Novel mechanisms regulating the netrin-1 receptor deleted in colorectal cancer (DCC) during cortical axon outgrowth

Philippe M. Duquette

Degree of Doctorate of Philosophy

Department of Anatomy & Cell Biology

McGill University

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“The whole problem with the world is that fools and fanatics are always so certain of themselves and wiser people so full of doubts”

Bertrand Russell

DEDICATION

I dedicate this thesis to the loving memory of my father, Richard Duquette - Papa, whose enthusiasm and interest in my research forced me to put the scientific jargon aside and practice my elevator pitch on a regular basis. I am forever grateful for the sacrifices you made so I wouldn't have to. My accomplishments are as much yours as they are mine!

« Prend le temps de le faire comme il faut! »

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LIST OF ABBREVIATIONS

4EBP	eIF-4E-binding protein 1
5HT3aR	Ionotropic serotonin receptor
A2b	adenosine 2B receptor
AC	anterior commissure
ACC	agenesis of the corpus callosum
ADAM	A Disintegrin and Metalloprotease
AIS	axon initial segment
BDNF	brain-derived neurotrophic factor
BMP	bone morphogenetic factor
C region	central region
Ca^{2+}	calcium
CAM	cell adhesion molecule
cAMP	cyclic adenosine monophosphate
CC	corpus callosum
CFuPN	corticofugal projection neuron
CGE	caudal ganglionic eminence
cGMP	cyclic guanosine monophosphate
CMM	congenital mirror movement
CNS	central nervous system
CPN	callosal projection neuron
CRIB	Cdc42/Rac1 interactive binding
Comm	Commissureless
CSMN	Corticospinal motor neuron
CST	Corticospinal tract

C-terminus	carboxy terminus
CThPN	corticothalamic projection neuron
CTPN	corticotectal projection neuron
DAG	diacylglycerol
DCC	Deleted in Colorectal Cancer
DLC1	deleted in liver cancer 1
DIV	days of culture in vitro
DSBS	developmental split brain
dSTORM	direct stochastic optical reconstruction microscopy
DSCAM	Down Syndrome Cell Adhesion Molecule
ECM	extracellular matrix
EGF	epidermal growth factor
Ena/VASP	enabled/ vasodilator-stimulated phosphoprotein
ER	endoplasmic reticulum
ERM	ezrin-radixin-moesin
ERK	extracellular signal-regulated kinase
ESC	embryonic stem cell
ExAC	Exome Aggregation Consortium
FAK	focal adhesion kinase
F-actin	filamentous actin
FGF	fibroblast growth factor
FN	fibronectin
GAP	GTPase-activating protein
GDNF	Glial Cell Line-Derived Neurotrophic Factor
GEF	guanine nucleotide exchange

	factor
GFP	green fluorescent protein
GPI	glycophosphatidylinositol
GST	glutathione S-transferase
ICD	intracellular domain
Ig	immunoglobulin
IGF-1	insulin-like growth factor 1
IN	interneuron
IPC	intermediate progenitor zone
IZ	intermediate zone
LRR	leucine-rich repeat
MAP	microtubule-associated proteins
MGE	medial ganglionic eminence
MMP	metalloprotease
mRNA	messenger RNA
MT	microtubule
NGF	nerve growth factor
Nrp	neuropilin
NT-3	neurotrophin-3
N-terminus	amino terminus
NTR	netrin-like domain
N-WASP	neural Wiskott-Aldrich syndrome protein
P region	peripheral region
PAK	p21 activated protein
PALM	photoactivated localization microscopy
PDGF	platelet-derived growth factor
PI3K	phosphatidylinositol 3-kinase
pERM	phosphorylated ERM proteins
PIP2	phosphatidylinositol 4,5-

	bisphosphate
PIP3	phosphatidylinositol 3,4,5-triphosphate
PKA	cAMP-dependent protein kinase
PKC	protein kinase C
PlTPα	phosphatidylinositol transfer protein-α
PN	projection neuron
PNS	peripheral nervous system
PTEN	phosphatase and tensin homolog deleted on chromosome 10
PV	parvalbumin
RA	retinoic acid
RAP1B	Ras-related protein 1B
RBD	Rho binding domain
RG	radial glia
RPTP	receptor protein tyrosine phosphatase
RGC	retinal ganglion cell
Rho	Ras homologous
ROCK	Rho kinase
ROI	region of interest
R-Ras	Related to Ras
RTK	Receptor tyrosine kinase
ScPN	subcerebral projection neuron
SEM	standard error of the mean
Sema3A	semaphorin 3A
SFK	Src family kinase
SH2	Src homology 2
SH3	Src homology 3

Shh	sonic hedgehog
SIM	Structured Illumination Microscopy
Smo	smoothened
SNP	small nucleotide polymorphism
SNARE	soluble N-ethylmaleimide-sensitive factor attachment protein receptor
SNAP25	synaptosomal-associated protein of 25 kDa
Sos	son of sevenless
SST	somatostatin
STED	Stimulated Emission Depletion
SVZ	subventricular zone
TAG-1	transient axonal glycoprotein-1
TGFβ	transforming growth factor β
TRIM	Tripartite Motif Containing
TRITC	tetramethyl rhodamine isothiocyanate
TUBB3	βIII-tubulin
T zone	transition zone
UNC	uncoordinated
VAMP	vesicle-associated membrane protein
VEGF	Vascular Endothelial Growth Factor
VZ	ventricular zone
WASP	Wiskott-Aldrich syndrome protein
WAVE	WASP-family verprolin-homologous protein
ZBP1	Zipcode binding protein 1

ABSTRACT

Neural circuits are the basis of the human experience. The long journey from birth to becoming a functional unit within the brain neural circuitry encompasses several processes which are the research interest of distinct neuroscientific sub-disciplines; from 1) neural induction, to 2) neurogenesis, followed by 3) neuronal polarization, 4) axon outgrowth and guidance, to 5) synaptogenesis, and finally, 6) pruning of excess connections. Following neurogenesis, newly-born neurons polarize and initiate axon outgrowth toward their future synaptic partner. Specialized embryonic structures, serving as intermediate targets throughout their journey, secrete guidance molecules that act as traffic lights, while adhesive molecules pave the road ahead. The molecular mechanisms mediating the cell's behavior in response to these signaling molecules is the subject of this thesis. Specifically, this thesis examines the molecular mechanisms involved in regulating the receptor for netrin-1, deleted in colorectal cancer (DCC), during cortical axon outgrowth and guidance.

DCC becomes phosphorylated in response to netrin-1 binding. The phosphorylation of the conserved tyrosine 1418 (Y1418) is required for netrin-1-mediated axon outgrowth and guidance in vertebrates. Here we identified and characterized a novel SH2-containing binding partner to the phosphorylated Y1418, p120RasGAP. We show that upon netrin-1 stimulation, p120RasGAP is recruited to the DCC signaling platform and is required for netrin-1-mediated axon outgrowth and guidance.

In addition, netrin-1 binding to DCC induces receptor proteolysis. Here we identify a novel protease, calpain, activated in response to netrin-1 which cleaves DCC. We show that activation of calpain downstream of netrin/DCC is ERK1/2-dependent and essential for normal axon outgrowth.

Finally, since the majority of DCC phosphorylation occurs on ser/thr residues, we wanted to identify and investigate their function. One of these sites, threonine 1210 (T1210), which was found to be mutated in humans, regulates DCC stability. The negative charge provided by the phosphorylation modification prevents cleavage and maintains DCC in its full-length form. Removing this negative charge renders DCC susceptible to proteolysis and unable to induce cortical axon outgrowth. Taken together, the novel findings presented in this thesis contribute to and improve our knowledge of the mechanisms regulating DCC and the netrin/DCC signaling pathway during cortical development. The human diseases involving DCC reach well beyond the nervous system and preclinical trials are underway targeting the interaction between netrin-1 and DCC. Therefore, increasing our understanding of the mechanisms regulating DCC in health will undoubtedly improve our therapeutic strategies in disease.

RÉSUMÉ

Les circuits neuronaux forment la base de l'expérience humaine. Le long périple de la naissance jusqu'à devenir une unité fonctionnelle de la circuiterie neuronale du cerveau comprend plusieurs processus faisant l'objet de sous-discipline distincte dans le domaine des neurosciences : de 1) l'induction neuronale à 2) la neurogénèse, suivi de 3) la polarisation neuronale, 4) la croissance et le guidage axonal, jusqu'à 5) la synaptogénèse, et finalement 6) l'élimination du 'surplus' de connections. Suivant la neurogénèse, les neurones nouvellement nés polarisent et initient la croissance axonale vers leurs futurs partenaires synaptiques. Des structures embryonnaires spécialisées jouant le rôle de cibles intermédiaires durant leurs voyages sécrètent des molécules de guidages agissants comme des feux de circulation alors que des molécules d'adhésions pavent la route devant. Les mécanismes moléculaires à la base des comportements cellulaires en réponse à ces molécules de guidage font l'objet de cette thèse. En particulier, cette thèse examine les mécanismes moléculaires impliqués dans la régulation du récepteur pour la molécule de guidage axonal, nétrine-1, deleted in colorectal cancer (DCC) lors de la croissance et le guidage axonal des neurones corticaux.

DCC devient phosphorylé suivant sa liaison avec nétrine-1. La phosphorylation de la tyrosine conservée 1418 (Y1418) est requise pour la croissance et le guidage axonale induit par la nétrine-1 chez les vertébrés. Nous avons identifié et caractérisé un nouveau partenaire de liaison se liant avec la tyrosine phosphorylée de DCC par son domaine SH2, p120RasGAP. Nous démontrons que suivant la stimulation avec la nétrine-1, p120RasGAP est recruté vers la plate-forme de signalisation DCC et est requis pour la croissance et le guidage axonal dirigé par nétrine-1.

De plus, la liaison de la nétrine-1 à DCC induit la protéolyse du récepteur. Nous identifions une nouvelle protéase activée en réponse à la nétrine-1, calpain, qui clive DCC. Nous démontrons également que l'activation de calpain en aval de nétrine-1/DCC est dépendant d'ERK1/2 et est essentiel à une croissance axonale optimale.

Finalement, puisque la majorité de la phosphorylation de DCC se fait sur les résidus Ser/Thr, nous identifions quatre nouveaux sites de phosphorylations. Un de ces sites, Thr 1210 (T1210), qui est également muté chez la population humaine, régule la stabilité de DCC. La charge négative procurée par la phosphorylation prévient le clivage et maintient DCC dans sa forme complète. Une fois cette charge négative éliminée, DCC est plus susceptible à la protéolyse et incapable d'induire la croissance axonale. Pris ensemble, ces nouvelles découvertes présentées dans cette thèse contribuent et améliorent nos connaissances des mécanismes régulant DCC et la voie de signalisation nétrine-1/DCC lors du développement cortical. Les maladies humaines impliquant DCC s'élargissent plus loin que le système nerveux et des essais précliniques sont en cours ciblant l'interaction entre la nétrine-1 et DCC. Donc en améliorant notre compréhension des mécanismes régulant DCC chez l'individu en santé nous allons sans doute pouvoir améliorer nos stratégies thérapeutiques dans le contexte de la maladie.

This is a traditional thesis with the results section split into 3 chapters

ORIGINAL SCHOLARSHIP AND CONTRIBUTIONS TO KNOWLEDGE

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

1. "p120RasGAP Contributes as a Scaffolding Protein to Regulate Netrin-1-Mediated Axon Outgrowth and Attraction in a GAP-dependent Manner", Antoine-Bertrand, J, Duquette P.M., Alchini R., Li X., Kennedy T.E., Fournier A.E., and Lamarche-Vane, N. The journal of biological chemistry 2016 Feb 26;291(9):4589-602.

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

2. "A role for the calcium-activated protease calpain in the regulation of netrin-1/DCC-mediated cortical axon outgrowth and guidance", Duquette PM & Lamarche-Vane N, Manuscript ready for submission

This study provides the first description of calpain as protease cleaving and regulating the netrin-1/DCC signaling pathway during cortical axon outgrowth. This study is also the first to identify, in mammalian neurons, the intracellular domain (ICD) of DCC which increases in response to netrin-1 stimulation. Future studies will help determine the function of this DCC-ICD.

3. "Investigating the role of DCC-Thr 1210 phosphorylation during cortical axon outgrowth", Duquette PM, Piché C., Luangrath V., and Lamarche-Vane N.

This study offers the first report of a phosphorylation site necessary to maintain DCC in its full length form. It also shows that phosphorylation of Thr 1210 is required for downstream signaling and to promote cortical axon outgrowth. Preliminary results indicate that this phosphorylation might be protecting DCC from calpain-mediated proteolysis.

Published manuscripts not presented in this thesis

4. "Rho GTPases in embryonic development", Duquette PM & Lamarche-Vane N. Small GTPases. (2014);5(2):8

   (peer-reviewed review article)

5. "CUX2 protein functions as an accessory factor in the repair of oxidative DNA damage" Pal R, Ramdzan ZM, Kaur S, Duquette PM, Marcotte R, Leduy L, Davoudi S, Lamarche-Vane N, Iulianella A, and Nepveu A. The journal of biological chemistry (2015); 290(37):22520-31.

6. "CdGAP/ARHGAP31, a Cdc42/Rac1 GTPase regulator, is critical for vascular development and VEGF-mediated angiogenesis", Caron C, DeGeer J, Fournier P, Duquette PM, Luangrath V, Ishii H, Karimzadeh F, Lamarche-Vane N, Royal I. Scientific reports (2016);6:27485

CONTRIBUTIONS OF AUTHORS

CHAPTER 2: p120RasGAP Contributes as a Scaffolding Protein to Regulate Netrin-1-Mediated Axon Outgrowth and Attraction in a GAP-dependent Manner

Judith Antoine-Bertrand: Designed, executed and analyzed all experiments. Assembled all the figures and co-wrote the manuscript.
Philippe M. Duquette: Contributed to the execution of experiments for Figures 2.4 and 2.3.
Ricardo Alchini: Contributed to the design, execution and analysis of the Dunn chamber assay in Figure 2.5.
Timothy E. Kennedy: Provided the recombinant netrin-1 VI-V in Figure 2.5.
Alyson E. Fournier: Contributed to the design and analysis of the Dunn chamber assay in Figure 2.5.
Nathalie Lamarche-Vane: Designed and analyzed all experiments. Co-wrote the manuscript.

CHAPTER 3: A role for the calcium-activated protease calpain in the regulation of netrin-1/DCC-mediated cortical axon outgrowth and guidance

Philippe M. Duquette: Designed, executed and analyzed all experiments. Assembled all the figures and co-wrote the manuscript.
Nathalie Lamarche-Vane: Designed and analyzed all experiments. Co-wrote the manuscript.

CHAPTER 4: Investigating the role of DCC-Thr 1210 phosphorylation during cortical axon Outgrowth

Philippe M. Duquette: Designed, executed and analyzed all experiments except for Figure 4.1. Assembled all the figures.
Vilayphone Luangrath: executed and analyzed experiments in Figure 4.1
Chantal Piché: Designed and executed part of the experiments in Figure 4.1

Nathalie Lamarche-Vane: Designed and analyzed all experiments.

Preface to Chapter 1

This chapter is a literature review of the mechanisms regulating neuronal polarization and axon specification, outgrowth, and guidance during development of the central nervous system with an emphasis on cortical development. The functions of the growth cone cytoskeleton downstream of classical guidance cues are presented. Finally, the strategies involved in the regulation of guidance receptors are presented with an introduction of a novel protease implicated in netrin/DCC signaling, calpain.

Chapter 1: Introduction and literature review

"Men ought to know that from the brain, and from the brain only, arise our pleasures, joys, laughter and jests, as well as our sorrows, pains, griefs and tears. Through it, in particular, we think, see, hear, and distinguish the ugly from the beautiful, the bad from the good, the pleasant from the unpleasant. . . ." ***Hippocrates, 5th Century, B.C.***[1]

The brain is arguably the most complex structure to have ever evolved. How does the brain activity at the cellular and molecular level create what Plato defined as the Good, the True, and the Beautiful over 2500 years ago?[2] These three characteristics which define the human experience and what we stride towards in our everyday lives can be reduced to, according to modern neuroscience, the sum of the activity of each neuron within our neural network. Each thought and experience is the result of a specific combination of neural activity that creates the world we are 'immersed' in. This world is not independent of us and each creates its own…[3-6] To understand the process by which the right kinds of neurons are produced and connected to each other is not only fundamental to understanding how our brain activity at the neural network level mediates our thoughts and behaviors but more importantly, how our world is created.

1.1 - The Nervous System

"As long as our brain is a mystery, the universe, the reflection of the structure of the brain will also be a mystery." ***Cajal***[7]

The nervous system is responsible for relaying information from both the external environment and the internal state of homeostasis within multicellular organisms. This information is then assimilated to coordinate a response (behavior).[8, 9] Our current understanding of the nervous system and its structural elements stems from two fundamental discoveries made by Camilo Golgi's silver stain and Santiago Ramón y Cajal's histology. The first picture of neurons was made by Golgi's pioneering method for staining neurons. Using Golgi's method, Cajal investigated the histology of the nervous system that led him to conclude

that the brain and spinal cord were composed of discrete elements, neurons, that may touch one another but do not fuse. This came to be known as the 'neuron doctrine.' The term 'neuron' was coined by Heinrich Wilhelm Gottfried von Waldeyer-Hartz to refer to the nerve cell. Earlier theories of brain organization postulated that the structural elements were fused in a net-like meshwork; this was before the electron microscope and the discovery of the plasma membrane that separates each cell.[10] Eventually the space (or "zones for the transmission of stimuli") between neurons was termed 'synapse' by Sir Charles Scott Sherrington and therefore supported Cajal's theory.[11] Cajal's work,[7] which is still regularly consulted by modern-day scientists remains one of the greatest contributions, not only to neuroscience, but to science in general.

1.1.1 - The Cell Types of the Nervous System

1.1.1.1 - Neurons

Neurons (nerve cells) are among the most polarized cell type in the body.[12, 13] With its dendritic tree receiving input signals from neighboring cells through a specialized junction called a synapse, it sends a signal (action potential) of its own through its axon, depending on the strength of the many signals it receives from its synaptic partners.[9, 14] Integration of all input signals is done at the level of the cell body where the decision to propagate or terminate the signal is made. Once the threshold has been reached to initiate an action potential down its axon, an electrical signal mediated by an influx of positive sodium and efflux of positive potassium ions and negative chloride ions is sent along its axon terminal where the electric signal is converted into a chemical one.[8, 9] Axon terminals are specialized regions at the tip of axons where specialized vesicles are ready to release their content into the synapse to bind specific receptors located on the postsynaptic side of the synapse.[8, 9, 14] These specialized regions of the axon make up the presynaptic side of the synapse and are enriched in several proteins involved in

synaptic transmission.[8, 9, 14] Conversely, during development, the axonal growth cone located at the tip of the extending axon is also a specialized structure serving a different function. The highly motile, fan-shaped, axonal tip explores and guides the axon along its journey to its synaptic partner.[15]

1.1.1.2 - Glial cells

Although glia cells have traditionally been labelled as supportive cells for the main stars of the nervous system, neurons; they make up 90% of the cellular population in the human brain.[16, 17] Some of their functions are to insulate axons to increase the speed and efficiency of the electrical signal by forming a myelin sheath around axons similar to the way electric wires are insulated by rubber.[8, 9] Oligodendrocytes are responsible for insulating axons of the CNS while Schwann cells accomplish this in the PNS.[8, 9, 18] The third glial cell-type are astrocytes. They serve several homeostatic functions such as the establishment of the blood-brain barrier and regulation of ion concentration.[8, 9, 18, 19] Astrocytes are also involved in signaling to neurons by secreting various growth factors and regulating calcium signaling between neurons. Radial glial cells located in the ventricular zone of the developing telencephalon (see below) also serve important functions during neurogenesis and neuron migration, which I will come back to below.[20-23] Oligodentrocytes, Schwann cells, and astrocytes together make up the macroglia while microglia are immune cells within the nervous system.[18]

1.1.2 - The Central Nervous System

The CNS consists of the spinal cord and the brain. Starting from the base of the skull, the spinal cord is the most caudal (posterior) part of the CNS and the most accessible. As we will see below the spinal cord has been widely used as a model to study axon guidance due to its

relative simplicity and accessibility for manipulations.[24] It receives sensory information that is relayed to the brain for integration where the response is initiated and sent back down the spinal cord to create a motor response.[8, 9] Alternatively, reflex movements bypass the brain to allow a faster response time when minimizing the response time is crucial for survival (ex: removing your hand from a burning stove). The brain can be divided into six major regions: 1) *medulla*, 2) *pons*, 3) *midbrain*, and 4) *diencephalon*, which together make up the brain stem.[8, 9] The brain stem is responsible for controlling basic functions such as blood pressure, respiration, and sleep. Evolutionary speaking, it is the oldest part of the brain and is thus commonly called the reptilian brain.[25] The fifth subdivision, the *cerebellum* ("small brain" in latin) located in the back of the head, contains more neurons than any other part of the brain and is responsible for the control and coordination of motor movements as well as being involved in various cognitive tasks.[26] Finally, the most evolutionary recent addition to the CNS is the *telencephalon*, which includes the two cerebral hemispheres. The cerebral cortex, located dorsally, is responsible for higher cognitive functions and is the central governor of behavior, where information is integrated.[8, 9, 23, 27]

1.2 - Cellular Diversity in the Cortex

1.2.1 - Arealization

It is in the cerebral cortex that we see the most diversity in neuronal subtypes.[18, 21, 28, 29] Although the cerebellum contains the greater number of neurons, its neuronal population is relatively homogenous in comparison. The divisions of the neocortex into distinct functional areas[30-32] were first introduced by the neuroanatomist Paul Broca, which we owe the Broca Area (or Broca's language area).[8, 9] The rodent neocortex can be divided into four distinct primary areas: 1) somatosensory, 2) auditory, 3) visual, and 4) motor (Figure 1.1).[21, 29] Each of the first

three primary areas is dedicated in processing information coming from the PNS while the motor cortex, as the name implies, is responsible for initiating the command for movements that will go down the spinal cord and to the motor neurons of the PNS, to stimulate the appropriate muscle. Outside of these well-defined primary areas, other regions are mostly labelled "associative" for lack of any yet identified area-specific functions. These areas tend to be activated under various different tasks and relay information between the well-defined areas.

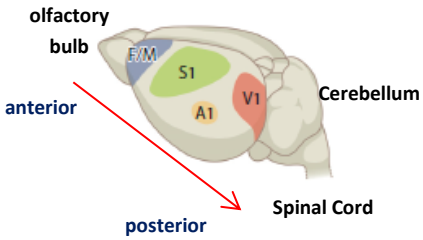


Figure 1.1: Four primary areas in the rodent neocortex

Schematic representation depicting the left hemisphere of the rodent neocortex. The olfactory bulb is the most anterior structure (upper left) with the spinal cord being the most posterior structure (bottom right). The four primary areas: 1) motor cortex (blue), 2) somatosensory (green), auditory (yellow), and visual (red). Adapted with permission from Annu. Rev. Cell Dev. Biol. 2015 (Lodato & Arlotta, 2015). Copyright © 2015 by Annual Reviews.

1.2.2 - Nomenclature

Within the various areas of the cortex, the cell bodies of neurons are organized into 6 layers where each layer shares common connectivity with other areas of the CNS.[18, 20, 21, 29, 33] The layers are organized such that as you move from the skull deeper into the brain you encounter different layers of neurons. The superficial layers (I/II) are closest to the skull while the deep layers are the furthest from the skull and closest to the inner ventricles. The great endeavour of classifying all cortical neurons started over a hundred years ago and remains incomplete. Employing the method developed by Camillo Golgi, Santiago Ramon y Cajal –

widely recognized as the Father of Neuroscience, was able to sketch some detailed drawings of the cerebral cortex, highlighting its great heterogeneity and cellular diversity.[7, 34-36] Today's nomenclature of cortical neurons is still largely based on connectivity which was first proposed by Cajal in 1909,[34] although molecular characterization through gene expression profile, is now adding some complexity in the identification of the various cortical neuron subtypes. Cortical neurons are now defined by a combination of characteristics such as molecular markers, morphology, and electrophysiological characteristics, in addition to their connectivity.[18, 21, 29, 37]

1.2.3 - Cortical neuron subtypes – projection neurons

There are two types of neurons in the cerebral cortex: projection neurons (PN) and interneurons (IN) organized in six distinct layers. Projection neurons are excitatory glutamatergic and connect the cerebral cortex to the rest of the CNS. They make up around 80% of the neuronal population in the cortex and make long range connections.[18, 21, 29] Although shorter than axons of the sciatic nerve that can measure more than one meter, PN typically have long axons that can measure tens of centimeters. They can be classified as either intracortical or corticofugal.[18, 21, 29, 37, 38] Intracortical neurons are predominantly located within superficial layers II/III. They are either "associative" and establish connections within the same hemisphere or "commissural" and cross the midline to establish connections with neuron in the other hemisphere. The axons of these commissural projection neurons project to the contralateral side through two commissures: the anterior commissure (AC) and the corpus callosum (CC).[18, 21, 29] The AC is the most evolutionary conserved commissure but is responsible for a small minority of crossing axons in rodents and humans. Most commissural neurons cross the midline through the corpus callosum and are thus termed callosal projection neurons (CPN) (Figure 1.2A & 1.4).[18, 21, 39] Corticofugal PNs (CFuPNs) are located in deep cortical layers (V-VI) and send their

axons to distal targets outside the cortex. They can be subdivided into corticothalamic (CThPNs) in layer VI (Figure 2B) and subcerebral (ScPNs) in layer V (Figure 1.2C & 1.4). ScPNs project mainly to the pons and other nuclei of the brainstem (corticopontine), to the superior colliculus (corticotectal), and the spinal cord (corticospinal motor neurons) (Figure 1.2C & 1.4). Together with this basic framework, each of these cortical neuron subtypes has been further characterized at the molecular level over the past 15 years. Thus, the nomenclature based on connectivity initially proposed by Cajal together with differences in morphology and electrophysiological properties is now being combined with the specific expression of distinct molecular markers. Thus, the molecular profiling of cortical neurons enabled the identification of novel class-specific signature genes and identified distinct cortical neuron subtypes (Figure 1.2).

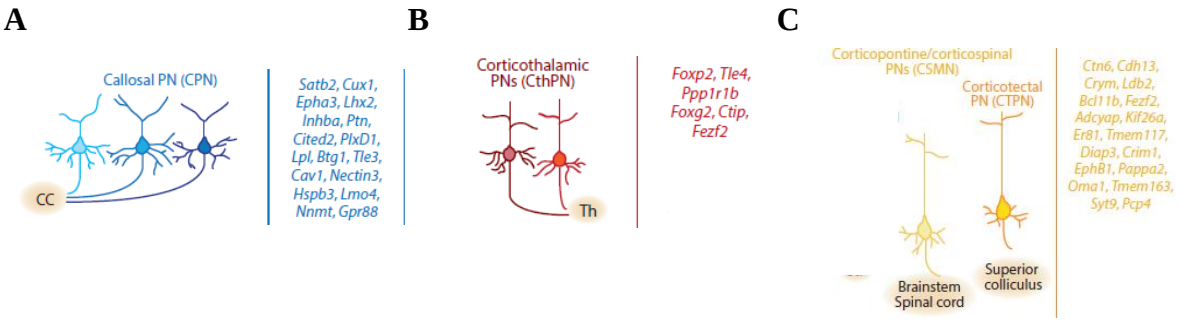


Figure 1.2: Cortical projection neuron subtypes

(A) Callosal projection neurons (CPN) located in superficial layers are commissural neurons of the neocortex establishing connections with neurons on the contralateral hemisphere through the corpus callosum (CC). A list of molecular markers characterizing this subtype is shown on the right. (B) Corticothalamic projection neurons (CThPN) are a subset of corticofugal neurons projecting to the thalamus and mainly located in layer VI. Some molecular markers identified are shown on the right (C) Subcerebral projection neurons (ScPNs) located in layer V send their axons outside the cerebral hemispheres to the superior colliculus (coritcotectal – CTPN), to the brain stem (corticopontine) and spinal cord (corticospinal motor neurons – CSMN). Some molecular markers shared by these neuronal subtypes are shown on the right. Adapted with permission from Annu. Rev. Cell Dev. Biol. 2015 (Lodato & Arlotta, 2015). Copyright © 2015 by Annual Reviews.

1.2.4. - Cortical neuron subtypes – interneurons

With only local connections, interneurons have not been characterized based on connectivity and no universally accepted classification exists. Some classification attempts have been made based on molecular markers, electrophysiological properties and dendritic tree morphologies.[18, 40] Briefly, three main subtypes have been identified based on non-overlapping expression of parvalbumin (PV+ - 40% of INs), somatostatin (SST+ - 30% of INs), and the ionotropic serotonin receptor (5HT3aR+ - 30% of INs)(Figure 1.3 & 1.4). Morphologically, PV+ are subdivided into basket and chandelier cells while SST+ INs are mainly Martinotti cells (Figure 1.3) . INs are heterogenous and further subdivisions based on combinations of other markers and electrophysiological/morphological differences have been used which are beyond the scope of this introduction.[41, 42]

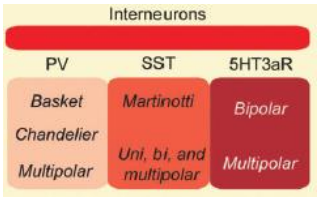


Figure 1.3: Cortical interneuron subtypes

Three main subtypes of interneurons based on non-overlapping expression of parvalbumin (PV), somatostatin (SST), and the ionotropic serotonin receptor (5HT3aR). Within these broad subtypes, they can be further categorized based on morphology (basket, chandelier, uni-, bi-, or multipolar). Adapted with permission from Annu. Rev. Cell Dev. Biol. 2015 (Lodato & Arlotta, 2015). Copyright © 2015 by Annual Reviews.

1.3 - From Neural Induction to Cortical Assembly

1.3.1 - Neural Induction

"Vertebrate embryonic cells will become nerve cells unless told otherwise" is the title of a 1997 Cell review that summarizes the default model of neural induction.[43, 44] It was the pioneer transplant experiments of Spemann and Mangold that led to the identification of the "organizer", an embryonic structure responsible for inducing neural tissue.[44-46] By grafting a piece of dorsal mesoderm taken from an early newt gastrula on the ventral side of a second embryo, Spemann and Mangold noticed the appearance of a well-organized second nervous system developing on the ventral side of the host embryo. The dorsal mesodermal structure was later termed the Spemann organizer for its neural induction properties. In rodents, the equivalent structure is the node. Following gastrulation, the embryo consists of three germ layers: endoderm, mesoderm, and ectoderm.[44, 47] Interestingly, it is the absence of instructive signals in the dorsal ectoderm that enables neural induction; the entry of multipotent ectodermal cells into the neural lineage. Among the molecules secreted from the node and responsible for neural induction in vivo are all inhibitors of BMP signaling: noggin, chordin, follistatin, and cerberus. Thus, it is the inhibition of BMP signaling in the dorsal ectoderm by the underlying mesoderm that is permissive to the development of the nervous system. Furthermore, in vivo ectodermal cells or embryonic stem cells (ESCs) cultured in vitro acquire a forebrain-specific neural fate in the absence of any factors added to the culture media. After neural induction, the dorsal ectoderm (i.e. neuroectoderm – neural plate) consists of a layer of neuroepithelial cells that will roll into a tube to form the neural tube, a process called "neurulation". The anterior part of this tube will become the forebrain in the absence of any signal while the acquisition of a more posterior identity (i.e. midbrain, hindbrain, and spinal cord), need additional factors such as Wnt, FGF8, and Retinoic acid (RA).[44] Being the dorsal part of the forebrain, the cerebral cortex is one

of a few structures that emerge from ESCs in the absence of any inductive signals. However, additional signals are required within the forebrain to fine-tune the differentiation into cortical neurons rather than other ventral structures of the forebrain such as the striatum. Throughout the anterior-posterior axis of the CNS, sonic hedgehog (Shh) is secreted ventrally to form a gradient along the dorso-ventral (DV) axis.[48] Depending on the location along this axis, progenitor cells will be exposed to different concentrations of Shh and will respond by activating the expression of different transcription factors that will lead to the expression of subtype-specific genes. Thus, Shh secreted from the ventral plate acts as a morphogen that competes with other morphogens secreted from dorsal structures (i.e. BMPs, Wnts).[48, 49] Inhibition of Shh signaling is therefore required to induce cells into the cortical lineage (i.e. dorsal identity). Taken together, the default model of neural induction tells us that ESCs are destined to become a forebrain but don't due to signals instructing them to become other structures. Therefore to become a cortical neuron, no "instructions" are necessary; it's the ESC's destiny.

1.3.2 - Cortical Assembly

Once the neural tube has closed, neurepithelial cells begin to differentiate into radial glia (RG) that will establish the ventrical zone (VZ). Radial glia acts as stem cells and can either divide asymetrically into neurons or intermediate progenitor cells (IPCs) that will migrate and establish a secondary proliferative region, the subventricular zone (SVZ) (Figure 1.4).[18, 20, 22, 23, 28] Each RG division self-renews to maintain the RG population while most IPCs divide into two postmitotic neurons that will migrate along RG into the cortical plate. As mentioned above, RG are involved in the migration of newly-born neurons. By spanning the entire thickness of the cortex, RG are used as scaffold by newly-born neurons to migrate into the cortical plate, their final destination where they will terminally differentiate. Neurons migrate in an inside-out

fashion such that early-born neurons populate deeper cortical layers while late-born neurons migrate past them to populate progressively superficial layers (Figure 1.4). From an evolutionary perspective, the SVZ, and its expansion in higher primates enabled the significant increase in the surface area (gyrification) and thickness of the cortex that expanded the relative number and diversity of cortical neurons.[50, 51] This led to the acquisition of higher cognitive functions. At the cellular level, human cortical neurons display distinct patterns of neuronal morphology due to an expanded SVZ leading to a longer relative proliferative period and neurogenic window. This is accompanied with a more prolonged period of neurite outgrowth, dendritic spine maturation, and synaptogenesis.[21, 52] Taken together, the prolonged developmental period at the cellular level correlates well with the increase weaning period and dependency of newly-born human babies on their parents. Interneurons, on the other hand, are born outside the developing cortex and have to migrate long distances tangentially from the medial and caudal ganglionic eminence (Figure 1.4).[53] At the molecular level several transcription factors have been identified as necessary for the generation of the cortical neuron subtypes mentioned above (Figure 1.2).[18, 20, 21, 23, 29, 38, 52] Briefly, Fezf2 is required for the generation of SCPN (subcerebral projection neurons) as no cortical neurons project to the brain stem and spinal cord in Fezf2-null mice.[54, 55] In these mice, Tbr1-positive CThPN expands into layer V, indicating a change in cortical neuron identity. Thus, Tbr1 acts in opposition to Fezf2, and its downstream target gene Ctip2, to specify CThPN by directly repressing Fezf2 expression.[55] Conversely, SATB2 is necessary for specification of CPN and repression of CFuPN.[56-59] In Satb2-deficient mice, almost no axons cross the corpus callosum even though the midline appears normal while CPN-specific genes are either completely lost or severely reduced.[56, 57, 59] Interestingly, it was shown that both receptors for the guidance cue netrin-1,

Unc5 and in particular *deleted in colorectal cancer* (DCC) - the focus of this thesis, are under the direct control of Satb2.[60] This highlights the importance of initiating the proper transcriptional program early to allow the expression of all necessary receptors necessary for normal axon outgrowth and guidance later in development. Finally, it is worth mentioning that CThPN and SCPN neurons share a common lineage, CFuPN, and its upstream master regulator, Sox5.[61] As such, their axons travel together for several days before their trajectory diverge and the expression of Tbr1 and CTIP2 becomes restricted to CThPN and SCPN, respectively. Taken together, signaling centers throughout the embryo secrete different factors (morphogens) that will establish different gradients.[49] Cells exposed to different concentrations of theses morphogens will respond by activating different combination of transcription factors that will activate subtype-specific genes necessary to differentiate into distinct cortical neural subtypes. Following neurogenesis, three key events must take place: 1) polarization through axon specification, followed by 2) axon outgrowth and guidance, and 3) branching and synaptogenesis

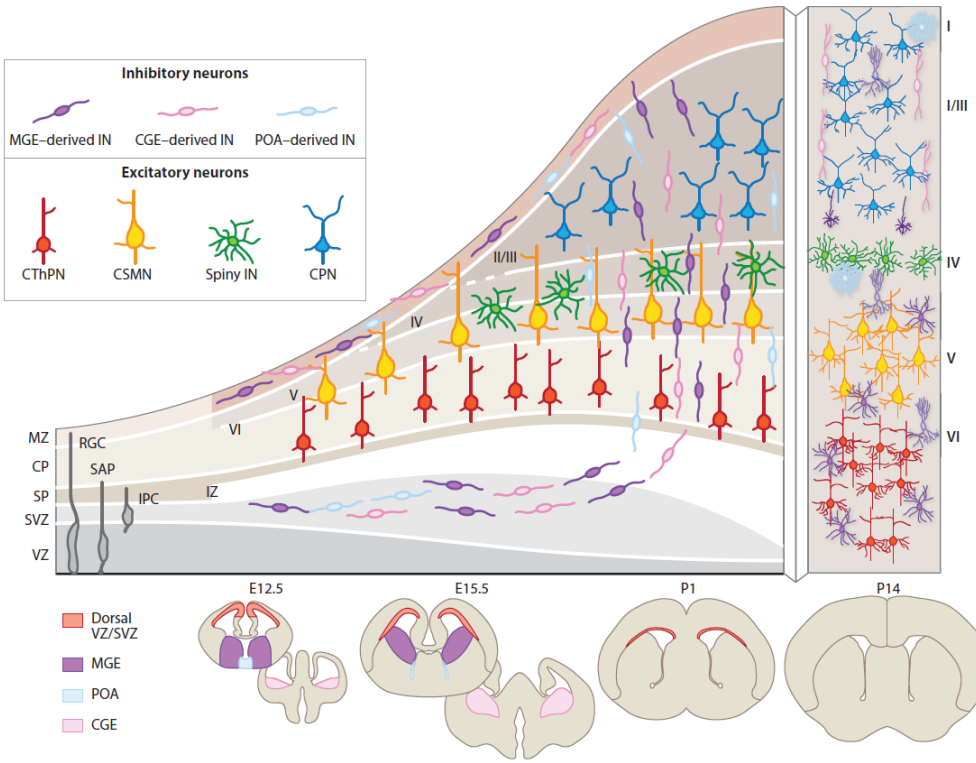


Figure 1.4: Development of the rodent neocortex

Neurogenesis in mice (E11-E19) and rats (E13-E21) occurs entirely before birth. Radial glial cells (RG) spanning the entire thickness of the cortex serve as scaffhold for newly-born neurons. Later in development, RGs generate intermediate progenitor cells (IPCs) giving rise to a secondary proliferative region, the subventricular zone (SVZ). At this stage, the intermediate zone (IZ) starts to enlarge, making room for axonal projections that have to pass through the IZ before exiting the cortex. Newly-born neurons migrate radially along RGs past earlier-born neurons to populate progressively superficial layers such that neurons located in superficial layers are born last. Interneurons, on the other hand, are born outside the cortex within the ganglionic eminences (GE, medial – MGE and caudal – CGE) and have to migrate tangentially. Adapted with permission from Annu. Rev. Cell Dev. Biol. 2015 (Lodato & Arlotta, 2015). Copyright © 2015 by Annual Reviews.

1.4 - Neuronal polarization, axon specification, outgrowth, and guidance

1.4.1 - Neuronal Polarization

The process of neuronal polarization has received so much attention over the last 30 years and tremendous progress has been made in characterizing the molecular mechanisms involved.[62] Axons are characterized by a uniform distribution of microtubules with the plus-end facing away from the cell body while dendrites have a mix orientation.[12, 63, 64] This orientation enables the polarized transport of various cargos involved in axon specification, differentiation, and maintenance.[65] This enables dynein, a minus-end-oriented microtubule motor protein, to bring cargos from the axon back to the cell body while transporting other cargos from the cell body into dendrites. In addition, axonal microtubules are mainly decorated by Tau and MAP1B whereas dendritic microtubules are labelled by MAP2a-c.[12, 63, 66] One of the pioneer papers that first proposed and characterized five distinct stages of neuronal polarization in vitro used cultured hippocampal neurons because of their homogeneity.[62, 67] At stage 1, freshly plated immature neurons display highly dynamic actin cytoskeletal structures such as lamellipodia and

protrusive filopodia (Figure 1.5). Stage 2 is characterized by the emergence of multiple immature neurites occurring within the first 2 days in culture (Figure 1.5). Accumulation of axon determinants (Ras, cAMP/PKA, PI3K/PIP3, and pLKB1)[68] in one neurite leads to a break in symmetry that triggers the first steps in axon specification at stage 3 (Figure 1.5). At this stage, one neurite starts to grow rapidly to become the axon (5-10 times faster than other neurites destined to become dendrites) in response to polarizing factors (ex: BDNF, laminin, TGF-β, Wnt5a, Sema3A, and Netrin-1). Actin waves, which migrate from the cell body into one neurite, transiently widen the neurite shaft to allow increased microtubule polymerization, lead to bursts of neurite extension into the future axon.[69] Orientation of organelles such as the Golgi, centrosomes, mitochondria, and endosomes correlates with the neurite that will become the axon.[12] This normally starts at the beginning of the second day in vitro (DIV2) for hippocampal/cortical neurons. Axon specification depends on actin destabilization and microtubule stabilization within the nascent axon, thus enabling rapid growth.[12, 63, 64, 66, 70] Stage 4 is characterized by rapid dendritic and axonal outgrowth (DIV4-15) (Figure 1.5) leading to terminally differentiated dendritic spines and the formation of the axon initial segment (AIS) at stage 5 (DIV15-25) (Figure 1.5). The AIS is critical for clustering voltage-dependent sodium channels that will initiate action potentials.[71, 72] This specialized structure is characterized by an actin meshwork composed of spectrins and ankyrin B and serving as a diffusion barrier preventing dendritic proteins from entering the axon.[71, 72] It is important to highlight the fact that these neurons which were dissected at embryonic day 14.5 were already polarized within the embryo and are thus repolarizing in vitro. It is thus possible that these neurons retain some of the molecular characteristics of polarized neurons and are not necessarily polarizing from scratch.[12] I will focus on molecular events occurring at stages 2-3 (neurite outgrowth, axon

specification and outgrowth) since the focus of the lab is to characterize the molecular mechanisms involved in the netrin-1/DCC signaling pathway during axon outgrowth at DIV2.

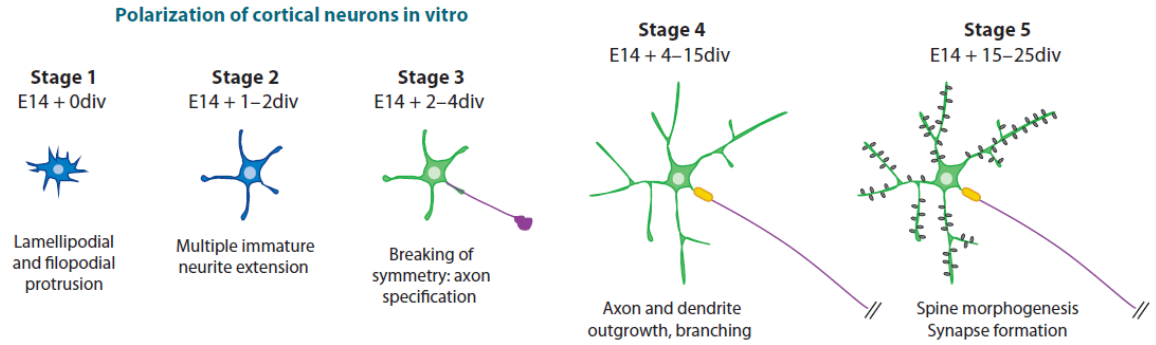


Figure 1.5: Polarization of cortical neurons in vitro

First described by Dotti et al., (1988) in hippocampal neurons, cortical neurons follow a specific sequence of morphological changes that characterizes polarization in dissociated cultures. Lamellipodia and protrusive filopodia characterizes stage 1, leading to the formation of multiple immature neurites at stage 2. The critical step when symmetry is broken and one neurite starts to grow rapidly to become the axon (axon specification- purple) happens during stage 3. Other neurites will become dendrites (green). Rapid outgrowth of both the axon and dendrites characterizes stage 4. Finally, terminal differentiation, which includes maturation of dendritic spines and the formation of the axon initial segment (yellow), defines stage 5. Adapted with permission from *Annual Review of Neuroscience* 2009 (Barnes & Polleux, 2009). Copyright © 2009 by Annual Reviews.

1.4.2 - Mechansims regulating neuronal polarization

1.4.2.1 – Super-resolution and TRIM46

Although the bulk of our understanding of how neurons establish their polarity have been discovered in vitro over the last 30 years, much of the in vivo studies of the last decade have confirmed these findings and provided support for the established molecular mechanisms.[73, 74] Intrinsic mechanisms exist that act in the absence of any external cues but the tissue environment play a dominant role in determining axon/dendrite polarity in vivo and will be discussed below.

Furthermore, new microscopy advances such as the different super-resolution techniques (i.e. *Structured Illumination Microscopy* (SIM), *Stimulated Emission Depletion* (STED), and *photo activated localization microscopy* (PALM)/ *direct stochastic optical reconstruction microscopy* (dSTORM) have allowed an even finer detailed description of the cytoskeletal structures involved at sub-200nm (limit imposed by light diffraction) resolution.[75] By combining live-cell imaging, electron microscopy, and dSTORM super-resolution, one study identified the microtubule-associated protein TRIM46 as the master regulator of microtubule orientation by forming cross-bridges between parallel microtubules in the nascent axon.[76] They show that TRIM46 is required for axon specification and neuronal polarity in vitro and in vivo.[76] They propose a model in which formation of uniform microtubules in the axonal shaft initiates neuronal polarity. Sub-diffraction microscopy techniques were also able to elucidate a novel actin structure within axons that went unnoticed by traditional light microscopy. In particular, circumferential rings of actin that wrap underneath the plasma membrane spaced at approximately 190 nm and connected by spectrin tetramers was revealed using STORM super-resolution microscopy.[77] Thus, a unique feature of the submembranous axonal cytoskeleton is its periodic, ladder-like structure. This periodic organization of actin was also seen in live neurons using STED and are present early in the most proximal axon segment and propagate as the axon grows.[77] Thus, similar to these examples, new super-resolution techniques will be able to characterize in finer details and identify novel structures within developing neurons to give us a better understanding of its molecular architecture.

1.4.2.2 -PI3K/PIP3

The activity of the lipid kinase phosphatidylinositol 3-kinase (PI3K) along with the accumulation of phosphatidylinositol (3,4,5)-triphosphate (PIP3) in the membrane of one neurite

seems to be necessary and sufficient for axon specification since blocking its activity prevents axon formation while expressing a constitutively active PI3K is enough to form multiple axons.[12, 68, 78-80] It was shown that local application of laminin leads to local accumulation of PIP3, suggesting that it lies upstream of PI3K, together with EGFR/Ras.[78] Finally, two PI3K-interacting proteins, Shootin1 and Singar1/2 were also shown to act upstream of PI3K during axon specification.[81, 82] In direct opposition to PI3K is the lipid and protein phosphatase PTEN (phosphatase and tensin homolog deleted on chromosome 10) that dephosphorylates PIP3 into PIP2.[79, 80] RNAi-mediated PTEN knockdown leads to the formation of multiple axons, similar to the expression of constitutively active PI3K.[79] Among the various proteins recruited to PIP3-enriched membrane regions through their PH domains, AKT appears to be crucial in axon specification.[80, 83] Following translocation to the membrane, AKT is activated by PKD1/2 and ILK-mediated phosphorylation. Expression of a constitutively active AKT is sufficient to induce multiple axons independent of PI3K, consistent with AKT acting downstream of PI3K.[83] GSK3β, a negative regulator of axon formation and major hub in the polarity pathways, undergoes inactivating phosphorylation by AKT/ILK.[84-86] This inactivation in turn relieves inhibition of several microtubule-binding proteins such as CRMP-2, APC, and microtubule-associated proteins (MAPs) such as MAP1b and Tau.[87-90] Importantly, GSK3β inhibition not only leads to multiple axons but can also convert dendrites into axons.[79] Of the downstream MAPs, CRMP-2 overexpression is sufficient to induce multiple axons while expression of a truncated form impairs axon formation.[90] Specifically, CRMP-2 links the Sra-1/WAVE1 complex to the plus-end orientated microtubule-based motor protein Kinesin 1, leading to actin dynamics at the tip of nascent axons.[91] Similar results were observed with the other GSK3β targets APC, MAP1b, and Tau. Thus, once this particular break is removed (GSK3β inhibition),

selective transport through various kinesins can take place and enrich axon-specific proteins within the nascent axon.[12, 68]

1.4.2.3 - BDNF → TrkB → PKA → LKB1 → SAD-A/B/MARK-2

The Par proteins play a critical role in epithelial cell polarity and the Par3/Par6/aPKC complex is a well-known mediator of PI3K-dependent axon formation.[92] Another Par protein, LKB1 (or Par4), also lies upstream in this polarity pathway. Similar to PI3K, phosphorylated LKB1 (pLKB1) is one of the few upstream proteins that has been labelled an axon determinant (i.e. necessary and sufficient).[68] Strong evidence by different groups demonstrated the requirement of LKB1 in axon specification during neuronal polarization in cultured cortical neurons as well as in vivo within the embryonic cortex.[93-97] Local exposure of an undifferentiated neurite to brain-derived neurotrophic factor (BDNF) promotes axon specification that is dependent on PKA-mediated LKB1 phosphorylation on serine 431.[95] Activated LKB1 in turn phosphorylates SAD-A/B and MARK-2 which reduces the affinity of these microtubule-binding proteins, thus destabilizing microtubules.[93, 96] Here we have a well characterized pathway from an extra-cellular cue (BDNF) activating a series of kinases (TrkB → PKA → LKB1 → SAD-A/B/MARK-2) converging on microtubules similar to the PI3K/AKT pathway. Interestingly, PI3K also gets activated by BDNF within the growth cone, suggesting parallel pathways being activated by the same extra-cellular cue.[74, 98] Several studies have observed defects in axon formation in vivo but almost all of them displayed migration defects.[68, 99-103] LKB1 knockdown or overexpression of a phospho-null mutant is one of a few examples of a defect in axon formation without the accompanied migration defect, highlighting the specific requirement of LKB1 in axon specification but not during radial migration into the cortical plate.[96] In addition, this suggests that although there is great overlap with the molecular

mechanisms involved, radial migration can still take place normally even when the neuron cannot grow an axon.

1.4.2.4 - Ras superfamily of small GTPases

Several members of the Ras- and Rho-family of small GTPases have been shown to regulate neuronal polarity.[12, 104] Small GTPases are molecular switches that are active when bound to GTP. With their relatively slow intrinsic GTPase activity they remain active until their GTPase activity is stimulated by interacting with GTPase activating proteins (GAPs).[105] Thus, GAPs act as negative regulators of small GTPase activity by promoting the GDP bound (inactive) state. In contrast, they are activated by the exchange of GDP for GTP which is regulated by guanine nucleotide exchange factors (GEFs). The Ras-related protein 1B (RAP1B) was shown to act downstream of PI3K during neuronal polarization since an active RAP1B form was able to rescue axonal loss following PI3K inhibition.[106] Furthermore, overexpression of RAP1B induces multiple axon-like neurites while RAP1B knock-down with RNAi causes a complete loss of axons; a phenotype that is partially rescued by expression of an active form of Cdc42.[106] Thus, the accumulation of Rap1B at the tip of prospective axons follows PI3K activation and contributes to the recruitment of several downstream molecules involved in axon specification such as Cdc42.

Ras activation is coupled to several cell surface receptors, thereby relaying extracellular information to several downstream effectors such as PI3K and the canonical MAPK pathway. [107] Several members of the Ras-family of small GTPases have been shown to regulate neuronal polarity including H-Ras, R-Ras, K-Ras, and N-Ras.[83, 106, 108, 109] Overexpression of these Ras members lead to production of multiple axons. The PI3K-mediated PIP3 enrichment induced by

Ras activation in turn leads to activation of Cdc42 and Rac1, two Rho family small GTPases well known for regulating the actin cytoskeleton.[104]

1.4.2.5 - Ras homologous (Rho) GTPases in neuronal polarization

Ras homologous (Rho) family proteins are less than 25kDa in size and are members of the Ras superfamily of small GTPases.[105] Among the 22 members of the Rho family of small GTPases, Cdc42, Rac1, and RhoA have been the most extensively characterized.[104, 105, 110] The great diversity of their regulators (82 GEFs, 67 GAPs, and 3 GDIs) highlights the diverse and tight regulation of these molecular switches in cell biology.[104, 105] Expression of a cdc42 mutant (Cdc42L28), which autonomously cycles between a GDP- and GTP-bound state, leads to multiple axons while neurons expressing a constitutively active Cdc42 fail to form any neurites.[106] Similarly, loss of Cdc42 leads to severe defects in axon specification at least partially due to increased levels of phosphorylated cofilin (inactive), a regulator of actin dynamics enriched in developing axons.[111] Taken together, this indicates that cycling between GDP- and GTP-bound state is essential to normal GTPase function and the requirement of both GAPs and GEFs are necessary to promote this cycling. Among the different GEFs involved in neuronal polarization, the Rac1-specific GEFs DOCK7, TIAM1, and STEF have been shown to be necessary to promote actin dynamics and microtubule stability.[112, 113] Lastly, the RhoA-specific GAP, p190RhoGAP, is inactivated by GSK3β phosphorylation.[114] As previously mentioned, GSK3β is inhibited during neuronal polarization by AKT phosphorylation downstream of PI3K.[79, 86] This GSK3β inactivation in turn leads to increased p190RhoGAP activity and consequently a reduction in active RhoA-GTP.[115] This highlights the negative role RhoA plays during axon outgrowth. Mice lacking either of the two p190RhoGAP (A and B) genes display axon outgrowth defects.[116] Taken together, Rho GTPase regulation is a crucial

intermediary step in cytoskeletal regulation allowing proper axon specification and outgrowth. They are regulated by several GAPs and GEFs which remain poorly characterized. Since only a handful of GTPase regulators (GEFs and GAPs) have been described in the context of neuronal polarization so far and the fine-tuning of GTPase activity is necessary for proper physiological functions (ex: axon defect in Cdc42-deficient and constitutively active-Cdc42-expressing neurons); it will be interesting to see future studies addressing the role of other GAPs and GEFs.

1.4.2.6 - In vivo

Unlike neuronal polarization in culture in a homogenous 2D environment containing only neurons, newly-born neurons are exposed to multiple signals and have to polarize and migrate along a specific trajectory. During cortical development, newly-generated projection neurons in the SVZ extend multiple neurites into the intermediate zone (IZ) similar to stage 2 cultured neurons (compare Figures 1.5 & 1.6).[12, 117] These multipolar cells can migrate laterally before extending a trailing process that will become the axon and a leading process that will become one of several dendrites. It is these bipolar cells that migrate toward the cortical plate to develop into mature neurons.[12, 117] This multipolar-to-bipolar morphology is a critical step in neuronal polarization in vivo and the formation of the trailing process (future axon) is the initial step, similar to cultured neurons (Figure 1.6). Several environmental cues have been shown to regulate this transition but a crucial step in regulating polarization in vivo is cell-cell interaction.[118] A 'Touch and Go' model in which multipolar cells extend and retract immature neurites in the IZ suggests that once a neurite 'touches' the pioneering axons from early-born neurons, the neurite is stabilized and extend rapidly (Go).[118] This 'Touch' mechanism is mediated by transient axonal glycoprotein-1 (TAG-1) expressed along pioneering axons while the 'Go' phase involves Lyn kinase and Rac1 activation.[118] Several environmental cues such as

neurotrophins (NT-3, BDNF),[95] Wnts,[119] insulin-like growth factor (IGF-1),[120] TGF-β,[121] Semaphorin,[122] Unc-6/netrin,[123, 124] and Reelin,[101] extracellular matrices, such as laminin,[125] and TAG-1[118] promote axon specification in vitro and in vivo. The most compelling evidence for the role of an extracellular cue in regulating neuronal polarization and axon specification is that of TGF-β.[121] TGF-β2 is highly enriched in the VZ and forms a gradient along the radial axis where projection neurons migrate. High TGF-β2 within the VZ/SVZ and exogenous TGF-β2 is sufficient to direct the rapid growth and differentiation of an axon while cortical neurons lacking the type II TGF- β receptor fail to form any axons in vitro and in vivo. This study also showed that these TGF- β-dependent events were mediated by site-specific phosphorylation of Par6.[121] Other than TGF- β, Semaphorin-3A secreted by superficial layers diffuses to form a gradient opposite to that of TGF-β.[122] Sema3A and its receptors neuropilin-1 and plexins have been shown to play an important role in keeping bipolar neurons polarized toward the cortical plate.[122] Another molecule forming a descending gradient, reelin, is secreted by Cajal-Retzius cells located in the marginal zone above the cortical plate and plays an important role in orienting multipolar cells towards the cortical plate.[101] This orientation is mediated by activation of Rap1, which maintains N-cadherin levels at the cell surface. In addition, ephrin guidance cues and their Eph receptors were shown to play a similar role in regulating lateral migration of multipolar cells before radial migration of bipolar cells take place. Another classical axon guidance cue shown to regulate neuronal polarity is UNC-6/Netrin. UNC-6/UNC-40(Netrin/DCC) were shown to be required to break symmetry of HSC neurons in *C. elegans* while a recent study examined the role of netrin and its receptor DCC during cortical polarization in mice.[124] Netrin/DCC signaling was shown to be involved in the multipolar-to-bipolar transition, thereby regulating both polarization and radial migration.[123] Specifically, Netrin/DCC-mediated Fyn

activation led to tyrosine phosphorylation and activation of the adaptor protein Dab1. The best-studied pathway regulating cortical migration is the Reelin-Dab1 signaling cascade and this highlights an alternative means to activate Dab1 and promote cortical neuron polarization and migration. Taken together, several cues have been identified in the regulation of neuronal polarity and axon specification in vitro and in vivo. The intracellular mechanisms involved in vivo largely recapitulated what had been characterized in vitro.[74, 117] A thorough description of all the players involved is well beyond the scope of this chapter and a brief selection of some well-defined pathways were mentioned here as examples. A common feature of these pathways is their convergence onto the microtubule and actin cytoskeleton. This convergence on the cytoskeleton drives the changes in cellular morphology necessary for neurons to polarize into their final and distinct polarized shape. In contrast to microtubules and the actin cytoskeleton, intermediate filaments (or neurofilaments) are relatively static and do not play any major role in the changes in morphology involved during neuronal polarization.

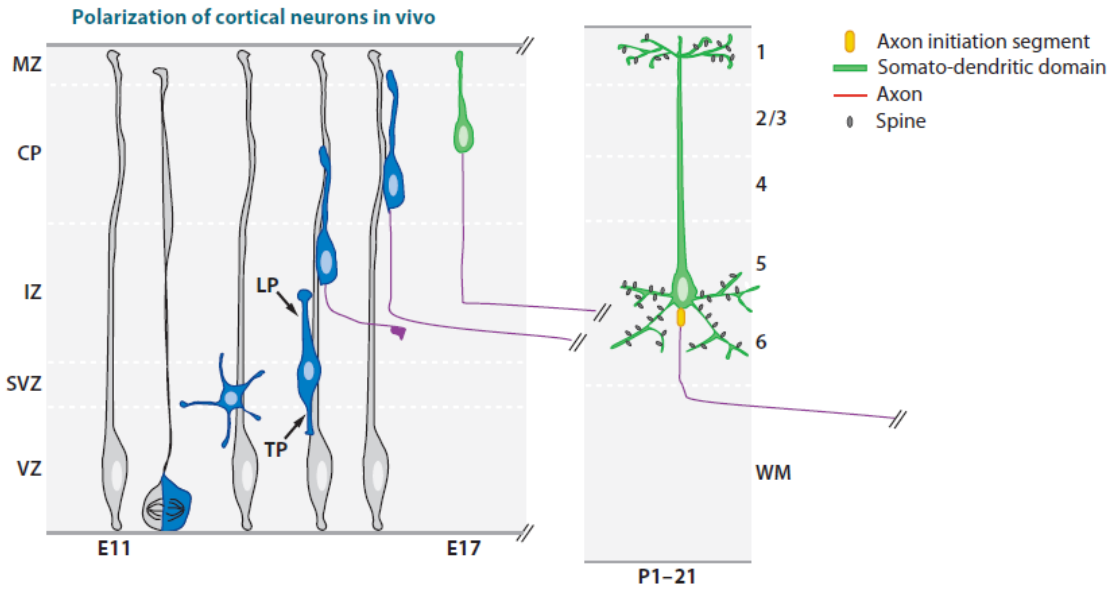


Figure 1.6: Polarization of cortical neurons in vivo

Newly-born neurons (blue) go through an initial multipolar morphology consisting of multiple neurites, similar to polarization in vitro. The critical multipolar-to-bipolar transition is defined by the emergence of a major process in the radial direction (leading process – LP). Radial translocation along radial glia (grey) leaves behind a trailing process (TP). This TP will become the axon (purple) that will grow into the intermediate zone (IZ) before exiting the cortex. Following the neurogenic period (E11~E17), dendrites (green) and axons terminally differentiate which include formation of the axon initial segment (yellow) and dendritic spine maturation. Adapted with permission from *Annual Review of Neuroscience* 2009 (Barnes & Polleux, 2009). Copyright © 2009 by Annual Reviews.

1.4.3 - Axon Outgrowth

"It appears therefore, that the innumerable processes and intercellular connections offered by the adult nervous system can be interpreted as the morphologic expression of the infinite routes traced in space by currents of inducting or positive chemotropic substances during the entire developmental period. Thus the total arborization of a neuron represents the graphic history of conflicts suffered during its embryonic life."[10] ***Cajal, 1890***

Neural circuits are the basis of neural function in health and disease. Investigating the molecular mechanisms involved in neural circuit assembly will help us understand neurodevelopmental disorders such as intellectual disability, autism spectrum disorders, and schizophrenia, enabling earlier diagnosis and develop a better therapeutic strategy. The molecular insights from these studies could also be exploited to improve therapies in the context of neural circuit disintegration such as Parkinson's and Alzheimer's disease. It is a long journey for the newly-born neuron in the VZ/SVZ to being part of the complex cortical neural circuitry and it all starts with neurite initiation.[126] Polymerized actin structures such as filopodia and lamellipodia form nascent growth cones that protrude away from the cell body. There are two basic requirements for neurite formation: 1) dynamic peripheral actin network and 2) bundling of microtubules arrays.[126] These dynamic cytoskeletal polymers provide the tips of growing neurites the ability to change shape and drive neurite outgrowth. As we have seen in the

previous section, intrinsic mechanisms are sufficient to initiate axon specification in one neurite.[73] In the 3D environment of the embryo, combinations of signaling molecules help dictate the orientation of axon initiation before radial migration takes place (Figure 1.6). Much of the communication that occurs between environmental guidance cues and the cytoskeleton takes place at the growing tip of the axon, the neuronal growth cone (Figure 1.7). Once axon specification has begun, the growth cone takes charge of where the growing axon will end its journey to form functional synapses.

1.4.3.1 - The Growth Cone

"The growth cone may be regarded as a sort of club or battering ram, endowed with exquisite chemical activity, with rapid amoeboid movements, and with certain impulsive force, thanks to which it is able to proceed forward and overcome obstacles met in its way, forcing cellular interstices until it arrives at its destination."[7] ***Cajal, 1890***

The growth cone is a terminal expansion of growing axonal and dendritic tips.[15] Under light microscopy, it can be divided into two distinct compartments: the peripheral and central regions (P and C regions)[15] (Figure 1.7). Using high resolution imaging, a third region termed 'transition zone' (or T zone) was identified with distinct functions.[127] As the name states it is the region between the periphery and central regions. The P region is a broad and relatively flat area characterized by dynamic filopodia and lamellipodia (Figure 1.7).[15, 128] The C region, connected to the axon shaft, is enriched in organelles such as mitochondria and exocytotic vesicles (Figure 1.7).[15, 128, 129] The majority of dense microtubule bundles terminate within the C region but single microtubules do venture out in the P region and interact with the actin cytoskeleton and cell signaling components to promote growth cone motility.[15, 130, 131] Taken together, the growth cone can be considered as the vehicle driving axonal outgrowth while reading the road signs (guidance cues) along the way with its cell surface receptors (Figures 1.7 & 1.8). The receptor-ligand interaction then triggers intracellular signaling cascades that will converge onto the

cytoskeleton to drive the proper turning response. Axon outgrowth is driven by the growth cone which goes through 3 different stages: 1) protrusion, 2) engorgement, and 3) consolidation.[15, 128, 132] Protrusion is the addition of new membrane at the edge of the growth cone, driven by filamentous actin (F-actin) structures such as filopodia and lamellipodia. Microtubule-driven transport of organelles and vesicles into the peripheral region characterizes the engorgement stage. Finally, consolidation results from the contraction and stabilization of the central region into the cylindrical-shape axon shaft. Consolidation involves bidirectional movement of organelles and vesicles. Even when the axon is not growing, a constant combination of F-actin treadmilling and retrograde flow provides the growth cone motor the means to respond quickly to any changes in the environment.[15, 128, 129] Retrograde flow is driven both by contractility of the motor protein myosin II in the T zone and polymerization in the P region. Overall the dynamic nature of the growth cone is driven by cyclical polymerization and depolymerisation of actin filaments. Thus, a thorough understanding of the molecular mechanisms involved in regulating the growth cone actin cytoskeleton is essential to understanding axon outgrowth and guidance.

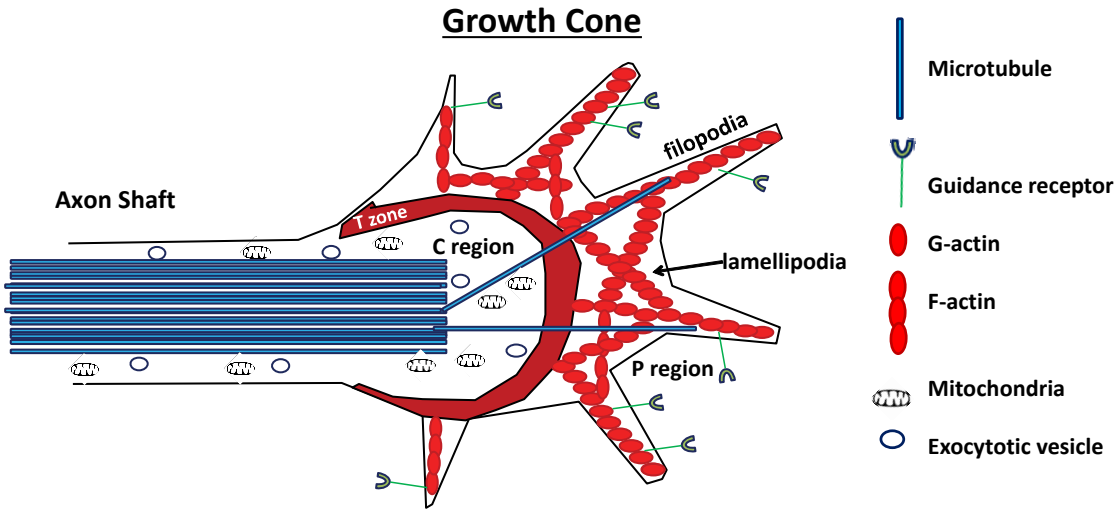


Figure 1.7: The neuronal growth cone

The terminal expansion of growing axonal and dendritic tips is called the neuronal growth cone. The leading edge (P region) consists of dynamic, finger-like filopodia that explore the 'road'. At the base of each protrusion, a mesh-like F-actin structure – lamellipodia, provide structural support and connects to the transition zone (T zone). Although the majority of stable microtubules remain in the central region (C region), single exploratory (dynamic – pioneer) microtubule can be seen within the peripheral region and into filopodia. Actin rings characterize the transition zone and 'barrier' that prevents organelles and vesicles from entering the peripheral region.

1.4.3.2 - Actin – the driver

Actin filaments (F-actin), like microtubules, are polar polymers.[133] The majority of actin monomers (G-actin) are added onto the barbed end and are bound to ATP while ADP-bound actin dissociates at the pointed end.[133-136] Bundles of actin filaments are formed with the help of bundling proteins, leading to the formation of Cdc42-dependent filopodia superstructures that protrudes from the growth cone reminiscent of antennas sensing the environment whereas lamellipodia is characterized by a meshwork of F-actin at the growth cone leading edge and is dependent on the action of Rac1 (Figure 1.7).[15, 104, 105, 128, 129] These actin superstructures, first characterized in Swiss 3T3 fibroblasts more than 25 years ago,[110, 137] are not unique to neurons and are a signature of migrating cells.[138] The clutch hypothesis proposed thirty years ago suggests that growth cone receptor binding to an adhesive substrate leads to the formation of a complex that acts like a molecular clutch, mechanically coupling the receptors and F-actin, thus anchoring Factin to prevent retrograde flow and driving actin-based protrusion of the growth cone on the adhesive substrate.[139, 140] This interaction creates traction which is also dependent on myosin-II.[141] Filopodia, the finger-like F-actin structures that protrude from the growth cone, act as guidance sensors at the front line of the growth cone and have a major role in establishing growth cone – substrate adhesive contacts during environmental exploration.[15, 128] Actin

dynamics are necessary for directed axon outgrowth but overall dispensable for axon outgrowth.[129, 142, 143] Neurons treated with agents that depolymerize F-actin are still capable of axon elongation but cannot initiate a turn in response to guidance cues.[144, 145] A neuron without F-actin can still grow but doesn't know where it is going, reminiscent of a driverless car. Thus, the growing axon can be thought of as a car with actin as the driver, microtubules as the engine, the road is the various substrates and extracellular matrices it encounters while the various road signs are the secreted guidance cues (Figure 1.8). Interestingly, one group that specifically targeted filopodia structures found that these actin-based protrusions are indeed dispensable for accurate growth cone guidance but required for normal growth cone motility.[146] It will be interesting to investigate at a super-resolution level the behavior of the growth cone when filopodia structures are eliminated. The balance between the rate of retrograde flow and polymerization determines if the growth cone extends or withdraws protrusions. If polymerization exceeds 3-6μm/min (retrograde flow rate), there is protrusion.[134, 141] In addition, when cell-substrate adhesions increase, retrograde flow slows down and polymerization drives protrusions.[134] Within the T zone, myosin-II plays an important role in severing actin filaments by exerting a contractile force on anti-parallel F-actin that contracts the actin meshwork and breaks it into small pieces.[141] In terms of growth cone turning, it is widely assumed that repulsive guidance cues leads to disruption and loss of actin superstructures and actomyosin contraction while attractive cues leads to asymmetrical incorporation of actin on the side of the growth cone closest to the cue,[15, 129, 134] although super-resolution cytoskeletal dynamics during growth cone turning is still lacking. The exquisite control of actin nucleation, polymerization/depolymerisation, bundling, and contraction involved in shaping the growth cone

is mediated by a complex repertoire of actin regulatory proteins which is beyond the scope of this introduction.[129, 134]

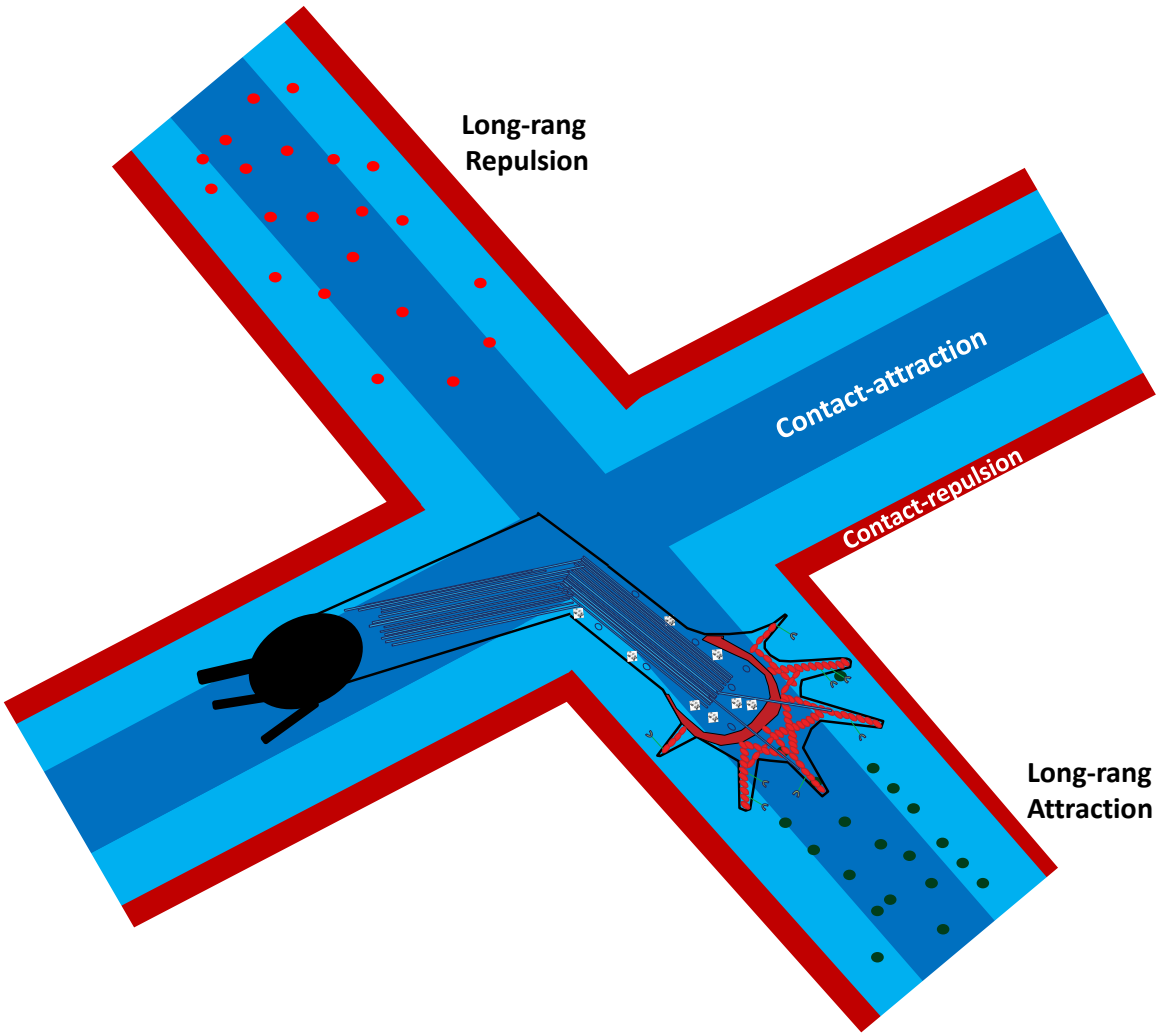


Figure 1.8: The road signs throughout the road trip

The growth cone and its dynamic F-actin serve as the vehicle, driving directed-axon outgrowth, reading the signs along the way making assuring the axon grows in the right direction. The adhesive substrate-bound cues such as cadherins and extracellular matrix proteins such as laminin and fibronectin pave the road (blue) while repellant substrate-bound cues such as ephrins act as guard rails (red), making sure the growth cone doesn't deviate from its trajectory. In addition to the road (short-range/substrate-bound cues), several long-range cues (netrins & semaphorins) are secreted within the embryo, forming gradients that growth cone can respond to by turning towards or away from (traffic lights). The driver (F-actin) has to remain vigilant and ready to respond to the several road signs. The constant F-actin treadmilling and retrograde flow provide the growth cone the necessary flexibility to respond in a timely manner, not that different to the driver with his coffee to remain vigilant. The 'Go' versus 'Stop' signs does not derive from some intrinsic property of the guidance cue but rather depends on the receptors present at the membrane and consequently, the downstream signaling pathways activated.

1.4.3.3 - Microtubules – more than just an engine

Although microtubules have classically been considered secondary players in growth cone motility, axon outgrowth and growth cone turning requires the coordination of both the actin and microtubule cytoskeleton.[130, 147-152] It was largely accepted that actin filaments initiated the change in direction (driver) while MTs followed their lead to promote outgrowth in that direction (engine).[153, 154] As we will see, microtubules play a much more complex role than just driving outgrowth and can even steer the growth cone in response to guidance cues. Similar to actin polymers, MTs are polarized polymers consisting of α/β tubulin heterodimers held together in a head-to-tail fashion as their building block.[133] Most MTs within the axon shaft are bundled together by lateral interaction of 11-15 protofilaments while some single MTs do explore and invade the peripheral region of the growth cone (Figure 1.7).[147, 148] As mentioned above, axonal microtubules are homogenously oriented with their dynamic (+) ends towards the growth cone and away from the cell body.[75, 128, 134, 147, 148] This orientation drives the rapid and increased rate of axonal versus dendritic outgrowth since MTs polymerization occurs mostly at the (+) end and dendrites have 50% of their MTs (+) end oriented toward the cell body. Highly dynamic growth cone MTs switch from growth and shortening phases called dynamic instability and function as a sensor to steer the growth cone in the right direction.[134, 147, 148, 155] Furthermore, the dynamic pool of MTs are characterized by a post-translational modification in which a C-terminal tyrosine residue is added (tyrosinated MTs) that is required for directed outgrowth toward a target.[156, 157] The importance of MTs in axon outgrowth and guidance had been highlighted by the many pathfinding defects found in humans caused by α- and β-tubulin mutation.[158-160] An

increasing number of studies suggest that MTs play more than just a consolidation role for growth cone steering initiated by actin dynamics and play more of an instructive role than previously thought.[148, 161, 162] Application of low concentrations of the microtubule-stabilizing drug taxol promotes polymerization at (+) ends and increases axon outgrowth in vitro and in vivo.[163] Furthermore, disruption of MT dynamics in the growth cone completely abolished attractive and repulsive growth cone responses normally induced by diffusible cues such as Netrin-1 and Sema3A, respectively. These two guidance cues, together with the other two classical guidance cues, Slit and Ephrin, and their signaling pathways will be introduced in the following section.[155] It was shown that the local stabilization of MTs on one side on the growth cone induces attraction towards that side, highlighting the importance of MTs stabilization in growth cone turning. Interestingly, netrin-1-mediated growth attraction was shown to be mediated by the interaction of its receptor DCC with a β-tubulin isoform through direct TUBB3-DCC interaction leading to MTs stabilization.[164] This interaction is dependent on Src family kinase phosphorylation of TUBB3 which was also shown to phosphorylate DCC in response to netrin-1 stimulation.[165, 166] Lastly, TUBB3 mutations partially phenocopies the midline crossing defects observed in both DCC and Netrin loss-of-function, highlighting its role in this signaling pathway.[158-160] Taken together, the dynamic nature of growth cone microtubules and growth cone morphology makes it hard to visualize in real-time.[134] Novel super-resolution techniques will definitely add to our detailed understanding of microtubule-mediated growth cone motility.

1.4.3.4 - Actin-Microtubule interaction

Coordination of the two dynamic cytoskeletal polymers need to be tightly controlled to assure the growth cone responds to the environment in a coordinated manner. Three modes of coordination between actin filaments and MTs have been documented.[130] First, actin

polymerization and actomyosin contractibility influences MTs dynamics.[127, 130] As previously mentioned, actin retrograde flow restricts the entry but can also lead to the direct removal of MTs within the P domain and following filopodial F-actin bundle contraction and depolymerisation, actin-bound MTs can be transported rearward. In addition, contractile actomyosin arcs exert a pulling force that facilitates MT bundling and advancement into the C domain. Second, filopodia act as guides for single MTs from the C domain exploring the periphery.[127, 130, 167] Third, actin-mediated MT capture is thought to be important for the dynamic regulation of adhesion sites following coupling of cell-adhesion molecules with actin.[168] Other than these three modes of coordination, several actin/MT cross-linking proteins have been identified in the context of axon outgrowth and guidance. The +TIP protein spectraplakin has been shown to bind MT lattices via its GAS2 domain while binding actin with its CH domain.[130, 169] Deleting just one of the two mammalian spectraplakin, ACF7, in mouse cortical neurons led to unstable MTs leading to defects in leading process morphology and cortical migration.[170] Dynein was shown to target MTs to the peripheral actin network of neuronal growth cones through the formation of a Lis-dynactin-dynein-EB3 complex which is important for growth cone motility.[171] Finally, actin itself can transport MTs in the anterograde direction to regions of low MT density.[172]

1.4.3.5 - Intermediate filaments (Neurofilaments)

Although neurofilaments are an important structural component of the neuron's cytoskeleton,[173] they are largely dispensable during axon outgrowth and guidance due to their static nature.[174] If they do play an important role, it remains largely unknown.[175]

1.4.3.6 - Ras GTPases

Ras GTPases are activated by a large number of plasma membrane growth factor receptors and adhesion receptors leading to the activation of the MAPK and PI3K signaling pathways, among others.[104] Less is known about the role of the Ras family members in axon outgrowth compared to Rho GTPases although Ras was shown to be involved in NGF-mediated axon outgrowth through activation of Raf.[176] In addition, Ras inactivation downstream of semaphorin signaling is involved in growth cone collapse.[104, 177, 178] Ras has also been shown to play a role in Eph/ephrin-matrix adhesion[179] and netrin/DCC-mediated axon outgrowth and guidance.[180]

1.4.3.7 - Rho GTPases

Introduction of the mechanisms involved in regulating the actin and MT cytoskeleton wouldn't be complete without addressing the critical role of the small Rho GTPases.[104, 105] Their ability to coordinate multiple signal pathways with the help of their regulators (152 total) with precise spatial and temporal control drives most of the activities that underlie axon outgrowth and guidance.[104] The molecular cycle regulating cell motility was first proposed in migrating fibroblasts in a two-dimensional environment.[110, 137] First, there is protrusion at the leading edge, followed by adhesion of the leading edge (and de-adhesion of the trailing edge) and finally contraction of the rear that pushes the cell body forward.[138] In this model, the cycling activity of Rho GTPases is responsible for the protrusion-retraction cycle. Rac1 and Cdc42 promote actin polymerization and protrusions at the leading edge by stimulating the actin nucleation factor Arp2/3 through the activation of the Wiskott-Aldrich syndrome protein (WASP) family members, WASP-family verprolin-homologous protein (WAVE) and neural WASP (N-WASP), respectively.[105, 138] While Rac1 activity generates lamellipodia via the regulation of WAVE, Cdc42 activity induces the formation of filopodia via N-WASP. In contrast, RhoA/Rho kinase

(ROCK) activity at the trailing edge promotes contractibility by activating myosin light chain.[105, 138] In the context of axon outgrowth much of the same mechanisms apply to growth cone motility.[104] Briefly, RhoA can either promote or inhibit axon extension depending on the type of effector: mDia or ROCK, respectively.[104] Kalirin-9 (RhoGEF) activates while p190RhoGAP inactivates RhoA to promote axon outgrowth.[104, 181] Both ROCK and PAK (Rac1 effector) can inhibit cofilin through LIMK.[128, 134, 136] The balance of dephosphorylated (active) and phosphorylated (inactive) cofilin appears to be crucial for axon extension. Several GAPs (ex: α-chimerin, p190RhoGAP, & p120RasGAP)[180-182] and GEFs (ex: Tiam1, STEF, Dock180, & Trio)[112, 183-185] have been documented to be involved in axon outgrowth and guidance and we will see where some of them come into play in the context of the 4 classical guidance cues and their signaling pathways.

1.4.4 - Axon Guidance – The Road Signs

It appears that with this hypothesis we have shed light into a dark cave, when in reality we have explored only the entrance, from which its imposing abyss appears even more distant and black. On what bases are mechanical influences guiding the created ameboid streams? Which is the cause of certain preferences of time and location in the distribution of secretory phases? Why does the chemotactic sensitivity cease of decrease in certain periods? These are questions that present day Science can only pose: their clarification, i.e., their total reduction to physico-chemical mechanism, will be the work of the future.[7] ***Cajal, 1890***

The development of neural circuits comprising billions of neurons interconnected with trillions of synapses[17] depend on the correct pathfinding of axons to their final target. Today, most if not all, guidance molecules and receptors have been identified and in comparison to the complexity of neural circuits, the number is surprisingly small.[186-188] Similar to the multiple stops we take during a long road trip, axons navigate through a series of intermediate steps along the way to their final destination, their future synaptic partners. As highlighted by Cajal well over a century ago, how do neurons switch their chemotactic response at different intermediate steps? If growth cones are attracted to the intermediate target, why don't they form synapses within it? We now know that receptors on the cell surface of growth cones mediate the response

to guidance molecules present throughout the embryo and that instead of switching the guidance molecules present at different intermediate steps, which would require that all axons travel at the same time, neurons change the receptor population present at the cell surface.[186-188] Switching the response quickly within the intermediate target prevents staling and ensures the axon reaches its final target in a timely manner.[189] It was demonstrated that the correct navigation of axons depend on a combination of long and short-range attraction and repulsion (Figure 1.8).[190] Long range guidance molecules form gradients that can attract or repel axons far from its source while short-range are mostly contact-dependent (Figure 1.8). All four classic guidance cues first identified in the context of commissural axon guidance in the spinal cord have also been showed to be involved in cortical axon guidance.

1.4.4.1 - Classical guidance cues and commissural axon guidance

The best understood model for axon guidance is the dI1 interneurons of the dorsal spinal cord.[188, 191] Interneurons located on the dorsal side of the developing spinal cord, dI1 neurons, first send their axons ventrally toward their intermediate target, the floor plate, cross the midline before turning rostrally (Figure 1.9). The midline crossing of these dI1 interneurons create commissures along the spinal cord and are thus often called commissural neurons. This model for studying axon guidance represents a convenient model as the initial trajectory is easy to follow experimentally.[24] The spinal cord, along with the retina, are also two regions of the CNS with an easier accessibility for manipulation (ex: open-book preparation). It was in these commissural axons that the four major family of guidance cues were first identified: Netrins, Slits, Semaphorins, and Ephrins.[188, 190-192] Other factors first identified in a different context have also been identified in the spinal cord as having guidance properties.[186, 192, 193] Briefly, dI1 neurons born on the dorsal side of the spinal cord are attracted to gradients of netrin-1 secreted

from the the floor plate (Figure 1.9).[194] This attraction to the midline is mediated by the netrin-1 receptor DCC expressed at the cell surface of growth cones.[195, 196] Upregulation of the Slit receptor Robo1 within the floor plate switches the response from attraction to repulsion by favoring the Slit-Robo-mediated repulsion (Figure 1.9).[197] In addition, interaction between Robo1 and DCC attenuates the netrin/DCC-mediated attractive response.[198] Class-III Semaphorins derived from the floor plate provide a repulsive signal for post-crossing axons expressing Neuropilin-2 and PlexinA1 (Figure 1.9).[199] This particular repulsion is prevented in pre-crossing neurons by calpain-1-mediated PlexinA1 cleavage.[199] The role of calpain-1 in axon guidance in not limited to Semaphorin/Plexin signaling and chapter 3 will demonstrate that it plays an important role in netrin/DCC-mediated axon outgrowth. Finally, Ephrin-B3 expressed in the floor plate also mediates repulsion away from the midline through interaction with its receptor Eph-B3.[200] The complexity of neural circuits compared to the relatively small number of guidance molecules and receptors is achieved, at least in part, through the crosstalk of these signaling pathways, thereby increasing the diversity of cellular responses to the limited number of cues.[187, 188, 201] Since our lab focuses on cortical axon outgrowth and guidance, I will introduce these four guidance cues and their role in cortical axon guidance after taking a brief look at other molecules first identified in another context that were shown to have guidance properties.

1.4.4.2 - Morphogens and growth factors in commissural axon guidance

Some of the best-known morphogens have also been found to have guidance properties, independent of their role in providing positional information to initiate a differentiation cascade mediated by specific combinations of transcription factors.[48, 49, 193] Sonic hedgehog (Shh) secreted from the notochord and eventually the floor plate of the spinal cord forms a ventral-high

to dorsal-low gradient, providing neural progenitors positional information required to activate a specific series of transcription factors required for differentiation into a specific neural subtype.[202] Although Shh plays a similar function as netrin-1 in attracting commissural axons toward the midline, its effect is weaker than that of netrin-1 as the effect can only be detected in embryos defective in netrin-1 signaling (Figure 1.9).[203] Similarly, vascular endothelial growth factor (VEGF), also act as a chemoattractant to guide commissural axons toward the floor plate (Figure 1.9).[204] Interestingly, all three chemoattractant (netrin-1, Shh, and VEGF) converge on Src family kinases to mediate their chemoattractive properties.[165, 166, 204-206] In addition, Shh expressed in a caudal-high to rostral-low gradient acts as a repellent in post-crossing commissural axons, guiding them rostrally toward the brain (Figure 1.9).[207] Conversely, BMP7 and Draxin form an opposing gradient coming from the roof plate that repel commissural axons away from the roof plate and consequently toward the floor plate (Figure 1.9).[208, 209] Furthermore, Wnt4 expressed at the ventral midline in a rostral-high to caudal-low gradient attracts post-crossing commissural axons rostrally (Figure 1.9).[210] Finally, Wnt1 and Wnt5 have been implicated in chemorepulsion of the CST and corpus callosum.[211]

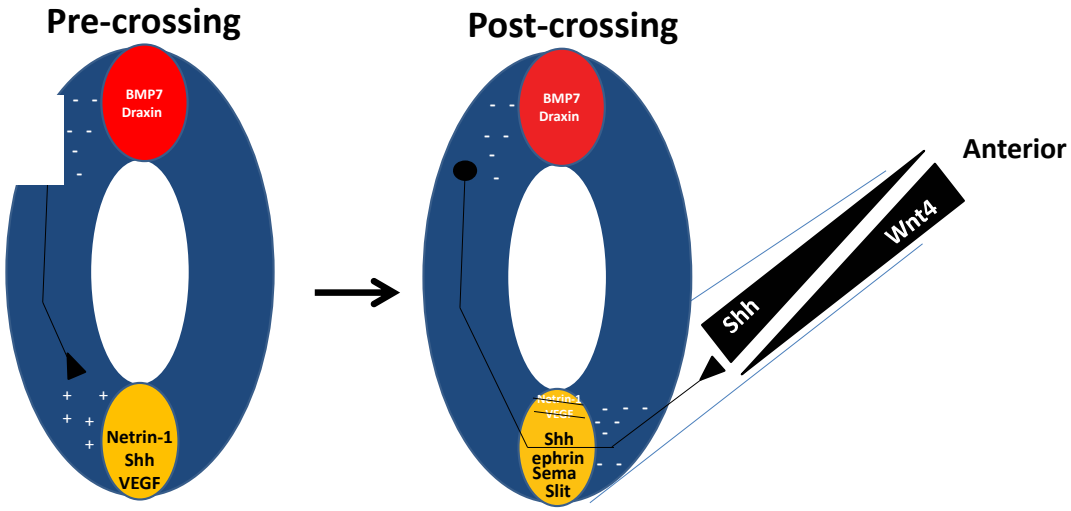


Figure 1.9: Commissural axon guidance in the spinal cord

DI1 neurons born on the dorsal side of the developing spinal cord first send their axons ventrally toward the floor plate, cross the midline before turning rostrally. Dl1 axons are attracted to the midline by a combination of floor plate-secreted netrin-1, Shh, and VEGF and repelled by roof plate-secreted BMP7 and Draxin. By regulating receptors present at the cell surface, the attractive netrin-1 and VEGF response is attenuated in favor of the repulsive response mediated by Slit, Sema, and ephrin while the initial attraction mediated by Shh is converted to repulsion. Post-crossing axons are attracted rostrally toward a Wnt4 gradient and

1.4.4.3 - Classical guidance cues

1.4.4.3.1 - Slit/Robo signaling at the roundabout

The highly conserved family of secreted repulsive guidance cues, Slits, were first identified through a genetic screen in Drosophila and found to be secreted at the ventral cord midline to repel ipsilateral projecting neurons and post-crossing commissural axons.[212-214] Three Slit genes have been identified in vertebrates encoding ~200kDa proteins. They consist of four stretches of leucine-rich repeat (LRR) domains (D1-D4), seven to nine epidermal growth factor (EGF) repeats, an Agrin-Perlecan-Laminin-Slit (ALPS)/Laminin-G-like domain, and a C-terminal cysteine knot.[197] The three Slit receptor genes, Robo, were also identified in Drosophila.[215, 216] Their name was coined due to the phenotype of Drosophila mutants in which commissural axons stalled at the midline of the ventral nerve cord, creating an axonal 'ROundaBOut.[217] Four mammalian orthologues exists with Robo1-3 containing five immunoglobulin-like domains and three fibronectin type-3 repeats within its extracellular domain, a single-pass transmembrane domain and a cytoplasmic tail containing several conserved regions termed CC (cytoplasmic conserved) domains.[197] These domains, however, are quite variable between species. Robo1/2 are well-conserved with overlapping functions during axon guidance while Robo3/4 do not actually bind Slits.[197, 218] Robo3 in particular was shown to be involved in opposing the repulsive response mediated by Slit. Interestingly, Robo3 also plays

a positive role in netrin/DCC-mediated growth cone attraction.[218] Upon Slit/Robo binding through Slit's LRR2 and the first Ig domain of Robo, the cytoplasmic tyrosine kinase Abelson play an important role by phosphorylating Robo's CC1 domain as well as downstream effectors required for growth cone repulsion.[187, 197, 219] Downstream, the activation of Rac1 and RhoA, together with the inhibition of Cdc42 mediate the required cytoskeletal rearrangements.[220] The adaptor protein Nck, which binds Robo receptors, was shown to mediate the link between Robo receptors and GTPase activation to mediate cortical axon outgrowth and branching.[221] In Drosophila, specific Rac GEFs and GAPs linking Robo receptors directly to the GTPases have been identified.[220, 222] The Slit-Robo-GTPase activating proteins (srGAPs) *Vilse/SrGAP* was shown to play an important role in growth cone repulsion in a dose-dependent manner.[223, 224] Too much or too little *Vilse/SrGAP* lead to defects in growth cone repulsion at the midline. Thus, the fine-tuning of Rac activity seems to be required to mediate midline repulsion and avoid either stalling or re-crossing at the midline.

One of the great enigmas in the field of axon guidance was the mechanism by which growth cones switch their attractive response to the midline to a repulsive one. The predicted consequence of midline attraction was either growth cone stalling within the floor plate or continuing re-crossing after exiting the midline. If Slits were expressed in the floor plate before axons crossed; why were the growth cones unresponsive to the repulsion that kept post-crossing axons away from the midline? The initial Drosophila screen that identified the Robo receptors also identified another mutant with severe midline crossing defects, *commissureless* (*Comm*).[225] *Comm*, specifically expressed in pre-crossing commissural axon (and not in ipsilateral-projecting axons) is a transmembrane receptor that targets Robo to the lysosome for degradation.[226] This maintains low levels of Robo receptors in pre-crossing axons and consequently prevents

repulsion. Following midline crossing, Comm expression is downregulated to enable Robo-mediated repulsion out of the midline and prevents re-crossing.[197, 226] No vertebrate homologue of Comm has been identified and alternative mechanisms have evolved to compensate to regulate midline crossing. As we will see below it involves the crosstalk between different signaling pathways.

1.4.4.3.2 - Semaphorin/Plexin signaling

The more than 20 semaphorin proteins identified thus far are categorised into eight subfamily classes (*Semaphorin Nomenclature Committee, 1999*).[227] Invertebrate semaphorins are grouped into classes 1, 2, and 5 while classes 3-7 represent vertebrate's semaphorins (except for Sema-5C only found in vertebrates).[227, 228] All semaphorins are defined by a ~400 amino acid sema domain which facilitates interaction with their receptors as well as mediating homodimerization. Their principal receptors, plexins, are transmembrane proteins divided into four classes (A-D).[229, 230] Two invertebrate plexins, PlexA and PlexB, and nine plexins have been identified in vertebrates. Unlike other guidance receptors, plexins contain a catalytically active intracellular domain, a GTPase-activating (GAP).[229, 230] Among the other receptors/co-receptors identified, class-3 semaphorins (except Sema3E) which cannot bind direclty to plexins require the high affinity binding of neuropilins as co-receptors.[230] Two neuropilins have been identified in vertebrates: neuropilin-1 and -2 (Nrp1/2). Both Nrp1 and Nrp2, together with plexins, form a holoreceptor complex in which they are obligate ligand-binding subunit of class 3 semaphorins.[228] Furthermore, Sema7A can signal independently of plexins through β1-integrins whereas Sema3A can also signal through Nrp1/L1cam receptor complex. Upon semaphoring binding, the GAP domain of plexin gets activated to reduce Ras and Rap activity in neurons.[228] This reduction in active Ras, in turn, leads to activation of the MT-depolymerizing

protein, CRMP-2, via PI3K-AKT-GSK3β. Binding of the small GTPase Rnd1 to plexin B1 was shown to stimulate its GAP activity while PKA-mediated GAP domain phosphorylation triggers 14-3-3ε recruitment and a concomitant reduction in GAP activity.[178, 231] In Drosophila, direct regulation of the actin cytoskeleton by plexins is accomplished via the Mical family of redox enzymes.[232] These enzymes can bind plexins and mediate F-actin subunit oxidation and disassembly. In addition, Sema3A regulate actin dynamics through the phosphorylation of cofilin by LIMK.[233] Although widely recognized as repellents, semaphorins can also mediate chemoattraction in the CNS through expression of various receptors/co-receptors.[228] The presence of Nrp1 and VEGR2 on subicular axons in the brain switches Sema3E-mediated repulsion to attraction through VEGFR2-mediated downstream signaling.[234] Interestingly, semaphorins and plexins can also engage in cis-interactions to prevent interactions in trans and/or activate plexin signaling in cis.[228] Taken together, this illustrates the versatility of semaphorins guidance cues during axon guidance.

1.4.4.3.3 - Ephrins/Eph signaling

A considerable backlog of supporting observations suggestive of chemotaxis has accumulated during the last two decades, but has not been emphasized owing to the prevailing unpopularity of anything suggestive of neurotropism and chemical guidance. With the demonstration of chemotactic regrowth in the optic system leaving little room for further doubt of the selectivity effects, the way seems cleared for retrospective reassessment of some of the earlier observations with an increased recognition of the importance of selective chemoaffinity in nerve growth and connection in general. ***R. W. SPERRY, 1963[235]***

It was ephrin/Eph signaling in the visual system that inspired Roger Sperry's chemoaffinity theory based on pioneering studies in the newt's retinotectal system.[235, 236] The discovery of Eph/ephrin signaling came over thirty years later in the context of topographical mapping of the visual system.[237, 238] Since then, Eph/ephrin signaling have been implicated in guiding axons of the auditory, somatosensory, and olfactory systems, as well as playing a critical role in midline crossing of spinal commissural and cortical callosal axons.[200, 239-243] They are the

largest family of receptor tyrosine kinase (RTK) encoded in the mammalian genome.[243, 244] The receptors are divided into two subclasses, EphA (EphA1-EphA8) which bind GPI-linked ephrin-As (ephrinA1-ephrinA5), and EphB receptors (EphB1-EphB6), which bind transmembrane ephrin-Bs(ephrinB1-ephrinB3). Ephrins are short range guidance cues since they are attached to the membrane.[243, 244] One unique feature of ephrin/Eph signaling is that it can occur in both directions; forward signaling is transduced by the receptor, Eph, while reverse signaling happens downstream of the ephrin ligands.[245] Although widely known as a repellant, both forward and reverse signaling can repel or attract the growth cone.[243, 244] As with the other guidance receptors, ephrin signaling influences the activation state of small Rho GTPases. Ephrin-A5 signaling activates RhoA and inactivates Rac1/Cdc42 through the constitutively-bound RhoGEF, ephexin, leading to growth cone collapse.[246] In addition, Eph receptor activation leads to the inhibition of Ras and the MAPK pathway.[247, 248] Direct link of Eph receptors to the cytoskeleton has been shown through the association and dephosphorylation of Abl upon ephrin binding.[249] Reverse signaling through ephrins on the other hand is initiated by Src-mediated tyrosine phosphorylation of ephrins that create binding sites for the SH2/SH3 adaptor protein Grb4. Grb4, in turn, regulates FAK activity and the redistribution of paxilin.[249] Ephrin/Eph signaling play an important role in mediating adhesion (Figure 1.8). Binding of the phosphotyrosine phosphatase Shp-2 to EphA2 leads to FAK inactivation and decreased intergrin-mediated adhesion whereas increased adhesion is induced by ephrin-A via Src kinases.[250] Crosstalk between Eph/ephrin and Semaphorin/Nrp1 during midline crossing has recenlty been demonstrated (see below).[242]

1.4.4.3.4 - Netrin - the one who guides

Direct support for Cajal's proposed chemotropic guidance of growth cones was only obtained a century after he published his chemotactic hypothesis with the identification of the netrin family of guidance cues.[194, 251, 252] Netrin was first discovered as a secreted molecule capable of guiding *C.elegans* axons towards Unc-6-secreting epidermoblasts located at the midline.[252] It was 2 years later that the verterbrate homologue of Unc-6 was identified and named netrin based on the Sanskrit word "netr" meaning "the one who guides".[194, 251] It was purified based on its ability to attract commissural axons of the spinal cord towards the floor plate. Similarly, the *C. Elegans* orthologue of DCC, Unc-40, was first identified in the same genetic pathway as Unc-6 in *C. Elegans*.[253] It was later characterized as an Unc-6 receptor in parallel to the characterization of DCC as a netrin receptor.[195] These pioneering studies paved the way for the identification of other guidance cues and the field of axon guidance in general. Vertebrates express 4 different secreted netrins (netrin 1-4) and two related GPI-anchored membrane proteins, netrin-G1 and netrin-G2.[196] All netrins are composed of ~600 amino acids and belong to the superfamily of laminin-related proteins composed of an N-terminal laminin VI domain, followed by a series of EGF-like domains and a C-terminal netrin-like domain (NTR) (Figure 1.10).[196] Netrins 1-3 are highly similar and are all capable of mimicking the chemoattraction of the floor plate. The best-studied member of the family is netrin-1. Netrin receptors include DCC, the DCC paralogue neogenin, UNC5A-D, the G protein-coupled Adenosine 2B receptor (A2b), and Down syndrome cell adhesion molecule (DSCAM). Netrins are bidirectional cues; they promote attraction when bound to DCC and repulsion when bound to DCC/UNC5. [196, 254]

Figure 1.10: Structural domains of netrin

Netrins are members of the laminin superfamily. All netrins are ~ 600 amino acids with their N-terminal laminin VI domain, followed by a series of EGF-like domains and a C-terminal netrin-like domain (NTR).

1.4.4.3.5 - Netrin - the one who guides... …locally

Netrin was thought to attract commissural axons by forming a gradient with the highest concentration close to its source, the floor plate. However, 20 years later, two independent laboratories reported that netrin-1 secreted from the floor plate is dispensable for commissural axons to reach the floor plate.[255, 256] Netrin secreted locally from precursor cells in the ventricular zone of the spinal cord is necessary and sufficient to guide commissural axons. Importantly, deletion of netrin specifically in the VZ phenocopies axon defects previously described in netrin-1 knockout mice.[255, 256] Thus, although netrin-1 does form a gradient along the dorsal-ventral axis of the spinal cord, it appears that at least for commissural axons, netrin-1 functions as a short-range contact-dependent attractive cue by promoting growth cone adhesion.

1.4.5 - DCC - The One Who Attracts

1.4.5.1 - Structure

DCC was first identified as a candidate for a tumor-suppressor gene on human chromosome 18q.[257] It was later characterized as the key receptor mediating the attractive response to netrin.[195] Inactivation of the DCC gene in mice causes severe defects in axonal projections, similar to netrin-1-deficient mice.[251, 258] DCC is a type I transmembrane protein consisting of four immunoglobulin-like domains (Ig) and six fibronectin type III domain (FN3) on its extracellular domain and three conserved P domains (P1, P2, & P3) on the intracellular side (Figure 1.11).[195, 196, 259] The structure of the cytoplasmic tail of DCC remains largely unknown while its extracellular domain has been resolved alone and in complex with netrin-1.[260, 261] The four N-terminal Ig-like domains of DCC fold into a horseshow-like conformation

(Figure 1.11).[262] This unique horseshoe conformation turns out to be a conserved structural feature in many neuronal receptors such as another netrin-1 receptor, DSCAM and the adhesion receptor TAG-1.[262] Several binding sites have been identified by two independent laboratories and the FN5 domain of DCC and the EGF domains of netrin seem to be crucial for downstream signaling (Figure 1.12).[259-261, 263] This highly specific first binding site involves netrin's EGF3 domain and DCC's FN5 domain. A second binding site between netrin's EGF1 and EGF2 domains and DCC's FN5 and FN6 was resolved.[261] Finally, netrin's laminin domain was found to interact with DCC's FN4 domain.[260] Thus, netrin-1 binds DCC mainly through DCC's FN5 domain and to a lesser extent with FN4 and FN6 domain. Importantly, netrin-1 always associates with two DCC molecules, confirming previous findings (Figure 1.12).[264] Importantly, in its dimerized form one DCC molecule never physically interacts with the other DCC molecule within the extracellular domain. The closest distance between the two DCC molecules is 20Å.[260, 261, 263] Physical interaction could be possible via its intracellular P3 domain as this domain is required for self-association (Figure 1.11).

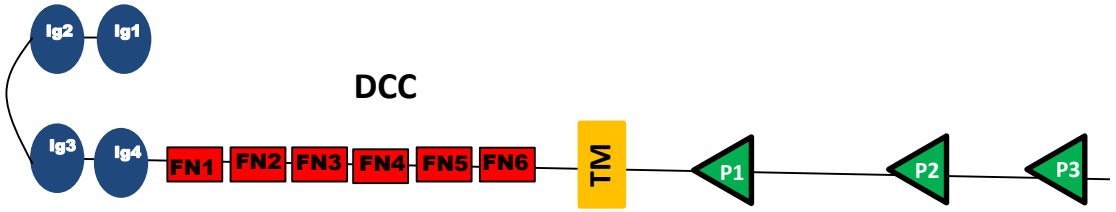


Figure 1.11: DCC is a type I transmembrane receptor part of the immunoglobulin superfamily

The extracellular domain consists of four N-terminal Ig domains folded in a horseshoe-like conformation followed by six fibronectin type-III domains. A short transmembrane domain links the intracellular tail consisting of 3 conserved P domains responsible for downstream signaling

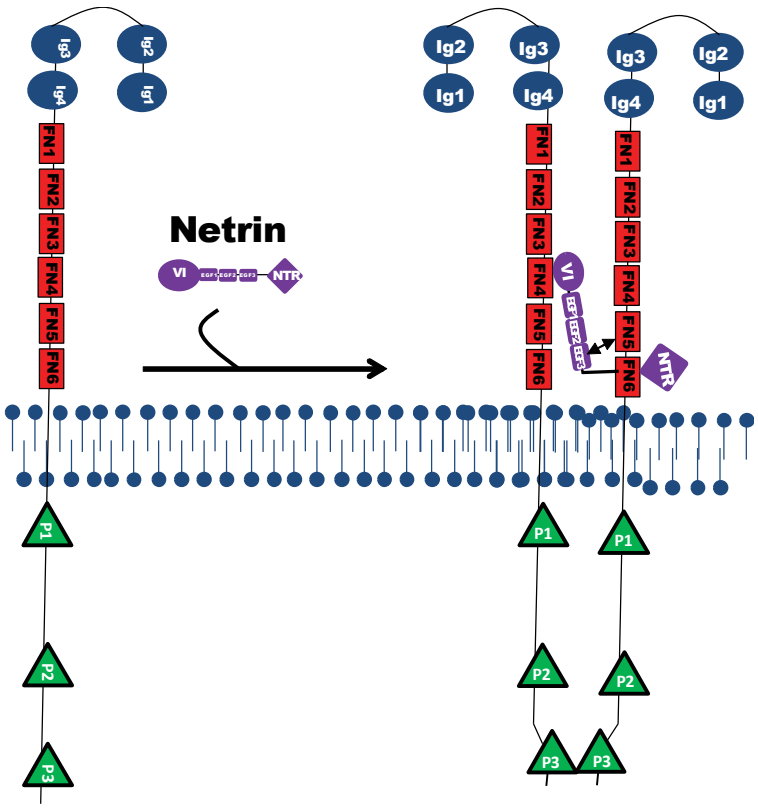


Figure 1.12: Netrin-1 associates with two DCC molecules

The first and highly specific binding site occurs between netrin's EGF3 domain and DCC's FN5 domains. Netrin's EGF1 and EGF2 also bind FN5 and FN6 while netrin's laminin domain can bind DCC's FN4 domain. The close proximity between the two P3 domains of each DCC molecules likely mediates dimerization on the intracellular side.

1.4.5.2 - Signaling

DCC plays two important roles in netrin-1-mediated axon outgrowth and attraction: 1) it serves as a transmembrane bridge linking extracellular netrin to the actin cytoskeleton and 2) as a signaling platform promoting actin dynamics and activating several downstream effectors.[196] In the absence of netrin-1 DCC is constitutively bound to the adaptor protein Nck1 and the focal adhesion kinase (FAK).[166, 265-267] Netrin-1 binding triggers DCC homodimerization via its P3 domain (Figure 1.11), autophosphorylation of FAK, as well as the recruitment and activation of Src family kinases.[166, 206, 265] Netrin-1-mediated traction force and FAK activation necessary for

axon outgrowth were also shown to be dependent on myosin II.[268] Surprisingly, activated Src kinases lead to Robo3 phosphorylation and activation that promote DCC interaction.[218] Interestingly, Robo3 does not mediate Slit-mediated repulsion and only weakly binds to Slit. Instead, Robo3 has been repurposed during mammalian evolution to promote netrin/DCC-mediated attraction instead of Slit-mediated repulsion.[218] Our lab has shown that activation of Fyn and the resulting Fyn-mediated tyrosine 1418 phosphorylation of DCC is crucial for downstream signaling and the overall growth cone response to netrin/DCC.[206] This led us to investigate one particular SH2-containing protein associating with pY1418, p120RasGAP, that will be discussed in chapter 2. Although phosphorylation on tyr 1418 is required for signaling, most phosphorylation on DCC occurs on ser/thr residues but their function has never been examined.[206] Ser/thr phosphorylation on DCC's cytoplasmic tail will be discussed in chapter 4. In addition to DCC phosphorylation, activation of Fyn leads to the activation of Rac1 and Cdc42 and inhibition of RhoA.[183, 269-271] Two Rac1-GEFs, Trio and DOCK180, promote Rac1 activation downstream of DCC activation.[183-185] Interestingly, several netrin-dependent projections were affected in Trio knockout mice. Projections that form the internal capsule and corpus callosum were defective while the anterior commissure was completely absent.[183] In addition, Trio-mediated Rac1 activation downstream of netrin/DCC was shown to be dependent on Hsc70 chaperone activity.[272] Downstream of Rac1 activation, p21(Rac1)-activated kinase-1 (Pak1) gets activated by phosphorylation and recruited to DCC-bound Nck1.[269] Downstream of the other activated GTPase, Cdc42, Ena/Vasp and N-WASP are activated and induces the formation of filopodia protrusions.[269] In parallel, Src kinases and RhoA/Rho kinase-mediated activation of ERM proteins downstream of netrin/DCC signaling leads to an increase in ERM-DCC located within filopodia.[273] ERM proteins in turn mediate a direct physical interaction of

PKA with DCC, leading to PKA activation.[274] Activated-PKA can then phosphorylate and activate Ena/Vasp proteins. Under basal conditions, Vasp is ubiquitinated by the E3 ubiquitin ligase TRIM9 and this reduces its filopodia tip localization, Vasp dynamics, and filopodia stability.[275] Upon netrin-1 stimulation, Ena/Vasp gets deubiquitinated, thus promoting its tip localization and filopodial stability.[275] Interestingly, the same group recently found that TRIM9-mediated ubiquitination of DCC blocks interaction and activation of FAK.[276] Upon netrin-1 stimulation, this ubiquitination is reduced, thus promoting FAK interaction and activation.[276] Interestingly, in addition to phosphorylating and activating Vasp, a novel Robo1 coreceptor, FLRT3, was shown to be required for netrin/DCC-mediated attraction by increasing the cell surface pool of DCC through activation of PKA downstream of Slit1/Robo1/FLRT3 signaling.[277] This crosstalk between netrin/DCC and Slit/Robo signaling is not unique and highlights the direction in which the field of axon guidance is going.[187, 188, 201]

Netrin/DCC signaling also activates the MAPK pathway leading to extracellular signal-regulated kinases 1 and 2 (ERK1(MAPK3) and ERK2(MAPK1) phosphorylation and recruitment to the DCC receptor complex.[278, 279] This ERK1/2 activation was shown to be required for netrin-1-induced protein synthesis (see below).[280] Netrin/DCC signaling also promotes the synthesis of phosphoinositide phosphatidylinositol (4,5) biphosphate (PIP2) that is phosphorylated by PI3K to produce PIP3.[281, 282] Just as PIP3 helps the recruitment of AKT to the plasma membrane to promote neuronal polarization and axon specification, this particular PIP3 enrichment helps recruit Rho GTPases to the plasma membrane where it regulates the actin cytoskeleton.[104, 105] Conversely, netrin-1 induces PIP2 hydrolysis by phospholipase Cγ (PLCγ) to generate diacylglycerol (DAG) that activates protein kinase C (PKC) and inositol 1,4,5-triphosphate (IP_3) that stimulates the release of intracellular Ca^{2+}.[281, 282] The calcium release is

required to induce netrin-dependent growth cone turning. Intracellular calcium concentration is also increased via the activation of transient receptor channels (TRPC) that allows Ca^{2+} influx from outside the cell.[283, 284] Calcium signaling serves many cellular functions such as changing a protein's conformation upon calcium binding.[285] In particular, the calcium-dependent cysteine protease, calpain, and its activation downstream of netrin/DCC signaling will be discussed in chapter 3.

1.4.5.3 - Netrin/DCC and the cytoskeleton

In addition to regulating the cytoskeleton indirectly through the various signaling effectors mentioned above, netrin-1 regulates the translation of β-actin to promote growth cone turning.[286, 287] Specifically, netrin-1 activates a translation initiation regulator, eIF-4E-binding protein 1 (4EBP) asymmetrically, leading to an increase in β-actin translation on the side of turning. The local transport and translation of β-actin mRNA downstream of netrin-1 stimulation is accomplished with the help of zipcode binding protein 1 (ZBP1), an RNA-binding protein which binds a specific sequence on the 3'UTR of β-actin mRNA.[286] This netrin-1-induced β-actin mRNA translation is likely mediated by DCC's direct physical interaction with several components of the translation machinery such as eIFs, ribosomal subunits, and monosomes.[288] The involvement of DCC in netrin-1-induced β-actin was also demonstrated indirectly by the fact that ERK1/2 activation, which depends on netrin/DCC signaling, is required for netrin-induced protein synthesis.[289] In addition to promoting β-actin translation, DCC interacts with MTs downstream of netrin-1 stimulation.[164] It was shown that DCC interacts directly with the most dynamic tubulin isoform in neurons, βIII-tubulin (TUBB3), in a netrin-1-dependent manner to promote MT dynamics. Reducing TUBB3 levels strongly impaired netrin/DCC-dependent projections.[164] Importantly, mutations in TUBB3 found in humans strongly affected netrin-

1/DCC signaling and mice harboring these mutations phenocopied the projection defects observed in netrin-1 and DCC knockout mice.[160]

1.4.5.4 - DCC in human axon guidance

DCC mutant mice exhibit a failure of the CST to cross the midline and a complete agenesis of the corpus callosum which is characterized by the absence of the corpus callosum.[195, 290] Similarly, germline DCC mutations in humans cause midline crossing defects that leads to abnormal corticospinal track (CST) and corpus callosum development.[291-294] Interestingly, monoallelic DCC mutations cause congenital mirror movements (CMM) associated with abnormal midline crossing of the CST and/or agenesis of the corpus callosum (ACC).[293] Congenital mirror movements are pathological involuntary movements that mirror intentional movements on the contralateral side. Biallelic DCC mutations on the other hand cause a more complex syndrome; developmental split brain syndrome associated with complete agenesis of the corpus callosum as well as widespread failure of commissural tracks throughout the CNS that also cause congenital mirror movements.[291-294] In addition to the absence of the corpus callosum, all other commissures (anterior, posterior, and hippocampal) of the brain are absent. Furthermore, two brothers with a 7 kb deletion that results in skipping exon 1 that encodes the membrane localization signal crucial for its function in axon guidance together with a separate individual harboring a homozygous 7bp deletion in exon 4 resulting in a truncated DCC all exhibit horizontal gaze palsy characterized by impaired conjugate horizontal eye movements, intellectual disability, and scoliosis characterized by a curved spine, while only the two brothers displayed CMM (Figure 1.13).[293] The combination of all individuals reported to have DCC mutations enabled an estimated 42% CMM penetrance and a 26% ACC penetrance.[293] Interestingly, males exhibit CMM twice as often as females. In contrast, five times more females

with a truncating DCC mutation display ACC.[293] This is in accordance with findings showing DCC expression levels to be dependent on testosterone.[295] Combining all studies thus far published, a new repository for all reported disease-associated with DCC sequence variants was created using the **Leiden Open Variation Database**https://databases.lovd.nl/shared/genes/DCC).[293] In line with DCC's role in guiding commissural axons of the spinal cord and similar to CMM; DCC mutations in humans lead to mirroring of somatosensory stimuli.[296] These individuals experience bilateral sensations evoked by unilateral somatosensory stimulation. Lastly, one particular DCC mutation, T1210A, identified through the Exome Aggregation Consortium (ExAC) databases[295] will be discussed in chapter 4. Although not predicted to cause CMM or ACC using an *in silico* prediction algorithm,[295] the spontaneous mutation, T1210A, identified in humans as well as T1210's role in DCC stability will be addressed in chapter 4.

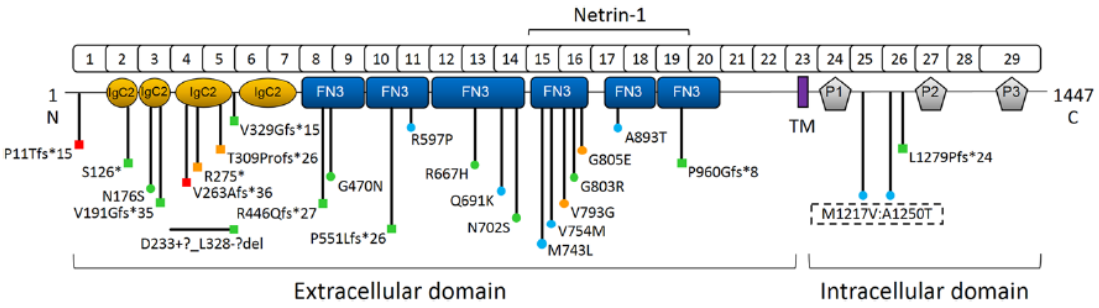


Figure 1.13: DCC mutation landscape identified in the human population

Location of all reported mutations depicted within the DCC protein structure. Square represents predicted loss-of-function; circles missense mutations. Red square mutations are associated with developmental split brain (DSBS), green represents CMM, blue ACC, orange CMM & ACC. Adapted with permission from *Human Mutation.* 2018 (Marsh et al., 2018). Copyright © 2018 by Human Mutation.

1.4.5.5 - DCC in neurological and psychiatric diseases

In addition to its requirements for the development of CNS commissures, DCC is also involved in wiring the prefrontal cortex, the most evolutionary recent addition to the brain, during adolescent.[297] Changes in DCC expression in humans have been linked to psychiatric conditions such as clinical depression and schizophrenia.[297] In addition to regulating dopaminergic connections targeting the prefrontal cortex, small nucleotide polymorphism (SNP) in the DCC gene has been associated with an increased susceptibility for Parkinson's disease.[298]

1.4.6 - Receptor regulation

1.4.6.1 - Regulated endocytosis/exocytosis

Changing the growth cone's response along the various intermediate targets require changes in receptors present at the plasma membrane as opposed to changing the different cues expressed along the growth cone's journey. The receptor population present at the cell surface is a highly dynamic process regulated by several different mechanisms.[186, 187] Among the several mechanisms involved, regulated exocytosis and proteolysis have been described for DCC.[299-303] A significant pool of axonal DCC resides within intracellular vesicles.[303, 304] It was demonstrated that netrin-induced depolarization of cortical neurons, and not spinal commissural neurons, lead to membrane insertion of DCC from these intracellular vesicles.[304] This depolarization-dependent plasma membrane insertion of DCC was dependent on PKA, PKC, PI3K, and the exocytosis machinery. Communication between neurons requires exocytosis of neurotransmitter-containing synaptic vesicles. This, in turn, depends on the assembly of the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex formed by Syntaxin-1 (Sytx1), synaptosomal-associated protein of 25 kDa (SNAP25 – t-SNARE), and vesicle-associated membrane protein 2 ((VAMP2)/Synaptobrevin – v-SNARE).[305]

Interestingly, developing neurons were shown to depend on the same molecular machinery to deliver guidance receptors to the growth cone plasma membrane. Specifically, DCC associates with Syntx1 and tetanus neurotoxininsensitive vesicle-associated membrane protein (TI-VAMP) in a netrin-1-dependent manner to mediate chemoattraction.[303] Furthermore, this SNARE-mediated exocytosis of DCC is suppressed by the interaction of TRIM9 with SNAP25.[303] Upon netrin-1 stimulation, TRIM9 releases SNAP25 to promote SNARE-mediated vesicle fusion to deliver DCC to the cell surface. Thus, TRIM9 is emerging as a master negative regulator of the netrin/DCC signaling pathway in the absence of netrin-1 by: 1) ubiquitinating Vasp,[275] 2) blocking DCC-FAK interaction and FAK activation,[276] and 3) blocking SNARE-mediated exocytosis of DCC.[303] In contrast, regulated endocytosis can switch a repulsive response to netrin-1 to an attraction by selectively removing UNC5 receptors from the cell surface.[306] As mentioned above, repulsion occurs in response to netrin-1 when DCC and UNC5 heterodimerizes. Endocytosis is accomplished through the activation of PKC downstream of another netrin receptor, the G protein-coupled Adenosine 2B receptor (A2b). Here, activation of protein kinase C (PKC) triggers the formation of a protein complex that includes UNC5H1, protein interacting with C-kinase-1 (Pick1), and PKC that specifically removes UNC5A from the growth cone cell surface leading to a switch in the response to netrin-1 from repulsion/growth cone collapse to attraction/growth cone expansion.[306] Importantly, PKC activation leads to colocalization of UNC5H1 with early endosomal markers.

1.4.6.2 - Proteolysis

Receptor proteolysis has been well characterized as a means to control the kinetics and quality of signal produced by receptor-ligand interaction. It can control pathway activation, the spatial distribution and number of receptors present at the plasma membrane at any given

time.[186, 187, 307] One of the first studies to demonstrate the role of cleavage of a guidance receptor as a means to attenuate the response to a guidance cue was ectodomain shedding of the netrin receptor DCC (deleted in colorectal cancer) by metalloproteases in response to netrin-1.[302] This cleavage by metalloproteases was shown to attenuate netrin-induced axon outgrowth. Metalloproteases are zinc-dependent enzymes of the Metzinccin superfamily.[308] They include the secreted matrix metalloprotease (MMP) and the transmembranous A Disintegrin and Metalloprotease (ADAM) families.[309] The disintegrin domain mediates interaction with cell surface receptors. In contrast to the negative regulation of netrin/DCC signaling, Kuzbanian/ADAM10 seem to play a positive role in Slit/Robo-mediated midline repulsion in Drosophila as Kuz mutants phenocopies the loss of Slit or Robo.[310] In addition, an increase in Robo1 expression was observed in Kuz mutant commissural axons. Kuz/ADAM10 also regulates the cleavage of both Eph receptors and ephrin-A2 ligands.[311] Ephrin-A2 binding to Eph receptors induces a conformational change that exposes a recognition sequence for ADAM10, resulting in cleavage of only ephrin-A2-bound to Eph and consequently converting an adhesion into repulsion.[312] In contrast, specific cleavage of Eph-B converts an ephrin-B/Eph-B adhesion into axon retraction. Thus, cleavage of both ligand and receptor is involved in converting an attraction (i.e. adhesion) into repulsion.[311]

Kuz was identified in Drosophila for its role in regulating Notch signaling in the context of neurogenesis.[313] Kuz-mediated shedding of Notch's extracellular ectodomain triggers γ-secretase intracellular cleavage of Notch, thereby releasing its intracellular domain (ICD) from the membrane and allowing it to translocate into the nucleus to regulate transcription.[314] This elegant mechanism mediated by γ-secretase cleavage of Notch during neurogenesis promotes heterogeneity in neuronal cell-types through lateral inhibition.[315] Differentiating cells inhibit

their neighbors from doing the same through a salt and pepper pattern of pro-neural versus proliferative gene expression.[316] Several studies have shown that following extracellular cleavage by metalloprotease, DCC gets cleaved intracellularly by presenilin-containing γ-secretase complex.[299-301] Evidence suggests that the DCC-ICD might be targeted to the nucleus following γ-secretase cleavage to regulate transcription similar to the Notch-ICD.[299] Specifically, it was shown that following DCC cleavage by γ-secretase, the intracellular domain was able to activate transcription of a reporter, suggesting a potential role for this cleavage event in regulating transcription.[299] Furthermore, the DCC drosophila homologue, Frazzled, was shown to be a required transcription factor for commissural axon guidance following γ-secretase cleavage.[317, 318] In addition, commissural neurons lacking presenilin acquire an inappropriate attraction to netrin-1 because of an accumulation DCC fragments at the plasma membrane and a corresponding insensitivity to Slit/Robo silencing.[301] Thus, γ-secretase cleavage of DCC might serve two functions: 1) clear the membrane of DCC fragments still capable of mediating downstream signaling and 2) create a DCC-ICD capable of shuttling into the nucleus and regulate transcription. In addition to DCC, several ephrin ligands and Eph receptors undergo a similar ADAM10/ γ-secretase sequential cleavage.[319-321] In contrast to the DCC-ICD shuttling to the nucleus, the ephrin-ICD binds to and activate Src family kinases and thus functions as a signaling molecule.[320] Regulated proteolysis has also been shown to prevent premature repulsion in pre-crossing commissural axons. Before reaching the midline, axons express both Nrp2 and Plexin-A1, two receptors for the repellant Sema3B, but Calpain-1-mediated Plexin-A1 cleavage prevents Semaphorin signaling to take place.[199] At the midline, secreted-glial cell line-derived neurotrophic factor (GDNF) inhibits calpain-1 activity through the cell adhesion molecule NrCAM, thereby enabling Sema3B/Nrp2/Plexin-A1-mediated repulsion out of the

midline.[322] Finally, it was shown that DCC gets cleaved by caspases in the absence of netrin leading the cell into apoptosis, thus highlighting the role of DCC as a dependence receptor. In chapter 3, we will show that a novel protease, calpain-1, already shown to play an important role in mediating Semaphorin/PlexinA1-mediated repulsion out of the spinal cord floor plate is also involved in regulating the netrin/DCC signaling pathway.

1.4.7 - Netrin-1-mediated cortical axon guidance

In terms of cortical axon guidance, netrin-1 is implicated in the guidance of all four major axonal tracts within the forebrain. Its receptor, DCC, is largely confined to projection neurons within the forebrain.[323] The first evidence that netrin plays a role in cortical outgrowth and guidance came from co-cultures of E15 rat neocortex explants with one of their intermediate target, the internal capsule.[324] In this scenario, cortical axon outgrowth was stimulated and directed toward the internal capsule. The same study confirmed the role of netrin-1 in attracting cortical axons. Around the same time a different group found that netrin-1 secreted from the ganglionic eminences promoted cortical axon outgrowth and guidance of corticofugal neurons.[325] Netrin-1 also plays a major role in attracting callosal axons. Following the attraction of pioneering axons toward the midline, netrin-1 secreted by midline glial structures equivalent to the spinal cord floor plate plays more of a secondary role in attenuating Slit/Robo-mediated repulsion in pre-crossing axons (Figure 1.14).[326, 327] Thus, the formation of forebrain commissures such as the corpus callosum, similar to spinal cord commissures, is largely dependent on netrin-1 signaling. Corticospinal projections are among the longest axons of the vertebrate CNS (Figure 1.15). It is the major output from the motor cortex that connects the cortex to the spinal cord (Figure 1.15).[328] Axon outgrowth and guidance of these axons happen almost exclusively after birth and reaches its final target around 3 weeks postnatally (Figure

1.14). Ventrally-derived netrin-1 diffuses from the ganglionic eminences to provide a chemoattractive gradient for pioneering corticofugal axons projecting to the intermediate zone where the majority of subcerebral projecting axons start their journey (Figure 1.15).[324, 325, 328] The internal capsule which also expresses netrin-1 serves as an intermediate target for both CST and corticothalamic axons where they separate from each other (Figure 1.14 & 1.15). CST axons then descend posteriorly through the midbrain and hindbrain toward the spinal cord (Figure 1.14). At the caudal-most part of the hindbrain they cross the midline dorsally, again with the help of netrin-1 expressed at the midline (Figure 1.15).[326, 328] After the initial guidance of pioneering corticospinal axons however, the cell adhesion molecules NCAM and L1 take over by mediating adhesion onto the already established axonal track of pioneering axons.[327] This midline crossing of CST axons is the reason why our limbs are controlled by the opposite side of our brain. Interestingly, a recent study comparing the CST in mice with a conditional deletion of DCC specifically in cortical neurons (Emx1-Cre;$DCC^{flx/flx}$) to mice with a germline DCC mutation ($DCC^{kanga/-}$) found that midline crossing happened normally in Emx1-Cre;$DCC^{flx/flx}$ mice.[329] In contrast, no axons crossed the midline in $DCC^{kanga/-}$ mice, indicating a non-cell-autonomous role of DCC during CST midline crossing since DCC was still expressed normally in the CST's trajectory in Emx1-Cre;$DCC^{flx/flx}$ mice.[329] The mechanism by which DCC functions to guide axons without being expressed at the growth cone cell surface is a novel idea that merits further investigation.

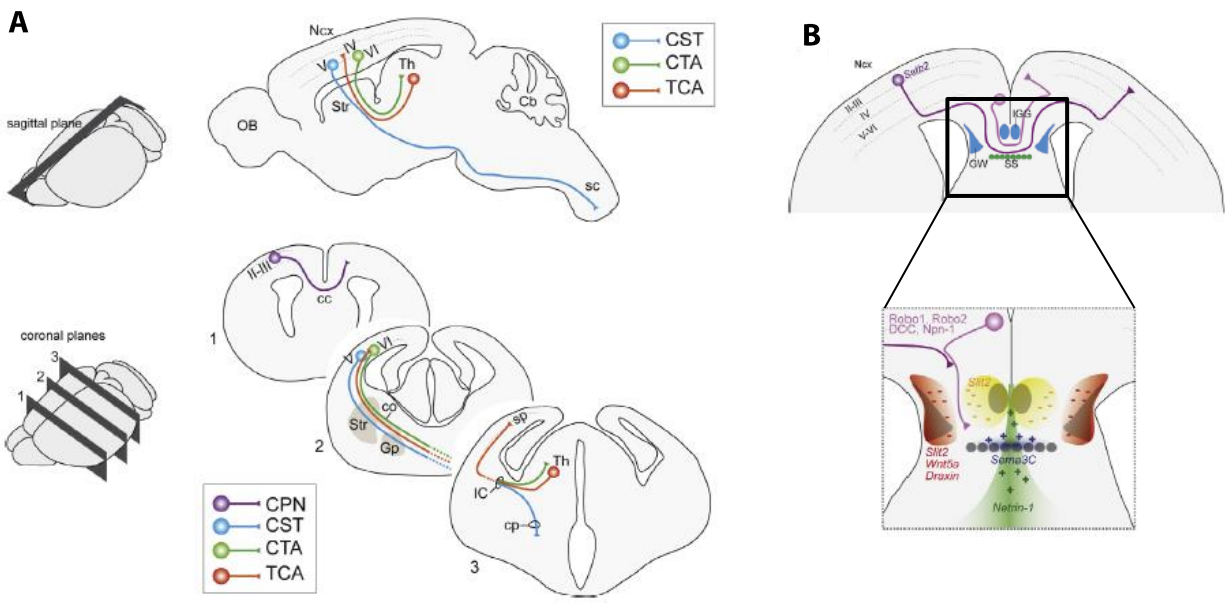


Figure 1.14: Cortical axon guidance

(A) Schematic representation of the four major forebrain axonal tracks. Callosal neurons (CPN) (purple) send their axons through the corpus callosum (CC) to the contralateral side; corticospinal track neurons (CST)(blue) project to the spinal cord via the internal capsule (IC); corticothalamic neurons (CTA)(green) follow a similar initial trajectory via the internal capsule towards the thalamus while thalamocortical neurons (TCA)(red) are born in the thalamus and project to the cortex. **(B)** Callosal neurons born in superficial layers (II/III) send their axons to the contralateral side through the corpus callosum. Inset, several guidance molecules guide these axons along the way such as Sema3C and netrin-1 expressed at the midline. Slit2, Wnt5a, and Draxin also participate in guiding axons toward the midline. Adapted with permission from *Neuroscience* 2013 (Leyva-Díaz & López-Bendito, 2013). Copyright © 2013 by Neuroscience.

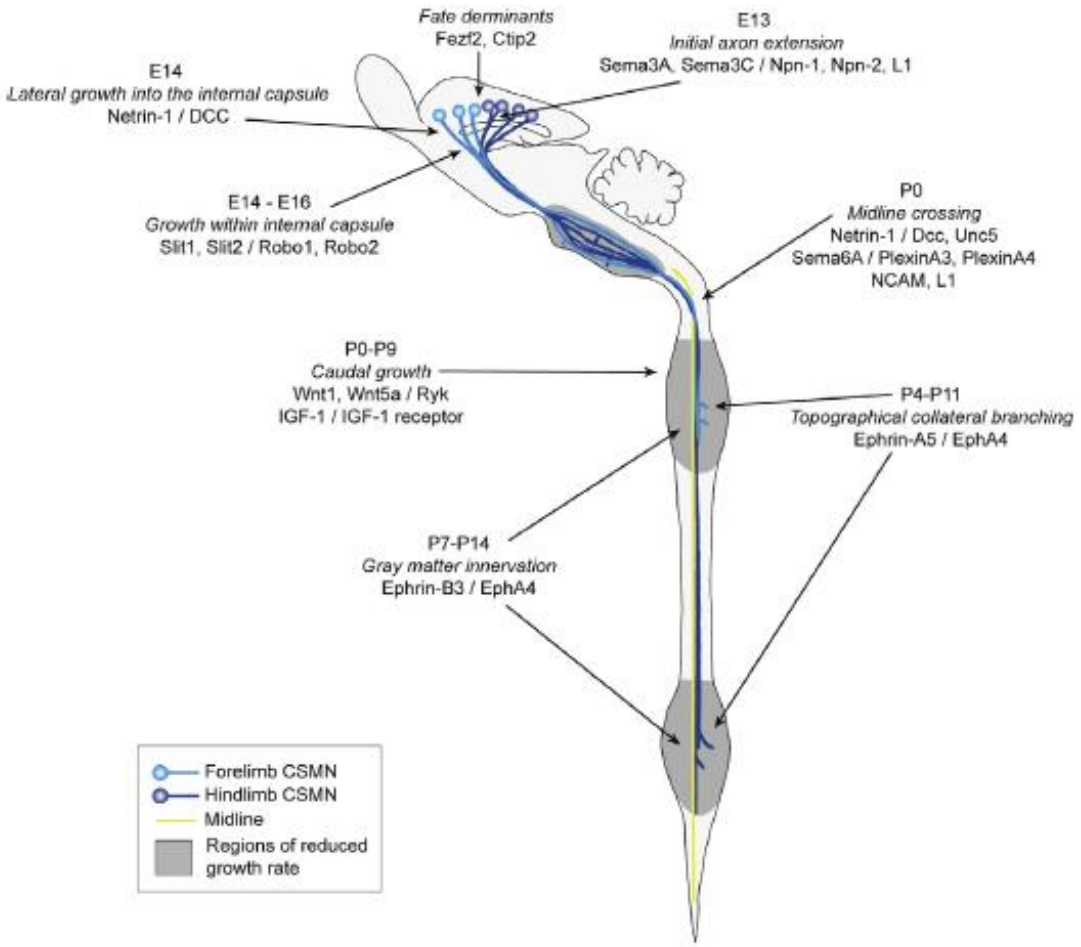


Figure 1.15: The long journey along the cortical spinal tract

Schematic representation indicating the major guidance decisions involved during corticospinal axon guidance from the motor cortex to the different targets within the spinal cord. Among the different molecules involved, netrin-1/DCC play important roles in guidaing axons toward the internal capsule as well as midline crossing. Adapted with permission from *Neuroscience* 2013 (Leyva-Díaz & López-Bendito, 2013). Copyright © 2013 by Neuroscience.

1.5 - Calpain

As previously mentioned, Calpain-1 has been implicated in commissural axon guidance by cleaving the Semaphorin receptor, PlexiA1.[199, 322] In chapter 3, we will demonstrate its role in regulating the netrin/DCC signaling pathway. There are 16 mammalian calpain genes with 14 encoding proteins with a cysteine protease domain (capn1-capn3, capn5-capn16) and the other two genes encoding regulatory subunits.[330, 331] Several isoforms are ubiquitously expressed while others display tissue-specific expression.[331, 332] Although generally thought of as intracellular proteases, some have reported active calpains in the extracellular space.[330, 333] The intracellular localization of calpains is complex and highly variable, suggesting that their subcellular localization might be dynamically regulated and therefore an important way to regulate their function. Typical calpains are characterized by penta-EF-hand motifs in their C-terminal calcium binding domain while atypical calpains lack this region (Figure 1.15).[330, 333] The ubiquitous calpains, calpain-1 and calpain-2, are the founding members of this family of proteases.[330] Paradoxically, they are commonly referred to by their in vitro requirements for calcium such that μ-calpain (calpain-1) require micromolar calcium concentrations while m-calpain (calpain-2) require millimolar concentrations whereas physiological intracellular calcium levels never even reaches 1μM.[330, 334] Only under pathological conditions such as apoptosis, axotomy, neurodegeneration, and ischemia do the calcium levels reach the in vitro requirements within the cell.[333] Thus, other regulatory mechanisms capable of lowering the calcium requirement in vivo have been the subject of intense investigations. Calpain-1/2 are the predominant calpains in the CNS, are ubiquitously expressed in the brain, and are referred to as modulatory proteases owing to their limited proteolysis of substrates leading to distinct functions for the cleaved products.[330, 335, 336] Their roles within the CNS are very diverse, ranging from

development,[199, 337-341] apoptosis,[342] synaptic plasticity,[335, 343-345] as well as in several pathologies such as Parkinson's disease,[335, 346] Alzheimer's disease,[347] Huntington disease,[348] and stroke.[336]

1.5.1 - Calpain regulation

Since intracellular calcium never reaches the levels required for activation in vitro, other means have been identified to compensate and lower the calcium requirement inside the cell. Much progress has been made in delineating the mechanisms involved in calpain regulation. Specifically, in the context of cell migration calpains play an important role in cell de-adhesion through cleavage of adhesion proteins.[333] For its role in cell migration, calpain has received much attention in the context of cancer metastasis.[349, 350] The ~80kDa Calpain1/2 large subunit requires heterodimerization with the small ~30kDa subunit (Figure 1.15), capns1, to form an active holoenzyme.[330] Autolysis of the large subunit lowers the calcium requirement but is suggested to function more in the progression of activation rather than its initiation.[333, 351, 352] Similarly, phospholipid and protein-protein binding lowers the calcium requirement.[353, 354] In addition, several phosphorylation sites control calpain activity, whether activating or inhibiting.[355, 356] Calpain-2 is activated by Erk1/2-mediated phosphorylation on Serine 50 even in the absence of calcium; a site that is absent in calpain-1.[356] Interestingly, FAK acts as a bridge between calpain-2 and ERK, thus promoting calpain-2 activation.[357] In contrast, PKA-mediated phosphorylation of calpain-2 is inhibitory.[355] Conversely, in mature neurons, calpain-1 is responsible for cleaving PHLPP1β, an inhibitor of ERK1/2, therefore indirectly activating ERK1/2.[358, 359] Finally, calpain activity is inhibited by their best-known interacting partner, the only known endogenous calpain inhibitor, calpastatin.[330, 333, 349] One calpastatin molecule can inhibit up to four calpain molecules.[360] Calpastatin preferentially binds activated and calcium-bound calpain and is therefore thought of as an attenuation mechanism.[333, 361, 362] Within the

CNS, calpain substrates include cytoskeletal proteins (e.g. spectrin, tubulin, tau, vinculin),[345, 363-368] synaptic/transmembrane proteins (glutamate receptors, calcium channels, amyloid precursor proteins), signaling proteins (e.g.PKC, CamKII, p35) and transcription regulators (e.g. p35, c-Jun, NFκB).[199, 335, 340, 343, 369-372] Specifically, cleavage of the αII-spectrin subunit, resulting in two breakdown products of ~150kDa and 145kDa, is temporally linked to calpain activation in a variety of cellular context.[77, 345, 364, 366, 373-375] Thus, assessing the state of the ~150kDa and 145kDa αII-spectrin breakdown products is a powerful tool to assess calpain activation.[364] No calpain consensus sequence has been found to have any significant value in predicting potential substrates.[333, 376] Therefore, calpains are thought to cleave their substrates in disordered regions between structured domains.

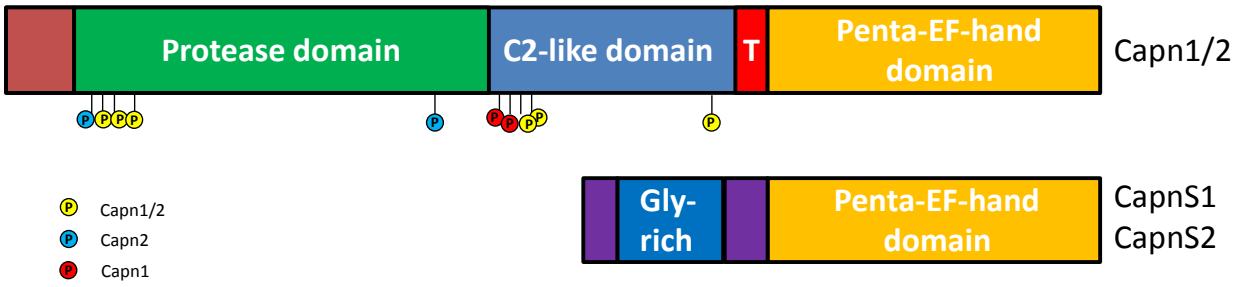


Figure 1.16: Domain organization of the classical calpains

The 80 kDa large subunits consist of a protease domain (green), a C2-like domain containing several phosphorylation sites, and four EF hands that participate in calcium binding as well as well as binding with the small subunit. The small 30 kDa subunit contain a highly flexible glycine rich region and four EF hands mediating interaction with the large subunits. The unique (blue & red) and common (yellow) phosphorylation sites are indicated.

1.5.2 - Calpain in axon outgrowth and guidance

An important signal that controls growth cone motility and therefore axon outgrowth and guidance is calcium.[284, 377, 378] In *Xenopus laevis* spinal cord, local Ca^{2+} within filopodia regulates growth cone motility through cleavage of two adhesion proteins, talin and FAK.[340, 378] Blocking calpain-mediated talin and FAK cleavage increased axon outgrowth in this system. Several growth factors have been shown to regulate calpain activity in neurons such as EGF, BDNF, and GDNF.[322, 339, 356, 379, 380] EGF was shown to activate calpain-2 through ERK activation while the role of BDNF seems to be context-dependent. In the context of learning and memory in mature cortical neurons, BDNF stimulates calpain activity[379, 380] whereas calpain activity is repressed during developmental axon branching.[339] Calpain is suggested to be involved in neurite consolidation by preventing excessive branching.[339] Branching factors such as BDNF, neurotrophin-3 (NT3), and netrin-1 inhibit calpain activity to promote branching.[339] Finally, although GDNF does not display any guidance properties for growing axons, it was shown to play an important role in promoting commissural axon exit out of the spinal cord midline.[322] In pre-crossing commissural axons, Sema3B receptors Nrp2 and Plexin-A1 are synthesized but calpain-1-mediated Plexin-A1 cleavage prevents it from being expressed at the growth cone cell surface.[199] This prevents premature midline repulsion. At the midline, secreted-GDNF inhibits calpain-1 activity through the cell adhesion molecule NrCam.[322] This initiates Sema3B-mediated repulsion and ensures a timely exit out of the midline. Finally, one study demonstrated the role of calpain downstream of another axon guidance molecule. Sema3A-mediated growth cone collapse in cultured hippocampal neurons was shown to be dependent on ERK-mediated calpain activation.[370] The role of calpain downstream of other guidance molecules will be interesting to investigate since many have the potential to influence calpain activity since they are expressed at the midline and throughout the axon's journey.

Rationale and objectives

This thesis examines the molecular mechanisms involved in regulating netrin/DCC-mediated axon outgrowth. The studies presented in chapter 2 were motivated by the discovery that phosphorylation of the conserved residue 1418 of DCC is required for netrin-1-mediated signaling and directed axon outgrowth.[206] In addition, the observation that the majority of DCC phosphorylation occurs on ser/thr residues led to the studies presented in chapter 4. Finally, the observation of several endogenous DCC fragments of low molecular weight led to studies presented in chapter 3. Here, the molecular mechanisms by which Y1418 phosphorylation regulates netrin/DCC signaling are explored via the characterization of a novel protein interaction with DCC. The role of a novel protease in netrin/DCC-mediated axon outgrowth as well as the role of thr 1210 phosphorylation is also explored.

In an effort to characterize novel players regulating this signaling pathway during rat cortical axon outgrowth, the specific objectives of the thesis are as follows:

Objective 1 (Chapter 2): Characterize the functional interaction between p120RasGAP and DCC downstream of netrin-1

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

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

Objective 2 (Chapter 3): Characterize the role of Calpain1/2 in netrin-DCC-mediated axon outgrowth

The observation presented in chapter 4 that calpastatin partially rescues the cleavage of a phospho-null DCC mutant (DCC-T1210V) as well as previous studies implicating calpain-1 in commissural axon guidance[199, 322] raised the possibility that some of the endogenous DCC fragments observed on SDS-PAGE was produced by calpain-1/2 cleavage. To address this possibility, we characterized the role of calpain-1/2 in netrin/DCC-mediated axon outgrowth.

Objective 3 (Chapter 4): Characterize the role of Thr 1210 phosphorylation during cortical axon outgrowth

The identification of four ser/thr phosphorylation sites led to the discovery of Thr 1210 and the observation of a DCC fragment when replaced with an alanine residue. This led to the hypothesis that Thr 1210 phosphorylation is essential for DCC stability. By introducing other mutations at this particular site and investigating their behavior in cell culture and primary neurons we characterized the role of this novel phosphorylation site.

Preface to Chapter 2

Post-translational modifications such as phosphorylation create binding sites that mediate dynamic protein-protein interactions. The phosphorylation of the conserved Y1418 residue of DCC is essential for netrin-1 signal transduction in the central vertebrate nervous system. The rationale of the study presented in Chapter 2 is to characterize the function of proteins that interact with DCC following the netrin-1-dependent phosphorylation of Y1418 in rat embryonic cortical neurons. Mass spectrometry has proven to be a valuable tool in the search for novel protein-protein interactions and was used here to search for rat embryonic brain proteins that interact with DCC upon the phosphorylation of Y1418. Members of the Ras subfamily of GTPases are essential regulators of cellular function and integrate signals from various cellular compartments, including signaling from plasma membrane receptors. It has been generally assumed that Ras activity mediates ERK activation downstream of netrin-1 and DCC. Yet, the regulation and the role of Ras activity have not been investigated in the context of netrin-1/DCC signaling. The study represents the first report of netrin-1-dependent Ras activation and proposes a mechanism for the role of p120RasGAP during netrin-1-mediated axon outgrowth and chemoattraction that includes Ras-dependent and Ras-independent functions. An edited version of this chapter was accepted for publication in 2016 by the Journal of Biological Chemistry.

Chapter 2 – p120RasGAP Contributes as a Scaffolding Protein to Regulate Netrin-1-Mediated Axon Outgrowth and Attraction in a GAP-dependent

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

To identify SH2-containing proteins that bind to the phosphorylated Y1418 (pY1418) residue of DCC, we screened an SH2 domain array using as bait a 15-amino acid (a.a.) synthetic DCC peptide comprising pY1418 (DCC-pY1418). Amongst an array of 46 SH2 domains, the N-terminal SH2 (N-SH2) domain of p120RasGAP (Fig. 2.1A) bound to the DCC-pY1418 peptide as revealed by a colorimetric enzyme-linked immunosorbent assay (ELISA). The N-SH2 domain of p120RasGAP displayed a 1.24- and 1.36-fold increase in absorbance relative to the GST control with 50 and 100 nM of DCC-pY1418 peptide, respectively (Fig. 2.1B).

To validate the interaction between the N-SH2 domain of p120RasGAP and DCC-pY1418, purified GST fusion proteins of the individual N-SH2 and C-terminal SH2 (C-SH2) domains of p120RasGAP (Fig. 2.1A and C) were tested for their ability to bind to immobilized DCC-pY1418 peptide in a dot blot assay (Fig. 2.1D). The N-SH2 domain was the sole domain capable of interacting with DCC-pY1418 and did not bind to a control spot of BSA (Fig. 2.1D). GST control protein did not bind to the DCC peptide or to BSA (Fig. 2.1D), and tyrosine phosphorylation of DCC-pY1418 was confirmed with a phosphospecific antibody raised against DCC-pY1418 (Fig. 2.1D). Thus, we conclude that the N-SH2 domain of p120RasGAP interacts directly with the synthetic DCC peptide via pY1418.

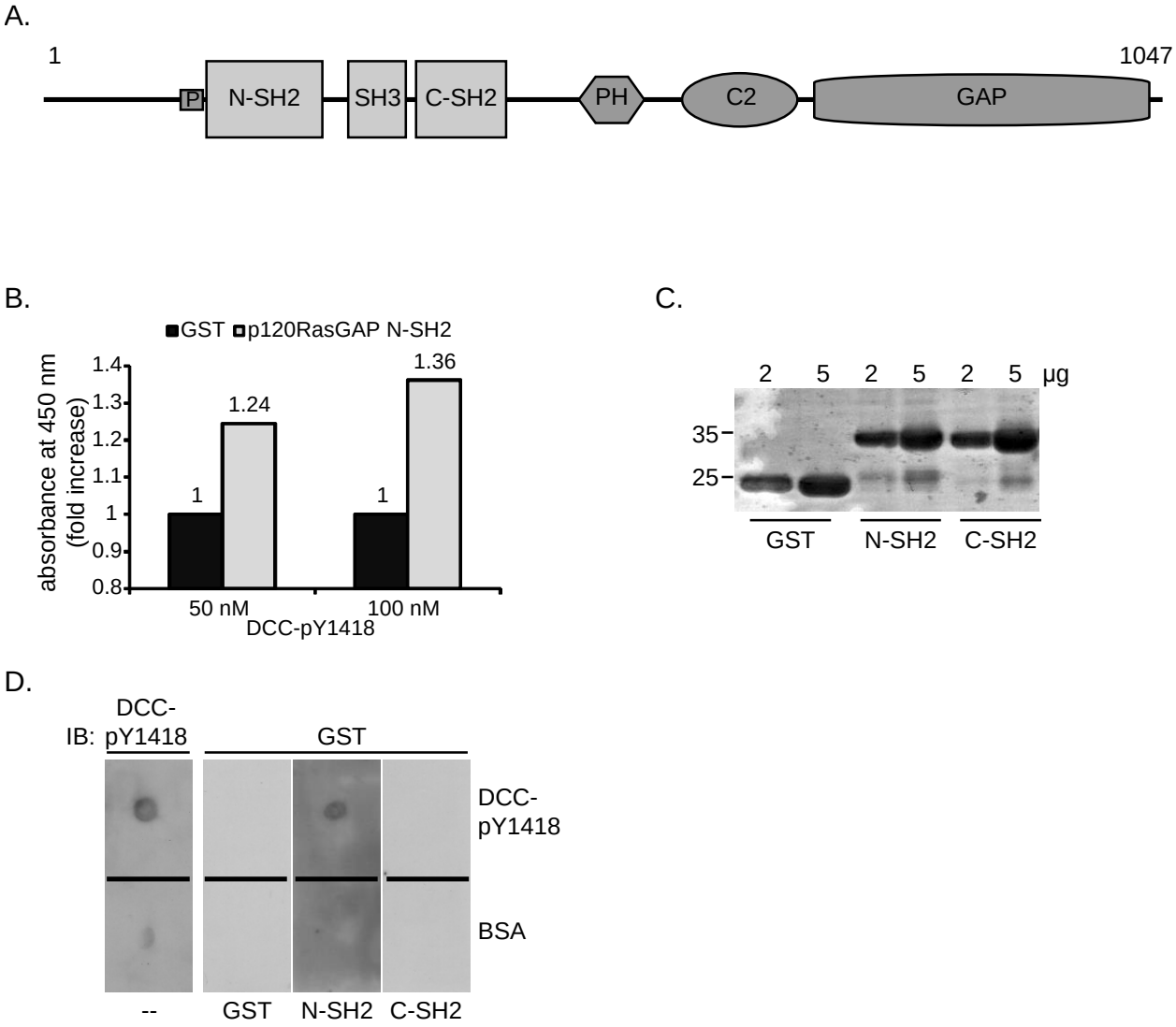


Figure 2.1: The N-terminal SH2 domain of p120RasGAP interacts in vitro with DCC via phosphorylated tyrosine Y1418

(A) p120RasGAP contains a proline-rich region (P), N- and C-terminal Src Homology 2 (N-SH2, C-SH2), Src Homology 3 (SH3), pleckstrin homology (PH), calcium-dependent phospholipid-binding (C2) and GTPase-activating protein (GAP) domains. (B) A synthetic DCC peptide containing phosphorylated Y1418 (DCC-pY1418) was used as bait to screen a SH2 domain array by ELISA. Binding of p120RasGAP N-SH2 with 50 and 100 nM of DCC-pY1418 peptide is represented as the fold increase in absorbance relative to the absorbance obtained with a GST control. (C) 2 and 5 ug of purified GST, GST-p120RasGAP N-SH2 and C-SH2 were resolved by SDS-PAGE and the proteins stained with Coomassie Blue. (D) The DCC-pY1418 peptide was spotted onto nitrocellulose membranes with BSA as a control, and each membrane was incubated with either purified GST, GST-p120RasGAP N-SH2 or C-SH2 (100ng/ml) followed by immunoblotting with anti-GST antibodies. One membrane was immunoblotted with phosphospecific anti-DCC-pY1418 (DCC-pY1418) antibodies (IB: immunoblot).

Netrin-1 promotes the association of p120RasGAP with DCC in embryonic cortical neurons

We next examined the interaction of p120RasGAP and DCC in dissociated embryonic day 18 (E18) rat cortical neurons, which are a good cellular model to investigate netrin-1/DCC-induced signaling pathways in the context of axon outgrowth and guidance [183, 273, 323-325, 381]. DCC and p120RasGAP co-immunoprecipitated and the interaction peaked after 10 minutes of stimulation with netrin-1 (Fig. 2.2A and B). Then, we evaluated the localization of DCC and p120RasGAP by immunostaining cortical neurons following netrin-1 stimulation. Visualization by confocal microscopy revealed that p120RasGAP and DCC were both present in the cell bodies, axons, and growth cones of cortical neurons (Fig. 2.2C). Netrin-1 increased the fluorescence intensity of p120RasGAP and DCC in the axons and growth cones (Fig. 2.2C). Quantification of the mean Pearson's correlation coefficient (r) revealed that the correlation between p120RasGAP and DCC fluorescence intensity was significantly increased in growth cones after 10 minutes of netrin-1 stimulation ($r=0.24$ versus $r=0.16$, $p=0.028$), while netrin-1 treatment resulted in no significant change of the co-association ($r=0.27$ versus 0.24, $p>0.05$) in whole cells (Fig. 2.2D). Altogether, we identified p120RasGAP as a novel DCC binding partner in embryonic cortical neurons, and we demonstrate that netrin-1 promotes the recruitment of p120RasGAP to DCC preferentially in growth cones.

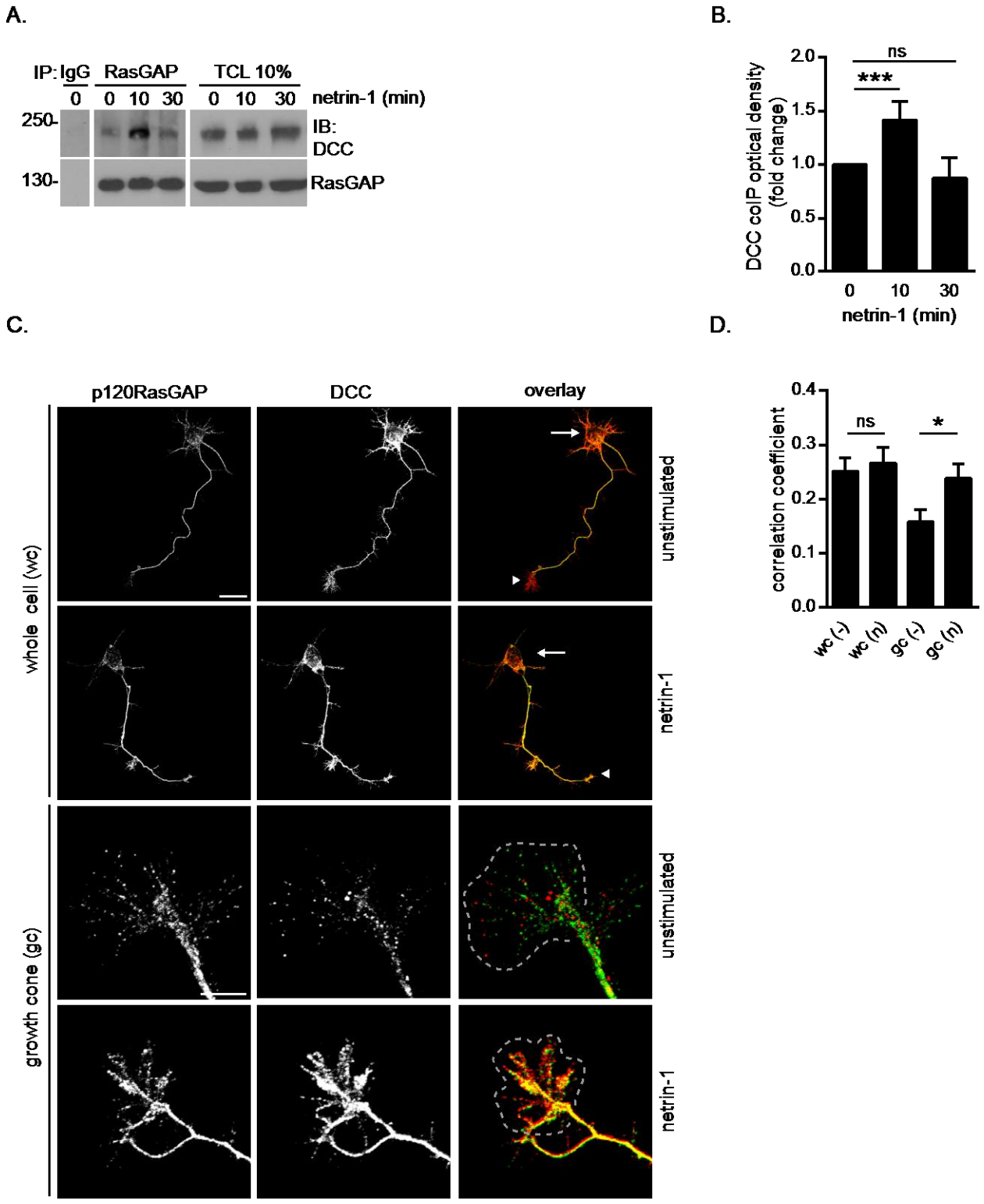

A.
IP: IgG
RasGAP
TCL 10%
0
0 10 30
0 10 30
netrin-1 (min)
250-
130-
IB: DCC
RasGAP
B.
DCC coIP optical density (fold change)
2.0
1.5
1.0
0.5
0.0
ns
***
0
10
30
netrin-1 (min)
C.
p120RasGAP
DCC
overlay
whole cell (wc)
growth cone (gc)
unstimulated
netrin-1
unstimulated
netrin-1
D.
correlation coefficient
0.4
0.3
0.2
0.1
0.0
ns
*
wc (-)
wc (n)
gc (-)
gc (n)

Figure 2.2 p120RasGAP interacts with DCC in netrin-1-induced embryonic cortical neurons

E18 embryonic rat cortical neurons were stimulated with netrin-1 (500 ng/ml) for the indicated times after being cultured 2 days in vitro (2DIV). (A) p120RasGAP was immunoprecipitated (IP) from cell lysates with anti-p120RasGAP antibodies or mouse immunoglobulin G (IgG) as control. IP proteins and total cell lysates (TCL) were resolved by SDS-PAGE and immunoblotted (IB) with antibodies against the indicated proteins. (B) Quantitative densitometry (±SEM) of DCC co-IP with p120RasGAP is represented as the fold change relative to 0 min of netrin-1 stimulation for at least three independent experiments. Unpaired Student's t test: ***$p<0.005$. (C) E18 embryonic rat cortical neurons (2DIV) were incubated with 500 ng/ml netrin-1 (n) or left unstimulated (-) for 10 minutes, immunostained with antibodies against DCC and p120RasGAP, and imaged by confocal microscopy. Arrows indicate cell bodies and arrowheads indicate growth cones. The gray dashed outlines represent the growth cones. Scale bars=50 μm (wc) and 20 μm (gc). (D) The correlation between DCC and p120RasGAP fluorescence intensities in (C) was measured with the Metamorph software using the Pearson's correlation coefficient (±SEM) in whole cells (wc) and growth cones (gc) in three independent experiments (number of neurons = 65, 51, 54, 40 from left to right). Unpaired Student's *t* test: ns, not significant; *$p=0.028$.

p120RasGAP associates with a DCC multiprotein signaling complex in netrin-1-stimulated cortical neurons

We next examined whether p120RasGAP is tyrosine phosphorylated in response to netrin-1. We observed that p120RasGAP was tyrosine phosphorylated in cortical neurons after 10 minutes of stimulation with netrin-1, concomitant with its association with DCC (Fig. 2.3A and B). Moreover, activated ERK (pERK) and FAK (FAK-pY397) co-immunoprecipitated with p120RasGAP and DCC in response to netrin-1 stimulation (Fig. 2.3A). Together, these results show that the assembly of a DCC-p120RasGAP protein complex with ERK and FAK is induced by netrin-1 in dissociated rat cortical neurons.

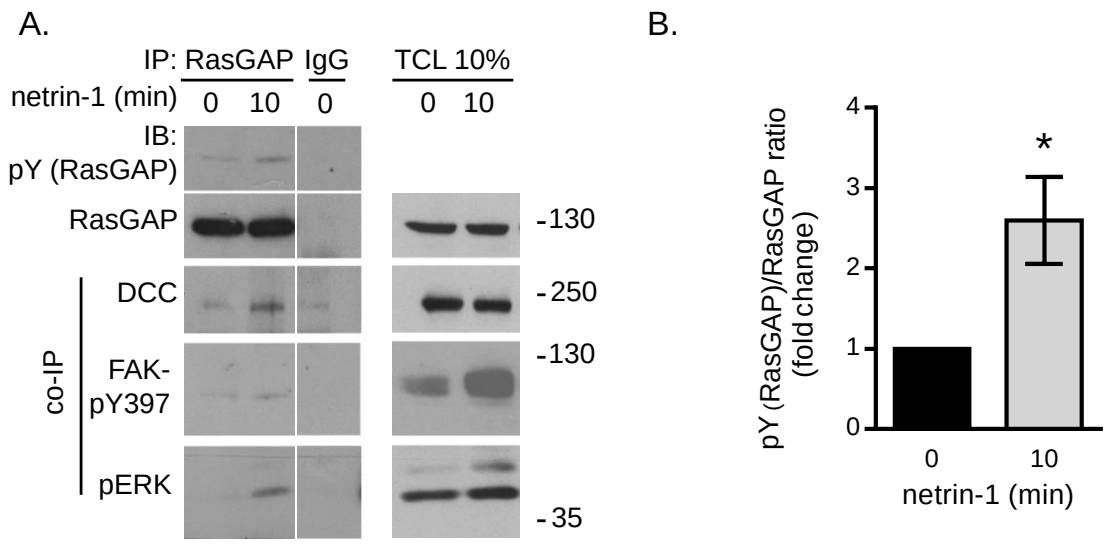


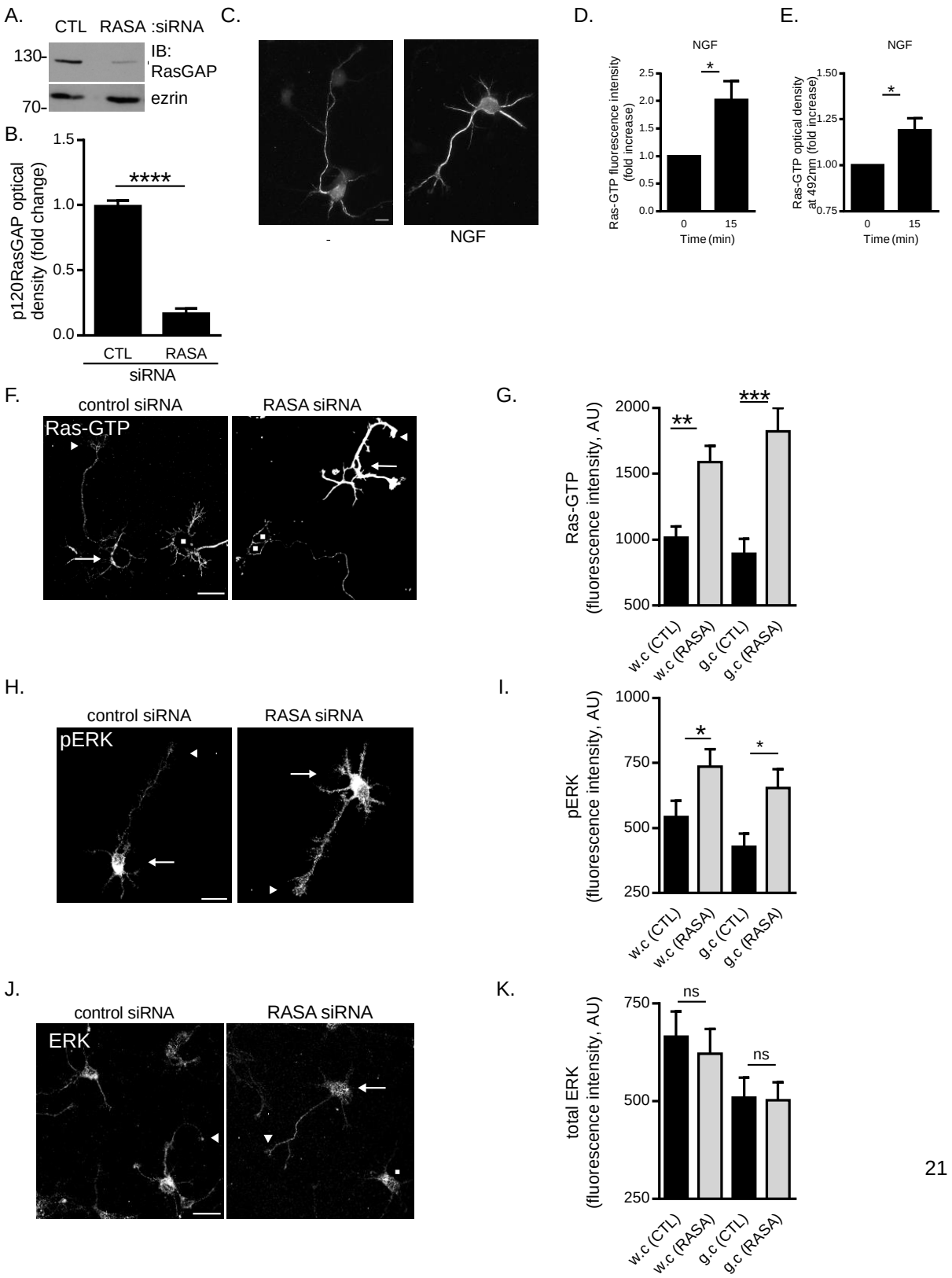
Figure 2.3 p120RasGAP associates with a DCC multiprotein complex in netrin-1-stimulated cortical neurons

(A) Cortical neurons were stimulated with netrin-1 for 10 minutes. p120RasGAP was IP from cell lysates with anti-p120RasGAP antibodies or mouse IgGs as control. IP proteins and total cell lysates (TCL) were resolved by SDS-PAGE and immunoblotted (IB) with antibodies against the indicated proteins (pY (RasGAP): anti-phosphotyrosine antibodies). Data were taken from the same film exposure. The white line between the RasGAP IP and IgG lanes is indicating that irrelevant lanes have been digitally removed from the original image. (B) Quantitative densitometry (±SEM) of pY (RasGAP)/RasGAP ratio is represented as the fold change relative to 0 min of netrin-1 stimulation for at least three independent experiments. Unpaired Student's t test: *$p<0.05$.

p120RasGAP is required to maintain basal Ras and ERK activities in cortical neurons

To further characterize the function of p120RasGAP in cortical neurons, endogenous p120RasGAP expression was downregulated in E18 rat cortical neurons by electroporating synthetic siRNA targeting the 5' end of p120RasGAP mRNA (RASA),[382] which led to a significant decrease of p120RasGAP expression compared to control siRNA (Fig. 2.4A and B). Then, we investigated the role of p120RasGAP, a negative regulator of Ras through its C-terminal GAP domain [383], in Ras activation in neurons by immunofluorescence using anti-Ras-

GTP antibodies and confocal microscopy (Fig. 2.4C). To validate the anti-Ras-GTP antibodies, we first monitor the levels of Ras-GTP in cortical neurons stimulated with nerve growth factor (NGF), a well-established activator of Ras in neurons [384, 385]. In cortical neurons stimulated with NGF for 15 minutes, we observed a significant 2-fold ($p<0.05$) increase in Ras-GTP fluorescence intensity (Fig. 2.5C and D), which was comparable to the activation of Ras detected by G-LISA assay in NGF-treated cortical neuron lysates (Fig. 2.5E). In unstimulated p120RasGAP-deficient cortical neurons, Ras-GTP levels showed a significant 1.56- ($p<0.005$) and 2.03-fold ($p<0.001$) increase in whole cells and growth cones, respectively (Fig. 2.4F and G). Next, we monitored the phosphorylation levels of ERK, a major signaling pathway activated downstream of Ras, by immunofluorescence and confocal microscopy in p120RasGAP-deficient neurons (Fig. 2.4H). We quantified the average fluorescence intensity of pERK at the plasma membrane of cortical neurons. In p120RasGAP-depleted neurons, pERK was significantly increased by 1.35- ($p<0.05$) and 1.53-fold ($p<0.05$) in whole cells and growth cones, respectively (Fig. 2.4I). No change in total ERK fluorescence intensity was detected in p120RasGAP-deficient neurons (Fig. 2.4J and K). Overall, p120RasGAP depletion in cortical neurons caused aberrant activation of Ras and ERK in whole cells and in the neuronal growth cones. Therefore, these results demonstrate the involvement of p120RasGAP for the proper regulation of basal Ras and ERK activities in cortical neurons.


A.
CTL
RASA :siRNA
130-
IB: RasGAP
70-
ezrin
B.
p120RasGAP optical density (fold change)
****
CTL
RASA
siRNA
C.
-
NGF
D.
NGF
Ras-GTP fluorescence intensity (fold increase)
*
Time (min)
E.
NGF
Ras-GTP optical density at 492nm (fold increase)
*
Time (min)
F.
control siRNA
RASA siRNA
Ras-GTP
G.
Ras-GTP (fluorescence intensity, AU)
**
***
w.c (CTL)
w.c (RASA)
g.c (CTL)
g.c (RASA)
H.
control siRNA
RASA siRNA
pERK
I.
pERK (fluorescence intensity, AU)
*
*
J.
control siRNA
RASA siRNA
ERK
K.
total ERK (fluorescence intensity, AU)
ns
ns

21

75

Figure 2.4 p120RasGAP is required to maintain basal Ras and ERK activities in cortical neurons

Control (CTL) or p120RasGAP (RASA) siRNA was electroporated in neurons at 0DIV with pGFP as a transfection reporter plasmid. (A) Total cell lysates were resolved by SDS-PAGE and immunoblotted (IB) with antibodies against p120RasGAP and ezrin as a loading control. (B) Quantitative densitometry of (A) is represented as the fold change (±SEM) relative to control siRNA measured in ten independent experiments. Unpaired Student's *t* test: ****p<0.0001. (C) Cortical neurons were stimulated with NGF (100 ng/ml) or left unstimulated (-) for 15 minutes. Neurons were immunostained with anti-Ras-GTP antibodies. Scale bar=10 μm. (D) Ras-GTP fluorescence intensity and the fold increase (±SEM) relative to unstimulated control neurons was measured in at least three independent experiments. (number of neurons > 50 per condition) Unpaired Student's t test: *p<0.05. (E) The levels of Ras-GTP in each cell lysate were evaluated by G-LISA assay by measuring the absorbance at 492 nm which is represented as the fold change (±SEM) relative to the unstimulated lysate (0 min) in at least three independent experiments. Unpaired Student's t test: *p<0.05. (F) Neurons were immunostained with anti-Ras-GTP antibodies. Arrows indicate cell bodies and arrowheads indicate growth cones of GFP-expressing neurons. Squares represent untransfected neurons. Scale bar=50 μm. (G) Ras-GTP fluorescence intensity (arbitrary units, AU; ±SEM) of GFP-expressing neurons in (F) was measured in whole cells (w.c) and growth cones (g.c) in three independent experiments (number of neurons=31, 32, 31, 32). Two-way ANOVA, Fisher's least significant difference (LSD) posttest: **p<0.0004, ***p<0.0001. (H) The neurons were immunostained with antibodies against pERK. Arrows indicate cell bodies and arrowheads indicate growth cones. Scale bars=50 μm. (I) pERK fluorescence intensity (arbitrary units, AU; ±SEM) of GFP-expressing neurons in (H) was measured in whole cells (w.c) and growth cones (g.c) in three independent experiments (number of neurons: 35, 36, 30, 35). Two-way ANOVA, Fisher's LSD posttest:*p<0.05. (J) Neurons were immunostained with antibodies against ERK. Arrows and arrowheads indicate the cell bodies and growth cones of GFP-expressing neurons. Squares indicate untransfected neurons. Scale bars=50 μm. (K) Total ERK fluorescence intensity (arbitrary units, AU; ±SEM) of GFP-expressing neurons in (J) was measured in whole cells (w.c) and growth cones (g.c) (number of neurons: 41, 38, 41, 38) in three independent experiments. Two-way ANOVA, Tukey's posttest: ns, not significant.

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

To determine whether p120RasGAP regulates netrin-1-dependent chemoattraction, we evaluated the impact of p120RasGAP depletion on cortical growth cone turning in response to a netrin-1 gradient using a Dunn chamber turning assay [207, 386, 387]. The growth cones of cortical neurons electroporated with control siRNA randomly turned with no particular preference for any direction when exposed to a control phosphate-buffered saline (PBS) gradient (Fig. 2.5A), but were attracted to a netrin-1 gradient (Fig. 2.5B). The introduction of p120RasGAP siRNA inhibited the attractive response to netrin-1 and growth cones reverted to turning randomly (Fig. 2.5C and D). 64% of the control growth cones were attracted to the netrin-1 gradient, whereas the percentage that turned towards the netrin-1 gradient in p120RasGAP-deficient neurons (46%) was similar to the percentage that turned towards PBS in control neurons (43%) (Fig. 2.5E). In fact, the turned angle of growth cones in response to netrin-1 (9.22° ± 4.03º: mean angle turned ± standard error of the mean (SEM)) was significantly reduced when p120RasGAP was depleted (-4.39° ± 3.86º) (Fig. 2.5F). p120RasGAP downregulation did not have an effect on displacement rates during the time the growth cones were imaged (Fig. 2.5G). These results demonstrate that p120RasGAP is required for netrin-1-dependent chemoattraction.

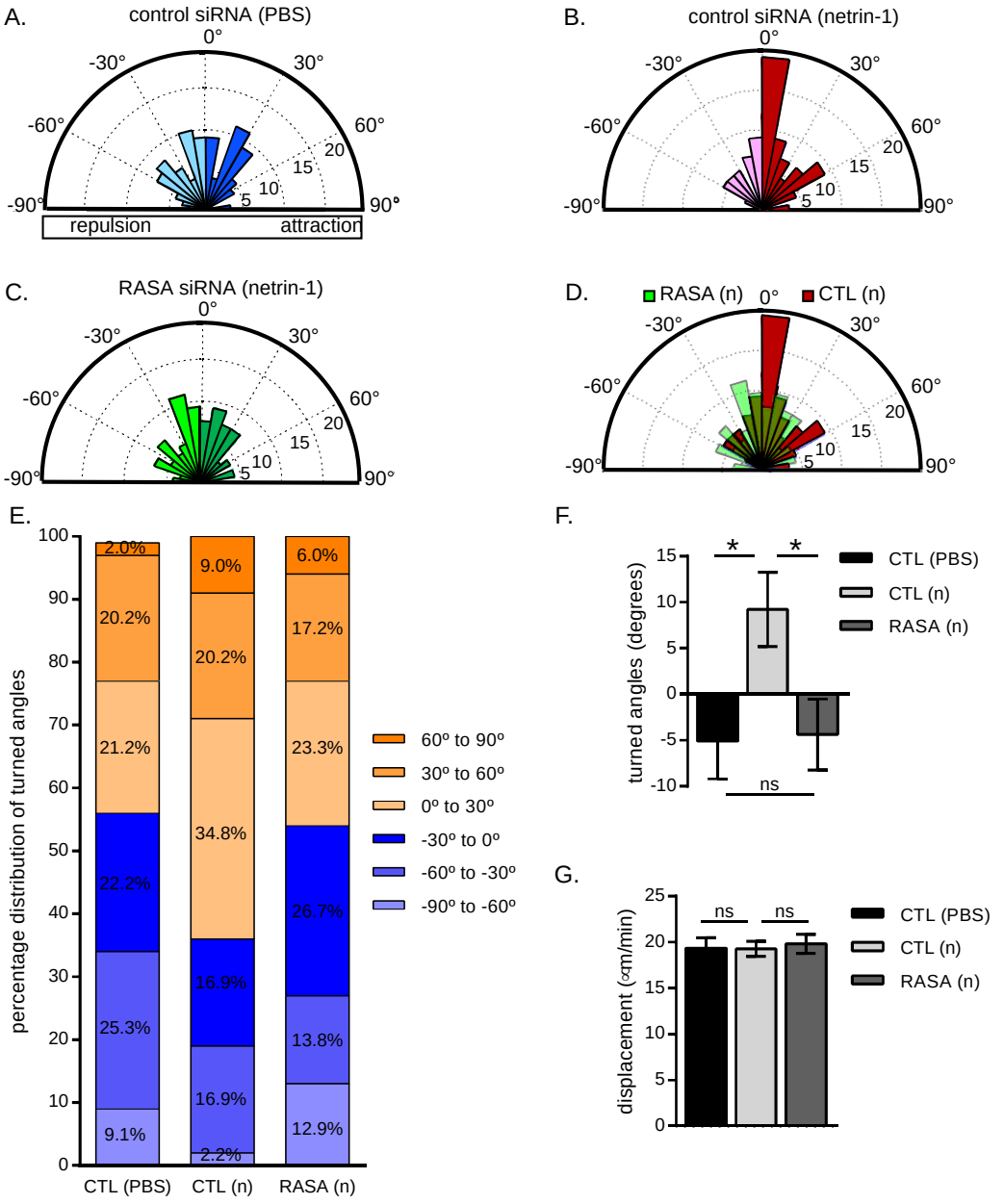

A.
control siRNA (PBS)
0°
-30°
30°
-60°
60°
-90°
90°
5
10
15
20
repulsion
attraction
B.
control siRNA (netrin-1)
C.
RASA siRNA (netrin-1)
D.
RASA (n)
CTL (n)
E.
percentage distribution of turned angles
2.0%
20.2%
21.2%
22.2%
25.3%
9.1%
9.0%
20.2%
34.8%
16.9%
16.9%
2.2%
6.0%
17.2%
23.3%
26.7%
13.8%
12.9%
60º to 90º
30º to 60º
0º to 30º
-30º to 0º
-60º to -30º
-90º to -60º
CTL (PBS)
CTL (n)
RASA (n)
F.
turned angles (degrees)
*
*
ns
G.
displacement (cm/min)
ns
ns

Figure 2.5. p120RasGAP is required for netrin-1-dependent attraction

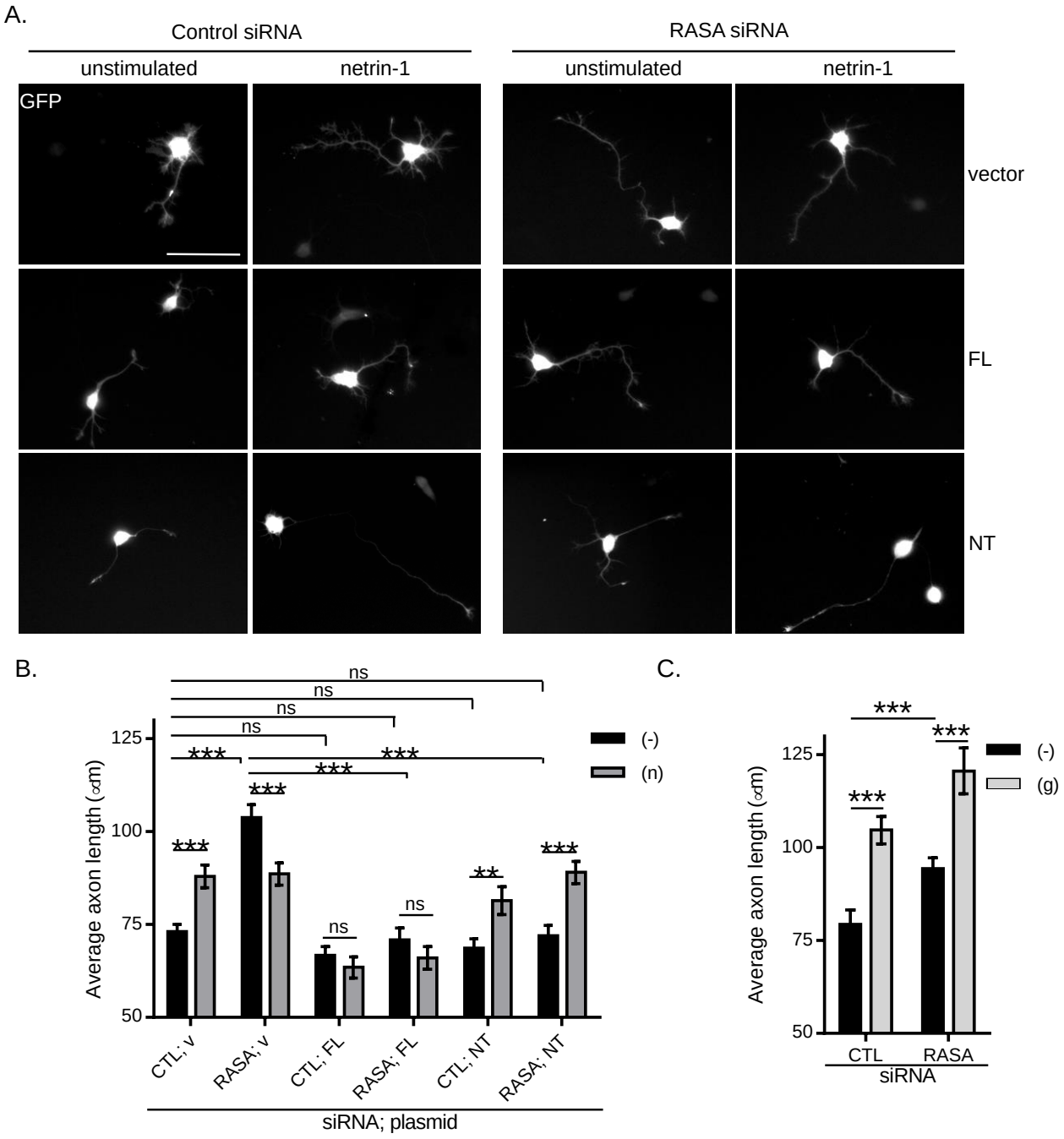
Control (CTL) or p120RasGAP (RASA) siRNA was electroporated with pGFP as a transfection reporter plasmid in E18 embryonic rat cortical neurons at 0 DIV. At 2 DIV, the neurons were exposed to a control PBS or a 200 ng/ml netrin-1 VI-V (n) gradient for 90 minutes. (A-C) Rose histograms represent the distribution of turned angles of cortical growth cones when exposed to a control PBS (A) or a netrin-1 gradient (B, C). Responses of individual neurons were clustered in 10° bins and the percentage of total neurons per bin is represented by the radius of each segment (number of neurons=99, 89, 116, respectively). (D) The overlay of the rose histograms presented in (B) and (C) is illustrated to compare the response to netrin-1 of control and p120RasGAP-deficient neurons. (E) The bar graph represents the turned angles percentage distribution of cortical growth cones in panels (A-C). (F) The mean turned angle (±SEM) toward the gradient was measured in degrees for each condition. (G) The mean displacement (±SEM) for a 90-minute netrin-1 treatment was calculated. One-way ANOVA, Fisher's LSD posttest: ns, not significant; *$p < 0.05$.

The N-terminus of p120RasGAP is sufficient to mediate netrin-1-dependent cortical axon outgrowth

We then explored the role of p120RasGAP in netrin-1-induced axon outgrowth in cortical neurons [183, 273]. p120RasGAP siRNA was electroporated together with GFP cDNA as a reporter to visualize the neurons. We measured the average axon length of GFP-expressing neurons after 24 hours of incubation with netrin-1. Netrin-1 significantly increased the average axon length compared to unstimulated control neurons (Fig. 2.6A, top panels and B), as previously described [183, 273, 381]. In unstimulated neurons, depletion of p120RasGAP resulted in significantly longer axons (103.8 μm, $p<0.0001$) (Fig. 2.6A, top panels and B), which correlated well with the higher levels of activated Ras and ERK detected in p120RasGAP-depleted neurons (Fig. 2.4). However, netrin-1 decreased the length of p120RasGAP-deficient neurons (88.6 μm, $p=0.0051$) (Fig. 2.6A, top panels and B). To assess the specificity of the p120RasGAP effects on axon extension, we analyzed the glutamate response in p120RasGAP-deficient neurons. Consistent with previous studies, glutamate stimulation increased axon outgrowth in cortical neurons and the effect was independent of p120RasGAP [183, 273] (Fig. 2.6C). Thus, the lack of stimulation in

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

To determine the domains within p120RasGAP responsible for the response to netrin-1, we expressed siRNA-resistant human full-length (FL) or the N-terminus domain (NT) of p120RasGAP in control or p120RasGAP-deficient neurons (Fig. 2.6A and B, and Fig. 2.7). Re-expression of p120RasGAP-FL restored the basal axon length in p120RasGAP-deficient neurons, while it inhibited netrin-1-induced axon outgrowth in both control and p120RasGAP-depleted neurons (Fig. 2.6A and B). In contrast, re-expression of the N-terminus (NT) of p120RasGAP lacking the GAP domain was sufficient to rescue netrin-1-induced cortical axon extension ($p<0.001$) in p120RasGAP-depleted neurons (Fig. 2.6A and B). Thus, the N-terminal scaffolding SH2-SH3-SH2 domains mediate the positive regulation of netrin-1-dependent axon outgrowth by p120RasGAP, while overexpression of p120RasGAP blocks the response of axons to netrin-1. This is in agreement with our findings that p120RasGAP is necessary to maintain basal Ras-GTP and pERK levels (Fig. 4), which is a requisite for axon extension [382].


A.
Control siRNA
RASA siRNA
unstimulated
netrin-1
unstimulated
netrin-1
GFP
vector
FL
NT
B.
ns
ns
ns
ns
***
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***
ns
ns
ns
125
100
75
50
Average axon length (∝m)
(-)
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CTL; v
RASA; v
CTL; FL
RASA; FL
CTL; NT
RASA; NT
siRNA; plasmid
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125
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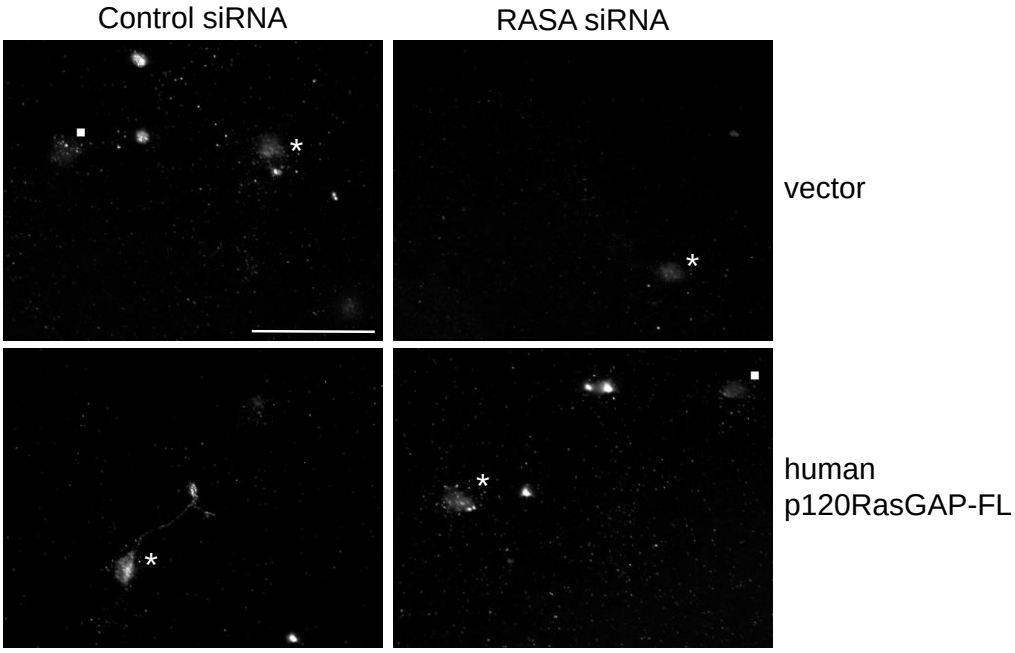
Average axon length (∝m)
(-)
(g)
CTL
RASA
siRNA

Figure 2.6. The N-terminus of p120RasGAP is sufficient to mediate netrin-1-dependent cortical axon outgrowth

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

A.



B.

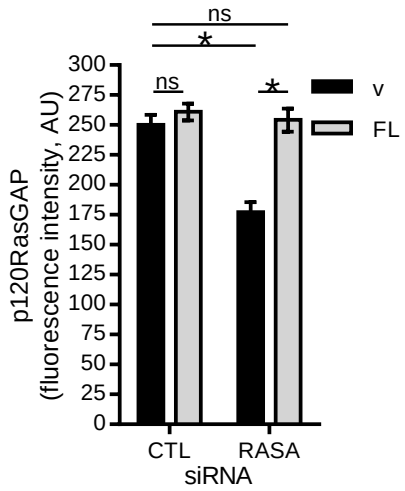


Figure 2.7. Human p120 RasGAP is expressed in p120RasGAP-deficient rat embryonic cortical neurons

Control (CTL) or p120RasGAP (RASA) siRNA was electroporated in E18 embryonic rat cortical neurons at 0 DIV with control vector or full-length (FL) human p120RasGAP with pGFP as a transfection reporter plasmid. (A) The cortical neurons from Fig. 6A were immunostained with anti-p120RasGAP antibodies. Asterisks indicate GFP-expressing neurons and squares indicate untransfected neurons. Scale bar=50 μm. (B) p120RasGAP fluorescence intensity (arbitrary units, AU; ±SEM) of GFP-expressing neurons in (A) was measured (number of neurons=38, 37, 45, 24). Two-way ANOVA, Tukey's posttest: * $p<0.0001$.

The N-terminus of p120RasGAP interacts with the C-terminus tail of DCC

Since the N-terminus of p120RasGAP mediates netrin-1-induced axon outgrowth, we next investigated the molecular interaction between the SH2-SH3-SH2 domains of p120RasGAP and DCC. GST-p120RasGAP domains were incubated with protein lysates from HEK293 cells overexpressing either wild-type DCC or DCC mutant proteins (Fig. 2.8A and B). DCC proteins did not bind to the GST protein control (Fig. 2.8C). p120RasGAP N-SH2 interacted with DCC, DCC-Y1361F, and with DCC 1-1421 lacking the P3 region (Fig. 2.8A and D). However, its binding to DCC 1-1327 truncated before the P2 region was strongly reduced (Fig. 2.8A and D). Surprisingly, DCC-Y1418F was still able to interact with p120RasGAP N-SH2 (Fig. 2.8D). Since FAK interacts with both the P3 region of DCC and p120RasGAP [265, 388, 389], we suspected that DCC-Y1418F might be indirectly pulled-down by p120RasGAP N-SH2 via an interaction with FAK (Fig. 2.8G). The phosphorylation of Y397 on FAK has been shown to mediate its interaction with the N-SH2 domain of p120RasGAP [389]. Indeed, GST-N-SH2 pulled down FAK-pY397 along with DCC and DCC mutant proteins (Fig. 2.8D), suggesting that p120RasGAP N-SH2 is also able to interact with DCC and FAK independently of DCC-Y1418. Furthermore, the

expression of either DCC 1-1421 or DCC 1-1327 severely impaired the phosphorylation of FAK on Y397 in total cell lysates, confirming that the P3 region of DCC is important for the phosphorylation of FAK on Y397 in HEK293 cells (Fig. 2.8B), as previously reported [166, 265]. Consequently, the expression of DCC 1-1421 or DCC 1-1327 impaired or completely abolished the interaction of FAK-pY397 with GST-N-SH2, respectively. It also suggested that phosphorylated Y1418 able to interact directly in vitro with p120RasGAP N-SH2 (Fig.1) may mediate the binding of DCC 1-1421 lacking the P3 region with GST-N-SH2 (Fig. 2.8D).

It has been previously shown that the 2 SH2 domains of p120RasGAP simultaneously bind to two adjacent tyrosine residues on binding partners such as p190RhoGAP and tyrosine kinase receptors [390-393]. Thus, we examined whether p120RasGAP C-SH2 could also interact with a phosphotyrosine residue in the intracellular domain of DCC. We selected Y1361 as a candidate binding site for the C-SH2 since it is the closest to DCC-Y1418 (Fig. 2.8A). GST-C-SH2 interacted with DCC, DCC-Y1418F, and DCC 1-1421 proteins, while its interaction with DCC 1-1327 and DCC-Y1361F was impaired (Fig. 2.8E), demonstrating that p120RasGAP C-SH2 preferentially interacted with DCC via the phosphorylated Y1361 residue. FAK-pY397 was also pulled-down indirectly with p120RasGAP C-SH2 via DCC or DCC-Y1418F interaction, but its interaction with DCC 1-1327, DCC-Y1361F, or DCC 1-1421 was severely reduced (Fig. 2.8E). Finally, the SH2-SH3-SH2 domains of p120RasGAP interacted with FAK-pY397 and DCC, DCC-Y1418F, -Y1361F, and 1-1421, but much less with DCC 1-1327 (Fig. 2.8F). Altogether, these data demonstrate cooperative binding of the N- and C-SH2 domains of p120RasGAP with the C-terminus region of DCC and FAK (Fig. 2.8G).

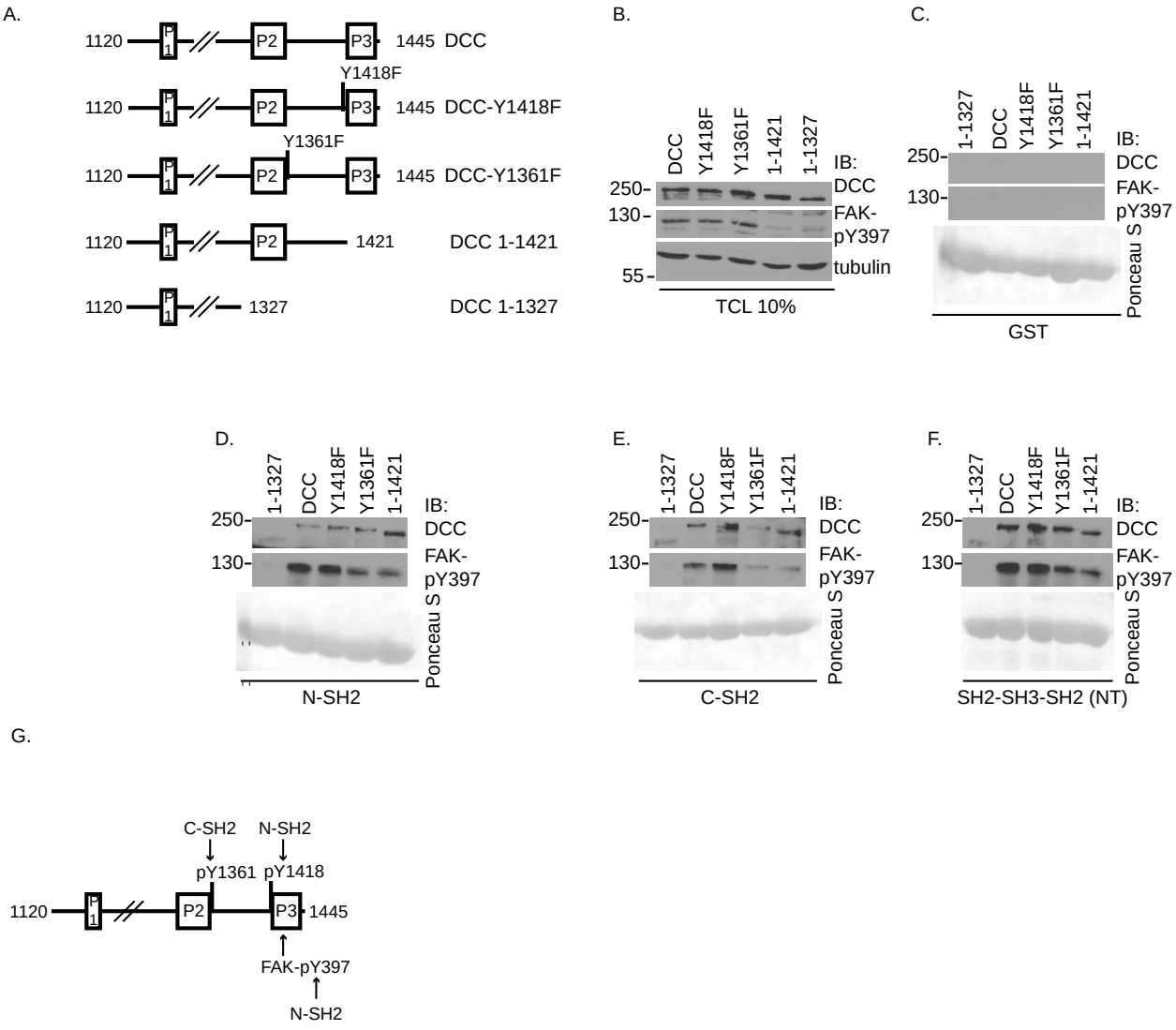


Figure 2.8. The N-terminus of p120RasGAP interacts with the C-terminus of DCC

(A) The intracellular domain of rat DCC (a.a 1120-1445) contains three conserved regions (P1, P2, P3). The conserved tyrosine residue in phosphodeficient mutants DCC-Y1418F and DCC-Y1361F was substituted for a phenylalanine residue. Truncation mutants DCC 1-1421 and 1-1327 are truncated before the P3 or the P2, respectively. (B) DCC, DCC-Y1418F, DCC-Y1361F, DCC 1-1421 and DCC 1-1327 were expressed in HEK293 cells. Proteins from cell lysates were pulled-down using purified GST control protein (C) or GST-p120RasGAP N-SH2 (D), C-SH2 (E), or SH2-SH3-SH2 (NT) (F). Associated proteins and total cell lysates (TCL) were resolved by SDS-PAGE and immunoblotted (IB) with antibodies against DCC, FAK-pY397 and tubulin. GST fusion proteins were stained with Ponceau S. (G) The N-SH2 and C-SH2 domains of p120RasGAP interact with phosphorylated (p) Y1418 and Y1361, respectively. Alternatively, the N-SH2 can also interact with FAK-pY397 and DCC independently of DCC-Y1418.

Preface to Chapter 3

The netrin-1 receptor, DCC, is a substrate for several proteases such as metalloproteases, γ-secretase, caspases and others not yet identified. The increase in DCC cleavage observed upon netrin-1 stimulation prompted us to investigate the nature of this cleavage and characterize its role in the context of cortical axon outgrowth. The identification of calpain-1 as a protease cleaving DCC in response to netrin-1 initiated the studies presented in chapter 3. Calpain's role in neurons has been well-characterized in the adult. However, few studies looked into calpain's role during nervous system development. This is the first report demonstrating a role for calpain-1/2 downstream of netrin-1 stimulation. This is also the first report characterizing calpain-1/2's role in mammalian cortical axon outgrowth during CNS development. The results presented in chapter 3 will be submitted for publication shortly.

Chapter 3 - A role for the calcium-activated protease calpain in the regulation of netrin-1/DCC-mediated cortical axon outgrowth and guidance

Calpain-1, Calpain-2, and Calpastatin are co-expressed with the netrin-1 receptor DCC during cortical axon outgrowth

Since the two major calpain isoforms, calpain-1 and calpain-2, have previously been shown to play an important role in cortical synaptic plasticity in mature neurons[335, 343, 344] and some evidence suggests a function in axon outgrowth and branching,[339, 340, 370, 378, 394] we first examined their subcellular expression along with their endogenous inhibitor, calpastatin in dissociated embryonic cortical neurons. We decided to look at early stages of neuronal development when axon specification and outgrowth occurs in dissociated cortical neurons (days in vitro (DIV) 1-10)[12, 13, 66, 68] (Figure 3.1). Interestingly, calpain-1, -2, and calpastatin expression was detected along with DCC throughout neuronal development during axon outgrowth (DIV1-3), branching (DIV3-5), and dendritic spine maturation (DIV7-10) (Figure 3.1). We found that calpain-1 was the major isoform expressed at DIV2 in the axon and to a lesser extent in the growth cone (Figure 3.2). However, its expression decreased during neuronal development with a lower expression at DIV7 (Figure 3.2B). In contrast, lower calpain-2 expression was detected at DIV2, which correlated with lower active-ERK1/2 (Figure 3.2B), although its expression increased as neurons matured in cell culture showing a higher expression at DIV7 when axon branching and dendritic spines are more developed, similar to DCC expression and active-ERK1/2 (Figure 3.2B). In contrast to calpain-1, calpastatin expression was highly enriched within the neuronal growth cone (Figures 3.1 & 3.2). Calpastatin was expressed in the axon shaft and at the periphery of the growth cone (Figure 3.2, top panel inset) whereas calpain-1 was expressed along the axon shaft but limited to the central region of the growth cone (Figure 3.1B,

middle panel inset). These results suggest that calpain-1 might be active in the growth cone, though calpastatin may be limiting its activity within the peripheral region of the growth cone to promote axon outgrowth. Finally, we also found that calpain-1/2 and calpastatin were expressed in the DCC-positive cortical axonal tracks within the developing cortex at embryonic day 17.5 (Figure 3.2C). Taken together, these results show that both calpain-1 and calpain-2 isoforms, together with their endogenous inhibitor calpastatin, are expressed during cortical development, which overlaps with the expression of the netrin-1 receptor DCC.

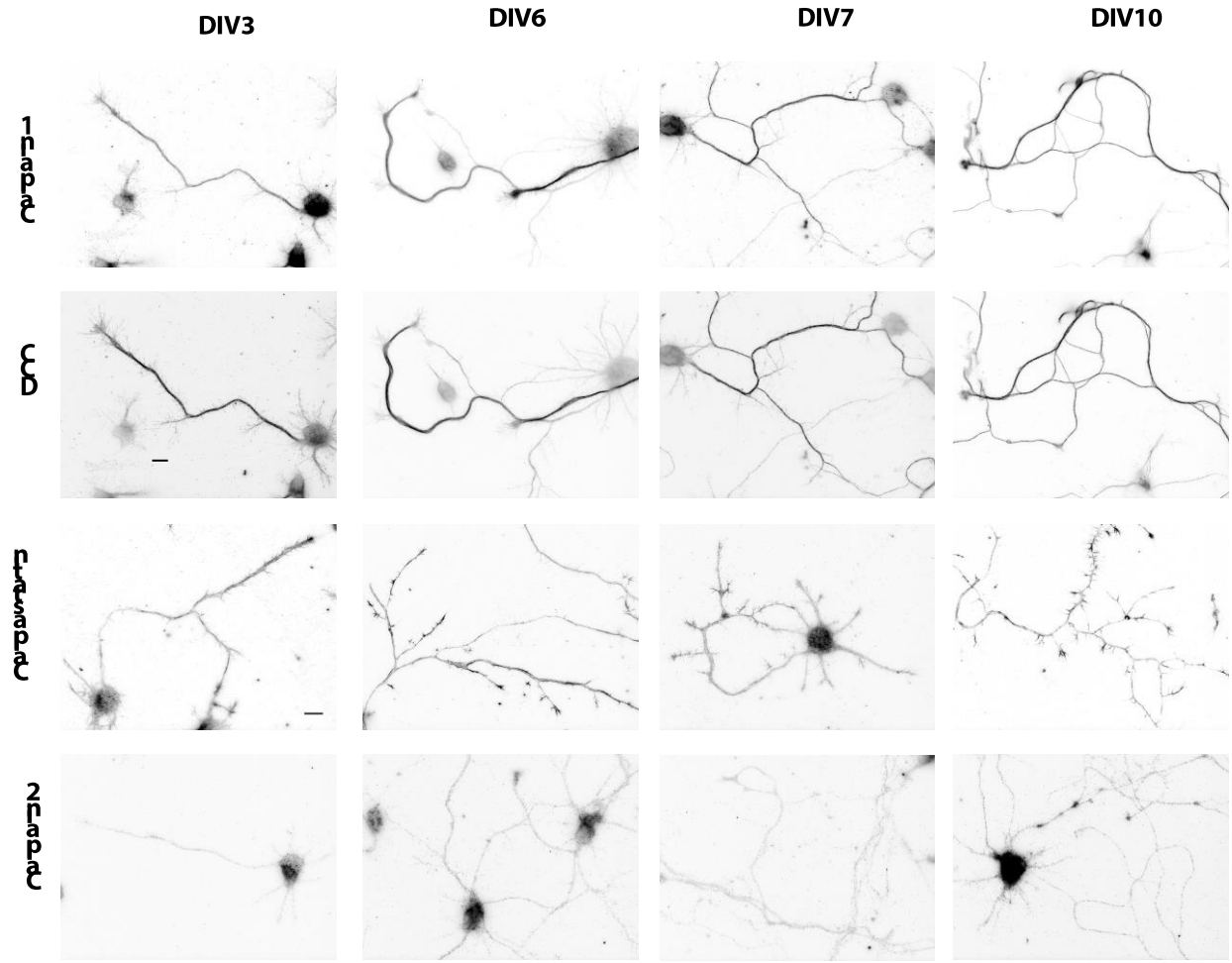
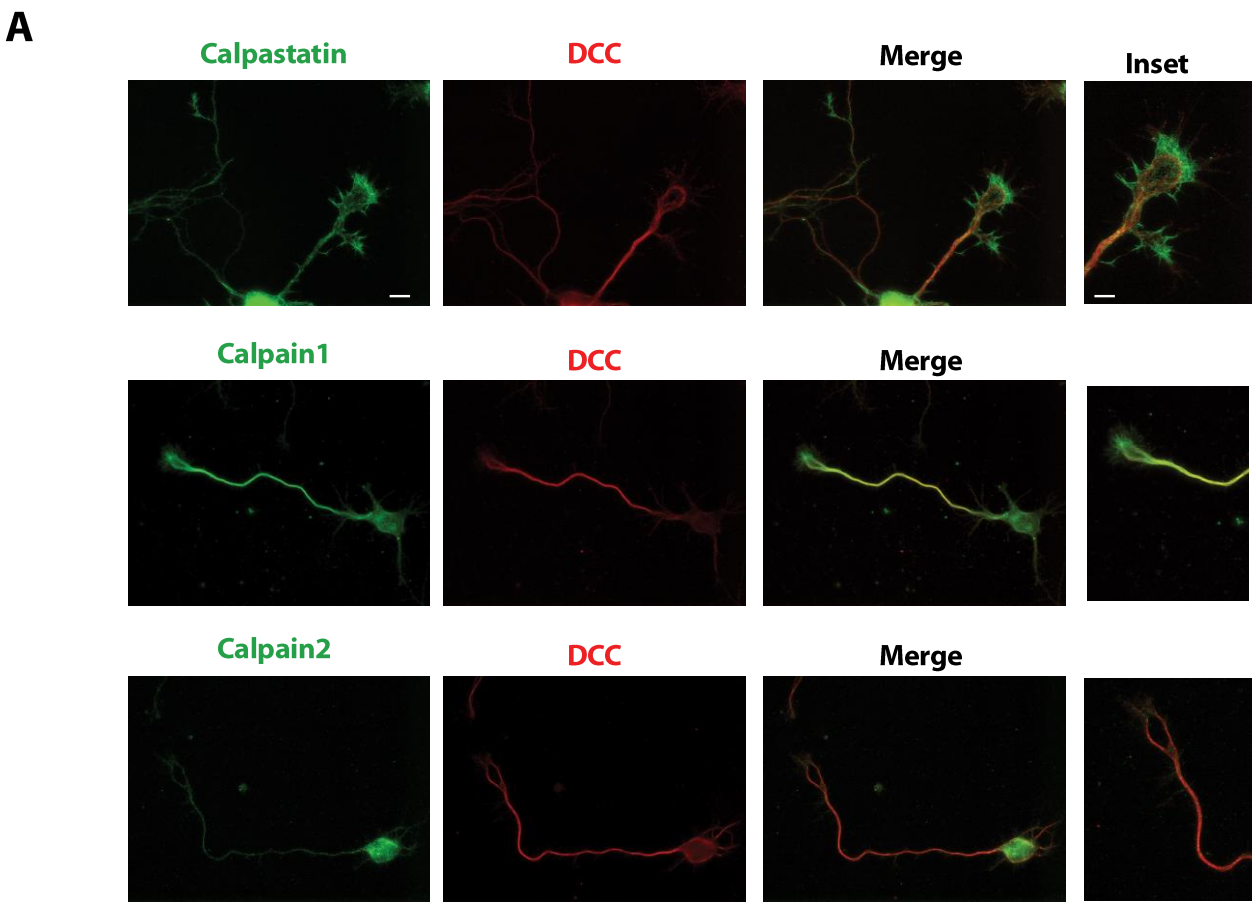
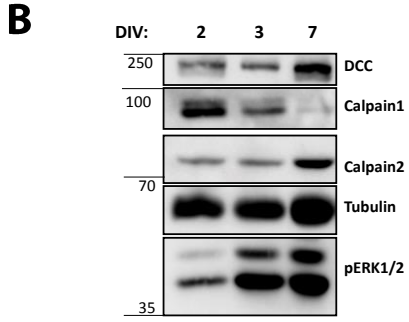


Figure 3.1: Expression of calpain-1, calpain-2, calpastatin and the netrin receptor, DCC during cortical neuron development in vitro

Dissociated E18 rat cortical neurons were plated at DIV0 and fixed at different DIVs. Calpain-1 (top panel), DCC (top-middle panel), calpastatin (bottom-middle panel), and calpain-2 (bottom panel) expression were examined at DIV3, DIV6, DIV7, and DIV10 by immunofluoresecence. Scale bar=10 μm


A
Calpastatin
DCC
Merge
Inset
Calpain1
DCC
Merge
Calpain2
DCC
Merge


B
DIV:
2
3
7
250
100
70
35
DCC
Calpain1
Calpain2
Tubulin
pERK1/2

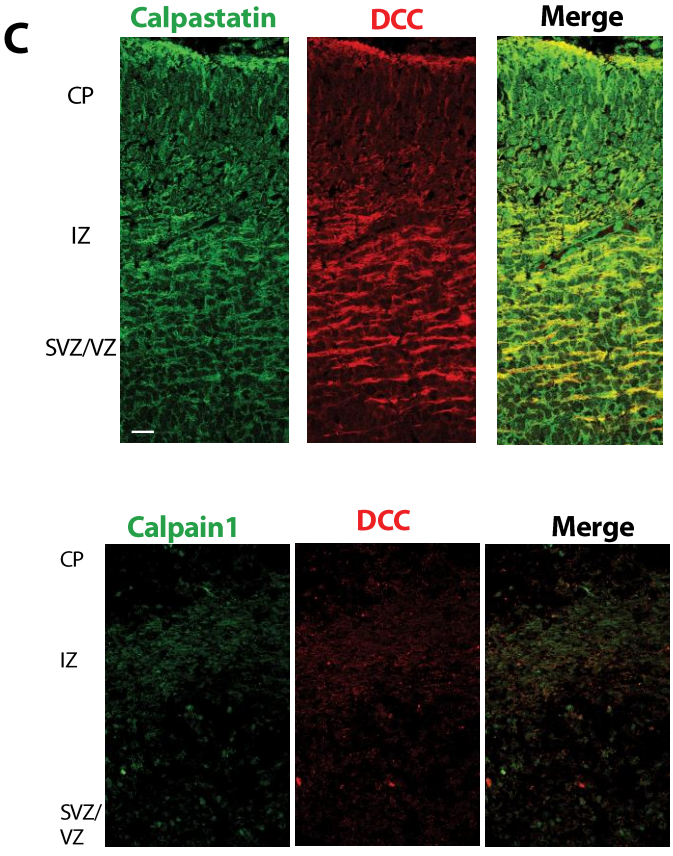

C
Calpastatin
DCC
Merge
CP
IZ
SVZ/VZ
Calpain1
DCC
Merge
CP
IZ
SVZ/
VZ

Figure 3.2: Expression of calpain-1, calpain-2, calpastatin and the netrin receptor, DCC, have distinct but overlapping subcellular localization during cortical axon outgrowth at 2 days in vitro (DIV2).

(A) E18 rat cortical neurons were fixed at DIV2 and immunostained with the indicated antibodies and visualized with fluorescent microscopy. Inset show a closer view of the growth cones. (B) Cortical neuron lysates were lysed and loaded on SDS-PAGE for western blot analysis with the indicated antibodies. Each lane represents different stages of development in vitro (DIV3, DIV7, and DIV10). (C) E17.5 rat cortices were fixed and immunostained with the indicated antibodies on coronal sections. scale bar = 10 um; Inset scale bar=5 um; scale bar in (C)=20 um.

Netrin-1 differentially regulates the localization of calpain-1 and calpastatin in growing cortical axons

Because it was previously shown that netrin-1 stimulation affects DCC expression within the growth cone and at the cell surface;[272, 303, 381] we next assessed whether netrin-1 regulates the localization of calpain-1/2 and calpastatin in dissociated cortical neurons by indirect immunofluorescence. Neurons were treated with netrin-1 over a 30-minute period, and then fixed and stained with antibodies against calpain-1, -2, and calpastatin. We observed a rapid and transient change in the expression/localization of calpain-1 and calpastatin but not calpain-2 upon netrin-1 stimulation (Figure 3.3). Calpain-1 was significantly increased along the axon shaft after 5 and 15 minutes of stimulation (Figure 3.3A, middle panel, and Figure 3.3B). On the other hand, calpastatin was significantly decreased in the growth cone after 5 minutes of netrin-1 stimulation, which was recovered to basal levels after 15 minutes (Figure 3.3A, top panel, and Figure 3.3B). Therefore, these data demonstrate that netrin-1 differentially influences calpain-1 and calpastatin expression in extending cortical axons.

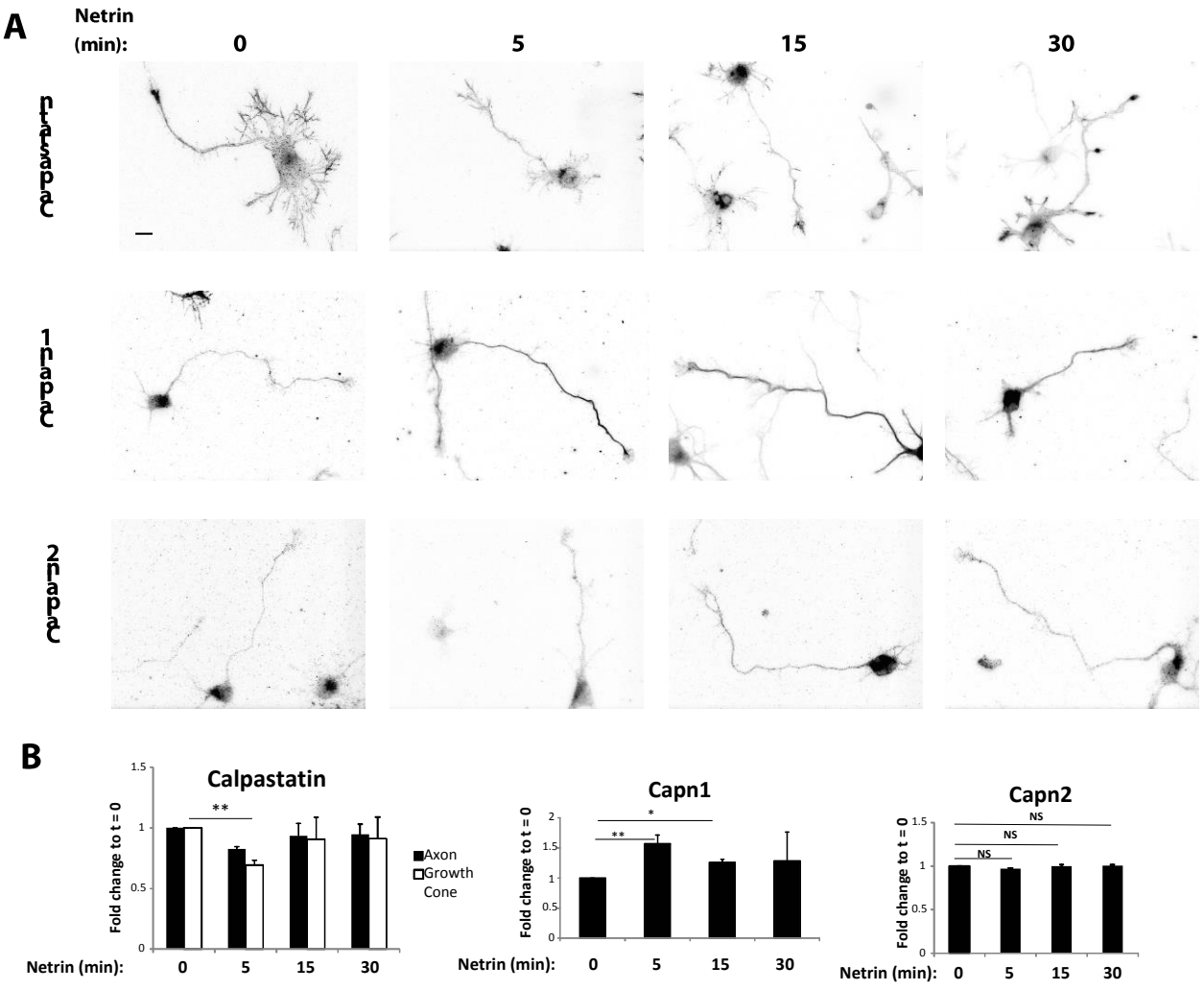


Figure 3.3: Netrin-1 stimulation increases calpain-1, but not calpain-2, expression while decreasing calpastatin expression in axons and growth cones

(A) Dissociated E18 rat cortical neurons were stimulated at DIV2 for the indicated time. Expression of calpain-1/2 and calpastatin were examined following netrin-1 stimulation. Pixel intensity was analyzed and quantified in (B). scale bar = 10 um. Unpaired Student's t test: $*p < 0.05$ $**p < 0.01$.

Netrin-1 activates calpain in cortical neurons and stimulates the cleavage of cytoskeletal proteins in a calpain-dependent manner

Netrin-1 stimulation is known to activate focal adhesion kinase (FAK),[165, 166, 206] the extracellular signal-regulated kinases (ERK1/2),[279, 395] the small GTPase Rac1,[183, 266, 270, 271] and the ezrin-radixin-moesin (ERM)[273] proteins within 5 minutes of treatment in cortical neurons. Because this activation of the netrin-1/DCC signaling pathway correlates with the changes in the localized expression of calpain-1 and calpastatin, we next determined whether netrin-1 regulates calpain activity in cortical neurons. To assess this, we took advantage of one of the well-characterized calpain substrate: α-spectrin, which migrates specifically as a doublet near 150 kDa upon calpain cleavage.[364] The levels of this doublet can be used as a means to measure calpain activity within whole cell lysates. Cortical neurons were treated with netrin-1 for 5 minutes and we determined the levels of calpain-mediated α-spectrin cleavage by western blot. We detected a 4-fold increase in the calpain-specific α-spectrin cleavage (Figure 3.4A and B). In addition, we also observed an increase in FAK cleavage, another well-known substrate of calpain, following 5 minutes of netrin-1 stimulation (Figure 3.4A and B). Both cleavage of spectrin and FAK were abolished in the presence of calpastatin, confirming that the observed cleaved products were calpain-specific (Figure 3.4A and B). To confirm that netrin-1 stimulation activated two of its known targets, we looked at the phosphorylation status of both FAK and ERK1/2 using phospho-specific antibodies as a readout of the activated netrin/DCC signaling pathway.[166, 180, 279, 381] Indeed, phosphorylation of both FAK and ERK1/2 were increased within 5 minutes of netrin-1 stimulation (Figure 3.4A). Interestingly, calpastatin treatment led to a reduction of ERK1/2 activation upon netrin-1 stimulation (Figure 3.4A). To determine the effect of netrin-1 stimulation and measure calpain activity in real time we incubated live cortical neurons with a

cell-permeable calpain substrate that emits a fluorescent signal only upon calpain cleavage (Figure 3.4C).[396] Within minutes of adding netrin-1 to the media we observed a significant increase in the fluorescence compared to the vehicle control (Figure 3.4D and E). Thus, netrin-1 stimulation activates calpain leading to the cleavage of cytoskeletal-associated proteins in cortical neurons concomitantly with FAK and ERK1/2 activation.

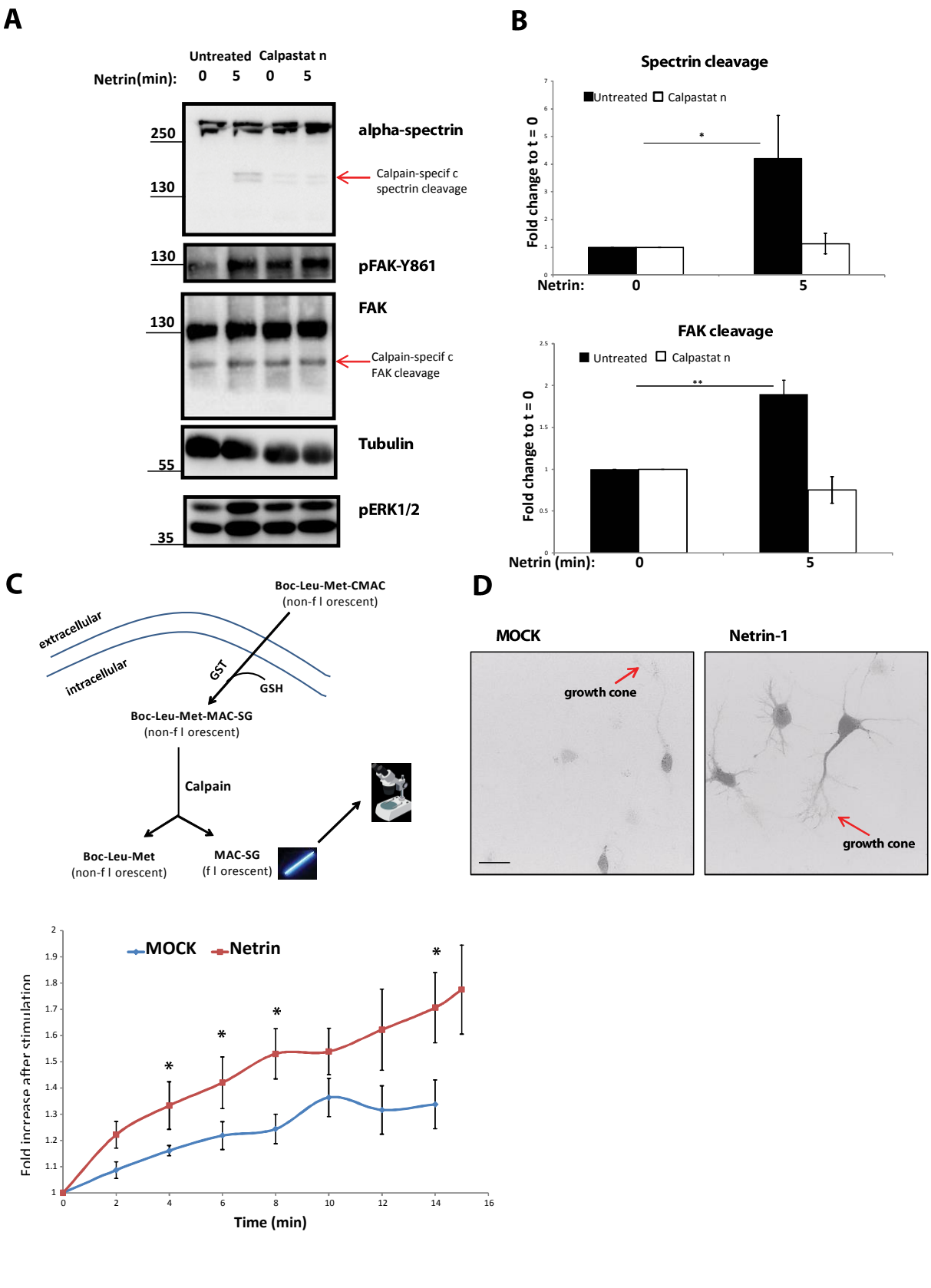

A
Untreated Calpastat n
Netrin(min): 0 5 0 5
250
130
alpha-spectrin
Calpain-specif c spectrin cleavage
130
pFAK-Y861
130
FAK
Calpain-specif c FAK cleavage
55
Tubulin
35
pERK1/2
B
Spectrin cleavage
Untreated Calpastat n
Fold change to t = 0
Netrin: 0 5
FAK cleavage
Untreated Calpastat n
Fold change to t = 0
Netrin (min): 0 5
C
Boc-Leu-Met-CMAC
(non-f l orescent)
extracellular
intracellular
GST
GSH
Boc-Leu-Met-MAC-SG
(non-f l orescent)
Calpain
Boc-Leu-Met
(non-f l orescent)
MAC-SG
(f l orescent)
MOCK
Netrin
Fold increase after stimulation
Time (min)
D
MOCK
Netrin-1
growth cone
growth cone

Figure 3.4: Netrin-1 activates calpain in dissociated cortical neurons

(A) Dissociated E18 rat cortical neurons were incubated with a purified calpastatin peptide or mock treatment for 1 hour prior to netrin-1 stimulation for 5 minutes at DIV2. Western blots of whole-cell lysates are showing FAK and ERK1/2 activation as controls together with spectrin and FAK cleavage upon netrin-1 stimulation. (B) Quantification of Spectrin and FAK cleavage depicted in (A). (C) Schematic representation of the fluorogenic calpain substrate t-boc depicting the fluorescent signal generated upon calpain cleavage. (D) Representative images of live cortical neurons stimulated with netrin-1 or DMSO in the presence of the t-boc . (E) Quantification of the time-lapse stimulation showing significant increases in the t-boc signal at different timepoints. Scale bar = 10um. Unpaired Student's t test:*$p < 0.05$.

Netrin-1 activates calpain in an ERK1/2-dependent manner in cortical neurons

Previous studies in non-neuronal cells have demonstrated the formation of an ERK1/2/FAK/Calpain-2 protein complex together with the direct phosphorylation and activation of calpain-2 by ERK1/2.[355, 357] Therefore, we next explored if the activation of calpain downstream of netrin-1 stimulation was dependent on ERK1/2 in cortical neurons. To assess this, cortical neurons were incubated with the ERK1/2 inhibitor U0126 prior to netrin-1 stimulation (Figure 3.5). In the presence of DMSO control, netrin-1 was able to activate calpain and ERK1/2 within 5 minutes of stimulation (Figure 3.5A and B) while netrin-1-induced calpain and ERK1/2 activities were both inhibited in the presence of the U0126 inhibitor (Figure 3.5A and B). Thus, netrin-1 activates calpain in an ERK1/2-dependent manner in developing cortical neurons.

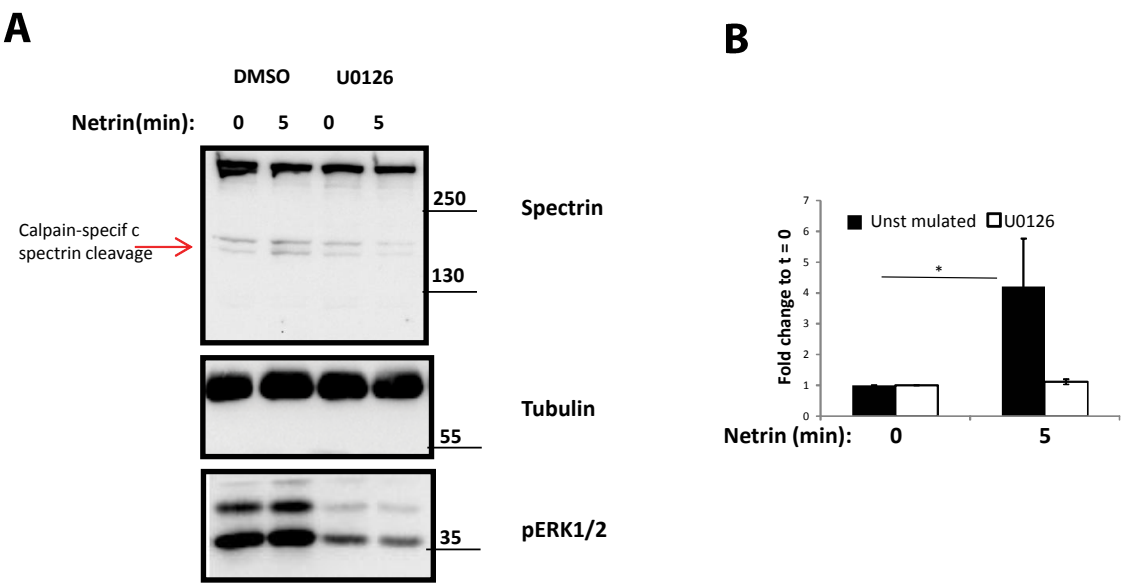


Figure 3.5: Netrin-1-mediated calpain activation is ERK1/2-dependent.

(A) Dissociated E18 rat cortical neurons were incubated with the ERK1/2 inhibitor, U0126, or DMSO for 1 hour prior to netrin-1 stimulation for 5 minutes at DIV2. Western blots of whole-cell lysates are showing phosphorylated-ERK1/2(activated), αII-spectrin cleavage and tubulin as loading control. Quantification of cleaved αII-spectrin is shown in (B). Unpaired Student's t test:*$p < 0.05$. N = 3

The netrin-1 receptor DCC is a novel calpain substrate in developing cortical neurons

Since netrin-1 activates calpain in cortical neurons leading to the cleavage of cytoskeletal-associated proteins and since we observed DCC proteolysis, we next explored whether DCC could be a substrate of calpain in response to netrin-1 stimulation. First, we examined whether calpain-1 was able to cleave DCC in vitro (Figure 3.6A). DCC was expressed in HEK293 cells and immunoprecipitated DCC was incubated with purified calpain-1 in the presence of calcium. Indeed, calpain-1 was able to cleave the entire pool of DCC, which was detected by immunoblotting using an antibody recognizing the extracellular domain of DCC (Figure 3.6A). Since the observed proteolytic fragment was larger than the 130kDa ectodomain

of DCC, we concluded that the cleavage took place within the intracellular domain (ICD) of DCC (Figure 3.6A). Then, we determined whether endogenous calpain was able to cleave overexpressed DCC in HEK293 cells. To assess this, DCC was co-transfected with a plasmid encoding calpastatin or empty vector in HEK293 cells. To identify the intracellular fragments of DCC, we used an antibody recognizing the cytoplasmic tail of DCC (Figure 3.6B). In the absence of calpastatin, we detected five major fragments of around 55, 50, 45, 35, and 30 KDa. Since the molecular weight of the intracellular domain of DCC is approximately 35 KDa, the proteolytic fragments of 55 to 45 KDa correspond to the cleavage of DCC at the extracellular side of the protein. In the presence of calpastatin, the presence of the fragments above 45 KDa were not affected whereas the lower fragments of 35 and 30KDa disappeared, indicating that these proteolytic fragments corresponded to calpain-dependent DCC cleavage (Figure 3.6B). On the contrary, the 35 and 30 KDa bands correspond to intracellular proteolytic fragments of DCC (Figure 3.6B). Next, we investigated whether DCC was also cleaved by calpain in a netrin-1-dependent manner in cortical neurons. After 5 minutes of netrin-1 stimulation, we observed a net increase in the fragments of approximately 55 and 35 KDa, corresponding to the extracellular and intracellular cleavage, respectively (Figure 3.6C). Interestingly, only the netrin-1-stimulated induction of the 35 KDa fragment was abolished in the presence of calpastatin (Figure 3.6C). These data indicated that the cytoplasmic domain of DCC was cleaved by calpain in cortical neurons, which correlated with the activation of calpain by netrin-1 at 5 minutes of stimulation (Figure 3.6A). Taken together, we show that netrin-1-mediated calpain activation induces the cleavage of its receptor DCC while calpastatin limits the extent of this proteolysis in cortical neurons.

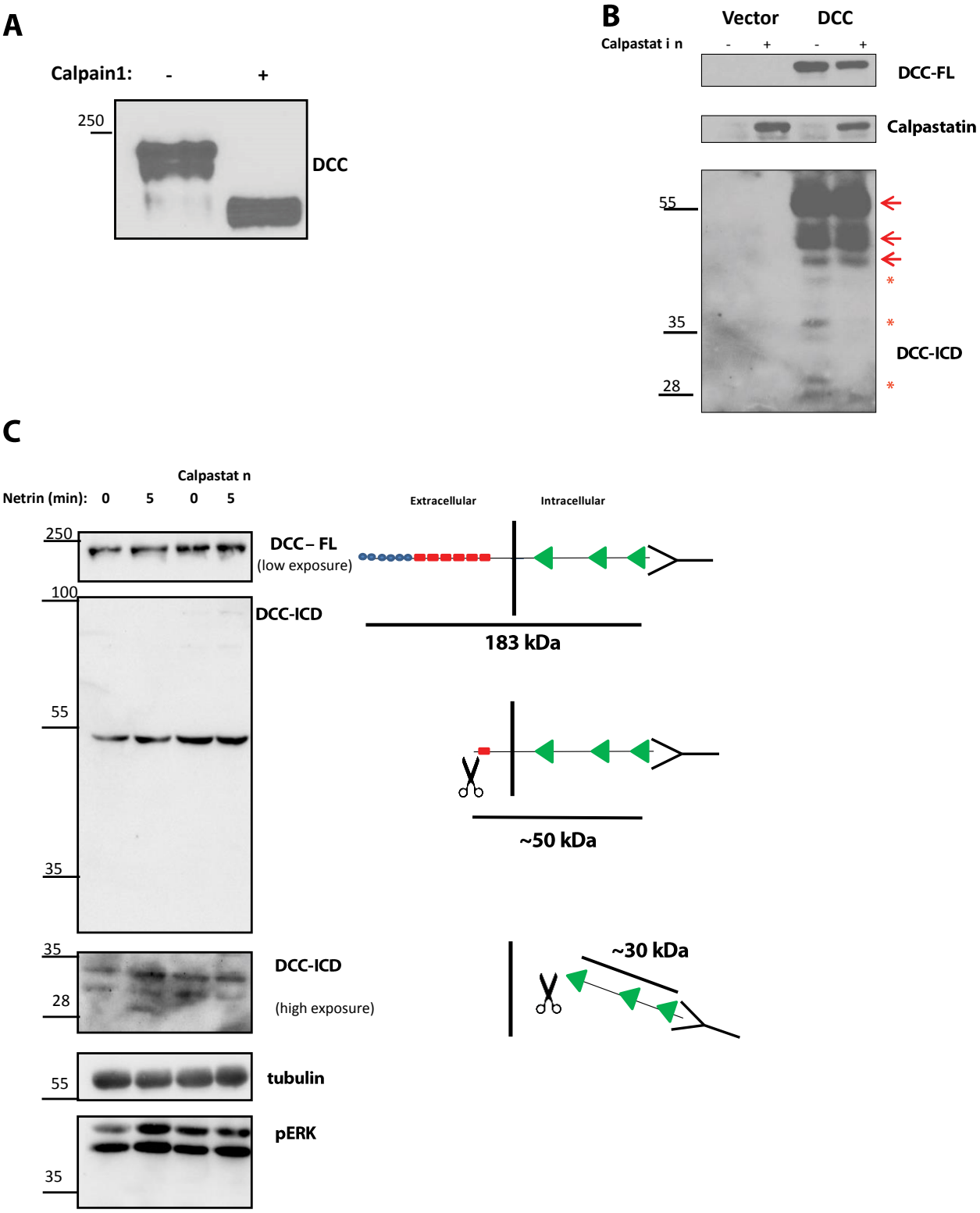

A
Calpain1: - +
250
DCC
B
Vector DCC
Calpastat i n - + - +
DCC-FL
Calpastatin
55
35
28
DCC-ICD
C
Calpastat n
Netrin (min): 0 5 0 5
250
100
55
35
DCC– FL
(low exposure)
DCC-ICD
Extracellular
Intracellular
183 kDa
~50 kDa
35
28
DCC-ICD
(high exposure)
~30 kDa
55
tubulin
35
pERK

Figure 3.6: Netrin-1 activates calpain leading to proteolysis of its receptor DCC

(A) Immunoprecipitated DCC was incubated with purified calpain-1 in the presence of excess calcium for 30 minutes. Proteins were then separated from agarose beads with 5X Laemmli buffer and loaded on SDS-PAGE for western bot analysis. **(B)** HEK293 cells were transiently transfected with DCC and calpastatin or control plasmid for 20 hours. Whole-cell lysates were loaded on SDS-PAGE for western bot analysis. An anti-DCC targeting the C-terminal of the intracellular domain was used to visualize intra-cellular cleavage events. Arrows indicate fragments with similar levels whereas asterix indicate rescued fragment in the presence of calpastatin **(C)** Dissociated E18 rat cortical neurons were incubated with calpastatin or a mock treatment for 1 hour prior to netrin-1 stimulation for 5 minutes at DIV2. Western blots of whole-cell lysates are showing ERK1/2 activation in in the mock treatment. Schematic representation of the different cleavage events previously reported to occur upon netrin-1 stimulation (metalloprotease & γ-secretase) and the observed bands. Predicted antibody binding on the C-temrinal tail is also displayed.

Calpain is a negative regulator of cortical axon outgrowth while calpastatin promotes axon extension

To investigate the role of calpain during netrin-1-induced cortical axon outgrowth, cortical neurons were electroporated with control or two siRNAs targeting calpain-1 and calpain-2 along with a GFP reporter to identify the electroporated cells. Neurons were fixed and immunostained for endogenous calpain-1/2 two days after electroporation, showing a significant reduction of the levels of calpain-1/2 in the GFP+ versus GFP- neurons (Figure 3.7A). Similarly, a significant decrease of calpain-1 expression was observed in the calpain-1/2 siRNAs-electroporated neurons compared with control siRNA (Figure 3.7B). We then measured the

average axon length of GFP-expressing neurons after 24h of incubation with netrin-1. As shown previously,[180, 183, 273] netrin-1 significantly increased the average axon length compared with unstimulated control neurons (Figure 3.7C and D). Depletion of calpain-1/2 in unstimulated neurons was sufficient to induce axon outgrowth to the same extent as netrin-1-stimulated neurons. However, netrin-1 was not able to further increase the axon length of calpain-1/2-deficient neurons (Figure 3.7C and D).

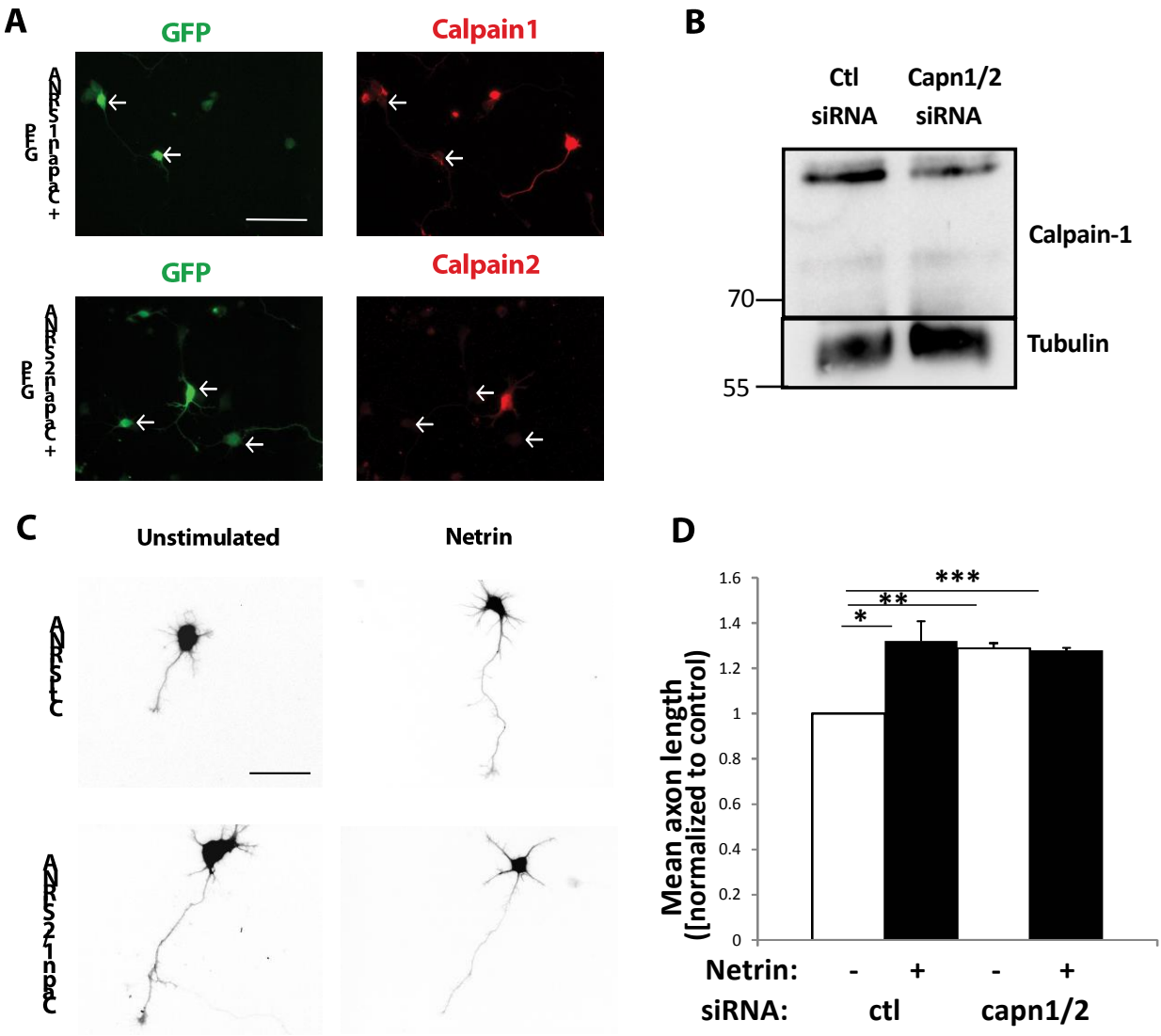


Figure 3.7: Calpain limits cortical axon outgrowth

(A) Control (CTL), calpain-1, or calpain-2 siRNA was electroporated in E18 embryonic rat cortical neurons at 0DIV with pGFP as a transfection reporter plasmid. Immunofluorescence was performed 48 hours later (DIV2) with the indicated antibodies to confirm the efficiency of knockdown in GFP+ cells. **(B)** Total cell lysates of DIV2 cortical neurons were loaded on SDS-PAGE and analyzed by western blot to confirm an efficient knockdown of calpain-1. **(C)** Control (CTL) or calpain-1/2 siRNA was electroporated in E18 embryonic rat cortical neurons at 0DIV with pGFP as a transfection reporter plasmid. Neurons at 1DIV were incubated with 500 ng/ml netrin-1 or left unstimulated for 24 hours and axon outgrowth was assessed in GFP-expressing neurons. **(D)** Axon outgrowth was measured and expressed as the average axon length normalized to E.V. in at least three independent experiments. scale bar = 50 um. Unpaired Student's t test: $*p < 0.05$ $**p < 0.01$.

Since calpastatin is an endogenous calpain inhibitor expressed throughout cortical neuron development (Figures 3.1 & 3.2), we next investigated its role in netrin-1-induced axon outgrowth. A calpastatin expression vector together with GFP cDNA were electroporated in cortical neurons left unstimulated or stimulated with netrin-1 for 24 hours. Neurons were fixed and immunostained for calpastatin, showing overexpression of calpastatin in the GFP+ neurons compared to empty vector (Figure 3.8A). Similar to calpain-1/2-depleted neurons, overexpression of calpastatin in unstimulated neurons led an increase in axon length comparable to netrin-1-stimulated neurons (Figure 3.8A and B). Moreover, netrin-1 stimulation did not promote axon outgrowth in calpastatin-expressing neurons similar to what we observed in calpain1/2-depleted neurons. Altogether, these results demonstrate that calpain activity negatively regulates axon outgrowth in cortical neurons. The lack of stimulation in axon outgrowth of calpain-1/2-deficient neurons or overexpressing calpastatin by netrin-1 further

supports that calpain activity is required for netrin-1 to promote axon extension in cortical neurons.

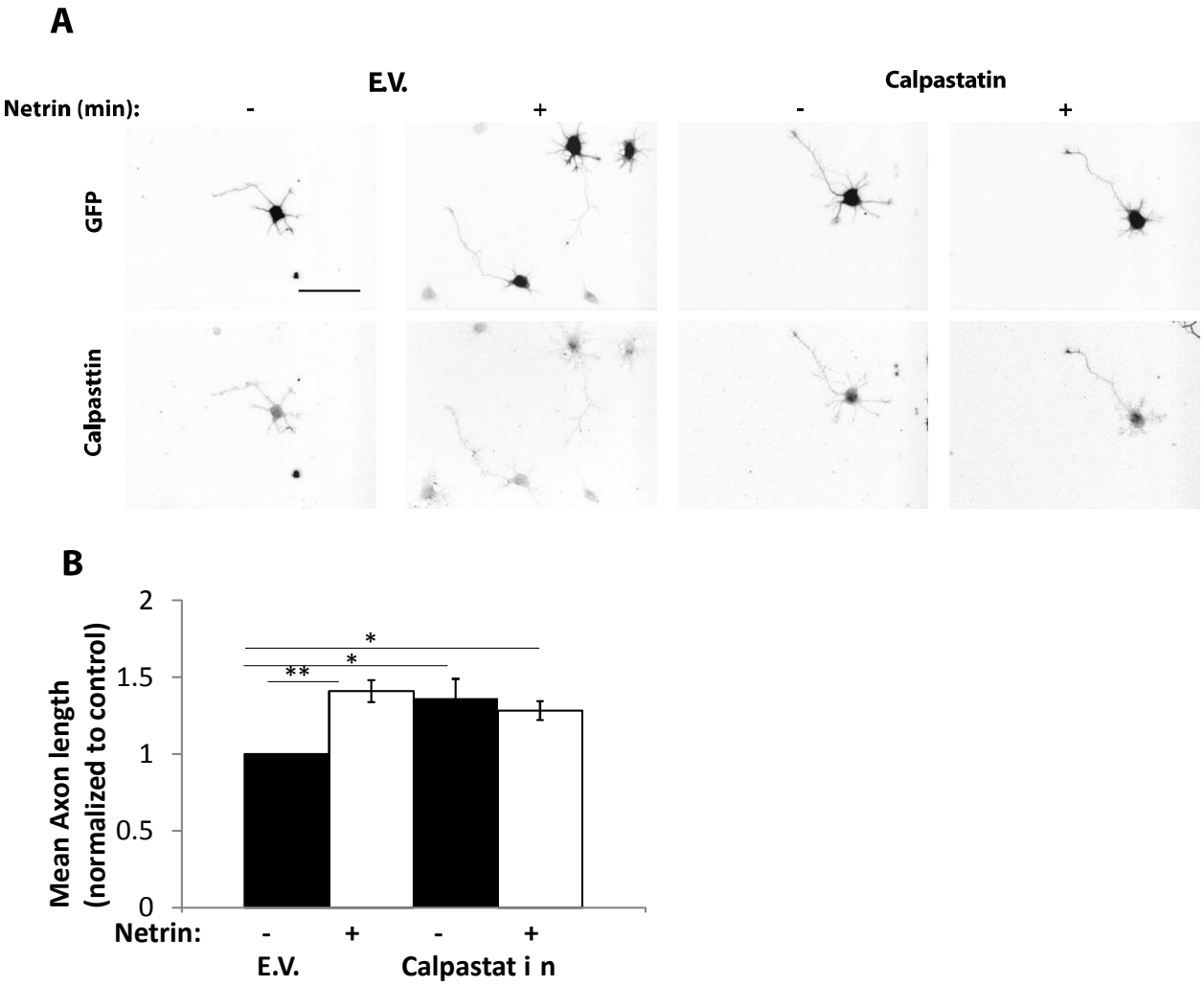


Figure 3.8: Calpastatin promotes axon outgrowth by inhibiting calpain1/2 activity

(A) Empty vector (E.V.) or calpastatin was electroporated in E18 embryonic rat cortical neurons at 0DIV with pGFP as a transfection reporter plasmid. Neurons at 1DIV were incubated with 500 ng/ml netrin-1 or left unstimulated for 24 hours and axon outgrowth was assessed in GFP-expressing neurons. **(B)** Axon outgrowth was measured and expressed as the average axon length normalized to E.V. in at least three independent experiments. scale bar = 50 um. Unpaired Student's t test: *$p < 0.05$ **$p < 0.01$.

Preface to Chapter 4

Previous studies from our lab have demonstrated the requirement Y1418 phosphorylation for netrin-1 signal transduction in the central vertebrate nervous system. The majority of DCC phosphorylation occurs on ser/thr residues. However, the role of these phosphorylated residues have has never been explored. The rationale of the study presented in chapter 4 is to identify and characterize the function of these phosphorylated residues. When one of the identified residues was mutated to an alanine, the cleaved fragment observed on SDS-PAGE prompted us to explore this particular residue. The results presented in chapter 4 are the first to provide evidence for a role of phosphorylation in regulating DCC stability and maintain a full-length DCC.

Chapter 4 - Investigating the role of DCC-Thr 1210 phosphorylation during cortical axon outgrowth

Identification of four novel Ser/Thr phosphorylation sites on the cytoplasmic tail of DCC

DCC is highly phosphorylated in response to netrin-1 stimulation[206] and tyrosine 1418 phosphorylation by Fyn, in particular, is crucial for netrin-mediated axon outgrowth and guidance.[206] The majority of the phosphorylation, however, occurs on serine/threonine residues[206] but its role in DCC function remains to be determined. Using mass spectrometry analysis, we identified 4 Ser/Thr phosphorylation sites in the cytoplasmic tail of DCC: Ser 1178, Ser 1182, Thr 1210, and Thr 1219 (Figure 4.1). Interestingly, Ser 1178 corresponds to an ERK1/2 consensus phosphorylation site which was later validated in a separate study.[278] Since it was previously been shown that netrin-1 stimulation leads to Erk1/2 phosphorylation and activation; Erk1/2 likely phosphorylates DCC in response to netrin-1-induced activation since direct interaction between the two proteins was also previously shown.[278] Surprisingly, expression of DCC-T1210A in several cell lines led to a truncated protein of 141.1 kDa compared to the full length protein of 183.2 kDa (Figure 4.1). Re-sequencing the cDNA encoding DCC-T1210A in the pRK5 plasmid confirmed that the complete coding region was intact, without any additional stop codon to explain the truncated protein. Furthermore, DCC-T1210A was only recognized with an antibody targeting the extra-cellular domain whereas an intra-cellular targeting antibody was unable to detect DCC-T1210A. Therefore, we conclude that DCC-T1210A is cleaved within the cytoplasmic tail and hypothesized that phosphorylation of T1210 protects DCC from proteolytic cleavage.

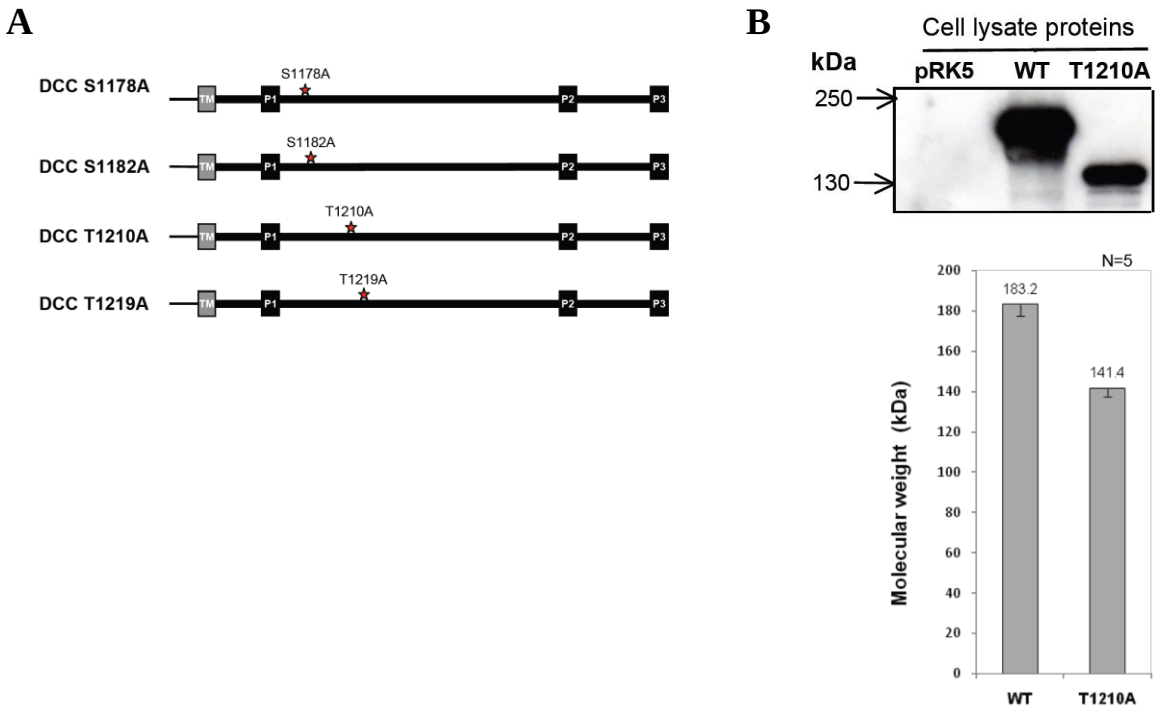


Figure 4.1: Identification of four novel Ser/Thr phosphorylation sites on the cytoplasmic tail of DCC

(A) Using mass spectrometry analysis, we identified three novel Ser/Thr phosphorylation sites on the cytoplasmic tail of DCC in overexpressed DCC in NIE-115 cells. The three sites were mutated with alanine for further investigation. **(B)** When overexpressed in HEK293 cells, DCC-T1210A mutant undergoes proteolysis, leading to a fragment of 141.4 kDa.

Phosphorylation on Thr 1210 protects DCC from proteolysis

To test this hypothesis we first replaced the alanine residue on T1210 with another hydrophobic residue, valine. Conversely, to mimic the phosphorylated state of DCC, we introduced a phosphomimetic residue, glutamic acid. Similar to DCC-T1210A, DCC-T1210V

gets cleaved but displays a different molecular weight on SDS-PAGE (Figure 4.2). In contrast, DCC-T1210E behaves similarly to DCC-WT and does not appear to undergo any proteolytic cleavage (Figure 4.2). Thus, the negative charge provided by the phosphate on Thr 1210 is sufficient to protect DCC from proteolysis.

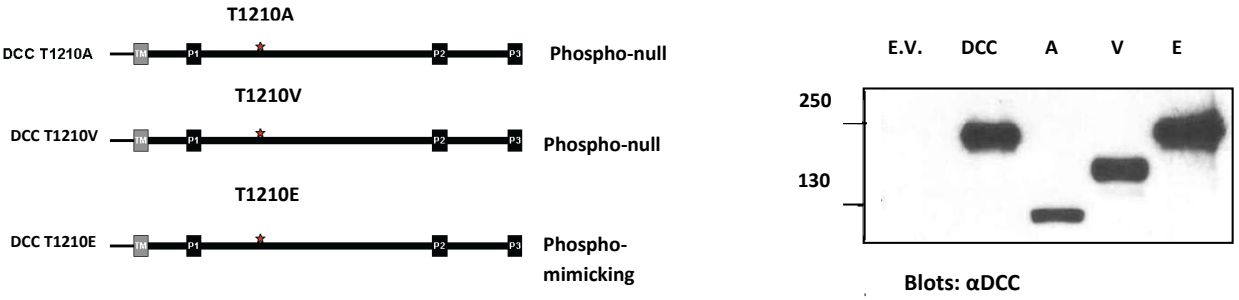


Figure 4.2 The negative charge provided by the phosphorylation of T1210 is sufficient to protect DCC from proteolysis

(A) Thr 1210 was replaced by a valine to validate the cleavage observed in DCC-T1210A and a glutamic acid was introduced to mimic to negative charge provided by the phosphorylation. **(B)** Total cell lysates of HEK293 cells overexpressing the three DCC mutants were loaded on SDS-PAGE and analyzed by western blot with an antibody recognizing the extracellular domain of DCC.

DCC-T1210V and DCC-T1210E are targeted to the plasma membrane

Before testing the functional relevance of this cleavage, we asked if these mutants were able to make their way to the cell surface where they can bind netrin-1. Using a cell surface biotinylation approach, we labelled all cell surface proteins with EZ-link Sulfo-NHS-LC-biotin. This compound is cell-impermeable and labels all accessible lysine residues. After labelling DCC-transfected HEK293 cells, we lysed the cells and pulled-down labelled proteins with avidin-conjugated agarose beads. Western blot analysis revealed that DCC-WT, DCC-T1210V, and DCC-T1210E were all being localized at the cell surface whereas DCC-T1210A was not

(Figure 4.3). Thus, DCC-T1210A gets cleaved before making it to the cell surface while DCC-T1210V either makes to the cell surface after cleavage or gets cleaved at the plasma membrane.

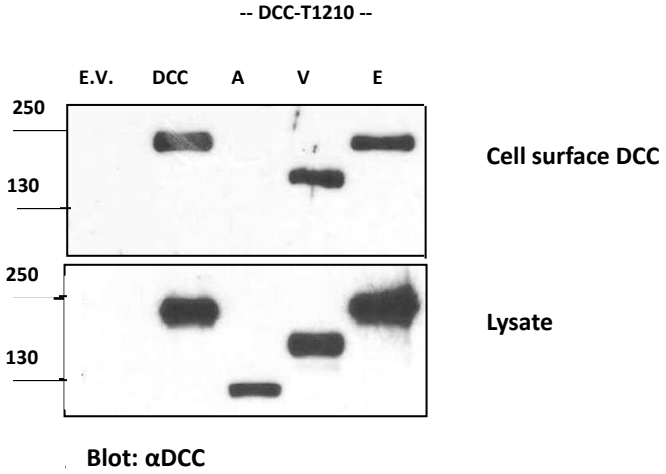


Figure 4.3: DCC-T1210V and DCC-T1210E are targeted to the plasma membrane

Transfected HEK293 cells were incubated with EZ-link-Sulfo-NHS-LC-Biotin for 30 minutes at 4^{o}C to label all extracellular proteins. Cells were then lysed and extracellular proteins were then purified using the biotin tag and streptavidin-conjugated beads. Proteins were then separated using SDS-PAGE and DCC detected by western blotting.

Ectodomain shedding of DCC

Since DCC was previously shown to be cleaved by metalloproteases, leading to ectodomain shedding in the extra-cellular space, we asked if DCC-T1210A was being secreted in the media. When the media of DCC-transfected HEK293 cells were immunoprecipitated with an antibody recognizing the extracellular domain of DCC, we observed a fragment of DCC being released in the media in all conditions (DCC-WT, DCC-T1210A, DCC-T1210V, and DCC-T1210E) (Figure 4.4). Interestingly, all fragments released in the media had the same molecular weight except for DCC-T1210A (Figure 4.4), suggesting that they get cleaved by a common

protease at the same site. However, DCC-T1210A likely gets cleaved intracellularly by a different protease at a different site than DCC-WT, DCC-T1210V, and DCC-T1210E.

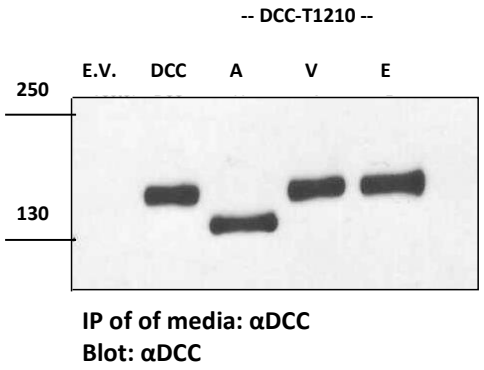


Figure 4.4: DCC ectomain shedding in the media

Immunoprecipitation of cultured media of HEK293 cells overexpressing the different DCC mutants with an antibody targeting the extracellular domain of DCC. Media was collected 20 hours after transfection and cell debris were removed by centrifugation. Following immunoprecipitation, proteins were separated from agarose beads with 5X Laemmli buffer and loaded on SDS-PAGE for western bot analysis.

DCC full-length promotes axon outgrowth in cortical neurons

Since both DCC-T1210V and DCC-T1210E were being targeted to the plasma membrane (Figure 4.3), we decided to test the function of Thr phosphorylation in the context of netrin-mediated cortical axon outgrowth using these two mutants and DCC-WT. Following dissection of E17.5 rat embryos and electroporation of DCC constructs with GFP as a reporter at DIV0, cortical neurons were plated and stimulated with netrin-1 the following day (DIV1). Twenty four hours later (DIV2), neurons were fixed and the mean axon length was calculated by measuring the axon length of at least 50 transfected cells (GFP+) per condition. Interestingly, overexpression of both DCC-WT and DCC-T1210E stimulated axon outgrowth compared to

vector control. In contrast, DCC-T1210V behaved similarly to the empty vector and did not stimulate outgrowth (Figure 4.5).

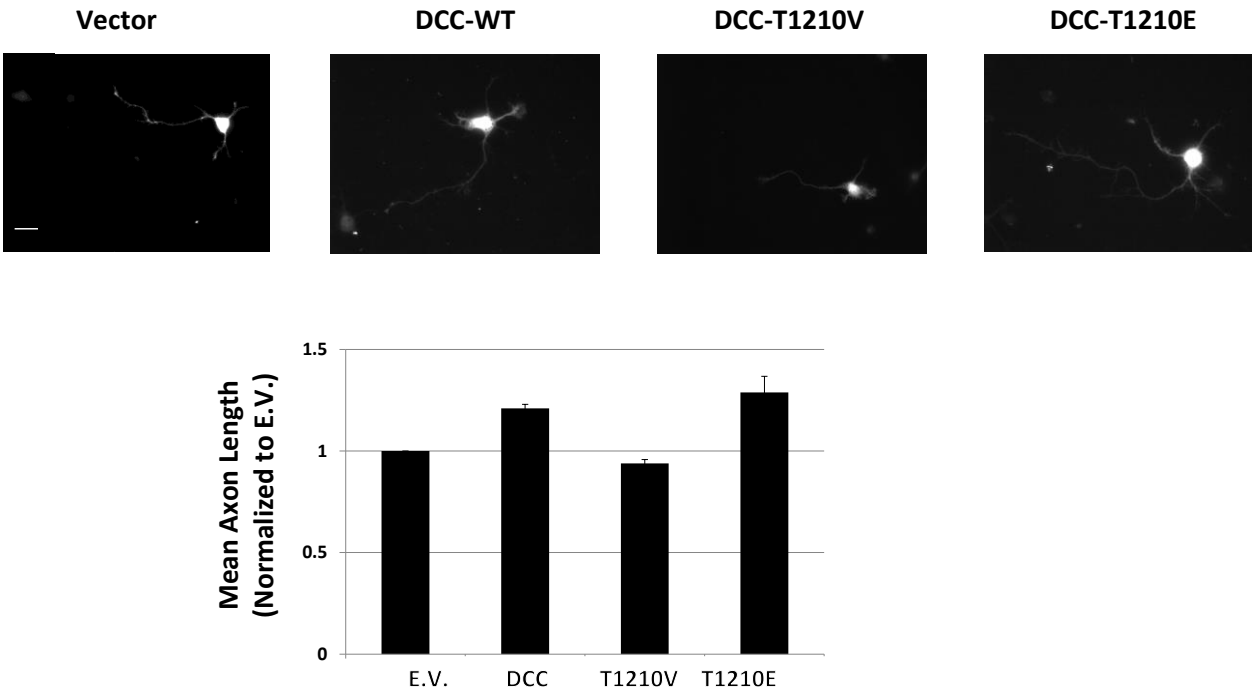


Figure 4.5: DCC full length promotes cortical axon outgrowth

Empty vector (E.V.), DCC-wt or DCC-T1210 mutants were electroporated in E18 embryonic rat cortical neurons at DIV0 with pGFP as a transfection reporter plasmid. Axon outgrowth was assessed in GFP-expressing neurons at 2DIV. **(B)** Axon outgrowth was measured and expressed as the average axon length normalized to E.V. in two independent experiments. scale bar = 10 um.

DCC-T1210V gets cleaved in proximity of the mutation

To identify the identity of the protease, we first determined the location of the cleavage site by performing mass spectrometry analysis of the DCC-T1210V fragment. DCC-T1210V-

transfected HEK293 cells were lysed and immunoprecipitated with a DCC antibody recognizing the extracellular domain. Interestingly, we found that the cleavage of DCC-T1210V was occurring around Thr 1210. We were able to conclude that cleavage of DCC-T1210V was happening between amino acids 1201 and 1222 (Figure 4.6).

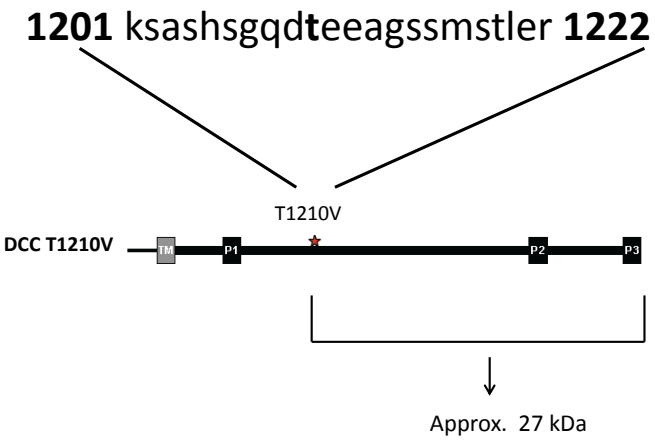


Figure 4.6: Cleavage site of DCC-T1210V mutant

Immunoprecipitated DCC-T1210V was sent for mass spectrometry analysis to determine the cleavage site. DCC-T1210V gets cleaved between amino acids 1201 and 1222.

Calpastatin partially rescues DCC-1210V cleavage

We then decided to investigate the identity of the protease cleaving DCC-T1210V by incubating DCC-transfected HEK293 cells with various protease inhibitors (Figure 4.7).

Interestingly, when the endogenous calpain inhibitor, calpastatin, was added to the cells we observed a partial rescue of DCC-T1210V cleavage (Figure 4.7). Since we have shown in chapter 3 of this thesis that DCC is a calpain substrate (chapter 3), we hypothesised that calpain cleaves DCC and that phosphorylating Thr 1210 protects DCC from calpain-mediated cleavage.

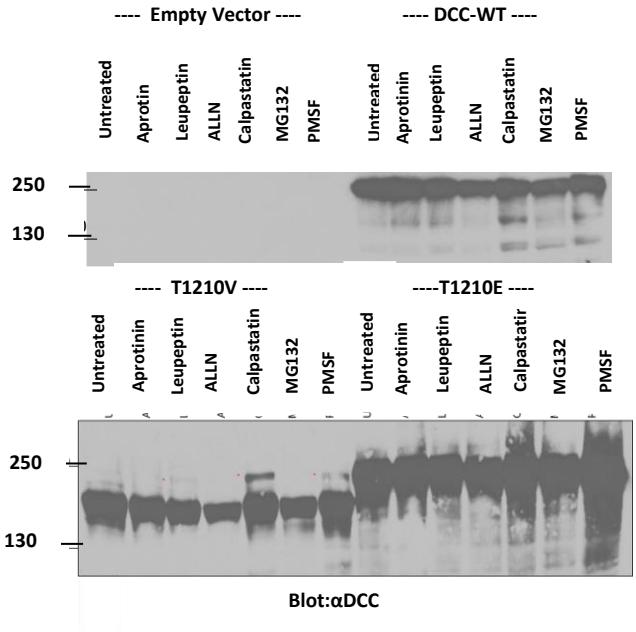


Figure 4.7: Calpastatin partially rescues cleavage of DCC-T1210V

HEK 293 cells were transfected with the indicated plasmid for 20 hours and incubated for 3 hours with various protease inhibitors before cell lysis. Total cell lysates were then loaded on SDS-PAGE and analyzed by western blot.

Chapter 5 - Discussion

5.1 Major findings

The results presented in this thesis were undertaken with the aim of identifying novel mechanisms regulating the netrin/DCC signaling pathway in the context of cortical axon outgrowth. This led to the identification of: 1) a novel DCC binding partner mediating crosstalk between distinct signaling pathways, 2) a novel protease regulating DCC, and 3) a novel phosphorylation site regulating DCC stability. The functional relevance was investigated in the context of rat cortical axon outgrowth.

5.2 p120RasGAP mediating Ras and netrin/DCC signaling

In the second chapter, we reported a novel interaction between p120RasGAP and DCC in the context of cortical neuron development in vitro. We demonstrate that p120RasGAP is essential for netrin-1-mediated cortical axon outgrowth and guidance. In addition, we show that the N-terminus of p120RasGAP positively regulate netrin-1-induced axon outgrowth by serving as a scaffolding protein mediating protein-protein interaction required for downstream signaling, independent of its GAP domain (Figure 5.1). To our knowledge, this is the first study implicating a positive role for p120RasGAP during axon outgrowth and guidance. In particular, p120RasGAP was considered an inhibitor of axon outgrowth and guidance due to its activity of its C-terminal RasGAP domain.[382, 397, 398]

Previous studies have implicated Ras GTPases downstream of ephrins, semaphorins, and neurotrophins during neuronal development but their role in netrin/DCC signaling had never been explored.[104, 399] In particular, p120RasGAP regulates the level and duration of PDGF-dependent Ras/ERK activation in fibroblasts.[400, 401] In contrast, the absence of p120RasGAP

recruitment to the EphB2 receptor delayed and reduced ephrin-B1-dependent ERK inhibition in neuroblastoma cells.[398] Similarly, p120RasGAP plays an important role in keeping Ras/ERK inactive until netrin-1 stimulation induces p120RasGAP tyrosine phosphorylation and sequestration, therefore relieving Ras/ERK inhibition.

It was previously shown that netrin-1/DCC activate ERK whereas the canonical MAPK pathway implicates activation of ERK1/2 downstream of Ras activation.[104, 279] How ERK1/2 gets activated downstream of netrin-1/DCC remained unclear. Here we show that under basal conditions, p120RasGAP puts a break on Ras, and consequently ERK activation via its GAP domain. Upon netrin-1 stimulation, p120RasGAP gets tyrosine phosphorylated along with DCC and other signaling proteins such as FAK. Interaction with DCC is achieved via p120RasGAP's N-terminus domain-containing SH2 domains and DCC's phosphorylated Y1361 and Y1418 residues. In addition, previous studies have shown that phosphorylated FAK-Y397 mediates interaction with both the N-SH2 domain of p120RasGAP and DCC.[265, 389] This sequesters p120RasGAP to DCC and within the multiprotein complex required for downstream signaling. p120RasGAP-bound to DCC can no longer stimulate Ras GTPase activity, which remains active, leading to ERK activation.

5.2.1 p120RasGAP's GAP-independent function

Our findings strongly support the requirement for a tight control of p120RasGAP expression and consequently Ras activity during cortical axon outgrowth. In p120RasGAP-depleted cortical neurons, basal axon outgrowth is increased and unresponsive to netrin-1-

stimulated axon outgrowth. Re-expression of p120RasGAP in these p120RasGAP-depleted neurons decreases axon length to unstimulated control neurons. However, these neurons remain unresponsive to netrin-1 stimulation when p120RasGAP is overexpressed. Netrin-1-mediated axon outgrowth is only restored when the N-terminal SH2-SH3-SH2 domains of p120RasGAP is expressed in p120RasGAP-depleted neurons. This suggests that when overexpressed, p120RasGAP's GAP domain takes over and netrin-1 stimulation cannot sequester enough p120RasGAP to prevent the GAP domain's inhibition of Ras activity. Since p120RasGAP play two very distinct functions, a tight control on its expression and localization is likely necessary to respond adequately to extracellular cues in a timely manner. Thus, the N-terminus of p120RasGAP is necessary and sufficient to mediate netrin-1/DCC-induced axon outgrowth and guidance. This is in line with a previous study that proposed a model in which p120RasGAP is sequestered and inhibited by the neogenin receptor via an interaction with the phosphorylated Y397 residue of FAK.[382] The repulsive guidance cue RGMa releases p120RasGAP from that interaction to stimulate Ras' GTPase activity thereby inhibiting downstream signaling. Other studies have shown that the GAP activity of p120RasGAP is inhibited when its N-terminus interacts with p190RhoGAP, FAK, p200RhoGAP, or SOCS-3.[389, 402-404] Here we show that axon outgrowth and attraction mediated by netrin-1/DCC involves the reverse mechanism whereby netrin-1 stimulation favors p120RasGAP sequestration within the multiprotein complex as opposed to releasing it upon ligand stimulation. p120RasGAP seem to have evolved two very different and seemingly opposite functions: 1) putting a break on axon outgrowth via Ras inhibition via its GAP domain under basal conditions and 2) mediating netrin-1/DCC-induced axon outgrowth upon netrin-1 stimulation via its N-terminus domain.

Other groups have also reported GAP-independent functions for p120RasGAP. Expression of the N-terminus increases Ras and ERK activation in fibroblasts[398] and promotes Ras-dependent differentiation of PC12 cells.[405, 406] In addition, cell migration is severely impaired in p120RasGAP-deficient mouse fibroblasts, which can be rescued by the N-terminus of p120RasGAP, similarly to the rescue in cortical axon outgrowth by the N-terminus in chapter 2. This suggests that p120RasGAP is required for cell motility, both in the context of cell migration and axon outgrowth and guidance. Our results presented in chapter 2 also support a positive role for the N-terminus of p120RasGAP in Ras/ERK activation. We show that Ras/ERK activation mediated by the N-terminus of p120RasGAP is required downstream of netrin-1/DCC signaling during growth cone navigation.

5.2.2 p120RasGAP and other GAPs

Although netrin-1 is well-known for promoting axon outgrowth;[206, 251, 279, 325] it reduced axon outgrowth in p120RasGAP-depleted cortical neurons. The mechanism by which netrin-1 reduces axon outgrowth in these p120RasGAP-depleted neurons is unclear. Since p120RasGAP interacts with three different RhoGAPs, it is possible that the observed decrease in axon outgrowth upon netrin-1 stimulation is due to a loss in RhoA regulation. First, p190RhoGAP was shown to be enriched in growth cones and that the p190RhoGAP-p120RasGAP-FAK could be necessary for netrin-1-mediated axon outgrowth.[407, 408] Second, interaction of p120RasGAP with another RhoGAP, deleted in liver cancer (DLC1),[409, 410] which is expressed during and required for embryonic brain development,[411] could also be involved in netrin-1-mediated axon outgrowth. In contrast, interaction of DLC1 with p120RasGAP inhibits DLC1's GAP activity, thereby promoting RhoA activation, and possibly reducing axon outgrowth.[412] Third, p120RasGAP interacts with the brain-specific p200RhoGAP.[403, 413] It was proposed that FAK,

p190RhoGAP, and p200RhoGAP interact with p120RasGAP to prevent Ras inhibition by p120RasGAP.[389, 403] Taken together, this suggests that Ras and RhoA regulation are interdependent and by removing a regulator of one, it likely affects regulation of the other. Therefore, interplay between Ras and RhoA regulation is plausible in the context of netrin-1/DCC signaling and axon outgrowth and guidance.

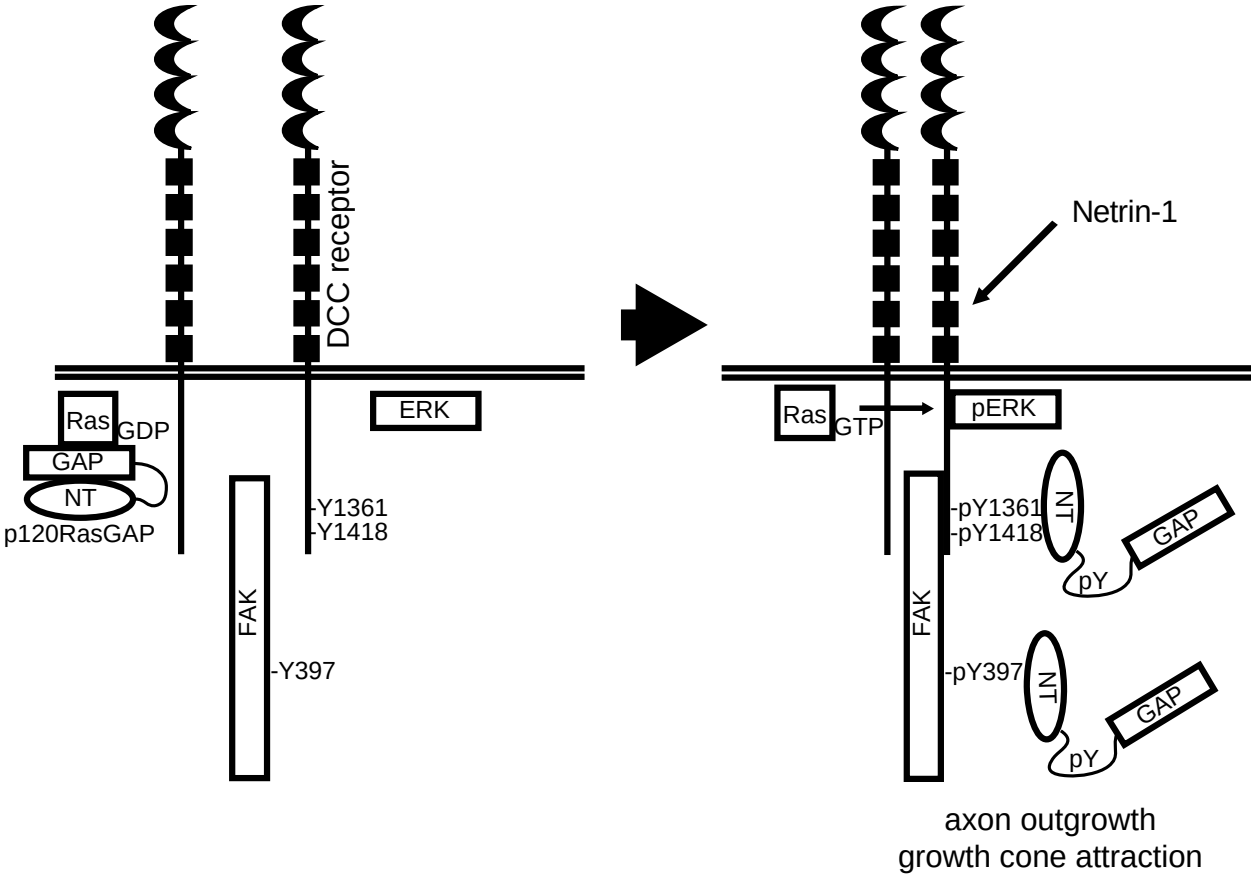


Figure 5.1. Proposed model for the role of p120RasGAP in netrin-1/DCC-mediated axon outgrowth and guidance

In the absence of ligand, the GAP domain of p120RasGAP maintains Ras inactive (Ras-GDP). In response to netrin-1 stimulation, DCC-pY1418/Y1361, FAK (pY397), pERK and tyrosine phosphorylated p120RasGAP associates in a multiprotein signaling complex. Through these interactions mediated by the N-terminal SH2-SH3-SH2 domains (NT) of p120RasGAP, Ras-GTP is released to induce sustained ERK activation in response to netrin-1, leading to axon outgrowth and growth cone attraction.

5.3 Calpain-1/2 in netrin-1/DCC signaling

In chapter 3, we show that calpain is a novel protease regulating netrin-1/DCC-mediated signaling pathways during axon outgrowth. We demonstrate that netrin-1 regulates calpain-1 expression and its endogenous inhibitor, calpastatin, in developing cortical neurons. The increased calpain-1 expression in the axons together with the decrease in calpastatin expression correlates with the positive regulation of calpain activity induced by netrin-1 in developing neurons. Moreover, we determined that netrin-1-mediated calpain activation is ERK1/2-dependent. Of interest, netrin-1-mediated calpain activation leads to the cleavage of its receptor DCC and cytoskeletal-associated proteins. Thus, we propose that calpain activation downstream of netrin-1/DCC-induced signaling pathways is an important mechanism to control axon outgrowth in developing neurons. In support of this, we find that calpain-1/2 is a negative regulator of cortical axon outgrowth independently of netrin-1. Both calpain-1/2 knockdown and overexpression of its endogenous inhibitor calpastatin lead to increased cortical axon outgrowth, which render the axons unresponsive to netrin-1 treatment. To our knowledge, this study is the first one to implicate calpain as a novel regulator of the chemoattractant cues netrin-1 and DCC during axon outgrowth (Figure 5.2). In the absence of netrin-1, calpain activity is a limiting factor in promoting axon extension. We propose a model in which netrin-1 activates ERK1/2, which in turn activates calpain, leading to proteolysis of its receptor DCC and cytoskeletal-associated proteins such as spectrin and FAK. The calpain-mediated intracellular fragment of DCC may be targeted either for degradation or to the nucleus to transcriptionally regulate proteins modulating axon outgrowth (Figure 5.2).

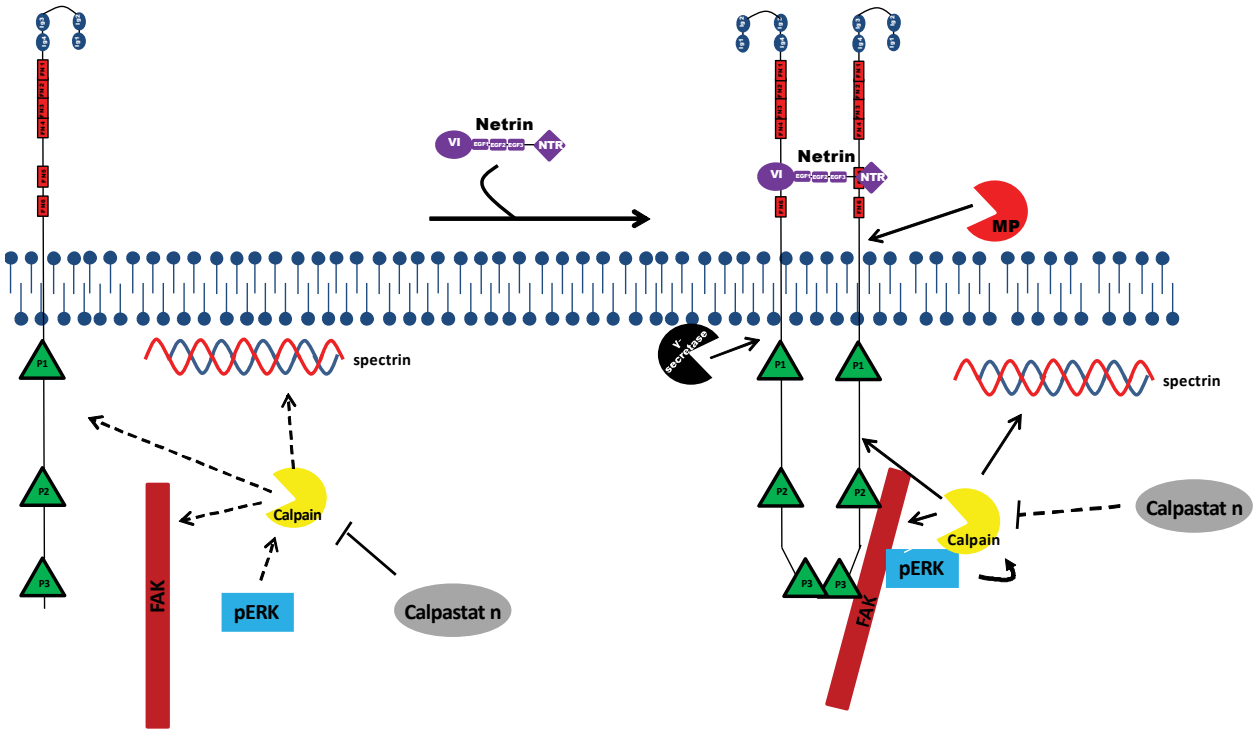


Figure 5.2: Proposed model for the role of calpain in netrin-1/DCC-mediated axon outgrowth and guidance

In the absence of netrin-1, calpain activity is mostly repressed by calpastatin. Upon netrin-1 stimulation, DCC homodimerizes via its P3 domain. Extracellular metalloprotease cleaves and releases the ectodomain of DCC which primes DCC for γ-secretase cleavage. In parallel, netrin-1 activates calpain via ERK1/2 activation which can then cleave several of its cytoskeletal targets such as spectrin and FAK, as well as DCC.

5.3.1 Netrin-1/DCC/Calpain in cortical axon outgrowth

In earlier studies calpain activity was also reported to reduce axon extension[340, 378, 394] and essential for neurite consolidation.[339] In particular, it was shown that calpain activity prevented axon branching through repression of the protrusive activity while branching factors such as BDNF, NT3, and Netrin-1 inhibited this repression by inhibiting calpain activity and thus allowing axonal branch formation.[339] On the other hand, our findings show that netrin-1 activates calpain in cortical neurons plated after two days in culture when axon specification and outgrowth takes place, preceding the formation of axon branches. This suggests that calpain

activity may be differently regulated by various extracellular factors at various stages of neuronal developmental. Netrin-1 is known for its branch-promoting activity[155, 414] and for promoting axon outgrowth.[183, 251, 279] It is important to note that when cortical neurons are stimulated at DIV1 for 24 hours, netrin-1 promotes axon outgrowth.[183, 206, 272, 279, 381] On the other hand, when neurons are stimulated for 72 hours, netrin-1 stimulates axon branching.[155, 414] Thus, it is plausible that netrin-1 activates calpain early to prevent aberrant branching and allow the cytoskeletal machinery to focus on axon outgrowth while later in development netrin-1 inhibits calpain to promote axon branching. Looking at different developmental stages and stimulating neurons for different length of time will help clarify this. Furthermore, the activation of calpain that we observed following netrin-1 stimulation was fast and brief. Thus, since calpain activation was not sustained and netrin-1 promotes significant axon outgrowth on a different timescale (≥24 hours), we conclude that calpain activation is brief to prevent pathological responses associated with sustained calpain activation while allowing a quick way to attenuate the pathway. Finally, PKA was shown to directly phosphorylate and inhibit calpain-2 activity[355] as well as being involved in the netrin-1/DCC signaling pathway by promoting DCC plasma membrane insertion in response to netrin-1.[274, 415, 416] Thus, removal of DCC from the cell surface through proteolysis in response to netrin-1 leads to PKA-dependent DCC membrane insertion from intracellular pool. In parallel, inhibiting calpain-2 activity in a PKA-dependent would promote axon outgrowth.

5.3.2 Netrin-1/DCC/Calpain in cortical axon guidance

The netrin-1/DCC signaling pathway is essential for the formation of commissures within the central nervous system, regulating the axonal projections of commissural neurons in the developing spinal cord and forebrain commissures such as the corpus callosum.[417, 418] Mice

lacking the expression of DCC have severe defects in the formation of brain commissures[419] that are very similar to the phenotype of mice lacking netrin-1.[420] Even a small truncation in the intracellular domain of DCC (DCC^{kanga})[290] or deletion of its downstream effector Trio leads to severe defects in axonal projections[290] with a complete absence of the anterior commissure in Trio-depleted mice.[183] Highlighting the importance of the formation of brain commissures in the human population, mutations in the *DCC* and *NETRIN-1* genes are causative of a rare developmental disorder caused by defects in commissure formation.[291, 293, 421-423] Congenital mirror movements (CMM) is characterized by early-onset, involuntary movements of one side of the body that mirror intentional movements on the opposite side in individuals with no other clinical signs or symptoms. Our findings suggest that the removal of calpain-1 and calpain-2 in the central nervous system will affect the axonal projections of the brain commissures. In support of this, biallelic mutations in CAPN1 (the calpain-1 gene) causes autosomal recessive hereditary spastic paraplegia (HSP), spastic ataxia, or both.[341, 424] HSPs are a heterogenous group of inherited neurological disorders characterized by pyramidal tract affection and variable other neurological manifestations. Furthermore, locomotor defects due to abnormal neuronal development were reported in C. elegans, *Drosophila*, and zebrafish[424] while calpain-1 null mice develop ataxia due to abnormal cerebellar development.[341] Using morpholino targeting the *capn1a* gene in zebrafish, one group showed strong axon guidance defects in capn1a-deficient animals.[424] Severe defects in motor neuron projections were observed as well as several migration defects. Specifically, in capn1a-knockdown animals, a reduction in acetylated-tubulin was observed at the level of the optic tectum and cerebellum, whereas a stronger staining was found in the dorsal telencephalon, suggesting an increase in axon outgrowth and/or branching. This is in accordance with calpain-1 limiting axon outgrowth and branching in mice cortical

neurons observed in vitro by our group and others[339, 340, 378, 394] It will be interesting to look into the different commissures in mice lacking the *CAPN1* gene or in patients harboring a CAPN1 mutation to assess if there are any overlapping defects between DCC and CAPN1 mutations. Alternatively, since calpain-2 might rescue the loss of calpain-1, targeting the small subunit necessary for both calpain-1 and calpain-2 activity will eliminate this problem. In fact, mice lacking the small subunit, capns1 (also known as calpain-4), have altered dendrite morphology leading to defects in synaptic transmission, learning, and memory.[343] Using this previously published mouse line in which the small regulatory subunit is deleted specifically in neural progenitors, Nestin-Cre; CAPNS1 flox/flox (CAPNS1-Nestin-cKO),[343] we looked at the development of cortical projections (Figure 5.3). When compared to Nestin-Cre control, there was a strong increase in TAG(+) projections within the cortex at postnatal day 3. This suggests an increase in axon outgrowth and/or branching which similar to what we and others have observed in vitro.[339] In support of capain-1's role in midline crossing, we observed a reduction in TAG+ projections within the corpus callosum, suggesting a callosal projection neurons axon guidance defect. Future studies in more animals will be needed to confirm these results. Since calpain-1 has also been implicated in commissural midline crossing in the spinal cord in the context of semaphorin signaling[199, 322] and netrin-1 is well-known for its role in commissural axon attraction to the midline,[194, 291, 326] it will be interesting to test if calpain-1 can mediate a crosstalk between the two signaling pathways. Using primary cultures of CAPNS1-Nestin-cKO mice, we could test if and how these signaling pathways are affected by looking at the activation state of downstream effectors. Furthermore, it will be interesting to look at different CNS projections such as the anterior commissure and commissures of the spinal cord in these CAPNS1-Nestin-cKO as well as our preliminary in vivo results in CAPNS1-Nestin-cKO (Figure

5.3). Taken together, the combination of the results presented in chapter 3, together with results found by others strongly supports a crucial role for calpain-1 in normal neural circuitry assembly.

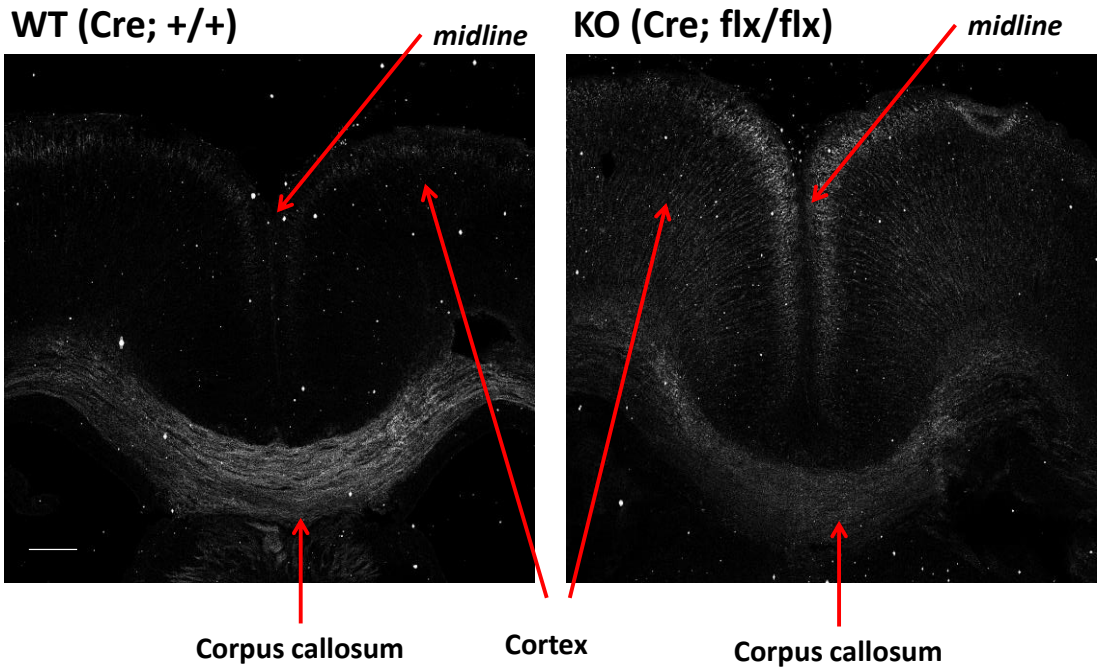


Figure 5.3: Axonal projections in CAPNS1-Nestin-cKO brain

Immunofluorescence on coronal sections of postnatal day 3 mouse forebrain with the transient axonal glycoprotein-1 (TAG-1) expressed along axons. The different structures ad regions are identified in red. Wilt-type (Nestin-Cre; CAPS1 wt/wt) is on the left whereas Nestin-Cre;CAPNS flox/flox (CAPNS1-Nestin-cKO) is on the right. Scale bar 20μm.

5.3.3 Netrin-1/DCC/Calpain and ERK activation

Similarly to our findings with netrin-1, growth factors such as EGF, BDNF, and the repulsive guidance cue semaphorin3A have been reported to activate calpain[356, 379, 380] in an

ERK1/2 activity-dependent manner.[356, 380].[370]. In contrast, we also show that netrin-1-induced ERK1/2 activation is reduced in the presence of the calpain inhibitor calpastatin, suggesting that calpain activity may regulate ERK1/2 activation by netrin-1 in cortical neurons. This potential positive feedback loop is supported by another study. Using a different calpain inhibitor, calpeptin, one group have implicated calpain activity as a requirement for ERK1/2 activation under pathological conditions.[375] Importantly, in mature neurons, calpain-1 is responsible for cleaving PHLPP1β, an inhibitor of ERK1/2, resulting in the indirect activation of ERK1/2.[341] Therefore, calpastatin may prevent calpain-1 from relieving ERK1/2 inhibition by PHLPP1β, thereby reducing overall ERK1/2 activation upon netrin-1 stimulation. Interestingly, the defects in cerebellar development observed in calpain-1 knockout mice were rescued by crossing these calpain-1 KO with PHLPP1β KO mice.[341] Thus, it will be interesting to see if removing PHLPP1β could rescue the increased axon outgrowth observed in calpain-1/2 knockdown and calpastatin overexpressing cortical neurons. Preliminary results using cortical primary culture of CAPNS1-Nestin-cKO E15.5 embryos, support the requirement of calpain activity for ERK1/2 activation (Figure 5.4). The mechanism by which calpain is activated in an ERK-dependent manner while ERK itself requires calpain activity could very well be mediated by calpain-1-mediated cleavage of PHLPP1β, thereby activating ERK1/2. One distinction between the inhibition of calpain activity using our calpastatin inhibitor that strongly decreased netrin-dependent ERK1/2 activation and the preliminary results showing an absence of ERK1/2 activation in CAPNS1-Nestin-cKO cortical neurons is the duration of calpain inhibition. In the first scenario, calpastatin inhibits calpain transiently for one hour (+5 min netrin-1 stimulation) whereas in CAPNS1-Nestin-cKO neurons, calpain activity is constitutively repressed due to the absence of the small regulatory subunit from the neuron's time of birth. Therefore, several

defects within the signaling network could affect ERK activation in these neurons, thereby preventing netrin-1-mediated ERK1/2 activation. Stimulating these neurons with other factors known to activate ERK will distinguish between a netrin-1-specific signaling defect or a general ERK inhibition in the absence of calpain activity. Again, repeating these experiments is necessary to make any substantive conclusions. Finally, since both Rac1 and calpain activity are required for cytoskeletal re-arrangements necessary for growth cone motility and Netrin-1-mediated ERK1/2 activation has previously been shown to be at least partially dependent on Rac1 activity;[269] manipulating Rac1 and looking at calpain activity will help determine if Rac1 plays a role in regulating calpain activity.

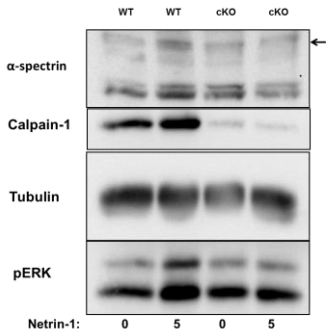


Figure 5.4: Netrin-1-mediated ERK1/2 activation is abolished in CAPNS1-Nestin-cKO cortical neurons

Dissociated Nestin-Cre; CAPS1 wt/wt (WT) or CAPNS1-Nestin-cKO (cKO) E16 mouse cortical neurons were stimulated with netrin-1 for 5 minutes at DIV2. Western blots of whole-cell lysates are showing calpain-mediated spectrin cleavage and ERK1/2 activation in WT upon netrin-1 stimulation.

Among the two ubiquitous calpain isoforms, only calpain-2 possesses the S50 ERK phosphorylation site. However, we show that calpain-1 is the predominant calpain expressed at DIV2, when we stimulated cortical neurons with netrin-1 and observed calpain activation. If the calpain activation we see upon netrin-1 stimulation is ERK1/2-dependent, then calpain-2 has to

play an important role in cleaving spectrin upon netrin-1 stimulation or calpain-1 also gets activated by ERK1/2. Alternatively, since it has previously been shown that calpain1/2 can be activated by autolysis,[332, 334, 351, 352, 425] it is possible that calpain-2 gets activated by ERK1/2 downstream of netrin-1 which can then activate calpain-1. Calpain-1 can then take over by activating other calpain-1 molecules. However, since we see calpain activation within 5 minutes of netrin-1 stimulation and calpain-2 levels are much lower than calpain-1, it is unlikely that autolysis between calpain-2 and calpain-1 play any significant role. Other means of activating calpain downstream of netrin-1 stimulation is likely involved.

5.3.4 Netrin-1-stimulated calcium influx

Although intracellular calcium never reaches the in vitro levels required to maximally activate calpain, calcium influx can nevertheless play a role in potentiating the calpain activation in vivo. Since calpain-1 requires micromolar calcium concentrations whereas calpain-2 requires millimolar concentration; calpain-1 is more likely to get activated by intracellular calcium influx either from the extracellular space or release from intracellular pools. In support of this, filopodial calcium influx has been shown to influence growth cone behavior through calpain activation in *Xenopus laevis*.[378, 426] Calcium influx was shown to decrease the extent of axon outgrowth through calpain activation in Xenopus spinal neurons. Furthermore, the influx of calcium through TRPC1-containing calcium channels located on growth cone filopodia activates calpain, which then cleaves the integrin adaptor protein talin to reduce Src-dependent axon outgrowth.[394] The reduction in axon outgrowth, due to reduced adhesion upon calcium-mediated calpain activation is achieved, at least partly, by calpain-mediated FAK and talin cleavage.[340]

Several studies have implicated Src family kinase activation downstream of netrin-1 in the context of cortical axon outgrowth and guidance.[165, 166, 206] Similarly, we showed calpain-mediated FAK cleavage downstream of netrin-1 stimulation. Importantly, netrin-1-mediated growth cone turning has been reported to be dependent on depolarization-dependent Ca^{2+} influx through TRPC calcium channels as well as Ca^{2+}-induced Ca^{2+} release from cytoplasmic stores.[283, 427-430] Specifically, blocking Ca^{2+} influx converts netrin-1-mediated growth cone attraction to repulsion.[430] This Ca^{2+} influx and growth cone decision in terms of attraction or repulsion was shown to depend on the ratio of the small cyclic nucleotide cAMP and cGMP such that high cAMP/cGMP ratios lead to calcium influx and growth cone attraction.[428, 429] Therefore, netrin-1-mediated calpain activation likely involves parallel mechanisms: 1) ERK-dependent calpain activation and 2) Ca^{2+} influx through TRCP1-contaning calcium channels. In addition, autolysis might further activate other calpain molecules. Thus, local calcium influx and consequently calpain activation, could promote local and asymmetrical adhesion disassembly which would lead to growth cone turning, similarly to Xenopus spinal neurons. Taken together, although not sufficient to fully activate calpain in vivo, netrin-1-induced calcium influx could participate in calpain activation via calcium influx in parallel to ERK-dependent calpain activation and allow the proper growth cone response to netrin-1.

5.3.5 DCC-ICD and transcription regulation

Following extracellular metalloprotease-mediated DCC ectodomain shedding, leaving a small transmembrane and intracellular domain (ICD) at the plasma membrane (DCC stub) with signaling capabilities.[300, 301] The DCC stub is then subject to presenilin 1(PS1)-mediated

processing at the plasma membrane.[299-301] PS1 is an essential component of the γ-secretase complex that cleaves amyloid-β precursor protein (APP), leading to the formation of toxic plaques that contribute to Alzheimer's disease (AD).[431] Inhibiting PS1 with a potent γ-secretase inhibitor or culturing primary cortical neurons from PS1-deficient mice leads to increased axon outgrowth due increased DCC signaling.[300, 301] Interestingly, following γ-secretase cleavage, the DCC-ICD was shown translocate to the nucleus and activate transcription of a reporter, suggesting the DCC-ICD possesses an intrinsic transcriptional activation domain.[299] In Drosophila, the conserved P3 domain of frazzled/DCC was shown to encode a transcription activation domain (Figure 5.5).[318] This P3-activation domain activates transcription of the endosomal protein Commissureless (Comm), which reduces sensitivity to the midline repellant Slit.[317, 318] Thus, at least in Drosophila, upon γ-secretase cleavage the frazzled/DCC-ICD shuttles to the nucleus to activate transcription of comm and consequently promotes midline crossing independent of netrin-1.[318] How Frazzled/DCC-ICD regulate transcription is unclear since it does not contain an obvious DNA-binding domain.[318] Therefore, Neuhaus-Follini and Bashaw (2015), hypothesized that the Fra ICD's DNA-binding activity and specificity arise from associations with DNA-binding partners, as in the case of Notch.[318]

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Rat DCC                    1421 d lseqmasleg lmkqlnaitg saf   1445
Drosophila DCC (Frazzled)  1350 e lnqemanleg lmkdlsaita nefec 1375
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Figure 5.5: Drosophila frazzled and rat DCC P3 domain alignment

The P3 domain of rat DCC shares 58% (14/24)homology to frazzled's P3 domain in amino acids, which encodes an intrinsic transcription activation domain.

In mammalian cells the role of DCC-ICD is not as clear. To my knowledge, the only group that was able to visualize the highly labile DCC-ICD in mammalian cell lysates was by pre-treating HEK293cells with the proteasome inhibitor lactacystin.[299] In chapter 3, we showed an increase in the production of the DCC-ICD in rat primary cortical neurons without any pre-treatment, suggesting that this DCC-ICD might be stable enough to regulate transcription (Figure 5.6). Future studies will be needed to determine if this DCC-ICD fragment produced upon netrin-1 stimulation can shuttle to the nucleus and regulate transcription (Figure 5.6). Since no *comm* gene has been identified in mammals, other target genes will be regulated by this mammalian DCC-ICD. Although basal levels of DCC-ICD could be detected in cortical neurons, which could be mediated by γ-secretase cleavage, the increase in DCC-ICD production upon netrin-1 stimulation that we showed is mediated by calpain activity since calpastatin abolished this increase (Figure 5.6). Therefore, we show that the production of this particular DCC-ICD detectable in cortical neurons is mediated by the novel protease, calpain, targeting DCC upon netrin-1 stimulation (summarized in Figure 5.6).

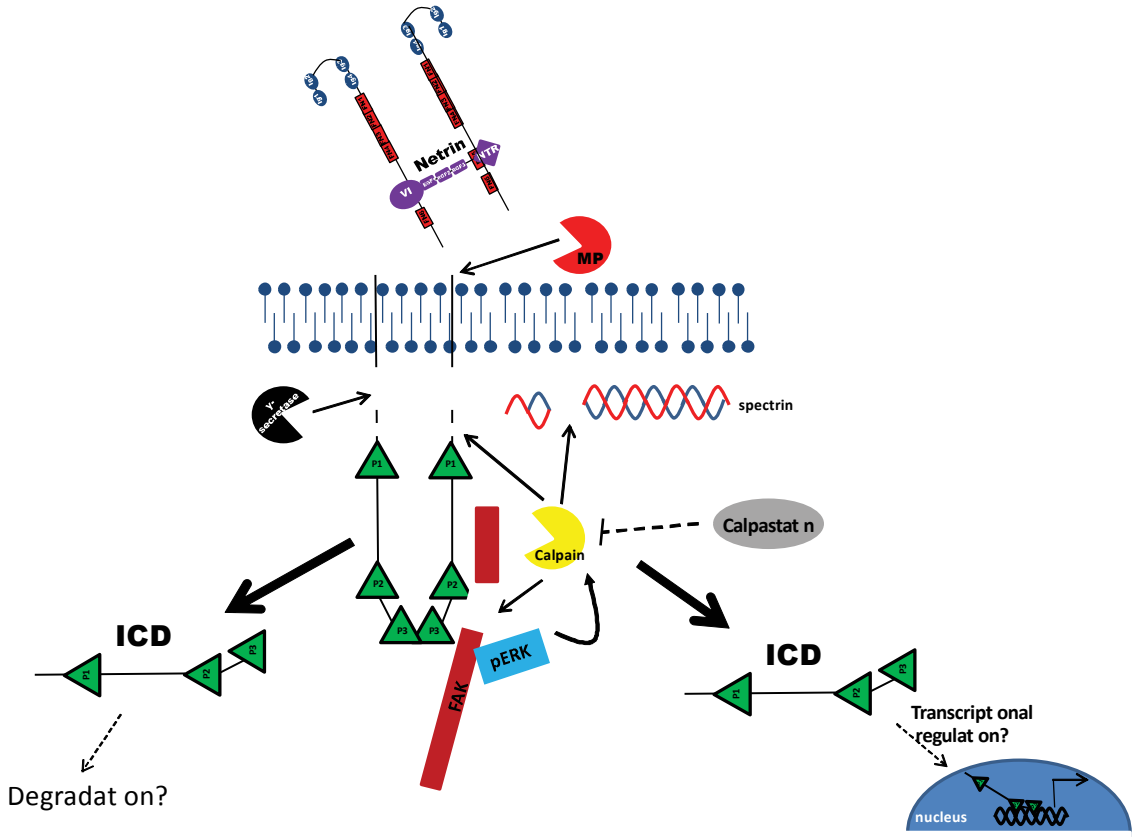

Figure 5.5: Cleavage events downstream of netrin-1 stimulation

Upon netrin-1 binding to DCC, extracellular metalloprotease (MP) releases the netrin-bound ectodomain. On the intracellular side, γ-secretase cleaves DCC at the plasma membrane. In addition, activated calpain cleaves DCC, releasing the DCC-ICD and potentially shuttling to the nucleus to mediate transcription.

Several calpain-targeted therapeutic strategies have already been developed and tremendous efforts are currently put in improving the specificity and bioavailability of calpain inhibitors to treat neurodegenerative disorders, brain ischemia, cardiovascular disorders, myopathies, ophthalmic diseases, cancer, infectious diseases, and recently identified calpainopathies.[350] Thus, a better understanding of the molecular mechanisms involved in calpain regulation will contribute at improving the therapies targeting these very diverse pathologies.

5.4. Phosphorylation of DCC Thr 1210 regulates its stability

In chapter 4 we identified a novel threonine phosphorylation site on the cytoplasmic tail of DCC. Our lab has showed that tyrosine 1418 phosphorylation in response to netrin-1 is required for downstream signaling and normal axon outgrowth and guidance.[206] Since the majority of DCC phosphorylation occurs on Ser/Thr residues, we decided to explore their function. When we mutated one of the identified sites (T1210) to an alanine we were intrigued by the lower molecular weight observed on SDS-PAGE. Furthermore, replacing the threonine 1210 with another hydrophobic residue, valine, seemed to prime DCC for proteolytic cleavage. By replacing the threonine with a glutamic acid we confirmed that the negative charge was sufficient to confer DCC the stability required to remain in its full-length form. Surprisingly, replacing Thr 1210 with an alanine or a valine did not result in DCC cleavage at the same location since the two mutants (DCC-T1210A and –T1210V) migrated differently on SDS-PAGE, indicating different molecular weights. With the aim of characterizing the function of this particular phosphorylation in the context of netrin-1-mediated axon outgrowth, we verified that the mutants were making their way to the plasma membrane where netrin-1-DCC interaction takes place and the signaling cascade is initiated. Since we couldn't detect any DCC-T1210A at the cell surface, we focused our efforts on the two mutants (DCC-T1210V and -T1210E) that were being localized at the plasma membrane for functional analyses. In addition, immunoprecipitation of the culture media of transfected cells confirmed the ectodomain shedding of DCC into the media. The DCC fragment being released into the media, along with the two mutants targeted to the cell surface were identical in size, suggesting a consensus cleavage site for ectodomain shedding. In rat embryonic cortical neurons, DCC-WT or DCC-T1210E, but not DCC-T1210V, overexpression was enough to increase axon outgrowth in the

absence of netrin-1 stimulation. We determined that cleavage of DCC-T1210V was occurring between amino acids 1201 and 1222, in the vicinity of Thr 1210. Finally, preliminary results suggests that the cleavage of DCC-T1210V might be mediated by calpain since incubation of cells expressing this mutant with calpastatin prior to cell lysis partially rescued DCC-T1210V cleavage.

5.4.1 Phosphorylation as a protective mechanism

Protein phosphorylation is one of the most common and important post-translational modifications (PTMs). This reversible PTM changes the polarity of the protein giving the modified protein hydrophilic properties and allowing it to change conformation and interact with different binding partners. Most cellular processes implicate protein phosphorylation such as in protein synthesis, cell growth, cell division, and signal transduction.[432] More than two-thirds of the 21,000 proteins encoded in the human genome has been shown to be phosphorylated and the majority of phosphorylation occurring on Ser/Thr. The majority of phosphorylation occurs on serine residues (86%) followed by threonine (12%) and tyrosine phosphorylation representing only 2%.[433] Similarly, Ser/Thr phosphorylation represents the majority of DCC phosphorylated residues.[206] DCC-T1210 is likely constitutively phosphorylated since removing the negative charge provided by the phosphate group renders DCC highly susceptible to proteolytic cleavage. Since phosphorylation can eliminate or introduce protein binding sites, it has the potential of regulating protein-protein interactions. These interactions can either protect the phosphorylated protein from proteases or can prime the modified protein for proteolysis.[434-445] Specifically, upon ligand binding, Erk1/2 phosphorylates the progesterone receptor, which targets the receptor for degradation.[445] Similarly, when Cubitus interruptus (Ci), a downstream effector of hedgehog signaling, gets phosphorylated by PKA, GSK3 and casein kinase I (CSKI), Ci gets targeted for

proteolysis which converts the transcription activator into a repressor.[439] In contrast, phosphorylation of neurofilaments, p35, and ezrin, among others, have been shown to be protective from calpain-mediated proteolysis.[434, 436, 441, 444] First, dephosphorylation of the 200kDa neurofilament increased its proteolysis by calpain.[441, 444] Second, a developmental switch in the phosphorylation status of p35 modifies its susceptibility to calpain-mediated cleavage.[434] Embryonic p35 is highly phosphorylated in the developing brain, which protects it from calpain proteolysis and prevents p25 production. In the adult brain, unphosphorylated p35 predominates which is readily cleaved by calpain into p25. Accumulation of this calpain-mediated cleavage of p35 into p25 has been observed in cultured neurons undergoing cell death.[446] Third, PKA-mediated phosphorylation on ser66 protects ezrin from calpain-1-mediated cleavage. Thus, Ser/Thr phosphorylation as a means to protect proteins from proteolysis could be a conserved mechanism regulating protein stability. Since the functional relevance of most ser/thr phosphorylation has yet to be characterized, future studies will be needed to determine if regulating protein stability through phosphorylation is a widespread mechanism inside the cell.

5.4.2 Phosphorylation modifies protein conformation

The DCC cleavage observed when Thr 1210 is replaced with a hydrophobic residue is likely due to a conformational change exposing a cleavage site normally hidden by the phosphorylated conformation. Phosphorylation can trigger transitions between conformations with different activity or binding specificity leading to distinct physiological consequences.[432, 433, 447] Since we observed two distinct fragments depending on the nature of the mutation (alanine or valine), different changes in conformation induced by the alanine and valine mutation

might expose different protease sites. Although preliminary results point to calpain as a candidate protease cleaving DCC-T1210V, we did not incubate cells expressing DCC-T1210V with an exhaustive number of inhibitors to conclusively determine the identity of the protease, as in Figure 4.7. In addition, we will need to repeat the experiment in Figure 4.7 to draw any solid conclusions on the rescue shown with calpastatin incubation. DCC-T1210A, on the other hand, is cleaved closer to the extracellular domain since we see a smaller fragment on SDS-PAGE as well as secreted in the cultured media when we label the fragment with an antibody targeting the extra-cellular domain. Therefore, γ-secretase might be cleaving DCC-T1210A, releasing the ectodomain and transmembrane domain into the media. Incubating cells expressing DCC-T1210A with a γ-secretase inhibitor will help clarify if this is the case. Alternatively, changes in conformation and 3D structure of the protein upon threonine 1210 mutation might render the protein unstable and cleaved within the secretory pathway which might explain why we cannot detect DCC –T1210A at the plasma membrane.[448]

5.4.3 Identity of the kinase phosphorylating Thr 1210

According to NetPhos 3.1's prediction algorithm, the likely candiate for the kinase responsible for phosphorylating T1210 is casein kinase II (CskII) (score: 0.518) and to a lesser extent GSK3 (score: 0.440). If this is the case, we predict that inhibiting CskII will render DCC unstable and susceptible to proteolytic cleavage, similarly to DCC-T1210A and DCC-T1210V.

5.4.4 The cytoplasmic tail of DCC is required for downstream signaling and axon outgrowth

The cytoplasmic tail of DCC is essential for downstream signaling and netrin-1/DCC-mediated axon outgrowth and guidance.[206, 266, 271, 449] The increased axon outgrowth measured when DCC-WT or DCC-T1210E, but not DCC-T1210V, was overexpressed in cortical neurons suggests that overexpression artificially mimicked netrin-1 stimulation by promoting DCC dimerization and consequently downstream signaling. The DCC-T1210V cleavage of the cytoplasmic tail indicates that without this signaling platform, DCC cannot induce the cytoskeletal changes required to potentiate axon outgrowth.

5.5 Conclusion

Cell signaling cascades involve various signaling mechanisms. In this thesis, we characterize three distinct means of regulating the netrin-1/DCC signaling pathway: 1) protein-protein interaction, 2) receptor proteolysis, and 3) receptor phosphorylation. The identification and characterization of RasGAP in the netrin-1/DCC signaling pathway highlights the crosstalk between Ras and Netrin-1 signaling. The GAP-independent functions identified in this thesis add an extra layer of complexity for the role of GTPase-activating proteins during cell signaling and the netrin-1/DCC signaling pathway in particular. The identification of Calpain-1/2 as a novel protease regulating netrin-1/DCC signaling and cortical axon outgrowth highlights the importance of signaling hubs in regulating crosstalk between pathways during axon outgrowth and guidance.[186, 187, 201] By implicating calpain-1 downstream of netrin-1/DCC signaling in addition to calpain-1's already documented role in semaphoring/Plexin-A1 signaling, we provide evidence to suggests that calpain-1 might be one of these signaling hubs regulating the expression of various guidance receptors. Playing a negative role in axon outgrowth as well as

being involved in cell death,[336, 342, 446, 450, 451] calpain could be targeted as a means to regenerate the nervous system, in addition to potential therapies in cardiovascular disorders, ophthalmic diseases, and cancer.[350] Since clinical trials targeting calpain are already under way, extending our knowledge of calpain function in health and disease will help minimize side effects due to calpain inhibition. Finally, by characterizing the role of a novel phosphorylation site that was found to be sporadically mutated in the human population,[295] we extend our knowledge of DCC regulation. This will help us have a better understanding of the causes of CMM and ACC in humans. Lastly, molecules targeting Netrin-1-DCC interaction are currently underway in the context of cancer[452] and a better understanding of DCC regulation will help improve therapeutic strategies.

Materials and methods

Plasmids

The plasmids pRK5, pRK5-DCC, pRK5-DCC-Y1418F, pRK5-DCC-Y1361F, pRK5-DCC 1-1327 and pRK5-DCC 1-1421 were previously described.[206, 271, 288] pRK5-DCC-P1336A/P1339A was generated by site-directed mutagenesis (Stratagene). The pCDNA3-GAP and pCDNA3-GAP-N (human) constructs were provided by T. Pawson.[401] The plasmids encoding GST and GST- human p120RasGAP (N-SH2, SH3, C-SH2 and SH2-SH3-SH2) were provided by L. Larose (McGill University, Montreal). pmaxGFP was purchased from Lonza (cat # VSPI-1003). The amino acid sequence alignment of DCC orthologs was generated with the BioEdit software Clustal W plugin. The following siRNAs were purchased: Silencer Negative Control No.1 siRNA (Life Technologies Ambion) and previously described 5'-GCAGGGAAATCTGGAAGCTACCTTA-3'p120RasGAP siRNA (Dharmacon).[382] Calpain-1 (5'-UACCUCUGUUCAAUUGCUCUA-3') and Calpain-2 (5'-GCGGUCAGAUACCUUCA UCAA-3') SiRNA were purchased from Bio Basic as previously described. pRK5-DCC-T1210A was generated by Mutagenex. The point mutations pRK5-DCC-T1210V and pRK5-DCC-T1210E were generated with "QuikChange II Site-Directed Mutagenesis Kit" (cat# 200523). For DCC-T1210V, the forward primer: 5'CCATTCAGGTCAAGATGTCGAGGA GGCAGGGAGCTCC-3' and the reverse primer 5'-GGAGCTCCCTGCCTCCTCGACATC TTGACCTGAATGG-3'. For DCC-T1210E, forward primer: 5'CCATTCAGGTCAAGATGAG GAGGAGGCAGGGAGCTCC-3' and reverse: 5'-GGAGCTCCCTGCCTCCTCCTCATC TTGACCTGAATGG-3' The calpastatin expression plasmid, pCMV-Sport6-Calpastatin, was kindly provided by Dr. Alyson Fournier (Montreal Neurological Institute, Montreal).

Cell culture and transfection

Cell culture was maintained in a humidified incubator at 37ºC with 5% CO2. HEK293 and COS-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Wisent Bioproducts) supplemented with 10% fetal bovine serum and antibiotics. Cells were transfected overnight with pRK5, pRK5-DCC, pRK5-DCC-T1210A, pRK5-DCC-T1210V, pRK5-DCC-T1210E, pCMV-Sport6-Calpastatin using polyethylenimine (PEI, PolyScience, cat # 23966-1) prepared according to the manufacturer's instructions.[273, 381] Briefly, HEK293 cells were plated in 100 mm-dishes and transfected when they reached 70-80% of confluency. cDNA constructs (4-8 μg) were incubated with PEI (40-80 μg) in 1 mL of DMEM for 15 minutes. The transfection mixes were then added to cells with 5 mL of fresh supplemented DMEM. After five hours transfection media was replaced with fresh media. Cells were harvested 16-20 hours post-transfection. 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 five hours and the media was replaced with Neurobasal-A medium supplemented with 2% B27 and 1% L-glutamine (Invitrogen). Neurons were treated with the following reagents: purified recombinant netrin-1(500ng/ml), Calpastatin (calbiochem), U0126 (Cell Signaling) DMSO or water.

Antibodies

The following antibodies were used for immunoblotting and immunofluorescence: mouse monoclonal anti-DCC, clone G97-449 (BD Biosciences Inc); mouse monoclonal (AF5) anti-

DCC (Calbiochem); anti-GST, anti-RasGAP B4F8 and anti-DCC A-20 (Santa Cruz Biotechnology); anti-phosphotyrosine (pY) 4G10, anti-tubulin (Millipore); anti-Calpain-1 (abcam); anti-Calpain-2 (Cell Signaling); anti-Calpastatin (Santa Cruz); anti-Spectrin, clone AA6 (milipore); anti-FAK (BS Biosciences); anti-phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) and anti-p44/42 MAPK (Erk1/2) (Cell Signaling Technology); anti-FAK [pY861] and anti-FAK [pY397] (Life Technologies Novex); anti-active Ras (NewEast Biosciences); anti-DCC [pY1418] (polyclonal antibodies raised in rabbit against the peptide KPTEDPASVpYEQDDL (DCC-pY1418)); anti-mouse Alexa 488, anti-mouse Cy3 and anti-rabbit Cy3, anti-rabbit Alexa 555 (Life Technologies Molecular Probes); anti-goat Immunoglobulin G (IgG) Cy3 (Sigma). The following reagents were used: recombinant chick netrin-1 and netrin-1 VI-V were produced and purified as previously described [251, 453], glutamate kindly provided by D. Bowie (McGill University), nerve growth factor (NGF) (Cedarlane).

Affinity purification and mass spectrometry

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

cut and sent to be identified by tandem mass-spectrometry. Protein identification was made with the Mascot software (Matrix Science).

Identification of the cleavage site DCC-T1210V

Overexpressed-DCC-T1210V in HEK293 cells was immunoprecipitated and loaded on SDS-PAGE for coomassie blue gel staining. The cleaved DCC-T1210V band was sent to the "Proteomics Research Discovery Platform" of the IRCM for tandem mass-spectrometry.

Purification of GST fusion proteins

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

Peptides, SH2 domain array and dot blot assays

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

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

GST pull-down

Transfected HEK293 cells were lysed in 1% Triton X-100 lysis buffer as previously described [183]. Protein lysates (1mg) were pre-cleared with 30 μl of glutathione–agarose beads (Sigma Aldrich) for 2 h at 4°C and incubated with 10 or 20 μg of fresh GST or GST-p120RasGAP proteins coupled to glutathione–agarose beads for 3 h at 4°C. Beads were washed three times in ice-cold lysis buffer and boiled in SDS sample buffer.

Immunoprecipitation

Cortical neurons (2 days in vitro (DIV)) lysates were prepared as previously described [273]. Protein lysates (1mg) were incubated with 4ug of anti-p120RasGAP with protein G Sepharose beads (GE Healthcare) for 3 h at 4°C.

Immunoblotting and quantitative densitometry

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

Ras G-LISA assay

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

Immunofluorescence

Cortical neurons (2DIV) were fixed 30 min with 3.7% formaldehyde in 20% sucrose PBS at 37°C, quenched 5 min in 0.1 M glycine at room temperature, permeabilized 5 min in 0.25% Triton X-100, and blocked 30 min with 3% BSA. Primary and secondary antibodies were incubated in 0.3% BSA. A 15 min fixation with 10% trichloroacetic acid in water was used for

phosphospecific antibodies [454]. Neurons were examined with the following: Olympus IX81 motorized inverted microscope (40× U PLAN Fluorite and 60× U PLAN S-APO oil objective lenses) with a CoolSnap 4K camera (Photometrics); Zeiss LSM780 confocal microscope (63×/1.40 oil Plan-Apochromat) with 488 nm argon and 561 nm DPSS lasers and a GaAsP detector. Pearson's correlation coefficient and fluorescence intensity quantification were measured with the Metamorph software.

Immunohistochemistry

E17.5 embryonic brains were fixed in 4% PFA overnight, cryopreserved for 2 days in 30% sucrose, and cryosectioned (10μm/section). Slides were then dried at 37^{o}C before washing with PBS. Antigen retrieval for Calpain-1 and Calpastatin was necessary for optimal staining: Slides were incubated with 10mM Sodium Citrate buffer, pH 6.0 at 90^{o}C for 20 minutes, then cooled at room temperature and washed several times with PBS. Blocking for one hour with 5% donkey (in PBS+0.1%Triton-X 100) serum at room temperature was performed before incubation with anti-DCC, clone G97-449 (BD Biosciences Inc), anti-Calpain-1 (abcam); anti-Calpain-2 (Cell Signaling); anti-Calpastatin (Santa Cruz) in PBS+0.1%Triton-X 100 overnight at 4^{o}C.

Axon outgrowth and Dunn chamber assays

Axon length of GFP-expressing cortical neurons (2DIV) was measured with Metamorph. Cortical neurons (2DIV) were plated on coverslips used for Dunn chamber assembly as previously described [387]. Gradients were generated with purified netrin-1 VI-V (200 ng/ml) or buffer containing PBS in the outer well. Cell images were acquired every 3–4 min for at least 90 min on a temperature controlled stage. Neurites of at least 10 μm in length were tracked in GFP-expressing neurons. The final position of the growth cone was used to determine the angle turned

over 90 min relative to the gradient position. Measurements are presented in rose histograms in bins of 10° with the length of each segment representing the frequency of measurements in percent. Percentage distribution of turned angles, average turned angle and average displacement are also represented.

Time-lapse fluorescence imaging

Live cell imaging was performed with a Zeiss inverted microscope LSM780 using a 40x oil objective and 10x digital zoom. Images were acquired every 60 seconds and played every 25msecs. To maintain cell viability, the microscope was equipped with a chamber to regulate temperature and CO2. We also limited the time the cells were incubated in the microcope chamber to 45-50 minutes. For the addition of netrin-1, 50µl of solution containing the amount of netrin-1 for a final dose of 500nM was applied.

Cell surface biotinylation assay

Transfected HEK293 cells were washed three times with cold PBS (+ 1mM $MgCl_2$ +0.1mM $CaCl_2$) and incubated at 4^{o}C for 20 minutes with 5 ml of EZ-Link-Sulfo-NHS-LC-Biotin (0.5mgml) in PBS. Cells were then washed four times with PBS (10mM glycine) at 4^{o}C before lysis with RIPA buffer. After centrifugation, supernatants were loaded on SDS-PAGE for western blot analysis.

Calpain Assay

DCC-transfected HEK293 cells were lysed with HEPES-based RIPA buffer containing 2mM $CaCl_2$ without protease inhibitors. Immunoprecipitated DCC was then incubated with 1.6 ug of

Calpain-1 (purified from *Porcine Erythrocytes* - cat# 208712) for 30 minutes at 37^{o}C. Reaction was stopped by adding 50 ul of 5X laemmli buffer.

Calpain activity assay

20 µM of BOC-LM-CMAC (Molecular Probes) was added to living cortical neurons 20 minutes prior to visualization of Calpain activity. Exposure times were kept constant and at a medium grey scale saturation to detect both increase and decrease in fluorescence. When imaged at 20 minutes after addition of tBoc, the observed intensity was highly reproducible between experiments and therefore the data were combined. After some initial trials, we determined that measurements from neuronal somas and proximal axon, as opposed to distal neurites and growth cones, would be more easily measurable. A compartment of less variable size (or width), as in the case of the soma, is more desirable than distal neurites because it minimizes the differences in fluorescence due to changes in neurite thickness. In addition, the highly dynamic nature of growth cones and distal axons make it difficult to follow and measure accurately over an extended period of time. In contrast, within the 15 minutes imaging period, the cell soma and proximal axon are relatively static and allow for more accurate measurements. After subtracting background levels, we referred every data point of the "control" and netrin-1 coverslips to a fold change to pre-treatment activity.

Statistical analysis

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

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