A role for p190RhoGAP in the signaling mechanisms mediated by the axon guidance cue netrin-1 and its receptor deleted in colorectal cancer (DCC) in primary cortical neurons

Sadig Niftullayev

Department of Anatomy and Cell Biology
McGill University, Montreal, Canada

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CONTRIBUTION OF AUTHORS

This thesis is a manuscript-based structure. According to the faculty regulations, manuscripts co-authored by others must be accompanied with an explicit statement as to who contributed to such work and to what extent. Copyright waivers from the co-authors appear in the appendix.

Chapter 2

My contribution to the manuscript entitled “A role for p190RhoGAP in the signaling mechanisms mediated by the axon guidance cue netrin-1 and its receptor deleted in colorectal cancer (DCC) in primary cortical neurons” involves performing all the experiments. Philippe Duquette has also assisted me during the dissection of rat embryos, as well as, dissociation, culturing, and maintenance of cortical neurons. I have written the manuscript with the support of Dr. Lamarche-Vane.

ABSTRACT

Axon outgrowth and path-finding are crucial points in the development of the Central Nervous System (CNS), where neurons use the distal tip of their axons— the growth cone— to navigate towards their final destination. The growth cone contains highly dynamic actin cytoskeleton and it carries the machinery to respond to various guidance cues, one of which is netrin-1— a secreted, laminin-like protein family. Netrin-1 signal through a cell membrane receptor protein— deleted in colorectal cancer (DCC)— to induce an attractive response. Mutations and small nucleotide polymorphisms (SNPs) in different components of netrin-1/DCC signaling pathway have been implicated in neurological disorders such as congenital mirror movement, schizophrenia, Parkinson's disease, aggressive behavior, and Alzheimer's disease. Although our understanding of the netrin-1/DCC signaling pathway is far from complete, work from different research group has strongly hinted at the substantial role of the Rho family of small GTPases downstream of this pathway. Among other functions, small Rho GTPases regulate the dynamics of actin cytoskeleton, which is an important component of the growth cone movement. The activity of small Rho GTPases is regulated by three classes of upstream proteins— Guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs), and GDP-dissociation inhibitors (GDIs)— roles of which in axon guidance have been studied only to a limited extent.

In the first chapter of this thesis, we have reviewed the classic guidance cues and their receptors, as well as, the role of Rho GTPases and their regulators— GAPs and GEFs— in axon outgrowth and guidance. In this chapter, we have particularly focused on the involvement of GAPs and GEFs in neurological disorders.

In the second chapter, we have demonstrated our findings related to the role of p190— a GAP protein that is active towards RhoA— in the development of cortical neurons downstream of netrin-1/DCC signaling pathway. It has already been shown that p190 is an important protein for CNS development as it is involved in fear memory formation, axon outgrowth and guidance; however, its role in netrin-1/DCC pathway has not been addressed yet. In this chapter, we report that p190 forms a complex with RasGAP and DCC in both HEK293 cells and cortical neurons. We have also shown that, in cortical neurons, upon netrin-1 stimulation, p190 is phosphorylated by Src family kinases. We have documented a similar effect in HEK293 cells upon overexpression of DCC. In addition, we have noted that the tyrosine 1418 (Y1418) residue of DCC is crucial for its binding to p190. Furthermore, we observed that p190 is highly expressed in cortical neurons, where it has similar localization patterns with, actin, DCC, and Rho-GTP at the growth cone. Finally, we have reported that along with its SH2 domains, the SH3 domain of p120RasGAP is also interacting with p190. Together, these findings suggest a role for p190 downstream of netrin-1/DCC signaling pathway.

Résumé

La croissance et le guidage axonal sont des événements essentiels au cours du développement du Système Nerveux Central (SNC), pendant lesquels les neurones utilisent l'extrémité distale de leurs axones— le cône de croissance— afin de naviguer vers leurs destinations finales. Le cône de croissance renferme un cytosquelette d'actine hautement dynamique et la machinerie nécessaire pour réagir aux différents facteurs de guidage dont la nétrine-1— une protéine sécrétée qui ressemble aux lamines. Nétrine-1 agit via son récepteur « deleted in colorectal cancer» (DCC), qui est une protéine transmembranaire, lui permettant d'induire une réponse attractive des neurones environnants. Des mutations et des « small nucleotide polymorphisms (SNPs) » dans les gènes codant pour différentes protéines de la voie de signalisation nétrine-1/DCC ont été impliqué dans les troubles neurologiques tels que le rare syndrome « congenital mirror movement", la schizophrénie, la maladie de Parkinson, les comportements agressifs et la maladie d'Alzheimer. Bien que notre compréhension de la voie de signalisation de nétrine-1/DCC soit bien loin d'être complète, la recherche faite par différents laboratoires a suggéré fortement un rôle substantiel de la famille des petites GTPases Rho en aval de cette voie de signalisation. Entre autres fonctions, les petites GTPases Rho régulent la réorganisation des filaments d'actine qui est une composante importante du mouvement du cône de croissance. L'activité des petites GTPases Rho est contrôlée par trois classes de protéines, soient les « Guanine nucleotide exchange factors» (GEF), les « GTPase-activating protein» (GAP) et les « GDP-*dissociation inhibitors*» (GDI). Les fonctions de celles-ci dans le guidage axonal ont encore été peu étudiées jusqu'à ce jour.

Dans le premier chapitre de cette thèse, nous présentons les facteurs classiques de guidage et leurs récepteurs, ainsi que les rôles des GTPases Rho et leurs régulateurs— GAPs and GEFs— durant la croissance et le guidage des axones. Dans ce chapitre, nous nous sommes concentrés tout particulièrement sur l'implication des GAPs and des GEFs dans les troubles neurologiques.

Dans le deuxième chapitre, nous présentons nos résultats sur le rôle de p190— une GAP qui est active en vers RhoA— dans le développement des neurones corticaux en aval de la voie de signalisation de nétrine-1/DCC. Il a déjà été démontré que p190 est une protéine importante pour le développement du SNC, car elle est impliquée en formation du mémoire de peur, l'excroissance et le guidage des axones; cependant, son rôle dans la voie de signalisation de nétrine-1/DCC n'a pas encore été étudié. Dans ce chapitre, nous démontrons que p190 forme un complexe avec DCC et RasGAP dans les cellules HEK293 et les neurones corticaux primaires. Nous montrons également que, dans les neurones corticaux, p190 est phosphorylée par les kinases de la famille Src suite à la stimulation avec la nétrine-1. Nous avons documenté un effet similaire dans les cellules HEK293 suite à la surexpression de DCC dans ces cellules. De plus, nous avons remarqué que le résidu tyrosine 1418 (Y1418) de DCC est capital pour son interaction avec p190. En outre, nous avons observé que les neurones corticaux ont une haute expression de p190 et que, dans ces neurones, la localisation subcellulaire de p190 dans le cône de croissance est similaire à celle de l'actine, DCC et Rho-GTP. Fianlement, nous avons remarqué qu'en plus de son motif SH2, le motif SH3 de RasGAP interagit également avec p190. Ainsi, ces résultats suggèrent fortement un rôle pour p190 en aval de la voie de signalisation de nétrine-1/DCC.

LIST OF ABBREVIATIONS

AB	Apicobasal
AD	Alzheimer’s disease
ADCA	Autosomal dominant cerebellar ataxia
ADHD	Attention deficit hyperactivity disorder
AKAP13	A-kinase anchor protein
ALS	Amyotrophic lateral sclerosis
AMP	Adenosine monophosphate
APP	Amyloid precursor protein
AQP4	Aquaporin 4
ARAP	ArfGAP and RhoGAP with ankyrin repeat and PH domains
ARF	ADP ribosylation factor
ARHGAP	Rho GTPase activating protein
ARHGEF	Rho Guanine nucleotide exchange factor
ASD	Autism spectrum disorder
ASEF1	APC-stimulated guanine nucleotide exchange factor 1
Aß	ß-amyloid
BBB	Blood-brain-barrier
cAMP	Cyclic adenosine monophosphate
Cdc42	Cell division cycle 42
cGMP	Cyclic guanosine monophosphate
CICR	Calcium-induced calcium release
CIN/PDXP	Haloacid dehydrogenase family phosphatase chronophin

CIPN	Chemotherapy-induced peripheral neuropathy
Clg	Common-site lymphoma/leukemia
CMT	Charcot-Marie-Tooth
CNS	Central nervous system
CNV	Copy number variation
Comm	Commissureless
CPG	Central pattern generator
CSC	Cancer stem cell
Cyk4	Cytokeratine 4
DAG	Diacylglycerol
DBL	Diffuse B-cell lymphoma
DCC	Deleted in colorectal cancer
DH	DBL homology
DHR	DOCK-homology regions
DLPFC	Dorsolateral prefrontal cortex
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DN	Dominant-negative
DNMBP	Dynamin-binding protein
DOCK	Dedicator of cytokinesis (domain)
Dock	Dreadlocks-ortholog (Drosophila Nck ortholog)
DRG	Dorsal root ganglion
DRS	Duane’s retraction syndrome

DS	Down syndrome
DsCAM	Down syndrome cell adhesion molecule
EAE	Experimental autoimmune encephalomyelitis
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ERK	Extracellular signal-regulated kinase
FAK	Focal adhesion kinase
FARP2	FERM, RhoGEF, and pleckstrin domain-containing protein 2
FBS	Fetal bovine serum
FERM	Four-point-one/ezrin/radixin/moesin
FGD1	Faciogenital dysplasia protein 1
fMRI	Functional MRI
Fn	Fibronectin
JPLS	Juvenile-onset primary lateral sclerosis
GABA	^{3}-aminobutyric acid
GAP	GTPase-activating protein
GB	Glioblastoma
GDI	GDP-dissociator inhibitor
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
GMIP	GEM-interacting protein
GMP	Guanosine monophosphate

GPI	Glycosylphosphatidylinositol
GST	Glutathione-S-Transferase
GTP	Guanosine triphosphate
GWAS	Genome-wide association study
HD	Huntington’s disease
HEPES	20mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HGPPS	Horizontal gaze palsy with progressive scoliosis
HIE	Hypoxic ischemic encephalopathy
HMHA-1	Histocompatibility Minor HA-1
HTT	Huntingtin
IAHSP	Infantile-onset ascending hereditary spastic paraplegia
ICD	Intracellular domain
ID	Intellectual disability
Ig	Immunoglobulin
IPTG	Isopropylthiogalactopyranoside
IQ	Intelligence quotient
ITSN1	Intersectin1
Kuz	Kuzbanian
LARG	Leukemia-associated RhoGEF
LDP	Long-term depression
LGN	Lateral geniculate nucleus
LMN	Lower motor neurons
LOAD	Late onset AD

LTP	Long-term potentiation
MB	Medulloblastoma
MDD	Major depressive disorder
MF	Microfilaments
MgcRacGAP	Male germ cell RacGAP
MMD	Mirror movement disorder
MR	Mental retardation
MRI	Magnetic resonance imaging
MRX	X-linked mental retardation
MS	Multiple sclerosis
MSN	Medium spiny neurons
MT	Microtubules
MWO	Migraine without aura
MYO9B	Myosin IXb
NAc	Nucleus accumbens
NCAM	Neural cell adhesion molecules
Nck	Non-catalytic region of tyrosine kinase
NOS	Nitric oxide synthase
Nox1	NADPH oxidase homolog 1
Npn	Neuropilin
NrCAM	Neuronal cell adhesion molecule
OPHN1	Oligophrenin-1
PBS	Phosphate-buffered saline

PCR	Polymerase chain reaction
PD	Parkinson’s disease
PDZ	PICK1/PSD95/Dlg/ZO-1
PEI	Polyethylenimine
PH	Pleckstrin homology
PI-PLC	Phosphatidylinositol-specific phospholipase C
PKA	Protein kinase A
PKC±	Protein kinase C±
PLEKHG	Pleckstrin homology domain containing, family G
PMSF	Phenylmethylsulfonyl fluoride
PP2	Pyrazolopyrimidine 2
PSD95	Postsynaptic density protein of 95 KDa
qPCR	Quantitative PCR
QT	Quantitative trait
Rac	Ras-related C3 botulinum toxin substrate
Ral	Ras like
Ras	Rat sarcoma
RGC	Retinal ganglion cell
Rho	Ras homologous
RICH	RhoGAP interacting with CIP4 homologues
RIPA	Radioimmunoprecipitation assay
Robo	Roundabout
ROS	Reactive oxygen species

SDF1A	Stromal cell-derived factor 1A
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Sema	Semaphorins
SGEF	SH3 domain-containing guanine exchange factor
SHH	Sonic hedgehog
SH2	Src homology 2
SH3	Src homology 3
SH3BP1	SH3 domain binding protein 1
SNP	Single nucleotide polymorphisms
SNTA1	Syntrophin alpha 1
SrGAP	Slit-Robo GTPase-activating protein subfamily
SVZ	Sub-ventricular zone
TAG-1	Transient axonal glycoprotein-1
TCGAP	Tc10/CDC42 GTPase-activating protein
TGF² /BMP	Transforming growth factor ² (TGFb)/bone morphogenetic protein
TRAF2	TNF receptor-associated factor 2
TRITC	Tetramethylrhodamine
TWEAK	TNF-related weak inducer of apoptosis
UMN	Upper motor neurons
UNC or Unc	Uncoordinated
UTR	Untranslated region
WB	Western blot

WMI	White matter injuries
WT	Wild type
XLMR	X-linked mental retardation
Y	Tyrosine
YFP	Yellow fluorescent protein

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CHAPTER 1: INTRODUCTION AND LITTERATURE REVIEW

Will be submitted to the journal of Small GTPases

1.0 General introduction

One of the fundamental steps during the development of both the vertebrate and the invertebrate nervous systems is the formation of proper connections between neurons (specialized nerve cells) and their targets cells— a process called neural wiring [1, 2], failure of which causes neurological disorders ranging from autism to Down's syndrome [3]. The two main mechanisms of the neural wiring are pathfinding and target selection, pathfinding being the first of the two in chronological order. During pathfinding axons navigate through the complex environment of a developing embryo toward their targets, which can be far away from their cell bodies. As they reach their target areas, axons select their specific targets among many other cells. Successful implementation of the neuronal wiring, which is crucial for fulfillment of all the behavioral functions, is achieved through an intimate interplay between axon guidance and neural activity [4].

In this chapter, our focus will be on axon pathfinding and the implication of some of its downstream molecular components in neurological disorders. More precisely, we will talk about axon guidance and the molecules that are implicated in this process. After, we will briefly review the Rho family of small GTPases, their regulators, and their involvement in downstream signaling pathways of the axon guidance cues/receptor complexes. We will then proceed to the final and the main part of the chapter, where we will thoroughly review the implication the regulators of the Rho family of GTPases— GEFs (Guanine nucleotide Exchange Factors) and GAPs (GTPase-activating Proteins) — in neurological diseases and disorders.

1.1 Axon guidance

Species with bilateral symmetry, including humans, possess the midline axis, which is a very important structure for proper wiring of the nervous system [5, 6]. This feature becomes even more crucial in the vertebrates because of the complexness of the nervous system where neurons have to decide whether or not to cross the midline or towards which axis (for example, rostral versus caudal or ventral versus dorsal) they should send theirs axons to [7]. Naturally, all this complexity of developing nervous system raises a question: How do such an immense number of neurons manage to connect only with the certain number of target cells, while evading the. Currently, it is known that the axons of later born neurons fasciculate with the axons of early born neurons or simply follow the scaffold that had been laid by them. The early born neurons, however, have to navigate through an environment that is composed of undifferentiated neuroepithelial cells [8]. Pioneering studies addressing this issue dates back to late 19th century, to the work of the "father of neuroscience"— Ramon y Cajal. More than a century ago, he observed that the distal tip of the axon has a very irregularly shaped structure, which he called "the growth cone" [5, 9, 10]. Cajal proposed that the growth cone might be the structure that, in response to diffusible chemotropic signals, navigates through the developing brain to connect with the distant targets [9, 11]. Approximately two decades later, motility of the growth cone was shown by Harrison, using frog neurons [12-14]. The theory became more substantiated by the work of Sperry, where he showed that specific populations of retinal axons innervate only certain parts of the optic tectum, suggesting that there is some sort of molecular complementation between these axons and the tectum [15]. The influx of data supporting the possible chemoattraction

model encouraged the researchers to reveal the underlying molecular components. Many of the early candidates such as neural cell adhesion molecules (NCAMs), integrins, fasciculin, and cadherins did not fit the model since they were simply providing permissive environment rather than conferring directionality to the navigating axons [5]. Finally, a little less than a century after Cajal's initial work, using the combination of genetic, biochemical, *in vitro,* and *in vivo* approaches, scientists identified the classical guidance cues— Netrins, Slits, Semaphorins, and Ephrins— and their cognate receptors— deleted in colorectal cancer (DCC) and uncoordinated-5 (UNC5), Roundabout (Robo), Plexin and Neuropilin, and Eph, respectively [5, 16, 17]. As a result of the work done during the past few decades, it is now known that the growth cone is a very motile structure, mainly composed of highly dynamic cytoskeletal elements— actin and tubulin filaments—, as well as, a myriad of other proteins, which participate in the regulation of cytoskeletal assembly/disassembly. The past research has also disclosed the fact that the growth cone is furnished with one or more of the above-mentioned receptors to be able to respond to the guidance cues appropriately and to be able to navigate accordingly [3, 18-20].

The importance of the axon guidance cues and their receptors can also be seen from the fact that several studies have demonstrated a link between single nucleotide polymorphisms (SNPs) and mutations in the genes encoding these proteins and numerous congenital disorders and neurodegenerative diseases such as Parkinson's disease (PD), Alzheimer's disease (AD), Mirror movement disorder (MMD), Horizontal gaze palsy with progressive scoliosis (HGPPS), dyslexia, Kallmann's syndrome,

Hirschsprung's disease, Autism spectrum disorders (ASD), epilepsy, and Amyotrophic lateral sclerosis (ALS) [17, 20, 21].

Although some of the well-known morphogens such as Wnt, Sonic hedgehog (Shh), and transforming growth factor ² (TGF²)/bone morphogenetic protein (BMP) families have also been implicated in axon guidance [3, 4, 16, 17], we will only discuss the classical guidance cues that have been mentioned above. Also, it must be noted that the classical guidance cues are not restricted to the central nervous system (CNS), but also function in various molecular pathways outside the CNS [22, 23], however, this will not be discussed here.

1.1.1 Netrins

The netrins are members of a family of conserved secreted guidance cues. Netrins were uncovered in a convergent series of experiments that were carried out on invertebrates (circumferential axon guidance in *C.elegans*) and vertebrates (ventromedial guidance of commissural axons in vertebrates) trying to identify the molecules that function as chemoattractants in axon guidance [5, 16, 17]. The discovery of netrins in different branches of the animal kingdom proved that, despite the increasing complexity of the nervous system through evolution, some of the underling mechanisms have been well conserved [17]. So far, one netrin has been identified in *C.elegans* (UNC-6), two in *Drosophila* (netrin-A and B), and several in vertebrates— secreted netrins, netrin-1 to -4, and glycosylphosphatidylinositol (GPI)-anchored membrane-bound netrins— netrin-G1 and –G2. Mammals, including rats, mice, and humans, express netrin-1, -3, -4, -G1, and –G2. Netrin-2 expression, however, has only been detected in chicken and zebrafish [24]. The N-terminal domain of netrins is homologous to the domains V and VI found at the

amino terminal ends of laminins. In that sense, netrin-1, -2, and -3 show similarity to $_3$ chain of laminins, while netrin-4 and netrin-Gs are similar to the $_2$ chain of laminins [16, 24].

Further research has shown that, in vertebrates, netrin-1 is produced by the floor plate and can function as both short and long range chemoattractants [5, 16, 17]. Later, through the work done on rat embryo explants, it was demonstrated that netrin-1 can also act as repellant, repelling trochlear motor neurons and other dorsally projecting hindbrain motor neurons [5]. The chemoattractive functions of netrin-1 are mediated by "deleted in colorectal cancer" (DCC) family of proteins (UNC-40 in *C.elegans*, Frazzled in *Drosophila*), characterized by four immunoglobulin (Ig) and six fibronectin (Fn) type III repeats in their extracellular domains [5, 16, 17, 24]. Netrin-1 has also been shown to bind to another DCC family member, neogenin, and the Ig superfamily member, Down syndrome cell adhesion molecule (DsCAM), in other systems [16]. On the other hand, the repulsive functions of netrin-1 are mediated by the proteins of the UNC family, of which there is one (UNC5) in *C.elegans and Drosophila* and four in mammals (UNC5A-D). In *Drosophila*, UNC5 carries out the repulsive response of netrin alone, while in *Xenopus*, UNC5 and DCC form heterodimers to mediate the repulsion [25]. In *C. elegans*, however, there are multiple repulsive signaling mechanisms involving unc5 and unc40 [26].

1.1.2 Slits

Slits are large secreted chemorepellants. They contain four N-terminal leucine-rich repeats, as well as, epidermal growth factor (EGF)-like repeats [16]. Much of our understanding of slits are coming from work carried out in *Drosophila* where they

were discovered as ligands of the receptor protein roundabout (robo), which ensures that neurons cannot re-cross the midline, after having crossed it once [5, 16, 17]. There has been only one identified slit protein in *C.elegans and Drosophila*, versus three slits in mammals (Slit1-3). In parallel studies on *C. elegans*, robo was identified as a regulator of nerve ring formation [17]. There are three Robos in *Drosophila*, three robos in mammals, and only one (Sax-3) in *C.elegans* [16]. Slit-robo pathway was another evidence for the conservation of the axon guidance mechanisms through evolution [17].

In vertebrates, slit has been shown to control the midline crossing of the axons, working along with netrins. However, it was only after elimination of all three slit isoforms that its importance could be finally shown in term of commissure formation [17]. Furthermore, the expression of different robo combinations control the degree of responsiveness to slit and consequently, determines the exact position of the neurons, once they cross the midline [5, 16, 17].

The expression of both Robo and DCC in commissural axons begs the question of "How do these axons cross the midline?" or "How do these axons cross the midline only once?". In *Drosophila*, another protein, namely commissureless (comm), inhibits surface expression of Robo, by targeting it to proteasomal degradation, in the pre-crossing axons. Once the axon crosses the midline, the expression level of comm decreases, leading to an increase in the surface expression of robo, which prevents re-crossing of axons. Even though, there has not been any identified comm homolog in mammals, it has been noted that robo3 produces two isoforms by alternative splicing: robo3.1, which is highly expressed in the pre-crossing axons and inhibits the repulsive action of robo1 and 2; and robo3.2, which is highly expressed in the post-crossing axons and enhances the repulsive

action of the robo1 and 2 [5, 17]. Furthermore, it has been shown that upregulation of robo levels after midline crossing act in two ways: 1) by increasing the slit responsiveness 2) and by directly interacting with the cytoplasmic tail of DCC (in *cis* manner) to attenuate netrin-DCC signaling [5].

In addition to its role in midline crossing of axons, Slit-Robo signaling is also involved in the repulsion of retinal axons at the optic chiasm, in the repulsion of neuronal precursors migrating to the olfactory bulb, and in the repulsion of olfactory bulb axons. Another proven role of slit in the vertebrate nervous system is its role as a branching factor for sensory axons and cortical dendrites [17].

1.1.3 Semaphorins

Semaphorins (sema) represent a large family of phylogenetically conserved guidance cues that include both secreted and membrane-bound proteins, all of which contain the semaphorin domain of 500 amino acids that mediate receptor interaction. The first identified semaphorin— Fasciclin IV (Sema-1A) — was a transmembrane protein that is required for pathfinding in grasshopper limb sensory neurons [16]. The first identified vertebrate semaphorin, on the other hand, was a secreted protein— Collapsin-1 (Sema-3A), which was discovered from chick brain extracts because of its ability to cause growth cone collapse in cultured neurons [5, 16, 17]. Today, around twenty members of semaphorins have been discovered and divided into eight classes, based on their structural homology— class 1 and 2 are invertebrate semaphorins, whereas classes 3-7 are from vertebrates. However, emerging evidences make this lineation more and more ambiguous [17].

The first identified semaphorin receptor was neuropilin (Npn), of which there are only two members in vertebrates, neuropilin-1 and -2, and none in invertebrates. However, the lack of any signaling domains in neuropilins ignited the search for an alternative receptor. This led to the discovery of Plexins— a phylogenetically conserved family that is distantly related to the Semaphorins—, which contains more than ten members under four different sub-families (PlexinA-D). Different permutations of plexins and neuropilins receptors are expressed in distinct subset of neurons [17].

In vertebrates, the role of semaphorins in axon guidance has been studied mostly through secreted class-3 Semaphorins [17]. For example, Sema3A has been shown to be expressed in tissues that surround peripheral nerves, where it repels axons from entering into wrong trajectories by a mechanism called “surround repulsion” [16]. Generally, defects in distinct members of Sema3 signaling has been shown to cause aberrant projections of sensory neurons and specific motor cranial nerves (oculomotor, trochlear, trigeminal, and facial nerves), as well as defasciculation of nerve bundles [5]. The transmembrane semaphorins act also as repellents either through surround repulsion or by direct expression on the nerve bundles. In addition to their well-known repulsive roles, semaphorins have also been implicated in chemoattraction for cortical dendrites [17]. Another interesting aspect of the semaphorins is that they can also act as receptors in various processes such as photoreceptor targeting in *Drosophila*, cardiac development in chicken, and thalamic axon guidance in mammals [16].

1.1.4 Ephrins

Since Sperry’s experiments on frog retinal axon projections to the optic tectum [15], deciphering the underlying mechanisms has been the focus of many researchers.

Axon from the temporal and nasal part of the retina shows regional preference, respectively, for anterior and posterior parts of the tectum in lower vertebrates and for the lateral geniculate nucleus (LGN) of the thalamus in higher vertebrate. Co-culture experiments demonstrated that incubation with membranes from inappropriate region of the tectum causes growth cone collapse in retinal ganglion cells (RGCs) [27]. In addition, incubating the membranes with phosphatidylinositol-specific phospholipase C (PI-PLC) eliminated this effect, which suggested that the causative molecule is GPI-linked [28]. Purification of this molecule led to the discovery of EphrinA5, which is now known to be expressed in an increasing anteroposterior gradient across the tectum [5, 16, 17].

Since then, two subfamilies of ephrin molecules have been discovered: GPI-anchored EphrinA, represented by two classes and transmembrane EphrinB, represented by three classes. Both subfamilies are unable to elicit a response when they are released from the membrane, suggesting that ephrins are short range repulsive molecules. Class A ephrins signal through class-A Eph receptors, represented by eight members, while class B ephrins signal through class-B Eph receptors, represented by six members, with varying degree of selectivity. As opposed to the EphrinA-EphA pathway, which controls the topographic mapping through the anterior-posterior axis of the tectum (or LGN), EphrinB-EphB pathway is involved in the mapping of the dorsal-ventral axis [5, 16, 17].

EphrinsB can also be involved in the reverse signaling upon interacting with EphB receptors [5, 16, 17], which leads to phosphorylation of a tyrosine residue in the intracellular domain of the Eph tyrosine kinase receptor. Eventually the same idea was also proven for the class A ephrins, despite the fact that they lack an intracellular domain. It is believed that they act in *cis* manner through co-receptors [17]. Apart from their roles

in the topographic mapping, ephrins also function as short-range attractants and repellants in the guidance of many central and peripheral axons, as well as in the pruning of axonal trajectories. Regulation of dendritic morphology and synaptogenesis are also among the functions of ephrins [16].

1.2 Common mechanisms of axon guidance

1.2.1 Regulation of receptor complexes

As a result of the research addressing the molecular functions of guidance cues and their receptors, a common theme has emerged, which proposes that the receptor complex rather than the guidance cue itself determines the reaction of the growth cone to a given cue [18, 29]. Indeed, a wide range of intracellular mechanisms ensures that, in a given moment, the growth cone is well furnished with the correct combination of receptor complexes and is ready to respond to the guidance cues. However, the current knowledge we have about the regulatory mechanisms of the guidance cue/receptor complexes is incomplete as there are more mechanisms to be revealed. Nonetheless, there have been many advances in the elucidation of some of these mechanisms ranging from transcriptional regulation to proteolytic cleavage of the receptor complexes [29].

1.2.1.1 Trafficking and surface enrichment of guidance receptors

In theory, regulating the surface expression of a receptor should affect the strength of the signaling through its ligand. Indeed, several *in vivo* and *in vitro* studies have hinted at the importance of regulated receptor trafficking in axon guidance. Examples of this level of regulation would be the trafficking of Robo by Comm in flies [29]; trafficking of DCC by protein kinase A (PKA) and its surface enrichment through

Hsc70-TrioGEF in rat [29, 30]; and trafficking of UNC-40 (DCC), UNC-5, and SAX-3 (Robo) in *C. elegans* [29].

1.2.1.2 Regulated Endocytosis of Guidance Receptors

Another way of controlling the surface expression of a receptor, in parallel to its trafficking to the cell membrane, is the regulated internalization through endocytosis. Regulated endocytosis of receptor complexes is an integral part of axon guidance. For example, endocytosis of ephrin-Eph ligand/receptor complex has been shown to be important for repulsive responses in mice. In addition, interaction and co-endocytosis of receptor neuropilin-1 (Npn-1) with cell adhesion molecules L1 and transient axonal glycoprotein-1 (TAG-1) has been shown to be crucial for Sema3a signaling in mice. Finally, selective internalization of UNC5A, but not DCC, by active protein kinase C± (PKC±) switches from repulsive netrin-1 signaling to attractive one [29, 31].

1.2.1.3 Proteolytic Processing of Guidance Receptors

Several studies have documented that proteolytic cleavage of guidance cues and their receptors by ADAM metalloproteases, as well as, matrix metalloproteases is important for axon guidance *in vivo*, in both invertebrates and vertebrates. For instance, impairing metalloprotease-dependent ectodomain shedding of DCC results in potentiated netrin-1 signaling. Among these metalloproteases, the Kuzbanian/ADAM10 (kuz/ADAM10) family is of particular interest as they have been linked to the signaling pathways of more than one guidance cues. Studies in *Drosophila* have reported ADAM10 to be a positive regulator of Slit-Robo signaling. Furthermore, cleavage of the ligand ephrin-A2 and the receptor EphB2 by ADAM10 suggests that, in the context of

ephrin-Eph signaling, ADAM10 targets both the ligand and the receptor [29, 31]. In addition, more evidences are emerging to support a role for ADAM10/gamma-secretase-mediated sequential cleavage of receptors in axon guidance. DCC and a number of ephrins ligands appear to be the targets of a similar mechanism [29, 31].

1.2.2 Signaling from axon guidance receptor complexes

Upon assembly of the axon guidance cues with their appropriate receptor complexes, a myriad of signaling pathways are exploited to steer the growth cone. Although complete understanding of guidance receptor signaling is currently lacking, several components of downstream signaling pathways have been identified in recent years [29]. Examples of such intracellular components would be calcium, cyclic nucleotides, and Rho GTPases [18, 29, 31]. In this chapter, we will briefly talk about the involvement of calcium and cyclic nucleotides signaling in axon guidance. However, our main focus will be on the role Rho GTPases and their upstream regulators— GAPs and GEFs— in axon guidance.

1.2.2.1 Calcium and Cyclic Nucleotides

Based on *in vitro* studies, calcium and cyclic nucleotides (cAMP and cGMP) can directly mediate guidance receptor signaling and can modulate the response strength, as well. There is an intimate interplay between calcium and cyclic nucleotide signaling in modulation of axon guidance through the mechanisms that involve soluble adenylyl cyclases, nitric oxide synthase (NOS), plasma membrane Ca^{++} channels, and calcium-induced calcium release (CICR) [32, 33].

Overall, chemoattractants such as netrin-1 lead to membrane depolarization, while repellants such as Slit and Sema lead to membrane hyperpolarization by modulating Ca^{++} influx [34-37]. As to cyclic nucleotides, their levels or rather ratios— particularly the cAMP to cGMP ratio— determine the growth cone response to guidance cues: high cAMP/cGMP ratio favors attraction and low cAMP/cGMP favors repulsion [38-40].

Even though the main bulk of data related to the role of calcium and cyclic nucleotides in axon guidance is based on *in vitro* studies, more and more data is emerging that supports their importance *in vivo,* as well [31].

1.3 Linking axon guidance receptor signaling to the actin cytoskeleton: Rho GTPases and their regulators— GAPs and GEFs

1.3.1 Rho family of GTPases and their involvement in guidance receptor signaling

In order to mediate the cellular response of the growth cone to its extracellular environment, guidance cue/receptor complexes must alter the growth cone morphology by modulating the local cytoskeleton [29]. The growth cone has three zones based on the type of prevailing cytoskeletal elements: the central zone is occupied mainly by microtubules (MTs), the peripheral zone is occupied mainly by actin or microfilaments (MFs), and finally, the transition zone represents the part of the growth cone, where microtubule and actin filaments overlap [16, 41, 42].

In order to be able to respond to the guidance cues, the growth cone has to maintain proper dynamics of both actin and microtubule cytoskeletons. In support of this, drugs such as nocodazole and latrunculin, which inhibit microtubule and actin polymerization, respectively, cause unidirectional axon outgrowth. This data suggests that the dynamic nature of both cytoskeletal elements is crucial for changing the growth

cone direction [41]. Despite the equal importance of both cytoskeletal elements, actin is the primary target of guidance cue/receptor signaling as it predominates at the leading edge— the most motile part of the growth cone. In the leading edge, actin can form distinct type of superstructures: finger-like structures such as filopodia, and web-like structures as lamellipodia [2, 16, 29, 42-44]. During growth cone turning, the actin dynamics at the leading edge are exploited to direct the advance of microtubule [42]. The attractive cues promote actin polymerization at the periphery part of the growth cone, while the repulsive cues decrease it to produce turning effect towards or away from the source of a given cue, respectively [45]. In this regard, filopodia is particularly important as asymmetric filopodial growth is the starting point for the turning of the whole growth cone.

Although it is not fully understood how guidance cue/receptors signaling controls actin dynamics, it has been widely documented that they regulate, directly or indirectly, the activity of Rho family of small GTPases [2, 16, 29, 31, 42, 43, 45]. The Rho family of small GTPases, a subgroup of the Ras superfamily of small GTPases, are well-known for their roles in cell motility and regulation of cytoskeletal structures. The family contains well-studied members RhoA, Rac1, and Cdc42 along with some other relatively less-studied members. Pioneering work in fibroblasts have demonstrated that activation of RhoA, Rac1, and Cdc42 leads to formation of distinct actin based structures— stress fibers, lamellipodia, and filopodia, respectively [46-48]

Even though originally it was thought that the attractive cues induce leading edge actin polymerization through Rac1 and Cdc42 activation and the repulsive cues induce retraction through RhoA activation, further research in the field revealed that the real case

is far more complex than this [31]. Work in fibroblasts, rat commissural neuron, and N1E-115 neuroblastomas showed that Netrin-DCC signaling increases Rac1 and Cdc42 activity, while decreasing RhoA activity [49-51]. Surprisingly, as opposed to its well reported repulsive role [25, 52], overexpression of Unc5a in N1E-115 neuroblastoma cells has been shown to induce neurite outgrowth by increasing Rac1 and Cdc42 activity [53]. Moreover, it has also been reported that netrin stimulation of Unc5a overexpressing N1E-115 neuroblastoma cells lead to transient Rac1 activation in early stages and RhoA activation in the later stages of neurite outgrowth [53]. Upon Sema stimulation, plexin-B1 directly interacts and sequesters active Rac1, along with activation of RhoA [54, 55]. However, Sema3A-plexin-A signaling activates Rac1 [56]. Slit-Robo signaling causes decreased Cdc42 activity and increased RhoA and Rac1 activity [57, 58]. Finally, ephrin-Eph forward signaling causes increased RhoA activity, but it also leads to transient decrease of Rac1 activity in retinal ganglion cells (RGCs) [59, 60], while Eph-ephrin reverse signaling activates Rac1 and Cdc42 [61]. As shown in these examples, there is no solid pattern of Rho GTPase signaling downstream of the guidance receptors and further research is required to fully comprehend their role in axon guidance [29, 31, 43]. First step toward this end can be to understand how the guidance receptor complexes lead to activation or inhibition of the Rho GTPases. Except for the case of Plexin-B1, generally guidance receptors do not interact with Rho GTPases directly, but rather deploy signaling adaptors and kinases to modulate the activity of their upstream regulators [29]. Rho GTPases, like Ras GTPases exist in two states: active, GTP-bound, and inactive, GDP-bound. In their active state, Rho GTPases interact with their downstream effectors and modulate their functions. There are three groups of upstream regulators that control the

activity of Rho GTPases: GTPase-activating proteins (GAPs) stimulate the intrinsic low GTPase activity of Rho GTPases and act as negative regulators by switching the small GTPases from GTP-bound to GDP-bound state; guanine nucleotide exchange factors (GEFs) act as positive regulators by replacing the GDP with GTP, therefore, activating the small GTPases; finally, guanine nucleotide dissociation inhibitors (GDIs) binds GDP-bound form of the small GTPases and solubilize them, which inhibits the dissociation of GDP and keeps the small GTPase in its inactive state [43, 62, 63] (**Figure 1.1**).

1.3.2 General information about GEFs and GAPs

The first GAP protein— p50RhoGAP (ARHGAP1) — was identified almost thirty years ago, in 1989, from human spleen extract [64]. The first GEF protein— MCF-2/DBL (ARHGEF21)—, on the other hand, was identified four years earlier than that, in 1984, as an oncogene, using the NIH 3T3 mouse fibroblast focus formation assay [65]. Since then about 80 RhoGAPs and 82 RhoGEFs have been identified for 20 Rho GTPases in eukaryotes [45, 66, 67]. In all species, GAPs and GEFs outnumber Rho GTPases, which can be rationalized with four possible explanations: 1) despite the ubiquitous expression pattern of most GAPs/GEFs, some of them show tissue specificity; 2) some GAPs/GEFs are specific only for one member of the Rho GTPases, while the others are active towards multiple members, at least *in vitro*; 3) each GAP/GEF might be involved in specific RhoGTP-driven signaling pathways; 4) in addition to their conventional roles, GAPs/GEFs might act as scaffold proteins to mediate cross-talk between different GTPase pathways or the formation of protein complexes. Most likely, a combination of more than one of these possibilities is underlying the actual explanation [68].

There are two classes of RhoGEFs: diffuse B-cell lymphoma (DBL) and dedicator of cytokinesis (DOCK) families of proteins. There are 71 identified DBL GEFs, the classical GEFs, with characteristic DBL homology (DH) and pleckstrin homology (PH) domains. The DH domain catalyzes the GDP-GTP exchange, while the PH domain serves different functions such as localization to the plasma membrane, interaction with cytoskeletal proteins, and regulation of the DH catalytic activity. The DOCK family, however, is represented by 11 members, which contain DOCK-homology regions (DHR) 1 and 2. The DHR1 domain is involved in membrane localization and the DHR2 domain catalyzes the GDP-GTP exchange [45, 65-67, 69-73]. Another feature that distinguishes DOCK GEFs from DBL GEFs is that unlike DBL GEFs, DOCK GEFs activates only Rac and/or Cdc42, but not RhoA or the other members of Rho GTPases [70, 73]. RhoGAPs, on the other hand, are characterized by the presence of a conserved RhoGAP domain [68, 69]. In the current terminology RhoGAPs and DBL family of RhoGEFs are assigned a new, standardized names: Rho GTPase activating proteins (ARHGAPs) and Rho guanine nucleotide exchange factors (ARHGEFs), respectively. Both RhoGAPs and RhoGEFs are multidomain proteins with several lipid and/or protein interacting domains, which mediate their subcellular localization or formation of protein complexes. In addition, combination of different GAP and/or GEF domains can be found in a single GAP or GEF protein. This probably serves the purpose of linking different GTPase signaling pathways [68, 69]. For more in depth information about RhoGEFs and GAPs, their evolution, working mechanisms, and involvement in diseases we refer the readers to other review articles [45, 65-74].

1.3.3 RhoGAPs and GEFs in axon guidance

As the direct regulators of Rho GTPase, RhoGAPs and GEFs have been demonstrated to be implicated in axon guidance and pertaining signaling mechanisms [29-31, 42, 43, 45, 75, 76]. It is very cumbersome to identify the specific GAPs and GEFs that are involved in guidance receptor signaling pathways because of the following reasons: 1) GAPs/GEFs often show redundancy in their roles, 2) Individual GAPs/GEFs might be linked to several signaling pathways, 3) GAPs/GEFs generally participate only in part of a given pathway [29]. However, work done using cultured cells and in vivo animal models in past years have revealed several GAPs and GEFs as integral part of guidance receptor signaling [29-31, 42, 43, 45, 75, 76]. For example, mice lacking a Rho-specific GAP protein with high expression in the nervous system, p190RhoGAP (ARHGAP35), show clear guidance defects in the axonal projections of the posterior limb of the anterior commissure [77]. Here, we will briefly overview the well-established roles of RhoGAPs and GEFs in axon guidance, focusing particularly downstream of the four classic guidance cues and their receptor complexes (**Figure 1.2 and Table 1.1**).

1.3.3.1 Netrin-DCC

Although assigning a committed GEF and/or GAP protein to netrin-1/DCC signaling pathways have been elusive, recent work in the field has suggested two GEFs as potential candidates: Trio (ARHGEF23) and DOCK180 (DOCK1) GEFs [30, 75, 78-80]

Trio harbors two GEF domains: one is active toward Rac1 and RhoG and the second one is active for RhoA. Trio is a positive contributor of the embryonic *Drosophila* CNS and it can physically interact with both Frazzled and DCC [29, 31, 43]. The work by

Briancon-Marjollet et al. and DeGeer et al. has reported that Trio, together with Hsc70, mediates both Netrin-1/DCC-driven neurite outgrowth and DCC surface expression in the growth cone of embryonic rat cortical neurons [30, 75, 78]. Furthermore, cortical neurons from $Trio^{-/-}$ mice do not show netrin-1-induced Rac1 activation and the mice display guidance defects similar to those of the $DCC^{-/-}$ mice [78]. However, Trio cannot be the only GEF that is responsible for the netrin-1/DCC signaling as $Trio^{-/-}$ mice show milder commissural axon guidance defects than $DCC^{-/-}$ mice [31, 78]

DOCK180 is another GEF to be involved in netrin-1/DCC signaling. Like Trio, it interacts with DCC and activates Rac1. DOCK180 has been reported to participate in netrin-1/DCC induced and Rac1 mediated attraction, outgrowth, and turning of mouse cortical and commissural neurons. Its knockdown in the chick spinal cord leads to reduced midline crossing of commissural neurons [80]. Whether these two GEFs act in the same or parallel pathways is still not clear [29, 31, 43, 45].

1.3.3.2 Slit-Robo

The Slit/Robo-induced Rac1 activation provided an evidence for the fact that Rac activation can also contribute to the repulsive responses [57, 58, 81]. In *Drosophila*, Slit/Robo signaling and Rac activity is linked by specific RacGAP and GEF proteins. The GAP protein that is involved is a conserved RacGAP, vilse/CrGAP (ARHGAP39). Vilse/CrGAP is involved in slit-mediated midline repulsion of CNS axons. Interestingly, both increased and reduced levels of vilse/CrGAP causes dosage-dependent defects in slit/robo-induced repulsion, which indicates the importance of precise modulation of vilse/CrGAP activity [81, 82]. However, there should be other Rac regulators involved in slit/robo pathway as vilse mutants display only minor midline crossing defects [31].

Sos— a dual GEF with activity towards both Ras and Rho GTPases— is a promising candidate in that sense based on its expression in the *Drosophila* CNS, its genetic interaction with *slit* and *robo* mutants [83], and its interaction with the *Drosophila* ortholog of Nck adaptor protein— Dock [57] . Work using cultured human 293T cells has reported that upon Slit stimulation, Sos translocates to the plasma membrane, where it interacts with Robo and induces lamellipodia formation [84].

In line with the finding that slit/robo signaling leads to decreased Cdc42 activity [57, 58], Wu et al. [85] have reported that, upon its interaction with slit, robo interacts with and activates Slit-Robo Rho GTPase activating protein 1 (SrGAP1/ARHGAP13), which in turn inactivates Cdc42. The authors have reported that robo1 show tissue specific co-localization with SrGAP1 and 2 in the anterior sub-ventricular zone (SVZa) of neonatal mice. Moreover, they have shown that SrGAP1 co-immunoprecipitates with robo1 from rat neocortical extract. Further experiments using truncated SrGAP1 proteins have shown that the SH3 domain of SrGAP1 is sufficient to interact with the proline rich CC3 motif of robo1. In support of these data, using yeast two-hybrid system, Wong et al. [58] have demonstrated that the intracellular domain of the rat robo1 interacts strongly with SrGAP1, 2 and 3.

1.3.3.3 Sema-Plexin

In *Drosophila*, upon contact with Semaphorins, PlexinBs prevent the interaction of Rac with its effector by binding and sequestering active Rac [54]. Also, work from cultured cells suggests that active Rac increase both Plexin-B1 affinity towards Sema4D and its localization to the cell surface [86]. Meanwhile, Sema4D/Plexin-B1 signaling induces RhoA activity through two RhoGEFs— PDZ-RhoGEF (ARHGEF11) and

Leukemia-associated RhoGEF (LARG/ARHGEF12) [54, 55]. Both of these GEFs interact directly with the PDZ-binding domain of Plexin-B1. Dominant-negative (DN) versions of PDZ-RhoGEF and LARG abolish Sema4D-induced growth cone collapse in hippocampal neurons [55].

Unlike Plexin-Bs, Plexin-A induces Rac activation, to mediate growth cone collapse [87]. A recent work in cultured chick dorsal root ganglion (DRG) neurons has reported that Sema3A signaling through Plexin-A/Npn-1 leads to suppression of neurite outgrowth [88]. Further examination of the downstream pathway has shown that the processes requires Rac1 activation through a FERM domain-containing GEF— FERM, RhoGEF, and pleckstrin domain-containing protein 2 (FARP2). FARP2 is normally associated with PlexinA1/Npn-1 and it is released upon Sema3A stimulation. This release activates the GEF activity of FARP2 and leads to elevated Rac1 activity.

The first GAP that has been implicated in the sema/plexin signaling pathway is p190RhoGAP. Barberis and colleagues [89] have reported that p190RhoGAP mediates Sema4D/PlexinB1-induced neurite outgrowth in PC12 neuroblasts. They also report that p190RhoGAP plays role downstream of PlexinB1 signaling in various cell types such as fibroblasts, tumor epithelial and primary endothelial cells. Although this finding contradicts the mainly accepted idea that Sema4D/PlexinB1 signaling leads to RhoGEF-mediated, RhoA activation, authors claim that the two processes happen in temporally different manner [89]. Although they contribute to the formation of a more complete picture, the importance of these findings *in vivo* has yet to be validated [31].

1.3.3.4 Ephrin-Eph

Mutations in either of the genes encoding ephrinB3 or the receptor EphA4 lead to misrouting of interneuron axons of the mouse locomotor central pattern generator (CPG), the circuit necessary for coordinating alternating limb movement [90-95]. This subsequently leads to a particular phenotype called hopping gait [95]. The phenotype originates from defective ephrin-Eph forward signaling [92]. Interestingly, mutations in a gene encoding a Rac-specific GAP, ±-chimaerin result in an almost identical phenotype, hinting at the importance of ±-chimaerin-egulated Rac1 activity in ephrin-Eph forward signaling [90, 93, 94]. ±-chimaerin contains two domains that interact with EphA4: an N-terminal SH2 domain that binds to a phosphorylated juxtamembrane tyrosine residue of EphA4 and a C-terminal region that binds EphA4 constitutively. EphA4 mediates ephrinB3-induced tyrosine phosphorylation of ±-chimaerin, which increases its GAP activity towards Rac1 [93].

Surprisingly, the restoration of Rac1 activity also seems to be crucial for ephrin/Eph- driven growth cone collapse, particularly for class-B ephrin/Eph signaling [59, 87, 96, 97]. It seems that Rac1 activity-dependent endocytosis of ephrin/Eph complexes in *trans* to neighboring cells is an integral part of their repulsive mechanisms [98, 99]. Members of the Vav family of DBL GEFs— Vav2 and Vav3— have been reported to mediate Rac1-dependent endocytosis of ephrin/Eph ligand-receptor complexes [100].

Ephrin/Eph signaling also leads to RhoA activation through DBL family RhoGEFs— ephexins. Two members of ephexin, ephexin1/NGEF (ARHGEF27) and ephexin5/VSM-RhoGEF (ARHGEF15), are expressed in the mouse brain [101]. Depletion of mouse ephexin1 and chick ortholog c-ephexin leads to defects in ephrinA-

induced growth cone collapse and axon repulsion, as well as, defects in axon outgrowth [101].

In parallel to the forward signaling, ephrinB can behave as a receptor and induce reverse signaling upon contacting cognate Ephs [102]. DOCK180 has been reported to be implicated in ephrinB3-driven reverse signaling in stereotyped pruning of exuberant mossy fiber axons in the hippocampus. Nck2 adapter protein appears to link eprinB3 to DOCK180-mediated activation of Rac1 and Cdc42, which subsequently leads to axon retraction [61].

1.3.4 Regulators of Rho GTPases in neurological disorders

In this section, we will overview the GAP and GEF proteins that have been implicated in neurological diseases and disorders. We will focus mainly on the work published from mammalian research, in particular from human cases. In each subsection, we will briefly introduce the GAP or the GEF and then proceed to the work published about their implications in neurological diseases and disorders (**Table 1.2**). Finally, we will only review the DBL family of GEFs as the implication of the DOCK family of GEFs in neurological diseases has already been reviewed elsewhere [73].

1.3.4.1 GAPs

1.3.4.1a ARHGAP2 (±-chimaerin/CHN1)

The chimaerin subfamily of RhoGAPs has Rac1-specific GAP activity. The subfamily contains five members (±1-, ±2-, ²1-, ²2-, and ²3- chimaerins), all of which are the results of alternative splicing of two genes, ARHGAP2 (CHN1) and ARHGAP3

(CHN2). By controlling Rac1 activity, ±1-chimaerin plays a crucial role in the regulation of dendritic growth during neuronal development [103-105]. By comparing the expression levels of ±1- and ±2 chimaerin in postmortem brains of individuals with AD or unaffected individuals, Kato et al. [104] have demonstrated that ±1-chimaerin mRNA levels are significantly reduced in the temporal lobe of the AD patients in comparison to brains of unaffected individuals. However, no significant difference has been observed in the levels of ±2-chimaerin expression between the two groups.

±2-chimaerin, a close relative of ±1-chimaerin, has been implicated in axon guidance and is highly expressed in developing ocular motor neurons in rats and mice [90, 91, 93, 106]. Recently, two related studies [106, 107], have demonstrates that several missense mutations leading to enhanced dimerization and Rac1-GAP activity of ±2-chimaerin causes Duane's retraction syndrome (DRS) with strabismus phenotype. DRS is a congenital eye movement disorder caused by defects in the innervation of extraocular muscles by the axons of brainstem motor neurons. In support of this, a study by Ferrario and colleagues [108] have shown that Semaphorin 3A and 3C (Sema3A/C)/PlexinA signaling acts upstream of ±2-chimaerin to modulate the axon guidance of ocular motor neurons. However, implications of ±2-chimaerins in CNS is not limited to DRS. Using global and conditional knock-out mouse models, Iwata et al. [103] have demonstrated that deletion of *±2-chimaerin* gene but, not *±1-chimaerin* in early development leads to defects in contextual fear learning. The authors have also shown that this effect is not observed, if *±2-chimaerin* is deleted in adulthood, which suggests that ±2-chimaerin acts during development to establish adulthood cognitive abilities.

1.3.4.1b ARHGAP15

ARHGAP15 is a Rac-specific GAP protein. ARHGAP15 is expressed in both excitatory and inhibitory neurons of the adult hippocampus [109]. In a recent study by Zamboni and colleagues [109], ARHGAP15 has been associated with cognitive defects in mice. Authors have shown that loss of ARHGAP15 causes increased Rac1/3 activity, which leads to defects in the directionality and efficiency of migration of inhibitory neurons. These defects caused a reduction in numbers of inhibitory neurons and, subsequently, an alteration of the balance between inhibitory and excitatory synapses in favor of the latter, in hippocampus. As a result of the aforementioned changes, defects in hippocampus-dependent functions such as working and associative memories were observed in adult ARHGAP15$^{-/-}$ mice. The study stresses the importance of Rac activity and its precise regulation in developing hippocampal neurons.

1.3.4.1c ARHGAP18 (MacGAP, SENEX)

ARHGAP18 is a RhoA and RhoC-specific GAP protein [110]. Recently, in two related publications [111, 112], authors have used functional Magnetic Resonance Imaging (fMRI) of the dorsolateral prefrontal cortex (DLPFC) and genome-wide association study (GWAS) to identify novel schizophrenia associated genes. As a result, they have identified *ARHGAP18* as a Schizophrenia associated gene. Similar results have also been published by Guo et al., using Chinese-Han population as the target [113].

1.3.4.1d ARHGAP28

ARHGAP28 is a RhoA-specific GAP protein [114]. In a recent study by Jiang et al. [115], a point mutation in *ARHGAP28* gene has been associated to the most common type of migraines— migraine without aura (MWO). Migraine is a group of recurrent

headache disorder that is clinically characterized with neuropsychiatric aspects. Authors have targeted a cohort of 8 individuals— 4 with MWO and 4 without MWO. In 4 individuals with MWO, authors have detected mutations in six genes, one of which is *ARHGAP28.* A missense mutation in the *ARHGAP28* gene, which converts the threonine 31 residue to serine (T31S), reduced the ARHGAP28 expression, elevated RhoA and ROCK activities, resulting in cerebral vasoconstriction, spasm and migraine, as well as, an increase in the intensity of inflammatory reaction in the brain [115].

1.3.4.1e ARHGAP32 (p250GAP)

ARHGAP32 is a GAP protein with GAP activity towards Cdc42, Rac1, and RhoA [116, 117]. It is highly enriched in the CNS, where it is the primary target of the neuronal-specific microRNA— miR132. miR132 promotes neurite outgrowth by inhibiting ARHGAP32 expression [118]. A recent study [119] exploiting the mouse model of the Huntington's disease (HD) has shown that miR132 levels in this model is severely reduced, leading to increased expression of its primary target— ARHGAP32. HD is a dominant-inherited disease caused by the expansion of a CAG repeat in the *huntingtin (htt)* gene. The disease is currently incurable and eventually fatal. This study suggests that the cellular level of ARHGAP32 is important for proper brain development [119].

1.3.4.1f ARHGAP14 (srGAP3) and ARHGAP34 (srGAP2)

ARHGAP34 and ARHGAP14, better known as srGAP2 and srGAP3, respectively, are members of the Slit-Robo GTPase-activating protein subfamily (SrGAP) of RhoGAP with Rac1-specific GAP activity [120-122]. SrGAP2 is expressed in the

entire developing cortex and it prevents neuronal migration and promotes neurite outgrowth and neurite branching. SrGAP has also been shown to interact with robo1 and to regulate Cdc42 activity in slit-robo-dependent manner [58, 85]. A study by Saitsu and colleagues [122] has shown that a balanced translocation mutation that disrupts *srGAP2* gene leads to early infantile epileptic encephalopathy. SrGAP3 is highly expressed in the cortex and hippocampus, structures with crucial importance in the higher cognitive functions. It has been reported that the loss of one of the *srGAP3* copes as a result of a translocation mutation is associated with mental retardation (MR) [123]. In a more recent study [120], SrGAP has been demonstrated to be involved in spine development and its loss has been implicated in disruption of long-term memory.

1.3.4.1g ARHGAP33 (TCGAP)

ARHGAP33 is a Cdc42-specific GAP protein that is prominently expressed in developing and mature brain. It is involved in neurite outgrowth and branching, as well as, dendrite arborization and morphology [124, 125]. In two independent studies, ARHGAP33 has been implicated in neuropsychiatric developmental disorders, such as autism spectrum disorders (ASDs) and schizophrenia. Schuster et al., [126] have demonstrated that ARHGAP33 regulates dendritic spine maturation, synaptic transmission, and social behavior in mice, in a gender-specific manner. Authors show that loss of ARHGAP33 causes autism-like alteration in social behavior. In a more recent study [127], ARHGAP33-deficient mice show impaired dendritic spine morphology in the hippocampus, which leads to several behavioral defects such as working memory, learning, habituation, and anxiety, similar to the ones that are observed in neuropsychiatric developmental disorders [128, 129]. Using immortalized human

lymphocytes, the authors showed that the expression level of ARHGAP33 was lower in 45 schizophrenia patients in comparison to 45 sex/age-matched controls. They also showed that the genetic variation in the *ARHGAP33* gene is associated with Schizophrenia. In line with these data, authors have demonstrated that polymorphisms in ARHGAP33 may be linked to several schizophrenia related vulnerabilities in the brain morphology [127].

1.3.4.1h ARHGAP41 (oligophrenin-1/OPHN1)

ARHGAP41, better known as oligophrenin-1 (OPHN1), is a RhoGAP that is highly expressed in the brain. It was first identified as the gene that is mutated in patients with X-linked mental retardation (XLMR) [130-132]. Mental retardation (MR), recently renamed as intellectual disability (ID), is an early onset condition with subaverage intellectual functioning (IQ<70). It is defined as a non-progressive reduction in cognitive abilities [130, 132, 133]. Oligophrenin-1 was shown to have GAP activity towards RhoA, Rac1, and Cdc42 [131]. Studies on more XLMR cases by different research groups have proven that different deletion and duplications in the *OPHN1* gene cause X-linked mental retardation with a myriad of clinical and morphological phenotypes such as epilepsy, rostral ventricular enlargement, cerebellar hypoplasia, neonatal hypotonia, early onset seizures, marked strabismus, and language impairments [130-132, 134-136].

1.3.4.1i ARHGAP43 (SH3BP1/3BP1)

ARHGAP43, better known as SH3 domain binding protein 1 (SH3BP1), is a Rac1, Cdc42, and TC10-specific GAP protein [137, 138]. *SH3BP1* gene resides upstream of the gene encoding haloacid dehydrogenase family phosphatase chronophin (CIN/PDXP)

[138]. Recently, an mRNA transcript that is a partial fusion of SH2BP1 and CIN gene products has been linked to Alzheimer's disease (AD) [138]. The authors have named this mRNA and the protein it encodes BARGIN or BGIN. The study demonstrates that the C-terminal end of the BGIN non-covalently binds to poly-ubiquitin, which promotes its membrane localization, which, subsequently, leads to the inactivation of Rac1. In addition, the authors have observed BGIN-Ub interaction in tangled aggregates in AD brain. AD is caused by the accumulation of ß-amyloid (Aß) plaques and is the leading cause of dementia. Finally, using an amyloid precursor protein (APP) model, they have documented that BGIN mediates, at least partially, Rac1 inhibition and reactive oxygen species (ROS) generation.

1.3.4.1j ARHGAP44 (RICH2/Nadrin1)

ARHGAP44 is a Rac1 and Cdc42-specific GAP protein. It has important role in the CNS during dendritic spine morphogenesis. It was first identified as an interacting partner of SHANK3— a SHANK family member that coordinates structural and functional changes in the post-synaptic compartments [139, 140]. Since SHANK family has been closely associated with ASD and schizophrenia, its interaction with SHANK3 suggests an involvement of ARHGAP44 in neuropsychiatric diseases as well. To address this, Sarowar et al. have created an ARHGAP44$^{-/-}$ mouse line [140]. They have shown that depletion of ARHGAP44 leads to increase in the spine volume and the number of spines with multiple head. At the behavioral level, the authors have observed ASD like traits such as stereotypic behavior, specific phobia, and abnormal motor behavior [140].

1.3.4.1k ARAP1, ARAP3, ARHGAP12, ARHGAP29, ARHGAP40, and HMHA1 (ARHGAP45)

By analyzing the gene expression profile of skin fibroblasts obtained from either healthy individuals or individuals with bipolar disorder (BD), Logotheti et al. [141] found that genes involved in small GTPases-mediated signal transduction are downregulated. Among these genes, authors mention ARAP1 and 3, ARHGAP12, 29, 40, and Minor Histocompatibility Antigen HA-1 (HMHA1/ARHGAP45), all of which are GAPs for the Rho GTPases. ARAP (ArfGAP and RhoGAP with ankyrin repeat and PH domains) subfamily contains both ArfGAP and RhoGAP domains, which allow them to regulate both families of small GTPases [142, 143]. HMHA1 is a cancer therapeutic target with a RhoGAP activity [144, 145]. ARHGAP 12 and 29 are ubiquitously expressed and they carry GAP activities towards Rac1 and RhoA, respectively [146-148].

1.3.4.1l ARHGAP46 (GMIP)

ARHGAP46, better known as GEM-interacting protein (GMIP), is a ubiquitously expressed protein with RhoA-specific GAP activity. It is crucial for neurite growth, axonal guidance, neuronal migration, and synaptic functions [149, 150]. Tadokoro et al., [150] have shown that ARHGAP46-related SNPs are associated with major depressive disorder (MDD) in Japanese population, particularly in male subjects.

1.3.4.1m Myosin IXb (MYO9B)

MYO9B is a RhoA-specific GAP protein that has been associated with autoimmune disease— celiac disease [151, 152]. In their search for a candidate gene that could fit the long-thought association between autoimmune diseases and schizophrenia,

Jungerius and colleagues [151] have tested the hypothesis that MYO9B is the candidate gene. Interestingly, they show strong correlation between MYO9B associated SNPs and schizophrenia.

1.3.4.1n Ral binding protein 1 (RalBP1/RLIP76)

RalBP1 is a Rac1 and Cdc42-specific GAP protein. It also has GAP activity towards the Ras like GTPases (Ral GTPases), which renders RalBP1 crucial for crosstalk of Ras-Rho signaling [153, 154]. Interestingly, it can also activate Rac1 in GTP-R-Ras-dependent and Ral-independent manner, through Arf6 GTPase activity [155]. In forebrain, hippocampal CA1 neurons show the highest expression level of RalBP1. Using mouse RalBP1 hypomorphs— RalBP1 expression is only 18% of the WT levels—, Bae et al. showed that, although it does not cause seizures per se, reduction in the RalBP1 levels renders mice more susceptible to seizures by reducing the seizure threshold. Authors claim that the study is the first of its kind to implicate RhoGTPase signaling in seizures [153]. Also, more and more data are emerging, which supports a role for RalBP1 as a mediator of pharmacoresistance in the CNS; however, further research needs to be done in order to substantiate these data [156].

1.3.4.2 GEFs

1.3.4.2a ARHGEF2 (GEFH1)

ARHGEF2, better known as GEF-H1, is a microtubule-associated RhoA-specific GEF protein. It links actin and microtubule dynamics. ARHGEF2 plays important roles in mitotic spindle formation and orientation. It is expressed by neural precursors and immature neurons in mouse neocortex. ARHGEF2 play important roles in

the CNS in processes ranging from axonal re-networking, dendritic spine retraction, general gene expression, neurogenesis to neural tube closure by mediating RhoA activity [157-159]. A case study [158] has demonstrated that a loss-of-function mutation leading to truncated ARHGEF2 protein, causes intellectual disability (ID), mild microcephaly, and midbrain-hindbrain malformations. Authors have also demonstrated that mice lacking ARHGEF2 show significant reduction in the volume of the total brain size (microencephaly), the cerebellum, and the brain stem, as well as, absence of pontine nuclei. These defects recapitulate the phenotypes that have been observed in the human patients and proposes a conserved role for ARHGEF2 in humans and mice. In another study, Varma et al. [159] have demonstrated that ARHGEF2-mediated RhoA activation protects rat striatal neuronal cells against MT depolymerizing agents. Interestingly, this rescue phenomenon has been observed only when the neuronal cells express the mutant version of the Htt protein, but not the wild type (WT) version. Considering that the mutation in *Htt* gene leads to Huntington's disease (HD), this study suggests a possible neuroprotective role for the ARHGEF2-mediated modulation of RhoA activity in HD.

1.3.4.2b ARHGEF6 (αPIX/Cool-2)

ARHGEF6 is a Rac1/Cdc42-specific GEF protein. It is ubiquitously expressed in different tissues [160, 161]. In brain, ARHGEF6 has been shown to be expressed in hippocampal CA1-CA3 cells layer and in cultured neurons it has been shown to co-localize with PSD95 protein at postsynaptic densities of excitatory synapses [162]. In the CNS, ARHGEF6 plays a role in axonal and dendritic branching, regulation of spine morphogenesis, and synapse formation [160]. Loss of ARHGEF6 in mice leads to reduced active Rac1 and Cdc42 levels, altered dendritic morphology, decreased long-

term potentiation (LTP), increased long-term depression (LDP), and several behavioral abnormalities such as navigation errors, disinhibited object exploration, and impairment of complex positional learning [160]. Also, *ARHGEF6* is one of the three RhoGEF encoding genes to be involved in XLMR along with *faciogenital dysplasia protein 1 (FGD1)* and *ARHGEF9 (collybistin)*. A translocation mutation, which targets the first intron of the *ARHGEF6* gene and leads to the expression of truncated *ARHGEF6* transcript (lacking exon 2), was found to XLMR in the male individuals of a Dutch family by Kutsche et al [160, 163].

1.3.4.2c ARHGEF9 (collybistin)

ARHGEF9, also known as collybistin, is a Cdc42-specific GEF protein with high expression in the developing and adult brain [164]. ARHGEF9 has been shown to mediate the translocation of gephyrin to the postsynaptic membrane micro-aggregates, which in turn regulates clustering of the receptors for inhibitory neurotransmitters—glycine and ^{3}-Aminobutyric acid (GABA) [165]. Therefore, ARHGEF9 is an important component of the inhibitory synapses [164]. Several case studies have revealed mutations in the *ARHGEF9* gene leading to XLMR, ASD, along with phenotypes such as seizures and epilepsy [164, 166-171]. A dominant-negative missense mutation, p.G55A, has been linked to a severe mental retardation, hyperekplexia, drug-resistant seizures, and premature death in a male patient [164]. A separate study [171], have identified a microdeletion and a nonsense mutation in *ARHGEF9* gene causing loss-of-function in two male individuals. The common symptoms for both patients were mental retardation (MR) and epilepsy. Moreover, a missense mutation in *ARHGEF9* gene, p.R338W, has been identified, which causes structural disruption of the ARHGEF9 protein, in several

members of a family with XLMR, macrocephaly, and macro-orchidism [169]. Yet another missense mutation was identified by carrying out genetic analysis on an Ethiopian-Jewish family with four affected male individuals, the authors have found a p.G323R mutation. All the affected individuals had intellectual disability, focal epilepsy, and febrile seizures [168]. However, ARHGEF9 mutations are not limited to point mutation: two translocation mutations in two different female individuals have been reported. In one of the cases mutation led to epilepsy, anxiety, aggression, and MR, while in the other case, the patient had XLMR and sensory hyperarousal [167, 170]. Finally, an 82 kb deletion including *ARHGEF9* gene has been detected in an 8-years-old female with ASD, intellectual disability, and speech delay [166]. All these case studies stresses that ARHGEG9 and its function in gehphyrin mediated post-synaptic membrane organization in inhibitory synapses is of critical importance.

1.3.4.2d ARHGEF10

ARHGEF10 is a RhoA-specific GEF protein [172]. It is expressed in wide range of tissues with relatively high expression in the spinal cord and dorsal root ganglion. ARHGEF10 has been shown to be involved in neuronal growth, axonal migration, and to be implicated in human hypomyelination [173]. A missense mutation in the exon 3 of the *ARHGEF10* gene has been shown to cause non-clinical slowed nerve-conduction velocity. The mutation has shown to be inherited in autosomal dominant fashion and to cause increased GEF activity [174]. In addition, *ARHGEF10* gene has been shown to be associated with Charcot-Marie-Tooth (CMT) disease, a heterogeneous mix of hereditary motor and sensory neuropathies in humans [172, 175]. To check the association between CMT genes and chemotherapy-induced peripheral neuropathy (CIPN), Beutler and

colleagues have studied 49 CMT genes in a large cohort of cancer patients. During the study, the patients have been administered paclitaxel, a chemotherapy agent. The authors have identified three non-synonymous SNP in *ARHGEF10* gene to be highly associated with CIPN, one of them (rs9657362) showing the strongest association with neuroprotective effect [172]. This study has been successfully replicated in an independent cohort of patients [175]. Another mutation in the *ARHGEF10* gene, a deletion mutation leading to the loss of 50% of its protein product, has been shown to associate with juvenile-onset inherited polyneuropathy in Leonberger and Saint Bernard dogs. The affected dogs had severe symptoms such as axonal degeneration with progressive clinical signs of weakness and muscle atrophy leading to decreased nerve fiber density and chronic nerve fiber loss [173].

1.3.4.2e ARHGEF13 (A-kinase anchor protein 13 (AKAP13)/LBC/BRX)

ARHGEF13, better known as AKAP13, is a Rho-specific GEF protein that belongs to A-kinase anchor protein family (AKAP). This family is composed of more than 50 proteins encoded by 10 *AKAP* genes. These proteins share the ability of linking protein kinase A (PKA) to its targets. Recently, using bioinformatics and extensive manual literature mining, Poelmans et al. [176] have demonstrated that AKAPs can be candidate drug targets for the treatment of autism spectrum disorders (ASDs). By analyzing the results of 6 previously published GWASs and emerging signaling networks related to ASDs, they have shown that AKAPs are integrating signaling cascades within and between these networks. AKAP13 positively regulates RhoA, a small GTPase that is involved in neurite outgrowth, regulation of synaptic networks, and modulation of the effect of risperidone treatment for ASDs. In addition, AKAP13 gene has been found in

copy number variations (CNVs) in ASDs patients and its mRNA product is a target of ASDs related microRNAs. AKAP13 is also involved in two ASD-implicated biological processes— innate immunity and melatonin synthesis. Furthermore an AKAP13-derived peptide, Ht31, block 'masculinization' of a part of the rat brain. Finally, transgenic mice with conditional Ht31 expression in the hippocampus show ASD-like cognitive defects. Overall, these findings reveal AKAP13 as a promising candidate for ASD-targeted drug development.

1.3.4.2f ARHGEF23 (Trio)

ARHGEF23, better known as Trio, is a large, multi-domain protein with two separate GEF domains— GEF1 and GEF2. The GEF1 domains is active towards Rac1 and RhoG, while the GEF2 domain is active towards RhoA, which renders Trio a crucial signaling component in crosslinking RhoA-Rac1- mediated pathways [177, 178]. Trio is highly expressed in the developing brain of rodents and humans, especially in the cerebellum, cortex, hippocampus and thalamus [179, 180]. Interestingly, Trio expression decreases gradually in the adult brain [177-179]. Work done in *C. elegans, Drosophila*, rodents, as well as human patients has reported that Trio plays a crucial role in the regulation of dendrite, dendritic spine, and synapse development and function [177, 181-183]. Moreover, Trio has been documented to participate in netrin-1/DCC mediated-axon guidance in *Drosophila* and rodents [30, 75, 78, 184]. The importance of Trio can be highlighted with the fact that complete knockout of TRIO in the mouse nervous system is embryonically lethal [185]. Also, hippocampal or cortex-specific depletion of TRIO in the mouse brain results in the malformation of these structures, subsequently, leading to defective learning ability [186]. In line with its extensive involvement in the brain

development, mutations in the *TRIO* gene have been associated with neurological and neurodevelopment disorders such as intellectual disability (ID), schizophrenia, developmental delay, bipolar disorder (BD), and autism spectrum disorders (ASDs) [177, 178, 187-193]. The first such mutation has been reported by de Light et al. [187]. The authors have identified two disparate missense mutations in the *TRIO* gene of two patients with severe ID. However, the study has not been able to link the Trio gene to ID conclusively, as both patients have also harbored mutation in other well-established ID-related genes. A year later, a deletion mutation partially including *TRIO*gene has been reported to cause mild ID, developmental delay, behavioral defects, and facial dysmorphism [193]. Recently, more disease-causing *de novo* mutations have been identified in the *TRIO* gene. Ba and colleagues have identified four point mutations— three truncation and one frameshift mutation— leading to mild ID and behavioral defects [177]. Using rat hippocampal neurons, the authors have shown that the mutations leads to increased dendritic arborization and altered synaptic functions. In another study [191], several mutations in a part of the TRIO gene encoding GEF1 domain have been associated with ID and microcephaly. Finally, in an interesting study [178], Katrancha and colleagues have examined the effect of disease-causing mutations of the *TRIO* gene in its encoded protein. They have concluded that the majority of the mutations are localized to the GEF1 domain of the protein, thereby reducing its Rac1-GEF activity. Collectively, all these animal and human case studies underlie the importance of TRIO, in particular its Rac1-GEF activity, in the developing brain.

1.3.4.2g ARHGEF24 (Kalirin)

ARHGEF24, better known as Kalirin, is a Rac1-specific GEF protein [194-196]. Kalirin is a vertebrate paralog of Trio [178]. *Kalirin* gene has multiple promoters and transcriptional start sites, which lead to multiple isoform production [196, 197]. The most common Kalirin isoforms are Kalirin-7, -9, and -12. Kalirin is expressed in wide range of tissues such as brain, endocrine cells, liver, muscle, and heart. In neuronal tissue, Kalirin-9 and -12 are very abundant during the development. These two isoforms localize to the growth cone and neurites of immature neurons. The adult brain, on the other hand, expresses high level of Kalirin-7, which localizes to the postsynaptic compartments of excitatory synapses [197] Kalirin has also been implicated in ephrinB-EphB-mediated dendritic spine morphogenesis [198]. Cahill et al. [194] have demonstrated that Kalirin-7 knock-out mice shows reduced cortical Rac1 activity and spine density, which leads to disease-related behavioral phenotypes such as impaired working memory, sociability, and prepulse inhibition problems. These findings are in line with the suggested function of Kalirin-7 in spine/synapse formation both *in vitro* and *in vivo.* Kalirin-7 has also been implicated in cocaine addiction in mice— its expression is upregulated in medium spiny neurons (MSNs) of the nucleus accumbens (NAc)— a brain area associated with drug addiction and reward pathways—, upon chronic cocaine treatment. This upregulation leads to increased spine density, which is abolished in Kalirin-7 knock-out mice [197]. Furthermore, reduced forebrain Kalirin-7 expression has been observed in schizophrenia and AD [195, 199, 200]. Several studies have also associated *Kalirin-7* gene with attention deficit hyperactivity disorder (ADHD), depression, epilepsy, and ischemic strokes [194-197]. Overall, similar to its paralog Trio, Kalirin seems to be of substantial importance in the CNS.

1.3.4.2h ARHGEF36 (DNMBP)

ARHGEF36, better known as dynamin-binding protein (DNMBP), is a Cdc42-specific GEF protein [201]. DNMBP has high density at the periphery of presynaptic vesicle clusters, where it has been shown to co-localize with synapse-enriched proteins, amphiphysin-1 and dynamin-1 [201, 202]. As a scaffolding protein, DNMBP brings dynamin and actin regulators together at the synaptic membranes to mediate events such as synaptic vesicle recycling. Because of its role in the recycling of amyloid precursor protein (APP), DNMBP is a promising candidate to be involved in the AD pathology [201, 202]. Therefore, two independent studies, targeting cohorts of late-onset AD patients in Japanese and Belgian populations, have associated SNPs in the *DNMBP* gene to late-onset AD [201, 202]. Furthermore, it has been reported that the expression level of the *DNMBP* gene is reduced in AD brain by using post-mortem brain sections from AD and age-matched unaffected individuals [201].

1.3.4.2i ARHGEF42 (CLG/PLEKHG2)

ARHGEF42 is a Rac1 and Cdc42-specific GEF protein. It is only present in rodents and mammals [203]. Recently, a case study [203] has identified a missense mutation in *ARHGEF42* gene— p.R204W— that leads to reduced GEF activity and, consequently, impairment of Stromal Cell-derived Factor 1a (SDF1a)-stimulated actin polymerization. Five children, affected with the mentioned mutation, have shown profound MR, dystonia, postnatal microcephaly, and distinct neuroimaging patterns.

1.3.4.2j ARHGEF44 (Puratrophin-1/PLEKHG4)

ARHGEF44, better known as PLEKHG4, is a GEF protein that activates RhoA, Rac1 and Cdc42. It is expression levels vary in different tissues— from high expression in the testis to whole brain. In the mouse brain, PLEKHG4 is expressed in the brainstem, cerebellum, and midbrain [204, 205]. However, only the cerebellar expression is sustained in the adult brain, especially in the Purkinje neurons. In line with the cerebellar expression of PLEKHG4, using positional cloning, Ishikawa and colleagues [205] have demonstrated that single nucleotide substitution in the 5' untranslated region (UTR) of the *PLEKHG4* gene is associated with autosomal dominant cerebellar ataxia (ADCA), a group of heterogeneous neurodegenerative disorders.

1.3.4.2k Alsin

Alsin is the product of *ALS2* gene. Alsin has GEF activity towards Rac, Ran, and Rab5, which makes it an important regulator of signaling by distinct small GTPases [206, 207]. The *ALS2* gene produces two products by alternative splicing: the long (6394 nt) and the short (2651 nt) variants [206-208]. Mutations in the *ALS2* gene have been shown to cause infantile-onset ascending hereditary spastic paraplegia (IAHSP), juvenile-onset primary lateral sclerosis (JPLS), and amyotrophic lateral sclerosis (ALS). HSP, PLS, and ALS are genetically heterogeneous neurodegenerative diseases characterized by primary degeneration of motor neurons [206-208]. Initially it was proposed that the mutations that affect only the long variant cause JPLS, while the mutations that affect both variants cause the more severe form, ALS [206]. However, a study by Eymard-Pierre et al. [206] has argued against this by showing that a mutation affecting both variants of *alsin* transcripts shows no sign of lower motor involvement in affected patient.

1.3.4.2l Faciogenital dysplasia protein 1 (FGD1)

FGD1 is a Cdc42-specific GEF protein [209, 210]. Mutations in the different domains of *FGD1* gene have been implicated in the Aarskog-Scott syndrome— a rare, X-linked recessive disorder that is characterized by short stature and exhibit unique skeletal and genital development [209]. In addition, *FGD1* is another one of the GEF encoding genes to be mutated in XLMR. A case study conducted by Lebel and colleagues has shown that a missense mutation in *FGD1* gene leads to the substitution of the leucine residue at the position 312 with a proline (L312P) is associated with severe cognitive impairments [211].

1.3.4.2m VAV1 and 3

The VAV family proteins carry GEF activity towards RhoA, RhoG, Rac1, and Cdc42. There are three VAV proteins in mammals: VAV1, 2, and 3. While VAV2 and 3 are expressed in several tissues, VAV1 is mainly restricted to haematopoietic lineage [212]. Using experimental autoimmune encephalomyelitis (EAE), a rat model of multiple sclerosis (MS)— a chronic inflammatory disease that damages myelin sheaths and nerve fibers—, an association has been detected between VAV1 and MS [213]. The authors of the work have observed that VAV1 expression is elevated in rats and have suggested that VAV1 may have a role in MS. Furthermore, pursuing a preceding Japanese GWAS analysis of schizophrenia [214], Aleksic and colleagues have revealed VAV3 to be a candidate gene for schizophrenia based on Voxel-Based Morphometry and Mutation Screening [215].

1.4 Conclusion

As described in the preceding sections, the Rho family of small GTPases and their regulators, in particular GAPs and GEFs, are crucial downstream components of guidance cue/receptor signaling pathways and dysregulation of their activity may lead to serious physiological complications. Consequently, more and more data are emerging which draw connections between the regulators of Rho GTPases and various diseases and disorders. In this chapter, we tried to amass published data describing the involvement of GAPs and GEFs in neurological disorders, especially in humans. However, our wok does not tell the whole story as more finding related to the role of GAPs and GEFs in the CNS are being uncovered and, even more have yet to be unveiled.

Figures and Tables for Chapter 1

Figure 1.1. The mechanism of the regulation of the small Rho GTPases

The Rho family of small GTPases shuttle between active, GTP-bound, and inactive, GDP-bound, states. This cycles is coordinated by three classes of proteins regulatory protein: 1) GEFs (Guanine nucleotide-exchange factors), which activates the small GTPases by perfoming the GDP to GTP exchange; 2) GAPs (GTPase-activating proteins), which inactivate the small GTPases by enhancing their intrinsic ability to hydrolyze the GTP to GDP; and 3) GDIs (Guanine nucleotide-dissociation inhibitors), which sequester the small GTPases in their GDP-bound, inactive state.

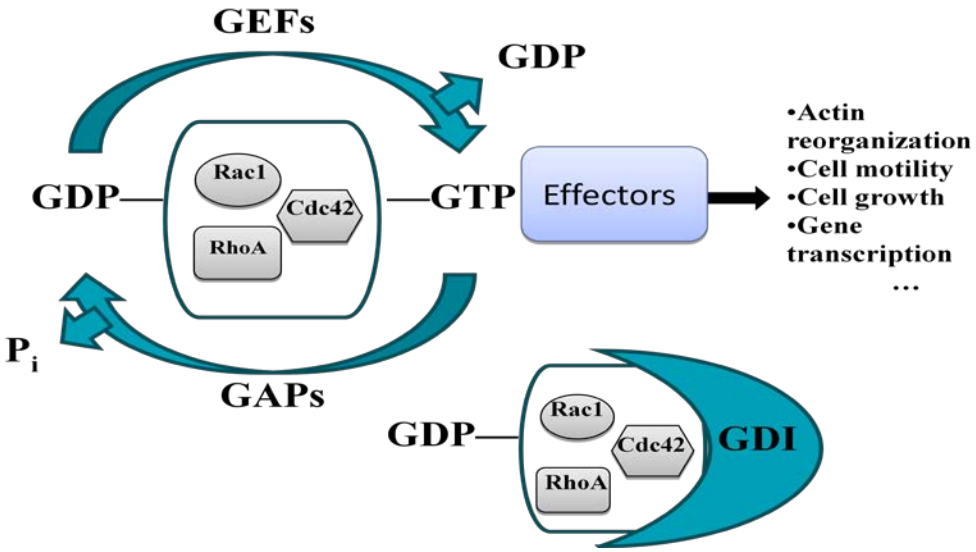


Figure 1.2. The classic guidance cues, their receptors, and their effect on the small Rho GTPases

A schematic representation of the regulation of the Rho GTPases by different guidance cue/receptor combinations.

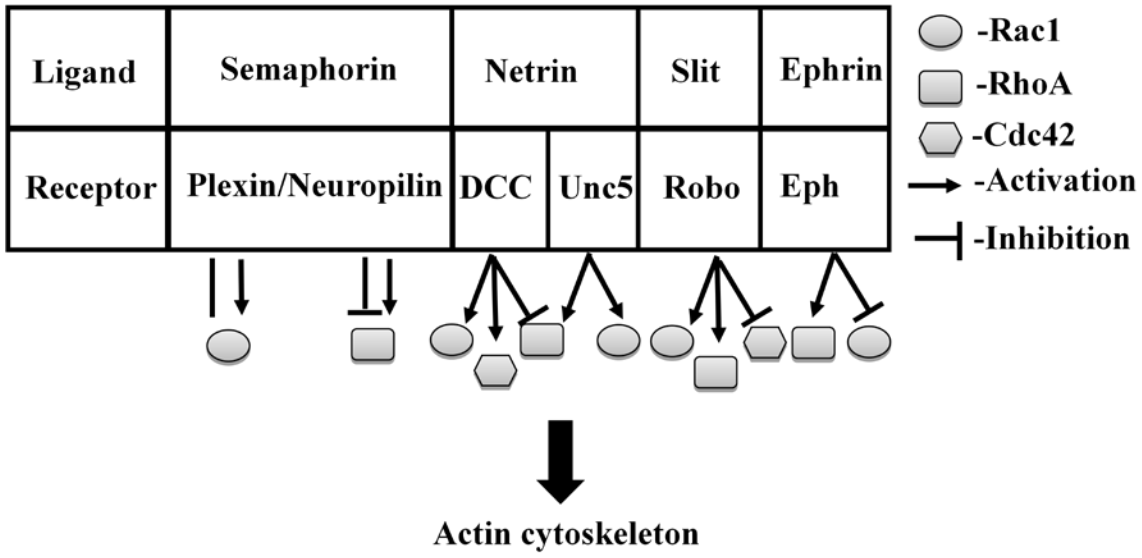


Table 1.1. GAPs and GEFs in guidance cue/receptor signaling pathways

Ligand	Receptor	GAPs involved	GEFs involved
Netrin	**DCC**	**-**	**Trio, (ARHGEF23) DOCK180**
Semaphorin	**Plexin/Neuropilin**	**p190RhoGAP (ARHGAP35)**	**PDZ-RhoGEF (ARHGEF11), LARG (ARHGEF12), FARP2**
Slit	**Robo**	**vilse/CrGAP (ARHGAP39), SrGAP (ARHGAP13)**	**Sos**
Ephrin	**Eph**	**±-chimaerin (ARHGAP2)**	**VAV2 and 3, ephexin1/NGEF, (ARHGEF27) ephexin5/VSMRho-GEF (ARHGEF15)**

Table 1.2. GAPs and GEFs in neurological diseases and disorders

Diseases and disorders	GAPs Involved	GEFs Involved	Studied organisms
Bipolar disorder	ARAP1, ARAP3, ARHGAP12, ARHGAP29, ARHGAP40, HMHA1 (ARHGAP45)	Trio	Human
Cognitive complications	ARHGAP15, ±-chimaerin (ARHGAP2)	ARHGEF6, Trio (ARHGEF23), Kalirin (ARHGEF24)	Mouse
Schizophrenia	ARHGAP18, ARHGAP33, Myosin IXb	Kalirin (ARHGEF24), Trio (ARHGEF23), VAV3	Human
Migraine	ARHGAP28		Human
Huntington's disease	ARHGAP32	GEFH1 (ARHGEF2)	Mouse, Rat
Austism spectrum disorders	ARHGAP33, ARHGAP44	AKAP13 (ARHGEF13), Collybistin (ARHGEF9), Trio (ARHGEF23)	Human, Mouse
Depressive disorders	GMIP (ARHGAP46)	Kalirin (ARHGEF24)	Human, Rat, Mouse
Intellectual disability	Oligophrenin (ARHGAP41), srGAP3 (ARHGAP14)	GEFH1 (ARHGEF2), ARHGEF6, FGD1, Collybistin (ARHGEF9), Trio (ARHGEF23), ARHGEF42	Human, Mouse
Seizure	RalBP1	Collybistin (ARHGEF9)	Human, Mouse

Alzheimer's disease	**SH3BP1 (ARHGAP43), ±-chimaerin (ARHGAP2)**	**DNMBP (ARHGEF36), Kalirin (ARHGEF24)**	**Human**
Infantile epileptic encephalopathy	**srGAP2 (ARHGAP34)**		**Human**
Duane's retraction syndrome	**±-chimaerin (ARHGAP2)**		**Human**
Amyotrophic lateral sclerosis		**Alsin**	**Human**
Epilepsy		**Collybistin (ARHGEF9), Kalirin (ARHGEF24)**	**Human, Rat**
Charcot-Marie-Tooth disease and polyneuropathy		**ARHGEF10**	**Human, Dog**
Attention deficit hyperactivity disorder		**Kalirin (ARHGEF24)**	**Human**
Cocaine addiction		**Kalirin (ARHGEF24)**	**Mouse**
Cerebellar ataxia		**PLEKHG4 (ARHGEF44)**	**Human**

RATIONALE AND OBJECTIVE

Rationale

As can be deduced from the vast number of studies that implicate them in a wide range of neurological diseases and disorders, GAPs and GEFs perform a multitude of crucial functions in the CNS of both developing and adult CNS. Furthermore, these studies span the animal kingdom from worms to human, suggesting evolutionarily conserved roles for GAPs and GEFs. Despite all these data, as we have already seen in the previous sections of this chapter, only few GAPs and GEFs have been identified to play role downstream of classic guidance cue/receptor complexes. In that sense, netrin/DCC-mediated axon guidance is of particular interest, as to date there has not been any identified GAP protein in this signaling pathway. To address this gap, in the Chapter 2 of this thesis, we set out to investigate the role of p190RhoGAP— a protein with Rho-specific GAP activity— in netrin-1/DCC mediated guidance and outgrowth of axons from cortical neurons.

Objective:

To investigate the possible role of p190RhoGAP in netrin-1/DCC-mediated axon guidance and outgrowth.

PREFACE TO CHAPTER 2

Decades of research has shown that the Rho family of small GTPases is one of the converging point of the guidance cue/receptor signaling. Interestingly, so far, only one example of a direct interaction between a guidance cue receptor and a Rho GTPase has been documented— PlexinB1/Rac1. In the rest of the reported cases, guidance cue receptors have been shown to recruit, directly or indirectly, the regulators of Rho GTPases— GAPs and GEFs. Work done in different model has revealed numbers of GAPs and GEFs downstream of guidance cur/receptor signaling pathway. Our main focus through the past years has been dissection of the signaling mechanisms downstream of the netrin-1/DCC-mediated axon guidance. Together with other research groups, we have shown that netrin-1/DCC signaling leads to a reduction of RhoA activity. This, in turn, suggests that netrin-1/DCC signaling machinery involves a RhoA-specific GAP protein. In the Chapter 2 of this thesis, we have used HEK293 cells and dissociated rat cortical neurons to examine the possible involvement of p190RhoGAP in netrin-1/DCC-mediated modulation of RhoA activity and axon guidance.

CHAPTER 2: A role for p190RhoGAP in the signaling mechanisms mediated by the axon guidance cue netrin-1 and its receptor deleted in colorectal cancer (DCC) in primary cortical neurons

Sadig Niftullayev [1,2], Philippe Duquette [1,2], Nathalie Lamarche-Vane [1,2]

1- Department of Anatomy and Cell Biology, Mcgill University, Montreal, Quebec H3A 0C7, Canada
2- The Cancer Research Program, The Research Institute of Mcgill University Health Centre, Montreal, Quebec H4A 3J1, Canada

Abstract

Netrin-1— a secreted protein with chemotropic property— is one of the classic guidance cues. It acts as both chemo-attractant and chemo-repellant in a receptor specific manner. In the CNS, chemo-attractive roles of netrin is mediated by DCC— a transmembrane receptor that is a type I glycoprotein with extracellular regions containing four Ig repeats followed by six type III fibronectin repeats (Fn). Netrin-1/DCC mediated axon guidance is crucial during development as the disruption of either netrin or DCC expression leads to the loss of spinal and cerebral commissures. In addition, mutations in *DCC* gene have been associated with neurological disorders such as congenital mirror movement. Despite vital importance of netrin/DCC signaling pathway during development, mechanism underlying it has still not been fully deciphered. Previous work from different groups has reported that, similar to other guidance cue signaling pathways, netrin/DCC signaling control the activity of the small Rho GTPases to modulate the dynamics of actin cytoskeleton. GTPase-activating proteins (GAPs) are one of the three main classes of the regulators of small Rho GTPase signaling. However, to date, the role of the GAPs in axon guidance, particularly downstream of netrin/DCC signaling platform, has not been extensively studied.

Previous work from our group has documented that p120RasGAP (hereafter RasGAP) is recruited to DCC, upon netrin-1 stimulation, where it modulates the activity of Ras GTPases and ERK. Interestingly, one of the well-studied binding-partners of RasGAP is p190RhoGAP (hereafter p190)— a Rho-specific GAP protein of 190kDa. p190 has been shown to be highly expressed in the CNS, where it is the principal target of Src kinase. In this study, we have hypothesized that p190, through its interaction with

RasGAP, is recruited to DCC, upon netrin-1 stimulation, and regulates Rho GTPase activity to modulate axon outgrowth and guidance. To examine the validity of our hypothesis, we have used the combination of biochemical assays and fluorescent microscopy. Our work has shown that p190 forms a complex with RasGAP and overexpressed DCC in HEK293 cells and that mere overexpression of DCC is sufficient to increase tyrosine phosphorylation of p190. Moreover, we have documented that the tyrosine 1418 residue (Y1418) and C-terminus end of DCC is important for its interaction with p190. We have also shown that along with its SH2 domains, the SH3 domain of RasGAP is interacting with p190, as well. Furthermore, we have observed that p190 is highly expressed in cortical neurons and it has similar localization patterns as actin, DCC, and Rho-GTP in the growth cones of cortical neurons. Our study has also demonstrated that, similar to the findings in the HEK293 cells, p190 forms a complex with RasGAP and DCC in cortical neurons. Finally, our work has shown that, in cortical neurons, upon netrin-1 stimulation, p190 is phosphorylated by Src family kinases, which increases its interaction with RasGAP and DCC. Therefore, our data strongly suggest a role for p190 in the netrin-1/DCC-mediated axon outgrowth and guidance.

Introduction

The functional integrity of the vertebrate nervous system depends on proper neuronal connectivity or wiring [21, 216], failure of which is linked to disorders such as autism spectrum disorder (ASDs) and Down's syndrome [3]. Correct target selection through axon guidance is one of the key mechanisms that ensure proper neuronal connectivity. During the development of the CNS, axons travel long distance, through complex extracellular environment, to reach their targets. On this journey, neurons rely on their growth cones— highly dynamic structure at the distal tip of the axon. The growth cone is equipped with appropriate machinery to be able to sense and respond to various extracellular cues [18]. The past few decades have led to the discovery of several classes of evolutionarily conserved axon guidance cues and their cognate receptors [5, 16, 17]. These cues can be either secreted or membrane-bound and can elicit attractive or repulsive responses in a receptor-dependent fashion [5, 16, 17, 19]. The netrin family of guidance cues has been shown to be crucial for the neuronal development in both invertebrates and vertebrates [24]. Netrin-1 has been reported to interact with several families of transmembrane receptor such as DCC and its paralog neogenin, DsCAM, the UNC-5 family, and amyloid precursor protein (APP) [5, 16, 17, 24, 217]. Acting through its receptors and receptor complexes, netrin-1 can induce both attractive and repulsive responses in a variety of neuronal types [5, 16, 17, 24]. Netrin-1-induced attractive responses in the developing spinal cord and cerebral cortex of vertebrates is achieved through the transmembrane receptor DCC [218-220]. The importance of netrin-1/DCC signaling pathways during development can be highlighted with the fact that disruption in either netrin-1 or DCC expression causes the absence of spinal and cerebral commissures

[221, 222]. Furthermore, mutations and genetic variability in the human *DCC* gene have been linked to neurological disorders such as congenital mirror movement [223, 224], schizophrenia [225], and Parkinson's disease (PD) [226]. Netrin-1 stimulation leads to the phosphorylation of DCC on serine, threonine, and tyrosine residues. Of particular interest is the phosphorylation of DCC on the C-terminal tyrosine residue 1418 (Y1418) by Src family kinases, which mediates axon outgrowth and guidance in vertebrates. The Y1418 residue is located in the C-terminal tail of DCC, in a motif called P3— a highly conserved region that modulates recruitment of several downstream molecules such as focal adhesion kinase (FAK), Src, Fyn, ezrin and Myosin X [227-234].

Although not fully deciphered, it has been shown that axon guidance cue/receptor signaling steers the growth cone by altering its morphology through the regulation of local actin cytoskeleton [29]. Actin is enriched in the peripheral domain of the growth cone and defines its dynamic nature by forming distinct structures— filopodia and lamellipodia [29, 42-44]. Rho GTPases are the main regulators of actin remodeling. They cycle between GTP-bound, active, and GDP-bound, inactive, states. Two classes of proteins— Guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs) — modulate the activity of Rho GTPases. GEFs activate Rho GTPases by inducing GDP-to-GTP exchange, while GAPs deactivate them by enhancing intrinsic GTPase activity of the Rho GTPases [29, 43, 62, 63]. Signaling through all four classic guidance cue/receptor complexes has been shown to regulate the activity of Rho GTPases [29, 31]. Netrin-1/DCC signaling has been documented to increase Rac1 and Cdc42 activity, while decreasing RhoA activity in embryonic rat spinal cord neurons (SCNs) [29, 31, 43]. So far, two GEF proteins have been implicated in the activation of Rac1 and

Cdc42 downstream of netrin-1/DCC— the DBL family GEF, Trio, and the DOCK family GEF, Dock180 [29-31, 43, 75]. The identification of a GAP protein(s) responsible for the deactivation of RhoA, however, has yet to be achieved.

A recent work from our group has reported that, upon netrin-1 stimulation, p120RasGAP (hereafter RasGAP), a Ras-specific GAP protein, is recruited to phosphorylated Y1418 residue of DCC through its N-terminal SH2 domain and modulates basal activity of ERK and Ras proteins [235]. Interestingly, one of the main binding partners of RasGAP is p190RhoGAP (hereafter p190)— a Rho-specific GAP protein. The N- and C- terminal SH2 domains of RasGAP interact with two highly phosphorylated tyrosine residues of p190, Y1087 and Y1105, to mediate translocation p190 to the plasma membrane where it can regulate Rho-mediated remodeling of actin cytoskeleton in response to different signaling pathways [236-238]. p190 is highly expressed in the developing CNS and has been shown to be the main substrate of Src kinase in the brain. Moreover, mice lacking p190 (*p190-/-*) displays defects in axon outgrowth, guidance, and fasciculation [77]. Finally, p190 has been shown to play role downstream of Sema4D/PlexinB1, where it negatively regulates RhoA activity to induce neurite outgrowth in PC12 cells [89]. Its implication in the netrin-1/DCC-induced regulation of RhoA activation, however, has not been addressed yet. To fill this gap, we decided to investigate the potential role of p190 downstream of Netrin-1/DCC signaling pathways, using cultured HEK293 cells and dissociated primary rat cortical neurons.

In this chapter, we have shown that p190 forms a complex with RasGAP and DCC in HEK293 overexpression system. We have also observed that overexpression of DCC in HEK293 cells is enough to increase tyrosine phosphorylation of p190. Moreover,

we have documented that the tyrosine 1418 residue (Y1418) and C-terminus end of DCC is important for its interaction with p190. In addition, our work has reported that along with its SH2 domains, the SH3 domain of RasGAP is also interacting with p190. Furthermore, we have observed that p190 is highly expressed in cortical neurons, where it has similar localization pattern as actin, DCC, and Rho-GTP in the growth cones of cortical neurons. Similar to the findings in the HEK293 cells, we have noted that p190 forms a complex with RasGAP and DCC in cortical neurons as well. Finally, we have shown that, in cortical neurons, netrin-1 stimulation leads to phosphorylation of p190 by Src family kinases, which increases its interaction with RasGAP and DCC. Altogether, we have reported that p190 is playing a role downstream of netrin-1/DCC-mediated axon outgrowth and guidance.

Materials and Methods

Antibodies and reagents

The following antibodies were purchased: anti-DCC A-20 (Santa Cruz Biotechnology); anti-phosphotyrosine (pY) 4G10, anti-tubulin and anti-DCC AF5 (Millipore); anti-DCC G92-13 and mouse anti-p190RhoGAP 610150 (BD Biosciences Pharmingen); antiphospho- p44/42 MAPK (Erk1/2) (Thr202/Tyr204) and rabbit anti-p190RhoGAP D8Q6C (Cell Signaling Technology); anti-FAK [pY861] and anti-FAK [pY397] (Life Technologies Novex); anti-p120RasGAP B4F8 (Abcam); anti-active RhoA (RhoA-GTP) (NewEast Biosciences); anti-mouse Alexa 488 and anti-goat Alexa 546 (Life Technologies molecular probe); TRITC-conjugated anti-mouse IgG (Sigma); horse radish peroxidase (HRP)-linked anti-mouse and anti-rabbit IgGs (GE Healthcare). The following reagents were used: recombinant chick netrin-1 was produced and purified as previously described [239, 240], recombinant mouse netrin-1 1109-N1 (R&D systems), PP2 (Calbiochem), dimethyl sulfoxide (DMSO) (Thermo Scientific), tetramethylrhodamine (TRITC)-conjugated phalloidin (Sigma).

Plasmids

The plasmids pRK5, pRK5-DCC, pRK5-DCCY1418F, pRK5-DCC-Y1361F, pRK5-DCC 1-1327, pRK5-DCC 1-1363, and pRK5-DCC 1-1421 were previously described [49, 231, 241]. The plasmids encoding GST and GST-human p120RasGAP (NSH2, C-SH2 and SH2-SH3-SH2) were provided by L. Larose. The plasmids encoding GFP-tagged p190RhoGAP and YFP-tagged p190RhoGAP-FF were generously provided by Dr. Keith Burridge and Dr. Tony Koleske.

Purification of GST fusion proteins

Production of GST and GST-p120RasGAP proteins was induced with 1.0 mM isopropylthiogalactopyranoside (IPTG) for 4 h at 37°C. Bacteria pellets were resuspended in the lysis buffer (Buffer A) (20mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer, pH 7.5; 120mM sodium chloride (NaCl); 2mM ethylenediaminetetraacetic acid **(**EDTA); 10% glycerol; and 1% Triton-X 100) supplemented with 20 mM sodium fluoride (NaF), 1 mM sodium orthovanadate (Na_3VO_4), 1 mM phenylmethylsulfonyl fluoride (PMSF), and 0.2 mg/ml lysozyme. Resuspended bacteria pellets were sonicated. Bacterial lysates obtained upon sonication were centrifuged at 3000 RPM, at 4ºC. Supernatant was kept and incubated with glutathione-agarose beads (Sigma) for 2h at 4ºC. The beads were washed three times with Buffer A and were incubated with the lysates from HEK293 cells expressing the protein(s) of interest. Purity and concentration were determined by Coomassie Blue-stained sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Cell culture and transfection

Cells were incubated at 37°C with 5% CO_2 in a humidified incubator. HEK293 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Wisent Bioproducts) supplemented with 10% fetal bovine serum (FBS) (Gibco®, Life Technologies) and antibiotics. The cells were transfected using polyethylenimine (PEI; PolySciences, PA) over the course of 4h. 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% Lglutamine (Invitrogen) as mentioned in [227]. Neurons were treated with the following reagents: recombinant chick

netrin-1 or recombinant mouse netrin-1 1109-N1 (R&D systems) (500 ng/ml), PP2 (Calbiochem) (10 nM) or DMSO (Thermo Scientific) as control.

GST pull down assays

HEK293 cells transfected with the plasmid of interest were lysed in Radioimmunoprecipitation assay (RIPA) buffer as (150 mM NaCl; 50mM HEPES, pH 7.5; 1% Triton-X 100; 10mM EDTA; 0.5% deoxycholate; 0.1% sodium dodecyl sulfate (SDS)) supplemented with 20 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, leupeptine, and aprotinin. Protein lysates were pre-cleared with 30µl of glutathione–agarose beads (50%w/v) (Sigma Aldrich) overnight at 4°C and incubated with GST or GST-p120RasGAP proteins coupled to glutathione–agarose beads for 3 h at 4°C. Beads were washed three times in ice-cold Ripa buffer and boiled in SDS sample buffer.

Immunoprecipitation

Rat cortical neurons and HEK293 cells were lysed using RIPA buffer supplemented with protease inhibitors. Protein lysates were centrifuged at 14000 RPM, at 4°C. The supernatants were incubated with 30µL protein-A sepharose beads (50% w/v) (Sigma) and the appropriate antibodies for 3h, at 4°C.

Immunoblotting and digital quantification

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 by ChemiDoc™ MP imaging system, using Clarity™ western ECL substrate (BioRad). All the analysis on the obtained images were carried out using Image Lab software.

Immunofluorescence

Cortical neurons (2DIV) were fixed 30 min with ice-cold 3.7% formaldehyde in 20% sucrose phosphate-buffered saline (PBS), permeabilized 5 min in 0.25% Triton X-100, and blocked 30 min with 0.25% bovine serum albumin (BSA). All the aforementioned steps were carried out at room temperature. Primary and secondary antibodies were incubated in 0.1% BSA. 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).

Statistical analysis

Statistical analysis was performed using two-tailed, unpaired Student's t-test. The data is presented as the mean ± the standard error of the mean (SEM).

Results

p190 and RasGAP are expressed in HEK293 cells.

To choose the optimal cell line to carry out our *in vitro* studies we examined the expression of p190 and RasGAP in three different cell lines— COS7, HeLA, and HEK293. Among the three cell lines we used, HEK293 showed the highest expression of both p190 and RasGAP (**Figure 2.1**). Therefore, we decided to use HEK293 cells for our in vitro studies.

p190 forms a complex with DCC in HEK293 cells.

To examine whether p190 is recruited to the DCC signaling complex, we immunoprecipitated p190 from the HEK293 protein lysates overexpressing full length DCC. We detected high levels of DCC in the same complex as p190 (**Figure 2.2A and B**). However, stimulation of HEK293 cells with netrin-1 for 10 minutes did not cause any further increase in the amount of co-immunoprecipitated DCC (**Figure 2.2A and B**). Increase in the extracellular signal-regulated kinase (ERK 1/2) phosphorylation is one of the readouts for netrin-1/DCC signaling, together with increased phosphorylation of focal adhesion kinase (FAK) [229, 242]. Indeed, western blot analysis showed phosphorylation of ERK1/2 in HEK293 cells, upon 10 minutes of netrin-1 stimulation (**Figure 2.2A**). This proves that the lack of further increase in the amount co-immunoprecipitated DCC is not due to failure of netrin-1 stimulation. (**Figure 2.2A**). Using a similar experimental approach, we attempted to examine tyrosine phosphorylation status of p190 upon netrin-1 stimulation in either regular HEK293 cell or in HEK293 cells expressing full length DCC. We immunoprecipitated p190. In line with the previous experiment, we observed that overexpression of DCC increases tyrosine phosphorylation of p190, which was not

further elevated upon 10 minutes of netrin-1 stimulation (**Figure 2.2C**). Moreover, the blot represented in **Figure 2.2C** shows a slight increase in the co-immunoprecipitated DCC amount in the condition with netrin-1 stimulation, however, this is due to the higher DCC expression level in the total cell lysate of the corresponding condition. Interestingly, we noted that netrin-1 stimulation leads to slight ERK1/2 activation in the absence of DCC, while DCC fails to induce ERK activation in the absence of netrin-1 stimulation (**Figure 2.2C**), which could be due to the presence of other netrin-1 receptors such as integrins. Furthermore, this experiment confirmed our finding that p190 and DCC are part of the same signaling complex (**Figure 2.2C**). Overall, these results show that p190 is a part of DCC signaling complex and that overexpression of DCC is sufficient to stimulate p190 tyrosine phosphorylation in the absence of netrin-1 stimulation.

The tyrosine 1418 (Y1418) residue of DCC is important for its interaction with p190

Having confirmed that p190 and DCC interact, we proceeded to dissect the mode of their interaction. To do so, we immunoprecipitated p190 from the HEK293 protein lysates expressing either full length or mutant DCC proteins (**Figure 2.3A**). We observed that while full length DCC and DCC 1-1421— truncation mutant that lacks the C-terminus extrimity— interact with p190 to similar extent, DCC Y1418F and DCC Y1361F point mutants show slightly lower interaction with p190 (**Figure 2.3B**). Finally, we observed very low interaction between p190 and DCC 1-1363— a truncation mutant that lacks the tyrosine 1418 (Y1418), but not the tyrosine 1361 (Y1361) residue (**Figure 2.3A and B**). Altogether, these data suggests that the tyrosine 1418 (Y1418) residue of DCC is crucial for its interaction with p190.

The SH3 domain of RasGAP interacts with both DCC and p190

To date, several studies have shown that the phosphorylated tyrosine residues Y1087 and Y1105 of p190 interact with two SH2 domains, the amino terminal (N)-SH2 and carboxy terminal (C)-SH2, of RasGAP (**Figure 2.4A**) [236-238]. Even though RasGAP also carries a SH3 domain, so far, it has not been investigated whether the SH3 domain of RasGAP interacts with p190. To address this question, we overexpressed GFP-tagged p190 in HEK293 cells and performed a GST-pulldown assay, using the following GST fusion proteins: N-SH2, C-SH2, SH3, and NT (domain of RasGAP that contains in tandem the SH2-SH3-SH2 domains) (**Figure 2.4B**). We used GST as a negative control. Using western blot analysis, we observed that the individual SH2 and SH3 domains bind p190 in comparable levels and, naturally, NT domain binds p190 in greater levels (**Figure 2.4C**). Together with the previously published data, this experiment reports that RasGAP interacts with p190 through its tandem SH2-SH3-SH2 motif.

p190 is highly expressed in rat cortical neurons.

Having confirmed its interaction with DCC in HEK293 cells, we wanted to study the possible involvement of p190 downstream of netrin-1/DCC in an endogenous system— in a primary culture of isolated rat embryonic cortical neurons. The cortical neurons are mechanically isolated and cultured from rat embryos at embryonic day 17-18 (E17-18) and all the experiments were performed at the day *in vitro* 2 (DIV 2)— a time point where axon specification and outgrowth take place (**Figure 2.5A**). First, we decided to verify the expression levels of p190, RasGAP, DCC, active ERK (phosphorylated ERK), and active FAK (pY861-FAK) at different DIVs. We noted that p190 is highly expressed in rat embryonic cortical neurons through the period of axon specification and outgrowth (**Figure 2.5B**). RasGAP (**Figure 2.5B**) and DCC (**data not**

shown) also revealed the same trend. The active ERK and FAK, however, showed increased expression towards the later DIVs (**Figure 2.5B**).

p190 localizes at the growth cone together with DCC and Rho-GTP.

Having confirmed high expression of p190 in cortical neurons, we wanted to investigate the sub-cellular localization of p190 in these neurons. Using immunofluorescence microscopy, we observed that p190 is present in all the compartments of cortical neurons— cell body, axon, and growth cone— with particularly high enrichment in the peri-nuclear region of the cell body and in the growth cone (**Figure 2.6A**). In addition, we also examined the possible co-localization of p190 with DCC and RhoA-GTP in the growth cone. We observed that indeed p190 localizes with DCC in the growth cone (**Figure 2.6B**). As to the localization of p190 and Rho-GTP, since the antibodies for both proteins have the same host— mouse—, we could not detect them in the same cortical neurons. However, examination of RhoA-GTP localization revealed that, similar to p190, it localizes to the growth cone (**Figure 2.6C**). Overall, these findings report that p190 is abundantly expressed in developing cortical neurons and it localizes together with DCC and RhoA-GTP in the growth cone, which suggests possible interplay between these proteins.

p190 forms a complex with RasGAP and DCC in rat cortical neurons.

After verifying the high levels of p190, RasGAP, and DCC expression in cortical neurons, we asked whether the three proteins form a complex in an endogenous system. To answer this question, we immunoprecipitated either p190 (**Figure 2.7A**) or RasGAP (**Figure 2.7B**) from DIV2 cortical neuron lysates. Using western blot analysis we observed that the two proteins co-immunoprecipitate with each other. Moreover, DCC

strongly co-immunoprecipitated with both p190 and RasGAP (**Figure 2.7A and B**). In general, this finding proves that p190, RasGAP, and DCC form a complex in rat cortical neurons— in an endogenous system.

Netrin-1 stimulation induces tyrosine phosphorylation of p190 in rat cortical neurons.

It has previously been shown that phosphorylation of p190 on tyrosine residues 1087 and 1105 mediates its interaction with RasGAP, which in turn targets p190 to plasma membrane [236-238]. Moreover, p190 has been shown to be phosphorylated in the CNS [77]. Considering this information, we set out to confirm whether netrin-1 stimulation leads to a change in the phosphorylation state of p190 in cortical neurons. We immunoprecipitated p190 from cortical neurons that were either left unstimulated or netrin -1-stimulated for 5 or 10 minutes. Using anti-phospho-tyrosine specific antibodies, we observed that netrin-1 leads to a 3-fold elevation in the tyrosine phosphorylation state of p190 (**Figure 2.8A and B**).

Src family kinases are responsible for netrin-1/DCC-mediated tyrosine phosphorylation of p190 in cortical neurons.

Our next aim was to identify the kinase that mediates netrin-1-dependent p190 phosphorylation. Brouns et al. have shown that p190 is the principal Src kinase substrate in the brain [77]. Furthermore, together with others, our group have reported that the Src family kinases have been implicated in netrin-1/DCC-mediated axon outgrowth and attraction [229-231, 243, 244]. Therefore, we decided to examine whether Src family kinases are responsible for the netrin-1-dependent tyrosine phosphorylation of p190. We

incubated cortical neurons either with PP2— a selective inhibitor of the Src family kinases [245, 246]— , or DMSO as control. Then, we either stimulated the neurons with netrin-1 for 10 minutes or left them unstimulated. Immunoprecipitation of p190 from protein lysates and immunoblotting with anti-phospho-tyrosine specific antibodies revealed that cortical neurons incubated with DMSO show significant increase in the tyrosine phosphorylation state of p190 upon netrin-1 stimulation (**Figure 2.9A and B).** The elevation in the tyrosine phosphorylation of p190 was accompanied by a significant increase in its interaction with RasGAP and DCC (**data not presented**). In PP2-treated cortical neurons, on the other hand, netrin-1-dependent increase in the tyrosine phosphorylation state of p190 was abolished (**Figure 2.9A and B)**, along with increased amount of co-immunoprecipitated RasGAP and DCC (**data not presented**). The PP2 treatment also abolished the netrin-1-mediated ERK and FAK phosphorylation (**Figure 2.9A**). Altogether, this set of experiments demonstrates that netrin-1 stimulation induces tyrosine phosphorylation of p190 by the Src family of kinases and strongly suggests a yet-to-be-determined role for p190 in the netrin-1/DCC signaling pathway.

Discussion

In this chapter, we have investigated the role of p190 in the context of cortical development downstream of netrin-1/DCC signaling. To carry out our investigation we have used two different systems: 1) HEK293 cells since they already express p190 and RasGAP in readily detectable levels and offer optimal conditions to perform *in vitro* experiments 2) Rat cortical neurons as they offer an environment where the role of p190 in axon guidance and outgrowth can be studied as the part of endogenous machinery.

Our work in the cortical neurons showed that p190, RasGAP, and DCC are highly expressed in the cortical neurons around the time point when axon specification and outgrowth is taking place [247]. However, we observed a gradual increase in active FAK and ERK levels towards the later DIVs. This finding can be explained with the fact that apart from their role in the neuronal migration and axon outgrowth, both FAK and ERK, play role in regulation of neuronal plasticity and survival— processes that are unique to mature neurons rather than developing neurons [229, 242-244, 248-253].

Furthermore, we also observed that p190 is localized in all the compartments of cortical neurons with particularly high expression in the peri-nuclear region of the cell body and in the growth cone. Of special interest is our finding that p190 localizes in the growth cone together with DCC and Rho-GTP, which suggests that these molecules can be part of the same signaling pathways.

Next, we demonstrated that p190 forms a complex with RasGAP and DCC in cortical neurons. We also showed that netrin-1 stimulation leads to tyrosine phosphorylation of p190, which augments its interactions with RasGAP and DCC. Our work reported that the phosphorylation is mediated by Src family kinases since the

increase in both p190 phosphorylation and interaction with RasGAP and DCC is abolished upon treatment of cortical neurons with Src family kinase-specific inhibitor, PP2. This is in agreement with the previous published literature, which demonstrate involvement of Src family kinases in netrin-1/DCC signaling pathway [229-231, 243]. We also noted that PP2 treatment abolishes netrin-1-dependent ERK and FAK phosphorylation. This finding demonstrates that Src family kinases are targeting more than one component in netrin-1/DCC pathway.

Our work with HEK293 cells demonstrated that p190, RasGAP and DCC form a complex. However, in case of HEK293 cells, we found out that even in the absence of netrin-1 stimulation and consequent ERK activation, overexpression of DCC is sufficient to create strong interaction between DCC and p190. Netrin-1 stimulation leads to dimerization and hence activation of DCC. Considering the fact that DCC is a transmembrane receptor molecule, its overexpression might lead to self-dimerization and subsequent activation, which may explain why netrin-1 stimulation does not further potentiate p190/DCC interaction. Also, while interpreting this piece of data, one has to keep in mind that we have worked with endogenous levels of p190, but overexpressed levels of DCC, which may lead to an imbalance in stoichiometry of the complex formation between the two proteins. In another word, overexpression of DCC beyond the physiological levels may exhaust available p190 pool, upon which netrin-1 stimulation could have further effect.

In addition, we reported that DCC point mutants DCC Y1361F and DCC Y1418F show slightly lower binding to p190 in comparison to DCC WT. However, DCC truncation mutant DCC 1-1363, which lack Y1418, but still has Y1361 showed rather

strong decrease in its binding to p190. Together these data suggest that Y1418, rather than Y1361, is critical for p190-DCC interaction. This is in line with the previous finding that phosphorylation of Y1418 is crucial for netrin-1/DCC-mediated axon outgrowth and attraction [227-232, 234, 235, 244].

Few previously published work has shown that individual SH2 domains of RasGAP interact with two tyrosine residues on p190— Y1087 and Y1105—, upon their phosphorylation [236, 237]. For instance, Bradley et al. have reported that interaction of p190 with SH2 domains of RasGAP causes a conformational change, which increases the affinity of latter's SH3 domain towards its targets [236]. However, to our knowledge, no one ever looked into whether SH3 domain of RasGAP interacts with p190 or not. Therefore, our work is the first to report a positive confirmation of the matter.

Although further research is indispensable, overall, our work suggest that p190 is involved downstream of netrin-1/DCC signaling pathway.

Figures for Chapter 2

Figure 2.1. HEK293 cells express both p190 and RasGAP in readily detectable levels.

50, 100, and 200 µg of protein lysates from HEK293, Cos7, and HeLa cells were resolved by SDS-PAGE and immunoblotted with antibodies against the indicated proteins.

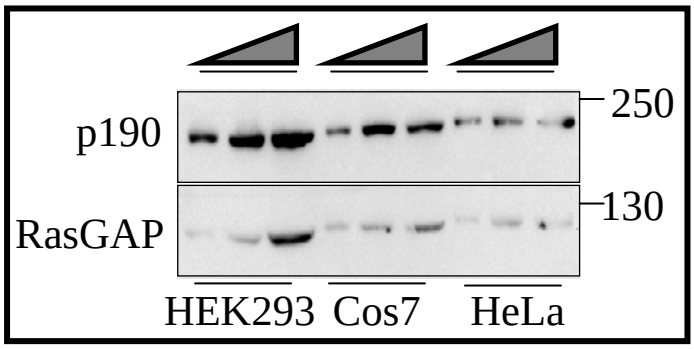
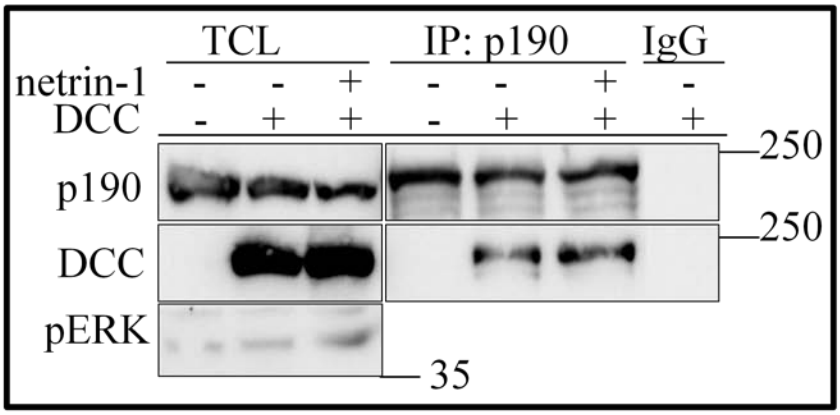

p190
RasGAP
250
130
HEK293
Cos7
HeLa

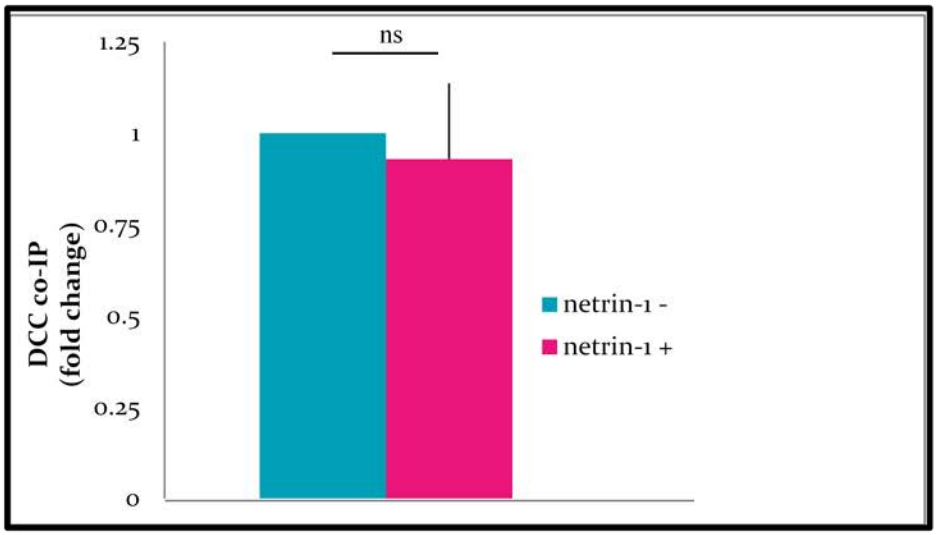
Figure 2.2. p190 forms a complex with RasGAP and DCC in HEK293 cells.

A. HEK293 cells were transfected with either empty pRK5 vector or pRK5 vector expressing DCC. The cells were then serum starved overnight and were either stimulated with netrin-1 (500ng/ml) or left unstimulated. p190 was immunoprecipitated from cell lysates with anti-p190 antibody or rabbit IgG as a control. Immunoprecipitated (IP) proteins and total cell lysates (TCL) were resolved by SDS-PAGE and immunoblotted with antibodies against the indicated proteins. B. The ratio of co-immunoprecipitated DCC to immunoprecipitated p190 is represented as the -fold change relative to the unstimulated condition for at least three independent experiments (n=3). C. HEK293 cells were transfected with either empty pRK5 vector or pRK5 vector expressing DCC. The cells were then serum starved overnight and were either stimulated with netrin-1 (500ng/ml) or left unstimulated. p190 and RasGAP were immunoprecipitated from cell lysates respectively with anti-p190 and anti-RasGAP antibodies or rabbit and mouse IgG as a control. Immunoprecipitated proteins and total cell lysates (TCL) were resolved by SDS-PAGE and immunoblotted with antibodies against the indicated proteins. Unpaired Student's t-test; ns, not significant.

A.


TCL
IP: p190
IgG
netrin-1
DCC
p190
DCC
pERK
250
250
35

B.


ns
1.25
1
0.75
0.5
0.25
0
DCC co-IP
(fold change)
netrin-1 -
netrin-1 +

C.

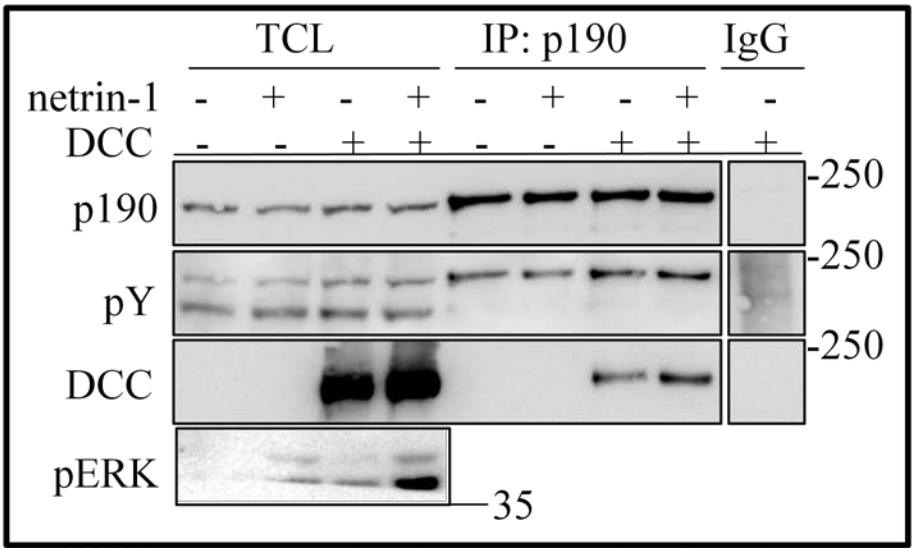
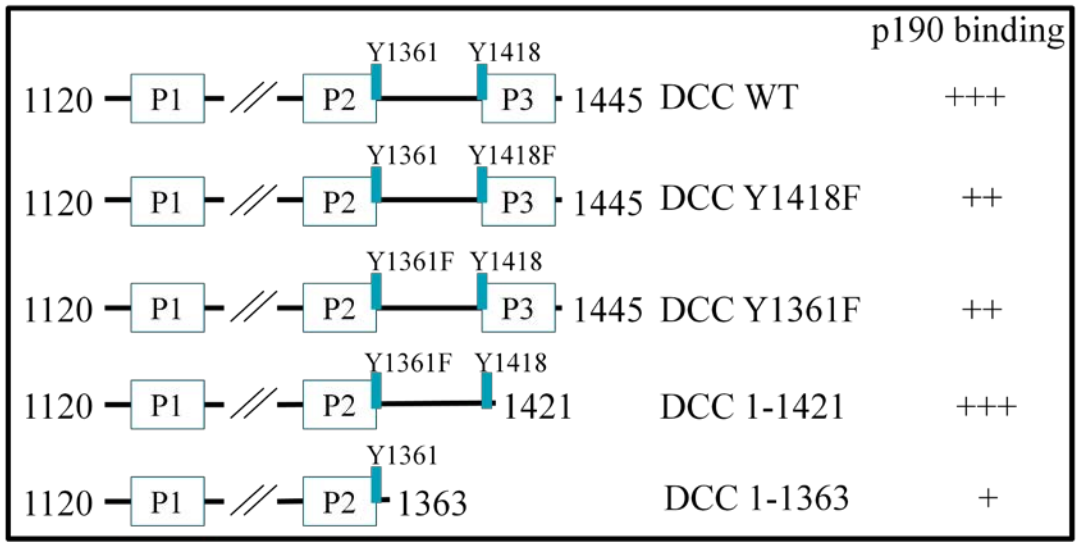

TCL
IP: p190
IgG
netrin-1
DCC
p190
pY
DCC
pERK
250
250
250
35

Figure 2.3. The tyrosine 1418 (Y1418) residue of DCC is important for its interaction with p190.

A. A schematic representation of the intracellular domain of wild type (WT) and mutant DCC proteins and their binding capacity to p190. B. HEK293 cells were transfected with either empty pRK5 vector or pRK5 vector expressing wild type or mutant DCC proteins. p190 was immunoprecipitated (IP) from cell lysates with anti-p190 antibody or rabbit IgG as a control. Immunoprecipitated proteins and total cell lysates (TCL) were resolved by SDS-PAGE and immunoblotted with antibodies against the indicated proteins.

A.


p190 binding
Y1361
Y1418
1120
P1
P2
P3
1445
DCC WT
+++
Y1361
Y1418F
1120
P1
P2
P3
1445
DCC Y1418F
++
Y1361F
Y1418
1120
P1
P2
P3
1445
DCC Y1361F
++
Y1361F
Y1418
1120
P1
P2
1421
DCC 1-1421
+++
Y1361
1120
P1
P2
1363
DCC 1-1363
+

B.

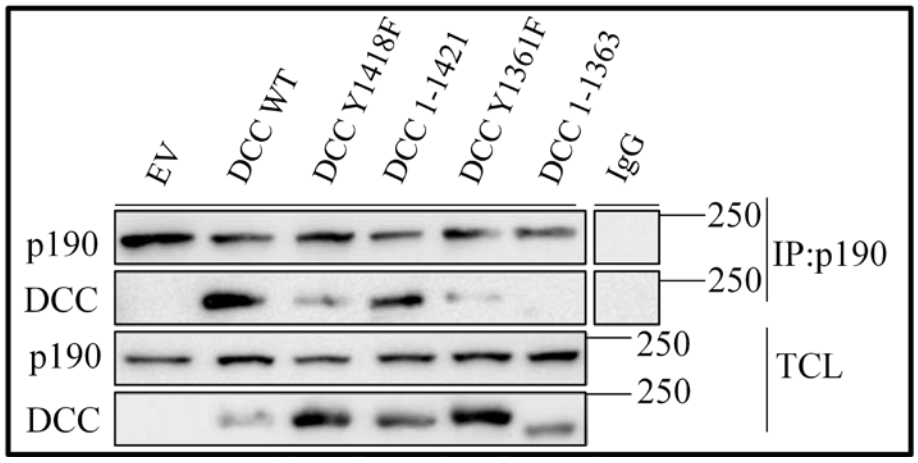

EV
DCC WT
DCC Y1418F
DCC 1-1421
DCC Y1361F
DCC 1-1363
IgG
p190
DCC
p190
DCC
250
250
250
250
IP:p190
TCL

Figure 2.4. The SH3 domain of RasGAP interacts with p190.

A. The schematic representations of RasGAP and p190. (P)- proline-rich region, (N-SH2)- amino-terminus Src-homology domain 2, (C-SH2)- carboxy-terminus Src-homology domain 2, (SH3)- Src-homology domain 3, (PH)- pleckstrin homology, (C2)- calcium-dependent phospholipid-binding, (FF)- two conserved phenylalanines, (Y1087 and Y1105)- tyrosine residues 1087 and 1105, and (GAP)- GTPase-activating domains. B. Schematic representations of GST constructs fused to different RasGAP domains. C. HEK293 cells were transfected with GFP-tagged p190. Proteins from cell lysates were pulled down using purified GST- C-SH2, -N-SH2, -SH3, -NT, or GST protein as a control. Associated proteins and total cell lysates (TCL) were resolved by SDS-PAGE and immunoblotted with antibody against GFP. GST fusion proteins were stained with Ponceau S.

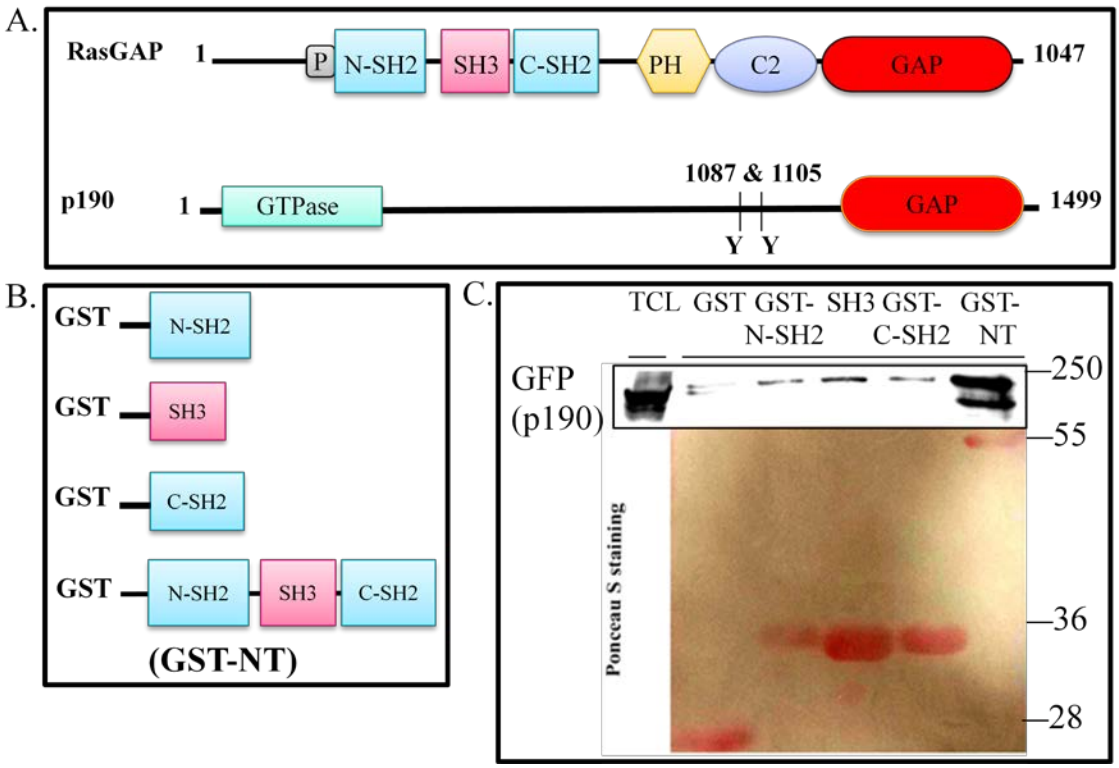
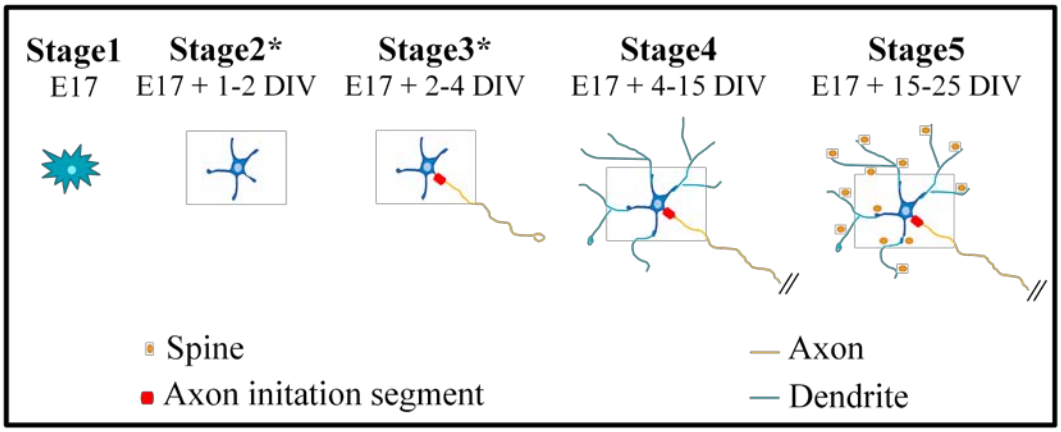

A.
RasGAP
1
P
N-SH2
SH3
C-SH2
PH
C2
GAP
1047
p190
1
GTPase
1087 & 1105
Y Y
GAP
1499
B.
GST
N-SH2
GST
SH3
GST
C-SH2
GST
N-SH2
SH3
C-SH2
(GST-NT)
C.
TCL
GST
GST-N-SH2
SH3
GST-C-SH2
GST-NT
GFP (p190)
Ponceau S staining
250
55
36
28

Figure 2.5. p190 is highly expressed in rat cortical neurons.

A. A schematic representation of the axon specification and neuronal growth based on Barnes et al., 2009 [247]. B. E18 rat cortical neurons were cultured for the indicated DIVs. Protein lysates from neurons were resolved by SDS-PAGE and immunoblotted with antibodies against the indicated proteins.

A.


Stage1
E17
Stage2*
E17 + 1-2 DIV
Stage3*
E17 + 2-4 DIV
Stage4
E17 + 4-15 DIV
Stage5
E17 + 15-25 DIV
Spine
Axon initation segment
Axon
Dendrite

B.

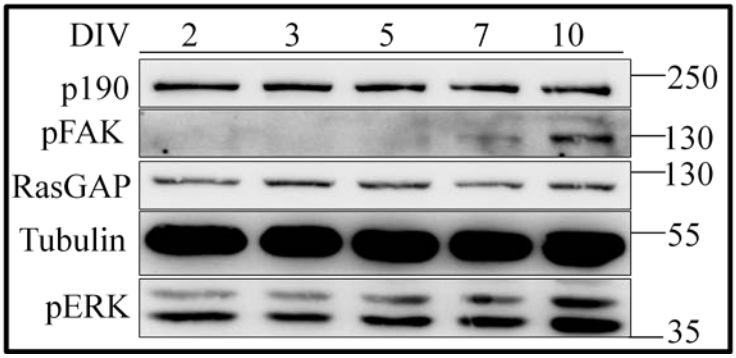
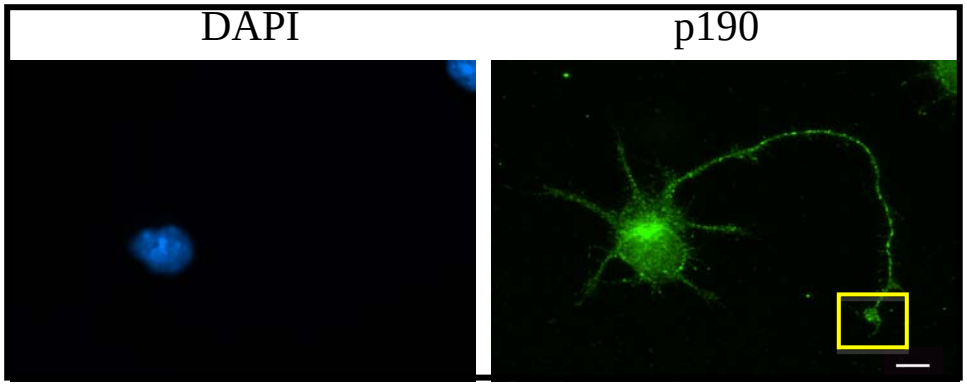

DIV
2
3
5
7
10
p190
pFAK
RasGAP
Tubulin
pERK
250
130
130
55
35

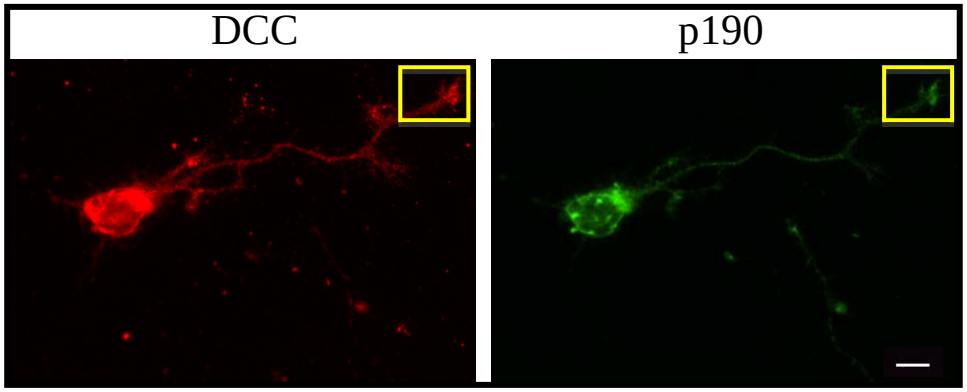
Figure 2.6. p190 localizes at the growth cone together with DCC and Rho-GTP.

A. E18 embryonic rat cortical neurons (2 DIVs) were immunostained with antibody against p190 and imaged by inverted fluorescence microscope. B. E18 embryonic rat cortical neurons (2 DIVs) were immunostained with antibodies against p190 and DCC, and imaged by inverted fluorescence microscope. C. E18 embryonic rat cortical neurons (2 DIVs) were immunostained with (TRITC)-conjugated phalloidin and antibody against RhoGTP, and imaged by inverted fluorescence microscope. The yellow rectangle represents the growth cone. The Scale bar= 25μm

A.



B.



C.

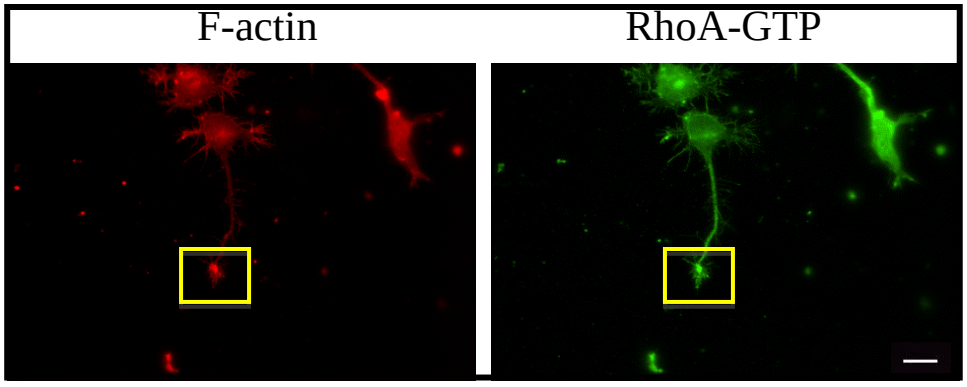
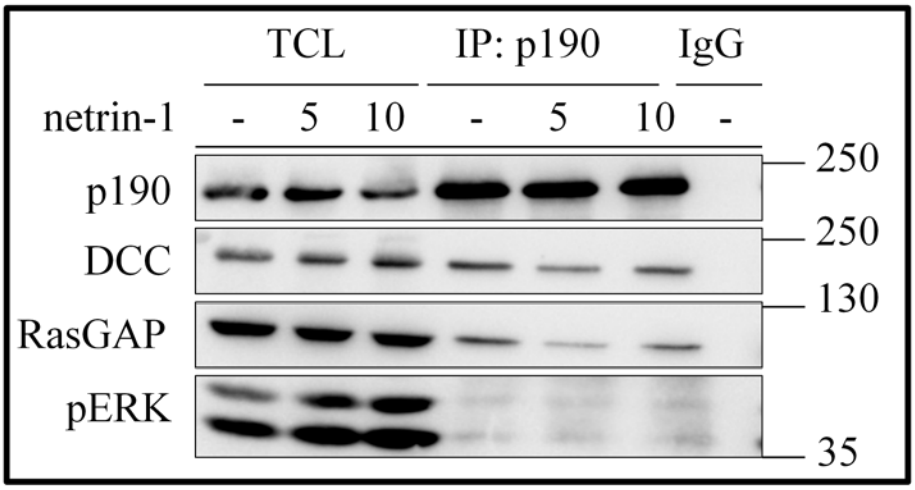


Figure 2.7. p190 forms a complex with RasGAP and DCC in rat cortical neurons.

E18 rat cortical neurons were either stimulated with netrin-1 (500 ng/ml) for the indicated times or left unstimulated after being cultured for 2 DIVs. A. p190 and B. RasGAP were immunoprecipitated (IP) from cell lysates respectively with anti-p190 and anti-RasGAP antibodies or rabbit and mouse IgG as a control. Immunoprecipitated proteins and total cell lysates (TCL) were resolved by SDS-PAGE and immunoblotted (IB) with antibodies against the indicated proteins.

A.


TCL
IP: p190
IgG
netrin-1
-
5
10
-
5
10
-
p190
DCC
RasGAP
pERK
250
250
130
35

B.

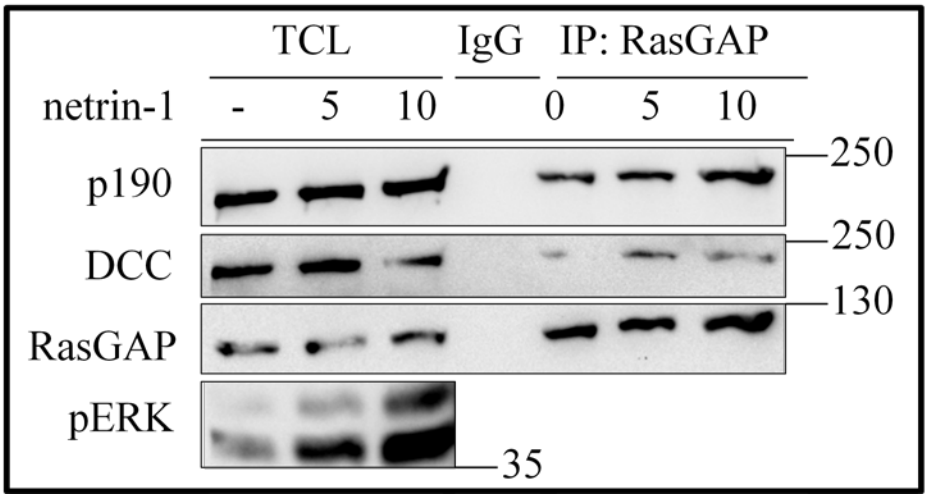
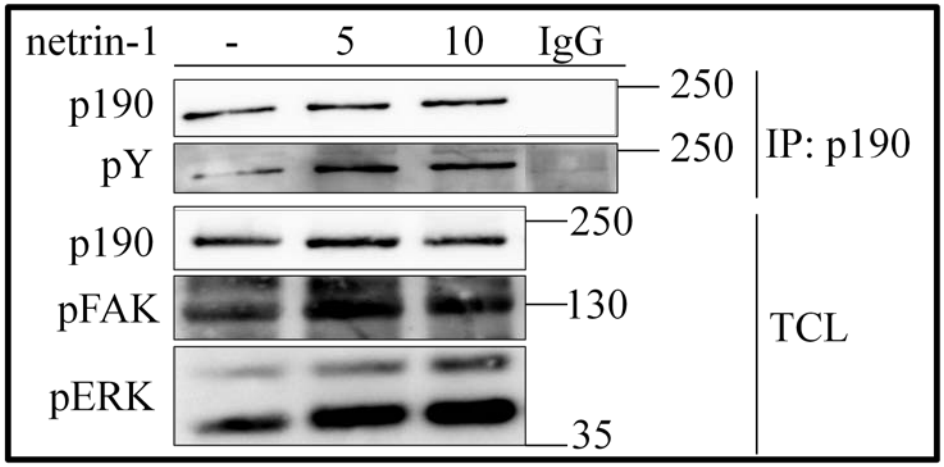

TCL
IgG
IP: RasGAP
netrin-1
-
5
10
0
5
10
p190
DCC
RasGAP
pERK
250
250
130
35

Figure 2.8. Netrin-1 stimulation induces tyrosine phosphorylation of p190 in rat cortical neurons.

A. E18 rat cortical neurons were either stimulated with netrin-1 (500 ng/ml) for the indicated times or left unstimulated after being cultured for 2 DIVs. p190 was immunoprecipitated (IP) from cell lysates with anti-p190 antibodies or rabbit IgG as a control. Immunoprecipitated proteins and total cell lysates (TCL) were resolved by SDS-PAGE and immunoblotted (IB) with antibodies against the indicated proteins. B. The ration of detected phosphor-tyrosine (pY) to immunoprecipitated p190 is represented as the -fold change relative to the unstimulated condition for at least three independent experiments. Unpaired Student's t-test; *, $p < 0.05$.

A.


netrin-1
-
5
10
IgG
p190
pY
p190
pFAK
pERK
250
250
250
130
35
IP: p190
TCL

B.

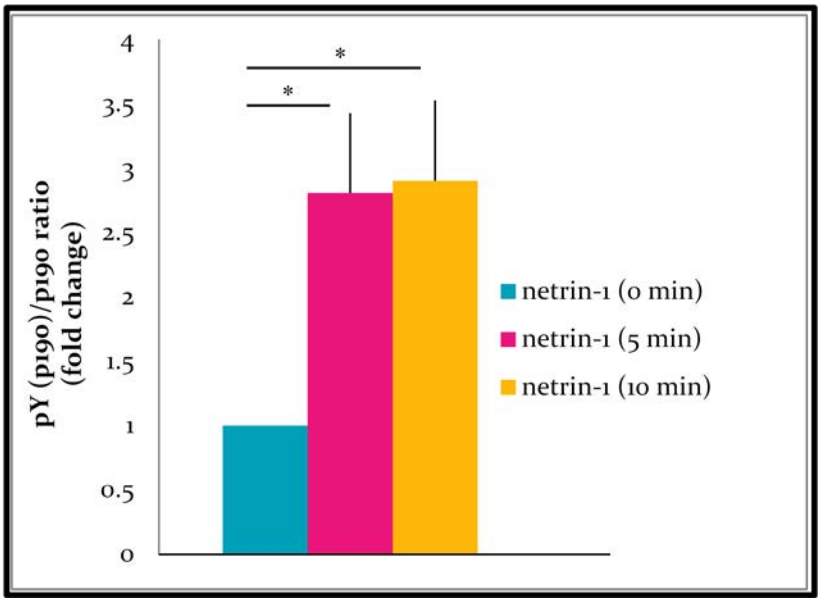
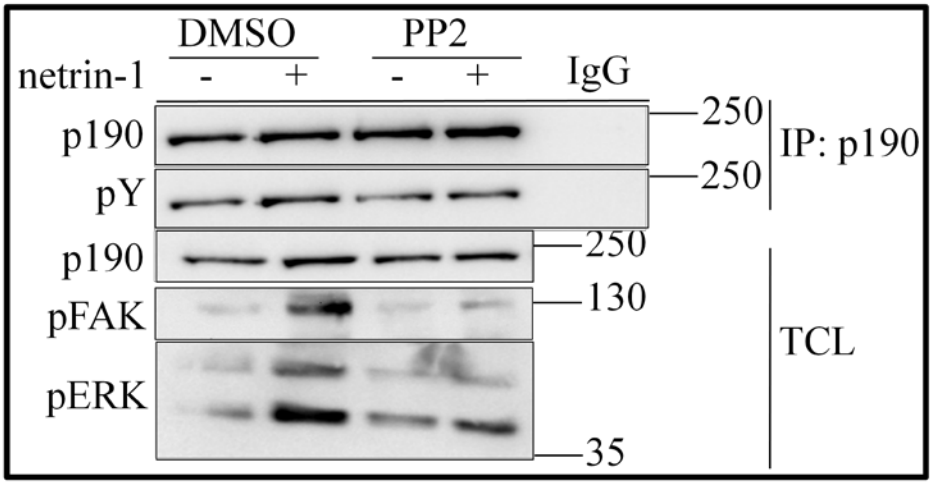

pY (p190)/p190 ratio
(fold change)
4
3.5
3
2.5
2
1.5
1
0.5
0
*
*
netrin-1 (0 min)
netrin-1 (5 min)
netrin-1 (10 min)

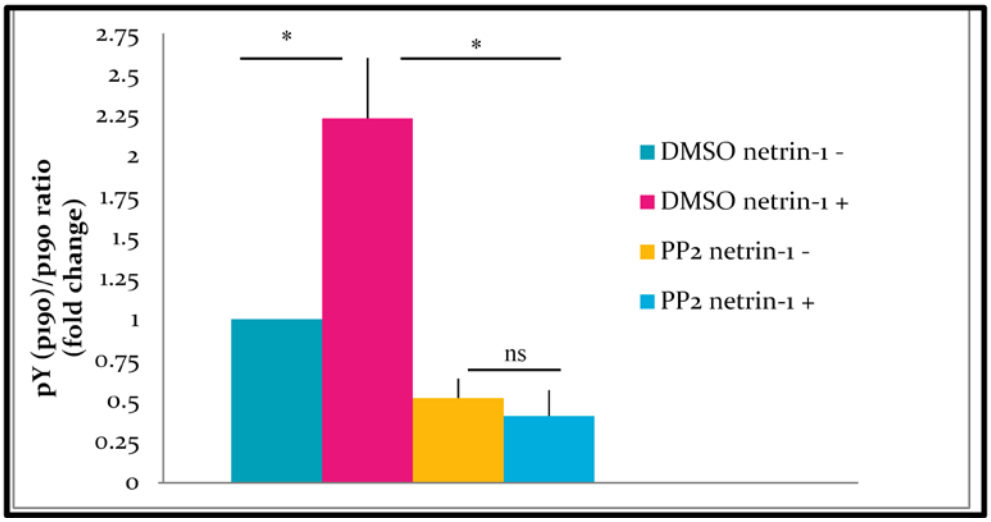
Figure 2.9. Src family kinases are responsible for netrin-1/DCC-mediated tyrosine phosphorylation of p190 in cortical neurons.

A. After being cultured for 2 DIVs, E18 rat cortical neurons were either stimulated with netrin-1 (500 ng/ml) or left unstimulated upon an hour of treatment with either DMSO or PP2. p190 was immunoprecipitated (IP) from cell lysates with anti-p190 antibodies or rabbit IgG as a control. Immunoprecipitated proteins and total cell lysates (TCL) were resolved by SDS-PAGE and immunoblotted (IB) with antibodies against the indicated proteins. B. The ration of detected phosphor-tyrosine (pY) to immunoprecipitated p190 is represented as the -fold change relative to the unstimulated-DMSO-treated condition for at least three independent experiments. Unpaired Student's t-test; *, $p < 0.05$; ns, not significant.

A.


DMSO
PP2
netrin-1
-
+
-
+
IgG
p190
pY
p190
pFAK
pERK
250
250
250
130
35
IP: p190
TCL

B.


pY (p190)/p190 ratio
(fold change)
2.75
2.5
2.25
2
1.75
1.5
1.25
1
0.75
0.5
0.25
0
*
*
ns
DMSO netrin-1 -
DMSO netrin-1 +
PP2 netrin-1 -
PP2 netrin-1 +

Chapter 3: SUMMARY AND FINAL CONLUSIONS

3.1 Summary of original findings

Until now, possible involvement of GAPs and GEFs in guidance cue signaling has not been extensively studied. In particular, to date, no GAP has been implicated in netrin/DCC signaling.

1. Our work suggests, for the first time, that a GAP protein— p190RhoGAP— is involved in the netrin-1/DCC signaling pathway. We have shown that p190 interact with the tandem SH2 and SH3 domains of RasGAP— a protein that our group had reported to interact with DCC and control Ras and ERK activity downstream of the netrin-1/DCC signaling pathway. We have also demonstrated that p190 co-immunoprecipitates with DCC in both HEK293 cells and cortical neurons. Furthermore, we have shown that netrin-1 stimulation leads to Src family kinases-mediated phosphorylation of p190, which leads to increased interaction with RasGAP and DCC. Altogether, these findings provide compelling evidences for us to suggest that p190 can be the first identified GAP to be involved in netrin-1/DCC mediated axon outgrowth and guidance. However, it must be noted that further experiments must be conducted to formulate more solid idea.

3.2 General conclusion

Although much more is known in the field of axon guidance since the discovery of the classic guidance molecules in the late 20^{th} century, our understanding of the field is far from complete. However, one thing is clear that all four classic guidance cue/receptor signaling pathways converge down to the Rho family of small GTPases [29, 31, 42, 43, 45, 53]. Decades of research has revealed that the actual scheme of the Rho GTPase involvement in axon guidance is not as simple as it is used to be thought—attractive cues activate Cdc42 and Rac1, while repulsive cues activate RhoA—, but rather is more

complex [31]. There is very limited number of the Rho GTPases and there is not any well-defined pattern in their regulation downstream of guidance cue/receptor complexes [31, 43]. This raises the question whether the key to modulate Rho GTPase activity lies in the tight control of their upstream regulators— GAPs and GEFs. Unfortunately, our current knowledge about the involvement of GAPs and GEFs in the context of guidance cue/receptor signaling is highly restricted to handful of studies [29-31, 42, 43, 76, 89, 235]. To this end, we have attempted to examine the possible role of p190 downstream of netrin-1/DCC-mediated axon outgrowth and guidance. p190 is highly expressed in the brain, where it is the principal Src kinase substrate [77]. Moreover, p190-mediated negative regulation of RhoA has been documented to be crucial for Sema4D/PlexinB1-induced neurite outgrowth in PC12 neuroblasts [89]. In this thesis, we report that p190 may also play role in netrin-1/DCC signaling pathway as it is phosphorylated upon netrin-1 stimulation for a short period. We also report that this netrin-1-induced stimulation of p190 strengthens its interaction with RasGAP and DCC. This might be the mechanism for targeting p190 to DCC signaling complex, where it can regulate RhoA activity.

A similar mechanism for the RasGAP protein has been published by our group. Antoine-Bertrand et al. have shown that netrin-1 stimulation leads to phosphorylation of RasGAP and its targeting to the plasma membrane, where it interacts with DCC and regulates the activities of ERK and Ras [235]. Furthermore, we have also observed that p190 is readily detectable in the growth cone of cortical neurons along with DCC, actin, and Rho GTP— an observation that hints at possible interplay between the mentioned proteins. Although far from decisive, our findings support the previously published data about p190 and

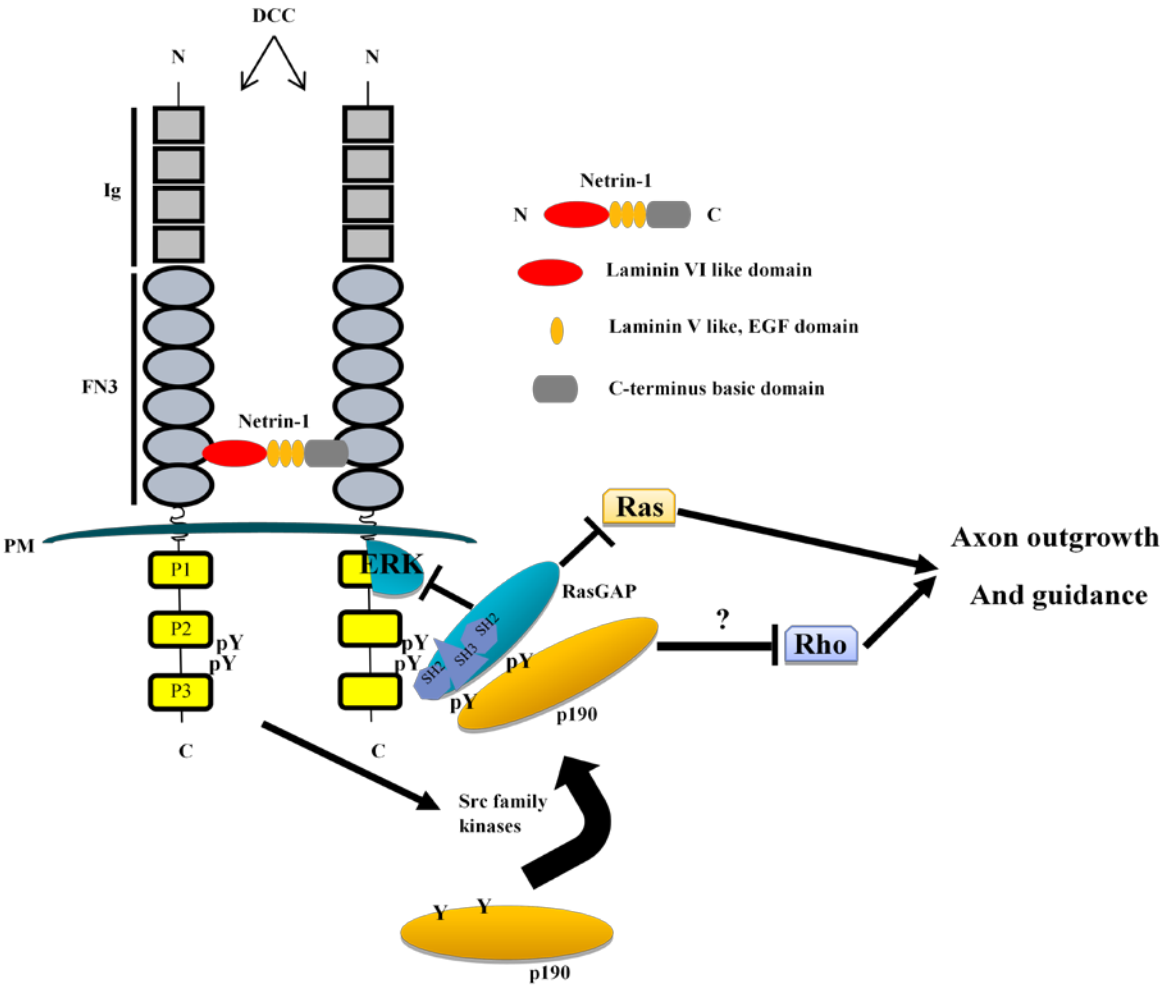
suggest that the Sema4D/PlexinB1 is not the only guidance pathway that utilizes p190. Our work is also in line with previous findings that mice lacking p190 show severe defects in axon outgrowth, guidance, and fasciculation [77]. Another promising aspect with our study is that, since p190 and RasGAP are well-established binding partners, it might cast some light on possible crosstalk between Ras-Rho signaling and regulation.

Overall, supporting the previous studies, concerning the role of p190 in outgrowth and guidance of the CNS axons, our work focuses on more specific pathway, the netrin-1/DCC signaling pathway, and is very encouraging for the future studies of our hypothesis. By performing further experiments in order to dissect and understand the mechanism of p190 involvement and its functional importance, we can obtain more in depth understanding of the subject, which can help us to visualize a broader image for the role of the Rho GTPases and their regulators in axon guidance.

Figure for Chapter 3

Figure 3.1. Preliminary model of the signaling mechanism of p190 downstream of the netrin-1/DCC signaling pathway.

Upon netrin-1 stimulation, p190 is phosphorylated by Src family kinases. The phosphorylation promotes the interaction of p190 with RasGAP, which in turn interacts with DCC and targets p190 to the plasma membrane, where it might negatively control RhoA activity in order to modulate axon outgrowth and guidance. (Ig)- immunoglobulin like domain, (FNIII)- fibronectin type III domain, (PM)- plasma membrane, (pY)- phosphor-tyrosine, and (EGF)- epidermal growth factor.


DCC
N
N
Ig
FN3
Netrin-1
PM
P1
P2
P3
pY
pY
C
C
ERK
Ras
RasGAP
SH2
SH3
SH2
pY
pY
pY
pY
p190
?
Rho
Axon outgrowth
And guidance
Src family
kinases
Y
Y
p190
Netrin-1
N
C
Laminin VI like domain
Laminin V like, EGF domain
C-terminus basic domain

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