and 4 are the

first reports to reveal that RhoA and Ras are activated downstream of netrin-1 and DCC in neurons. These findings raise questions regarding the spatiotemporal regulation of RhoA and Ras activities during axon guidance.

5.3.1 Spatiotemporal regulation and dual function of RhoA

The protrusion-retraction cycle of filopodia indicates that transient intracellular signals locally regulate proteins that influence filopodial dynamics. For instance, ERM protein activation in cortical and hippocampal neurons peaks within 10 minutes of exposure to the attractive cues netrin-1 and glutamate, respectively (Antoine-Bertrand et al., 2011a; Kim et al., 2010). In contrast, ERM proteins are transiently inactivated within 2 minutes of exposure to the repellent cue Sema3A in cortical neurons (Mintz et al., 2008). Since ERM proteins serve as linker proteins in the molecular clutch (Figure 5.1), their temporal regulation by attractive and repellent axon guidance cues likely mediates the protrusion and the retraction of growth cone filopodia, respectively.

While the molecular mechanism that mediates the inactivation of ERM proteins has yet to be identified in the context of netrin-1 signaling, Chapter 2 describes the requirement for RhoA activity in netrin-1-dependent ERM protein activation (Antoine-Bertrand et al., 2011a). The implication of RhoA and ROCK is intriguing because RhoA activity is commonly associated with growth cone repulsion and the inhibition of axon outgrowth (Govek et al., 2005; Hall and Lalli, 2010). Yet, Chapter 3 reveals that netrin-1

temporarily modulate RhoA activity oscillations in cortical neurons by increasing RhoA activity levels. These observations are in line with evidence that suggests that RhoA activation has a positive influence on growth cone motility (Fritz et al., 2013; Loudon et al., 2006; Nakamura et al., 2005; Picard et al., 2009). Notably, the assessment of RhoA activity in live neurons using FRET imaging, a method with greater potential for spatiotemporal resolution than biochemical assays, has uncovered RhoA activation in discrete cellular regions that had previously gone unnoticed such as in the growth cone filopodia and the nascent neurites of live unstimulated neuroblastoma cells and DRG neurons (Fritz et al., 2013; Nakamura et al., 2005; Picard et al., 2009).

The notion that netrin-1-dependent axon outgrowth and chemoattraction requires sustained neuron-wide inhibition of RhoA activity as well as transient growth cone-specific RhoA activation suggests that RhoA activity serves at least two different functions in the regulation of axon guidance (Antoine-Bertrand et al., 2011a; Moore et al., 2008a, Chapter 3). On one hand, widespread and sustained RhoA activation may increase actomyosin contractility and inhibit actin dynamics in response to repellent axon guidance cues (Aizawa et al., 2001; Gallo, 2006; Murray et al., 2010). On the other hand, early-onset transient activation of RhoA induced by attractive guidance cues in growth cone filopodia may mediate the dynamic regulation of proteins that control filopodial dynamics, including ERM proteins and cofilin (Antoine-Bertrand et al., 2011a; Marsick et al., 2010; Marsick et al., 2012b, Chapter 3). Additionally, the constant

fluctuation of RhoA activity levels reported in Chapter 3 indicates that RhoA is strictly regulated in unstimulated cortical neurons. This regulation is likely due to the tight coupling of GEF and GAP activities, a phenomenon that is thought to restrict the spatial spread of GTPase activation and could be responsible for the short-lived netrin-1-dependent activation of RhoA described in Chapter 3 (Bement et al., 2006; Duman et al., 2015; Pertz, 2010).

Most of our current understanding of the regulation of Rho GTPases by axon guidance cues is derived from biochemical assessments and functional assays in primary neurons. Despite being informative, these techniques paint an incomplete picture of Rho GTPase regulation since they cannot resolve GTPase activation in time and space. In contrast, the combination of GTPase activity reporters, high resolution microscopy and computational analysis has demonstrated that RhoA activation spatially and temporally precedes the activation of Rac1 and Cdc42 at the leading edge of migrating fibroblasts and wounded oocytes (Burkel et al., 2012; Machacek et al., 2009). Such a combinatorial approach could be used to evaluate correlations between growth cone dynamics and the spatiotemporal regulation of Rho GTPase activities and should facilitate the establishment of a more accurate model for the regulation of Rho GTPases downstream of axon guidance cues.

5.3.2 Regulation of RhoA activity by p120RasGAP

Chapter 4 demonstrates that p120RasGAP interacts with DCC and FAK in response to netrin-1 and that it is required for netrin-1-dependent axon outgrowth and chemoattraction in cortical neurons. Since it harbours two SH2 domains and one SH3 domain, the N-terminus of p120RasGAP likely mediates interactions with proteins, other than DCC and FAK, that also contribute to netrin-1 signal transduction (King et al., 2013; Pamonsinlapatham et al., 2009). Among the proteins known to interact with p120RasGAP, three RhoA GAPs represent interesting candidates as netrin-1 signaling effectors.

p190RhoGAP/ARHGAP35 interacts with p120RasGAP in the mouse brain and its expression is required for the establishment of axonal projections in tissue such as the posterior limb and the cerebral cortex (Brouns et al., 2001). In migrating fibroblasts, the complex formed by p190RhoGAP, p120RasGAP and FAK maintains cell polarity and motility by inhibiting RhoA activity and promoting focal adhesion turnover at the leading edge (Hu and Settleman, 1997; Kulkarni et al., 2000; McGlade et al., 1993; Moran et al., 1991; Tomar et al., 2009). The reported enrichment of p190RhoGAP in growth cones suggests that a p190RhoGAP-p120RasGAP-FAK complex could also be assembled to regulate growth cone motility during axon guidance (Brouns et al., 2001; Tomar et al., 2009). p120RasGAP also interacts with deleted in liver cancer 1 (DLC1)/ARHGAP7 (Jaiswal et al., 2014; Yang et al., 2009), a RhoGAP that is expressed in the murine brain and required for embryonic brain development (Durkin et

al., 2005; Sabbir et al., 2010). In contrast to p190RhoGAP, the GAP activity of DLC1 is inhibited by its interaction with p120RasGAP *in vitro*, which indicates that p120RasGAP potentially controls two different RhoA signaling modules to regulate motility-based cellular processes such as axon guidance (Braun and Olayioye, 2015; Jaiswal et al., 2014; Tomar et al., 2009; Yang et al., 2009).

In addition to p190RhoGAP and DLC1, p120RasGAP interacts with another RhoA GAP, the brain-specific p200RhoGAP/ARHGAP32 (Moon et al., 2003; Shang et al., 2007). It has been proposed that FAK, p190RhoGAP and p200RhoGAP interact with p120RasGAP to prevent it from inhibiting Ras activity (Hecker et al., 2004; Moran et al., 1991; Shang et al., 2007). Thus, the reciprocal regulation between p120RasGAP and RhoGAPs strongly suggests that the Ras and RhoA signaling modules are interdependent (Jaiswal et al., 2014). Consequently, there could be interplay in the regulation of Ras and RhoA activities during netrin-1/DCC signaling. For instance, p120RasGAP may control the activity of specific RhoGAPs and influence the spatiotemporal regulation of RhoA downstream of netrin-1. Reciprocally, RhoGAPs may inhibit the GAP activity of p120RasGAP in order to mediate Ras activation in response to netrin-1.

5.3.3 Regulation of Ras activity in axons

The scope of action of Ras GTPase signaling is broad and ranges from the regulation of the cytoskeleton to the regulation of cellular processes as diverse as adhesion, endocytosis, second messenger signaling, gene expression, cell survival and proliferation (Goitre et al., 2014; Prior and Hancock, 2012). The Ras subfamily of small GTPases includes the prototypical Ras proteins K-Ras, H-Ras and N-Ras, which are abundantly expressed in the embryonic and adult nervous systems (Chesa et al., 1987; Leon et al., 1987; Sudol, 1988). As in other organs, strict regulation of Ras activity is required for development. Whereas neurotrophins like NGF signal through Ras proteins to promote neuronal survival and axon elongation, overactivation of Ras proteins causes tumours in the nervous system (Karnoub and Weinberg, 2008; Skaper, 2012; Woods et al., 2002).

Chapter 4 demonstrates for the first time that Ras is activated by netrin-1/DCC signaling. The late-onset of Ras activation compared to Rac1 activation in cortical neurons attests to the temporal regulation of Ras activity downstream of netrin-1, and p120RasGAP potentially participates in this process since it regulates basal Ras activity and is recruited to DCC in response to netrin-1. Of note, protein interactions formed via the non-catalytic N-terminus of p120RasGAP enable the protein to serve as an effector that promotes Ras signaling in addition to being a RasGAP (Elowe et al., 2001; Gideon et al., 1992; Leblanc et al., 1998; Nakata and Watanabe, 1996; Tocque et al., 1997; Yatani et al., 1990). Thus, the interaction between DCC and the N-terminus of

p120RasGAP may contribute to the regulation of Ras downstream of netrin-1 by causing a functional change in p120RasGAP that inhibits the RasGAP activity in favor of the Ras effector function. Besides the presumptive de-inhibition of Ras activity owing to the recruitment of p120RasGAP to DCC, netrin-1 likely activates a RasGEF to promote Ras signaling. Sos is a viable candidate since its catalytic activity towards Ras is regulated by ezrin, an effector of netrin-1/DCC signaling in neurons (Antoine-Bertrand et al., 2011a; Geissler et al., 2013; Sperka et al., 2011). To this day, the mechanism of activation of Ras downstream of netrin-1 still remains to be determined.

Furthermore, the function of Ras activity during netrin-1/DCC signaling is not known. In axons, Ras activity contributes to axon initiation, outgrowth, branching and guidance (Hall and Lalli, 2010). Ras and Related to (R-)Ras proteins are activated in the nascent axon where they stimulate the production of PIP_3, a key determinant of neuronal polarization, via the regulation of PI3-kinase (Cheng and Poo, 2012; Fivaz et al., 2008; Oinuma et al., 2007; Yoshimura et al., 2006). Signal transduction downstream of the Ras/PI3-kinase pathway induces the activation of the kinase Akt, which increases axon caliber and branching when it is overexpressed in DRG neurons, whereas the activation of another Ras effector, Raf, produces axon elongation (Markus et al., 2002). Conversely, the loss of integrin-mediated cell adhesion is caused by the inactivation of Ras/R-Ras activity by the plexin receptor GAP domain and p120RasGAP downstream of semaphorins and ephrins, respectively, and results in growth cone collapse and

repulsion (Dail et al., 2006; Elowe et al., 2001; Hall and Lalli, 2010; Negishi et al., 2005; Oinuma et al., 2004a; Pascoe et al., 2015). This indicates that such as in non-neuronal cells, Ras activity may promote cell adhesion through the regulation of integrin trafficking in axons (De Franceschi et al., 2015). It also has been proposed that R-Ras/PI3-kinase signaling stabilizes microtubules in axons and promotes neuronal polarization, whereas the inactivation of R-Ras causes microtubule instability downstream of semaphorins (Buttrick and Wakefield, 2008; Hall and Lalli, 2010; Ito et al., 2006; Oinuma et al., 2007).

The key role of Ras GTPase activity in the control of cellular functions suggests that the spectrum of action of Ras may be broad during netrin-1/DCC-mediated axon guidance (Goitre et al., 2014; Prior and Hancock, 2012). In addition to the establishment of growth cone polarity and adhesion, Ras activity likely mediates ERK-dependent transcription and protein translation in response to netrin-1 (Campbell and Holt, 2003; Forcet et al., 2002). Furthermore, the interdependence between Ras and Rho GTPases extends to another Rho family member. The activation of Rac1 downstream of neurotrophins requires the interaction of active Ras with the Rac1GEF Tiam1 (Lambert et al., 2002; Shirazi Fard et al., 2010; Yamauchi et al., 2005). Thus, as growth cones navigate through shallow netrin-1 gradients during the development of the nervous system, late-onset Ras activation may contribute to a positive feedback loop that activates Rac1 and reinforces netrin-1/DCC signaling.

In conclusion, the timing and localization of RhoA and Ras activation appears to be a determining factor in netrin-1/DCC signaling. Together with the correlative measurement of GTPase activity and growth cone dynamics, the identification and characterization of the GEFs and GAPs that regulate GTPases in response to axon guidance cues will greatly improve our understanding of the spatiotemporal regulation and coordination of Rho and Ras GTPase activities during axon guidance (Duman et al., 2015; Pertz, 2010).

5.4 SFK-mediated tyrosine phosphorylation establishes a DCC signaling hub

The large number of proteins involved in netrin-1 signal transduction has certainly complicated the characterization of the various signaling cascades that are triggered downstream of DCC. Nonetheless, the study of these proteins in the context of netrin-1-mediated axon outgrowth and chemoattraction has garnered a wealth of data that can be used to identify the molecular mechanisms that are essential to netrin-1 signal transduction. In addition to the findings presented thus far, this thesis highlights two regulatory mechanisms that are important for netrin-1/DCC signaling in vertebrates: tyrosine phosphorylation and protein complex assembly via the P3 region of DCC.

5.4.1 SFK-dependent tyrosine phosphorylation

Tyrosine phosphorylation was identified as an important regulatory mechanism early into the “molecular age” of axon guidance research (Desai et al., 1997). Embryonic growth cones are enriched in tyrosine kinase and tyrosine phosphatase activities (Bixby and Jhabvala, 1993; Robles et al., 2005), which indicates that tyrosine phosphorylation is tightly regulated in growth cones during neuronal development. In fact, the catalytic activity of receptor protein tyrosine phosphatases (RPTP) is implicated in the regulation of neural morphogenesis, and many tyrosine kinases, including FAK and several SFKs, are expressed in the developing and adult nervous system (Armendariz et al., 2014; Ensslen-Craig and Brady-Kalnay, 2004; Johnson and Van Vactor, 2003; Lowell and Soriano, 1996; Stoker, 2015).

SFKs are nonreceptor tyrosine kinases that harbour two protein-protein interaction domains (SH2, SH3) and a catalytic domain (SH1) (Parsons and Parsons, 2004). They play an important role in the regulation of proteins involved in signal transduction downstream of many plasma membrane receptors, including RTKs and integrins (Parsons and Parsons, 2004; Thomas and Brugge, 1997). In the nervous system, the expression of the family members Src and Fyn peaks early during development and both proteins are enriched in embryonic growth cones (Bare et al., 1993; Bixby and Jhabvala, 1993; Maness et al., 1988). Axon pathfinding has not been closely characterized in mice that are deficient for Src, Fyn or both proteins (Soriano et al., 1991; Stein et al., 1992; Stein et al., 1994), and the only phenotypes reported *in vivo* are

axon fasciculation defects in the olfactory system of *src/fyn* double mutants and limb trajectory errors in the PNS of *src* null mice (Kao et al., 2009; Morse et al., 1998). The absence of overt phenotypes in the nervous system of single mutants has been attributed to the functional redundancy of SFKs during development (Lowell and Soriano, 1996; Stein et al., 1994). Nonetheless, the activity of SFKs, including Src and Fyn, was shown to be required for netrin-1 and ephrin-dependent axon outgrowth and guidance in primary neurons and explants originating from the vertebrate CNS, suggesting that SFKs are essential for axon guidance *in vivo* as well (Beggs et al., 1994; Ignelzi et al., 1994; Knoll and Drescher, 2004; Li et al., 2004; Liu et al., 2004; Meriane et al., 2004; Robles et al., 2005; Yue et al., 2008).

As described in Chapter 1, FAK, Src and Fyn are activated downstream of netrin-1 in vertebrates and SFK activity is required for netrin-1-induced axon outgrowth and chemoattraction (Li et al., 2004; Liu et al., 2004; Meriane et al., 2004; Ren et al., 2004). Upon netrin-1 binding, SFKs phosphorylate of functionally distinct proteins such as DCC, FAK, Trio, p130CAS and TUBB3, and promote the interaction of these proteins with DCC (DeGeer et al., 2013; Li et al., 2004; Liu et al., 2007; Meriane et al., 2004; Qu et al., 2013). Furthermore, Chapter 4 of this thesis demonstrates that SFK activity also induces the tyrosine phosphorylation of p120RasGAP and its interaction with DCC, along with ERK and FAK, in response to netrin-1. Furthermore, Chapter 2 reveals that ERM protein activation requires SFK activity downstream of netrin-1 (Antoine-Bertrand

et al., 2011a). All these SFK-dependent events are detected early, within 10 minutes of netrin-1 stimulation in time course experiments, indicating that tyrosine phosphorylation is a primary regulatory mechanism that initiates the signaling cascades downstream of netrin-1 and DCC.

5.4.2 Regulatory functions of the P3 region

The positive contribution of SFKs in axon guidance appears to be the result of functional evolution since SFK activity antagonizes netrin-mediated axon attraction in *Drosophila* (O'Donnell and Bashaw, 2013). The species-specific differences in netrin-1 signaling also include the absence of requirement for the tyrosine phosphorylation of the DCC *Drosophila* ortholog Frazzled, which incidentally lacks the tyrosine residues that are conserved and phosphorylated in vertebrates (Meriane et al., 2004; O'Donnell and Bashaw, 2013).

One of these conserved tyrosines, Y1418, is located in the evolutionarily conserved P3 region of DCC (amino acids 1392 to 1445 in *R. norvegicus*), which consists of two α-helices separated by a short linker region (Hirano et al., 2011; Wei et al., 2011). The longer C-terminal α-helix encompasses the P3 domain, for which the functional relevance in netrin-1-mediated axon guidance was confirmed by the discovery of a spontaneous recessive mutation that results in the deletion of the P3 in mice (Finger et al., 2002). Corticospinal tract axons fail to cross the midline in the spinal

cord of homozygotes, a defect that is considered to be the cause of a locomotor phenotype, a hopping gait, which inspired the name “kanga” for the mutation (Finger et al., 2002). Several forebrain commissures are also missing in adult *kanga* mice, a phenotype that is evocative of the midline crossing defects reported in the developing brain and spinal cord of netrin-1 and DCC mutant mice (Bin et al., 2015; Fazeli et al., 1997; Finger et al., 2002; Serafini et al., 1996; Yung et al., 2015). At the molecular level, the P3 domain of DCC is required for essential protein-protein interactions such as the homodimerization of the receptor and complex formation with Robo1 and Robo3, which silence and enhance netrin-1-mediated chemoattraction, respectively (Stein and Tessier-Lavigne, 2001; Stein et al., 2001; Zelina et al., 2014).

The Y1418 residue is the last amino acid in the shorter N-terminal α-helix of the P3 region and is phosphorylated by SFKs downstream of netrin-1 (Hirano et al., 2011; Li et al., 2004; Meriane et al., 2004; Wei et al., 2011). Several studies have demonstrated that Y1418 phosphorylation serves an important role in netrin-1 signal transduction in the CNS. In fact, the expression of the phosphodeficient DCC-Y1418F mutant blocks netrin-1-dependent axon outgrowth and chemoattraction in primary neurons (Li et al., 2004; Meriane et al., 2004; Ren et al., 2008). Specifically, Y1418 phosphorylation is required for netrin-1-dependent events such as the tyrosine phosphorylation of DCC, the recruitment of FAK, Rac1 activation and PIP_2 hydrolysis (Li et al., 2004; Meriane et al., 2004; Xie et al., 2006). Additional evidence of the regulatory

function of Y1418 phosphorylation in netrin-1 signaling is provided in Chapters 2 and 4, which demonstrate that the phosphorylation of the residue is required for ERM protein activation and mediates the recruitment of ezrin and p120RasGAP to DCC (Antoine-Bertrand et al., 2011a). Notably, the N-terminal SH2 domain of p120RasGAP directly interacts with the phosphorylated Y1418 residue, and similarly, Y1418 phosphorylation mediates the interaction of the SH2 domain of Fyn with DCC (Ren et al., 2008). These interactions confirm that protein complex assembly in the P3 region of DCC is mediated by the phosphorylation of Y1418, which creates a motif recognized by the phosphotyrosine-binding (PTB) and SH2 domains, both found in a number of catalytic and non-catalytic proteins, and initiates signaling cascades downstream of netrin-1 (Wagner et al., 2013).

5.4.3 Perspectives from the P3 signaling hub

Through its considerable influence over a variety of netrin-1-dependent molecular mechanisms and functions, the P3 region of DCC establishes a signaling hub upon netrin-1 binding (Figure 5.2). Yet, the P3 region mediates interactions with Src, FAK and myosin X that do not require the phosphorylation of Y1418 (Li et al., 2004; Ren et al., 2004; Wei et al., 2011; Zhu et al., 2007). This raises questions regarding the configuration and the function of the protein complexes associated with the P3 region. For instance, the constitutive interaction between DCC and the tyrosine kinases Src and FAK may be required upon the binding of netrin-1 to DCC to mediate conformational

changes that promote the rapid activation of downstream signaling proteins (Li et al., 2004; Ren et al., 2004). Besides creating a binding site for PTB and SH2 domains, netrin-1-dependent Y1418 phosphorylation may also induce a conformational change that alters the affinity of the P3 region for certain proteins.

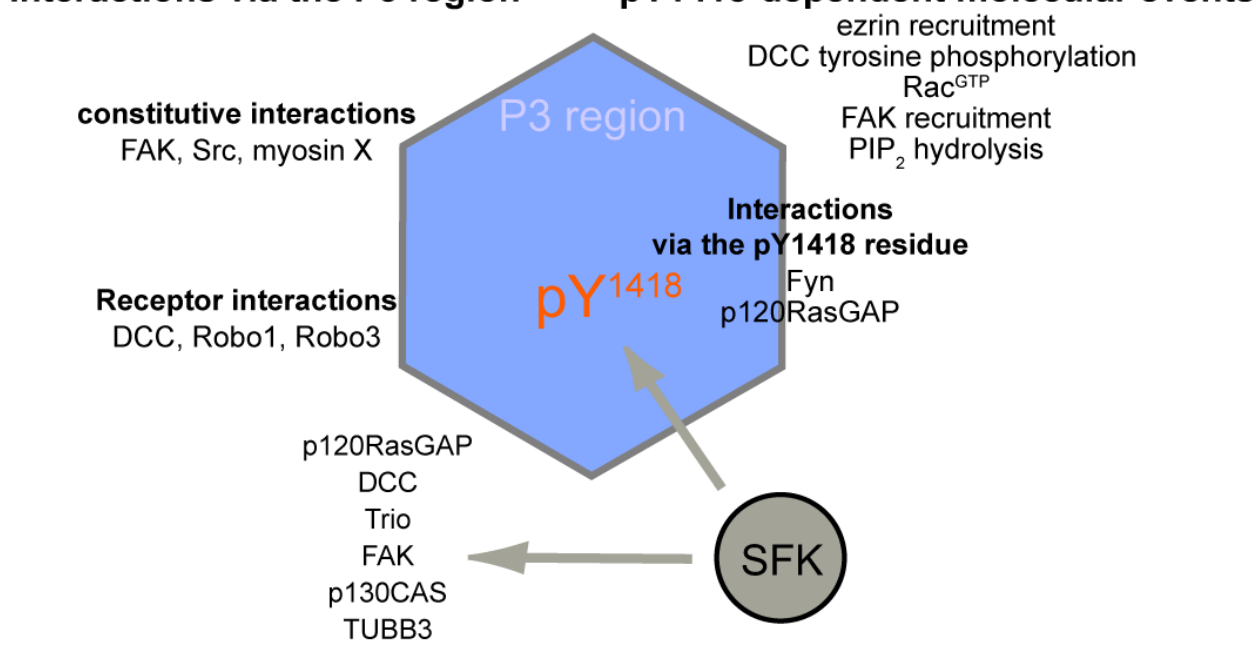


Figure 5.2 The P3 region of the DCC receptor is a netrin-1 signaling hub

The conserved P3 region (amino acids 1392 to 1445 in *R. norvegicus*, in the blue hexagone), represented by the blue hexagone, constitutively interacts with several proteins and mediates the dimerization of DCC with other receptors. Following the binding of netrin-1 to DCC, SFKs mediate the tyrosine phosphorylation (grey arrows) of the indicated proteins, including DCC on the Y1418 residue (in red). The

phosphorylation of Y1418 (pY1418) promotes netrin-1 signal transduction by mediating key molecular events.

Not only is DCC phosphorylated on tyrosines, it is also phosphorylated on serine and threonine residues in response to netrin-1 (Meriane et al., 2004). Thus, all three types of phosphorylation potentially regulate netrin-1 signal transduction. In recent years, the combination of X-ray crystallography and single particle cryo-electron microscopy has enabled the structural determination of macromolecules and protein complexes at the near-atomic level (Bai et al., 2015; Cheng, 2015; Milne et al., 2013). These high resolution structural imaging methods could be used in the future to evaluate the impact of netrin-1 binding and DCC phosphorylation on the three-dimensional structure of DCC protein complexes. In parallel, the requirement for the phosphorylation of specific residues, including the Y1418 residue, to transduce netrin-1-dependent axon guidance could be assessed *in vivo* using techniques such as *in utero* electroporation to express phosphodeficient DCC mutants in the CNS of mouse embryos.

Also, reversible post-translational modifications like phosphorylation generate dynamic signaling networks that can be resolved with high throughput applications such as protein microarrays and quantitative mass spectrometry (Gajadhar and White, 2014; Hennrich and Gavin, 2015; Newman et al., 2014). The computational integration of

datasets obtained from quantitative mass spectrometry studies can resolve dynamic phosphorylation-dependent signaling networks as complex as those propagated by EGF (Johnson and White, 2012; Olsen et al., 2006; Zheng et al., 2013), and could be used to establish a timeline of the phosphorylation events and protein-protein interactions that regulate key molecular mechanisms during netrin-1/DCC signaling.

In summary, SFKs play an important role in netrin-1 signal transduction by promoting the assembly of DCC protein complexes and regulating protein function. Notably, SFK-mediated phosphorylation of Y1418 within the P3 region of DCC serves as a trigger for the activation of signalling cascades that transduce axon outgrowth and chemoattraction downstream of netrin-1. The many molecular mechanisms that are regulated by the P3 region support the notion that this region of DCC creates a signaling hub. The presence of such a hub raises the possibility that others like it exist to regulate specific molecular mechanisms and functions downstream of netrin-1. The P1 and P2 domains of DCC, which like the P3 region are conserved amongst vertebrates, are likely candidates, primarily the P1 domain, since it is already known to mediate protein translation, ERK-dependent DCC phosphorylation and the interaction of DCC with UNC5 and ERK (Hong et al., 1999; Ma et al., 2010; Tcherkezian et al., 2010). The possibility that several DCC signaling hubs regulate netrin-1 signal transduction

represents an exciting avenue to explore to better understand the structural and spatio-temporal control of netrin-1/DCC signaling.

5.5 Conclusion

This thesis has provided evidence that supports the notion that spatiotemporal regulation of protein function is an essential characteristic of netrin-1/DCC signaling. The timing and localization of RhoA and Ras activation are shown to be tightly controlled downstream of netrin-1. The identification and characterization of proteins that contribute to netrin-1 signal transduction, such as ezrin and p120RasGAP, have revealed the great potential for interconnection that exists across the signaling cascades triggered by DCC downstream of netrin-1. Crosstalk between signaling proteins likely mediates the spatial and temporal coordination of the processes that support axon guidance, including receptor trafficking, protein synthesis, actin polymerization in the P domain and actomyosin contractility in the axon shaft (Vitriol and Zheng, 2012). Consequently, the spatiotemporal regulation of molecular and cellular processes should be included as a parameter in the design and interpretation of future studies in order to acquire a more accurate understanding of axon guidance signaling.

In addition to its contribution to the advancement of knowledge in the field of axon guidance signaling, the findings of this thesis have implications for netrin-1/DCC signaling in other cells and tissues. Netrin-1/DCC signaling regulates synaptogenesis

and oligodendrocyte development, and thus, mediates memory formation and myelination, respectively (Goldman et al., 2013; Horn et al., 2013; Jarjour et al., 2008; Rajasekharan et al., 2009). Still in the nervous system, the *dcc* locus is associated with neurological disorders such as congenital mirror movement, schizophrenia and Parkinson's disease (Depienne et al., 2011; Grant et al., 2012; Kim et al., 2011; Srour et al., 2010). Netrin-1/DCC signaling is also involved in many functions outside the nervous system. Notably, it regulates angiogenesis, a process that also requires the activities of p120RasGAP and ERM proteins (King et al., 2013; Layne et al., 2015; McClatchey, 2014). Moreover, the disruption of the expression of p120RasGAP, ERM proteins, DCC or netrin-1 contributes to cancer progression (Clucas and Valderrama, 2014; Mehlen et al., 2011; Pamonsinlapatham et al., 2009). Thus, the significance of the findings presented in this thesis extends beyond the signaling mechanisms underlying axon guidance downstream of the netrin-1 receptor DCC, and offers new perspectives on the regulation of development and disease downstream of netrin-1 and DCC.

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