Exploring the roles of RhoA and Collapsin Response Mediator Protein 4 (CRMP4) in the response to cellular damage

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ABSTRACT

The cytoskeleton is an essential structural component of eukaryotic cells that provides mechanical support, and mediates rapid responses to extrinsic and intrinsic stimuli. In neurons, this intricate meshwork underlies many cellular functions, including neurite outgrowth and guidance as well as synaptic plasticity. However, the cytoskeleton can become disorganized following injury and its reassembly is key to recovery. Several proteins regulate cytoskeletal dynamics in the developing and injured nervous system. In this thesis, I focused on two of these regulatory proteins, collapsin response mediator protein 4 (CRMP4) and RhoA, as they are key players in the failure of neuronal regeneration observed following injury. Primarily expressed in the nervous system, CRMP4 is a phospho-protein that possesses growth-promoting roles in developing neurons and growth-inhibitory functions in the injured central nervous system (CNS). However, its functions in the neuronal response to injury in the peripheral nervous system (PNS) remained unclear and thus, I investigated those in chapter 2. I found that CRMP4 facilitates the regrowth of the axons of damaged sensory neurons, while its calpain-dependent cleavage contributes to Wallerian degeneration. Through its combined effects, CRMP4 facilitates PNS regeneration. On the other hand, the ubiquitously expressed small GTPase RhoA is involved in many cellular functions during development, adulthood and in response to various insults. Due to its broad expression and functions, it is tightly regulated by several mechanisms. In chapter 3, I characterized a novel proteolytic event that regulates RhoA in response to cell stress. I found that the fragments generated upon RhoA cleavage affect the actin cytoskeleton by regulating the assembly of stress fibres and nuclear rods. Overall, the findings of this thesis further our understanding of the functions of CRMP4 and of the regulation of RhoA in the cellular response to damage. These findings could be extrapolated to other injury models and thus, they become key to the identification of novel targets that could be used to develop more specific therapies to improve the neuronal response to various insults.

RÉSUMÉ

Le cytosquelette est un composant structurel essentiel des cellules eucaryotes qui fournit un support mécanique et permet des réponses rapides aux stimuli extrinsèques et intrinsèques. Dans les neurones, ce maillage complexe sous-tend de nombreuses fonctions cellulaires, notamment la croissance et la navigation des neurites ainsi que la plasticité synaptique. Cependant, le cytosquelette peut devenir désorganisé suite à un dommage et son réassemblage est clé au rétablissement fonctionel. Plusieurs protéines régulent la dynamique du cytosquelette dans le système nerveux en développement et suite à une blessure. Dans cette thèse, je me concentre sur deux de ces protéines régulatrices, collapsin response mediator protein 4 (CRMP4) et RhoA, car celles-ci jouent un rôle important à l'échec de la regeneration neuronale observé suite à un dommage. Exprimée principalement dans le système nerveux, CRMP4 est une phospho-protéine qui joue un rôle favorable au development des neurones, alors qu'elle est inhibitoire dans le système nerveux central (SNC) endommagé. Cependant, ses fonctions dans la réponse neuronale suite à une lésion du système nerveux périphérique (SNP) demeurent inconnues et donc, nous investigons celles-ci dans le chapitre 2. J'ai constaté que CRMP4 facilite la repousse des axones des neurones sensoriels endommagés, tandis que son clivage par calpaïne contribue à la dégénérescence wallérienne. Grâce à ses effets combinés, CRMP4 facilite la régénération du SNP. D'autre part, omniprésente dans les cellules, la petite GTPase RhoA intervient dans de nombreuses fonctions cellulaires au cours du développement, à l'âge adulte et en réponse à diverses agressions. En raison de son expression et ses fonctions, cette protéine est étroitement régulée par plusieurs mécanismes. Dans le chapitre 3, j'ai caractérisé un nouveau mécanisme protéolytique régulant RhoA en réponse au stress cellulaire. J'ai constaté que les fragments générés lors du clivage de RhoA affectent le cytosquelette d'actine en régulant l'assemblage des fibres de stress et des bâtonnets nucléaires. Dans l'ensemble, les résultats de cette thèse nous permettent de mieux comprendre les fonctions de CRMP4 et la régulation de RhoA dans la réponse cellulaire suite aux dommages. Ces découvertes peuvent être extrapolées à d'autres modèles et, ainsi, elle deviennent essentielles à l'identification de nouvelles cibles pour le développement de thérapies qui améliorent la réponse neuronale face à diverses agressions.

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LIST OF ABBREVIATIONS

ADF: Actin-depolymerizing factor
ADP: Adenosine diphosphate
AEBSF: 4-(2-aminoethyl)benzenesulfonylfluoride
AKT: Protein kinase B
ALS: Amyotrophic lateral sclerosis
APC: Adenomatous polyposis coli
Arp2/3: Actin-related protein-2/3
ATP: Adenosine triphosphate
BDNF: Bone-derived neurotrophic factor
BSA: Bovine serum albumin
$CaCl_2$: Calcium chloride
Cdc42: Cell division control, protein 42
Cdk5: Cyclin-dependent kinase 5
CGRP: Calcitonin gene-related protein
ChABC: Chondroitinase ABC
ChAT: Choline acetyltransferase
CHO: Chinese ovary cells
CNS: Central nervous system
CO_2: Carbon dioxide
CRAM: CRMP3-associated molecule
CRMP: Collapsin response mediator protein
CRMP4L: Collapsin response mediator protein 4, long isoform
CRMP4S: Collapsin response mediator protein 4, short isoform
CSPG: Chondroitin sulfate proteoglycan
CTF: Carboxy-terminal cleavage fragment
Cul3: Cullin-3
DBE: Dibenzyl ether
DIV: Days *in vitro*
DMSO: Dimethyl sulfoxide
DLK: Dual leucine zipper kinase
DPI: Days post-injury
DRG: Dorsal root ganglion
DRP: Dihydropyrimidinase-related protein
DTT: Dithiothreitol
DYRK2: Dual specificity tyrosine-phosphorylation-regulated kinase 2
EDTA: Ethylenediaminetetraacetic acid
Ena: Enabled
ERK: Extracellular signal-regulated kinase
ES: Embryonic stem
F-actin: Actin filament
FBS: Fetal bovine serum

FDU: 5-fluoro-2'-deoxyuridine
FH1: Formin homology 1
FH2: Formin homology domain 2
FL: Full-length
G-actin: Globular actin
GAP: GTPase activating protein
GAP-43: Growth associated protein 43
GDI: Guanine nucleotide dissociation inhibitor
GDP: Guanosine diphosphate
GEF: Guanine nucleotide exchange factor
GSK3: Glycogen synthase kinase 3
GTP: Guanosine triphosphate
HCl: Hydrochloric acid
H_2O_2: Hydrogen peroxide
HPI: Hours post-injury
HRP: Horse-radish peroxidase
HSV: Herpes simplex virus
Htr2A: 5-hydroxytryptamine receptor 2A
IB4: Isolectin B4
IPTG: Isopropyl β-D-1-thiogalactopyranoside
JNK: c-Jun-terminal kinase
KCl: Potassium chloride
KRK-5: Kallikreins-related peptidases 5
LIMK: LIM kinase
LPA: Lysophosphatidic acid
LTD: Long-term depression
LTP: Long-term potentiation
MAG: Myelin-associated glycoprotein
MAI: Myelin-associated inhibitor
MAP1B: Microtubule-associated protein 1B
MAP2: Microtubule-associated protein 2
MAPK: Mitogen-activated protein kinase
mDia: Mammalian diaphanous-related formin
Mg^{2+}: Magnesium
$MgCl_2$: Magnesium chloride
NaCl: Sodium chloride
NAD+: Nicotinamide adenine dinucleotide
NaF: Sodium fluoride
MAG: Myelin-associated lycoproteins
$NaKPO_4$: Sodium potassium phosphate
Na_3VO_4: Sodium orthovanadate
NEFH: Neurofilament heavy chain
NFH: Neurofilament heavy chain
NFL: Neurofilament light chain
NFM: Neurofilament medium polypeptide
NGF: Nerve growth factor

NGS: Normal goat serum
NMNAT1: Nicotinamide mononucleotide adenylyltransferase 1
NT-3: Neurotrophin-3
NTF: Amino-terminal cleavage fragment
OMgp: Oligodendrocyte-myelin glycoprotein
PBS: Phosphate-buffered saline
PCR: Polymerase chain reaction
PFA: Paraformaldehyde
PKA: Protein kinase A
PKG: Protein kinase G
PKN: Protein kinase N
PLL: Poly-L-lysine
PNS: Peripheral nervous system
PVDF: Polyvinylidene fluoride
Rac: Ras-related C3 botulinum toxin substrate
RAG: Regeneration-associated gene
RBD: Rhotekin-binding domain
RFP: Red fluorescent protein
RGC: Retinal ganglion cell
RhoA: Ras homologous member A
ROCK: Rho-associated protein kinase
SCF^{FBXL19}: Skp1-Cul1-F-box (SCF) FBXL19 E3 ubiquitin ligase
SCG10: Superior cervical ganglion-10
S.E.M.: Standard error of the mean
Sema: Semaphorin
SDS: Sodium dodecyl sulfate
SMA: Spinal muscular atrophy
STMN2: Stathmin-2
TBS: Tris-buffered saline
tCRMP4: Truncated collapsin response mediator protein 4
THF: Tetrahydrofuran
TOAD-64: Turn on after division, 64 kDa
TrkA: Tropomyosin receptor kinase A
TrkB: Tropomyosin receptor kinase B
TUC: TOAD/Ulip/CRMP
Ulip: unc-33-like-phosphoprotein
WASP: Wiskott-Aldrich syndrome protein
WT: Wild-type
YopT: Yersinia outer protein

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CONTRIBUTIONS OF AUTHORS

Chapter 2: Collapsin response mediator protein 4 (CRMP4) facilitates Wallerian degeneration and axon regeneration following sciatic nerve injury

- **Marie-Pier Girouard:** Designed the project; performed the experiments and analyses pertaining to Fig. 2.1-4, 2.5 A-B, 2.6 C-D; wrote, edited and revised the manuscript.
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- **Ricardo L. Sanz:** Helped with the DRG dissections.
- **Isabel Rambaldi:** Performed the cloning to generate cleavage-resistant CRMP4 and the calpain digestion in Fig. 2.6 A; performed the cloning and purification of the TAT peptides in Fig. 2.6 B.
- **Marie-Eve Di Raddo:** Performed the analysis depicted in Fig. 2.1 B.
- **Kanchana K. Gamage:** Performed the analysis illustrated in Fig. 2.4 B.
- **Yu Yong:** Performed the analysis illustrated in Fig. 2.4 B.
- **Dianna Willis:** Provided training and conceptual support for the experiments using microfluidic devices; edited the manuscript.
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Chapter 3: RhoA proteolysis regulates the actin cytoskeleton in response to oxidative stress

- **Marie-Pier Girouard:** Contributed to the design of the project; performed experiments and analysis pertaining to figures 3.1 to 3.6 A-C; wrote, edited and revised the manuscript for publications.
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- **Ricardo Alchini:** Performed the analysis in figure 3.6 D.
- **Isabel Rambaldi:** Cloned the various DNA constructs used throughout this project.
- **Alyson E. Fournier:** Designed the project; contributed to the analysis of the data; wrote, edited and revised the manuscript.

INTRODUCTION

Eukaryotic cells are complex entities that are structurally strong and resilient, in order to withstand intrinsic and extrinsic forces, and maintain their morphology. However, they are also very dynamic, which enables them to rapidly respond to various internal and external stimuli. The cytoskeleton, an essential component of eukaryotic cells, plays a major role in those cellular properties. This array of protein filaments is organized as an elaborate network within the cell, which serves to maintain its integrity and morphology. In response to various stimuli, this network can rapidly reorganize itself and alter the structure of the cell. This rearrangement is driven by an array of regulatory proteins and post-translational modifications. In this thesis, I will highlight the mechanisms of action and functions of two cytoskeletal regulators, RhoA and collapsin response mediator proteins (CRMPs), in the cellular response to various insults.

1.1 Cytoskeleton

1.1.1 Structural components of the cytoskeleton

The cytoskeleton of neurons located in the vertebrate's central and peripheral nervous system (CNS and PNS, respectively) is composed of three types of filaments: actin filaments, microtubules and intermediate filaments. Although the properties of each vary, their inter-relations as a cellular network underlie many functions in intact and damaged neurons.

1.1.1.1 Actin filaments

Globular actin (G-actin) exists as multiple isoforms, with β- and γ-actin isoforms predominating in the vertebrate's nervous system (Choo and Bray, 1978). Asymmetric G-actin subunits assemble linearly to form actin filaments (F-actin), which are polarized by the presence of a fast growing barbed end and a relatively inert pointed end (Letourneau, 2009). However, the interaction between G-actin monomers is weak and thus, the turnover of F-actin occurs quickly. Subunits are continuously polymerized onto the barbed end that is oriented toward the leading edge of the cell, removed from the pointed end and recycled via replenishment of their ATP state (Okabe and Hirokawa, 1991; Mallavarapu and Mitchison, 1999; Pollard and Borisy, 2003; Ponti

et al., 2003; Letourneau, 2009). This cycling between polymerization and depolymerization is referred to as treadmilling (Letourneau, 2009). Several proteins regulate this cycling by binding and/or sequestering actin monomers, nucleating actin filaments, capping the barbed or pointed end, uncapping the barbed end, severing F-actin, bundling, cross-linking or stabilizing F-actin, or anchoring F-actin to the membrane (Dent and Gertler, 2003; Letourneau, 2009).

1.1.1.2 Microtubules

Microtubules are composed of α/β-tubulin heterodimers. In mammals, several tubulin isoforms exist; however, only 5 α-tubulins (α1A/B/C, α4A, α8) and 5 β-tubulins (β2A/B, β3, β4, β5) subunits are found in the brain (Luduena, 1998; Fukushima et al., 2009). This suggests that the neuronal population of microtubules is heterogeneous and that their subunit's composition could influence their dynamics (Panda et al., 1994; Derry et al., 1997; Schwarz et al., 1998; Bode et al., 2003; Khan and Luduena, 2003). α/β-tubulin heterodimers are linearly assembled as protofilaments, which then assemble to form hollow cylindrical structures. As the heterodimers are asymmetric, the microtubules formed have an inherent polarity with a fast-growing plus-end and an unstable minus-end. The plus-end of the microtubule is very dynamic, cycling through phases of growth and shrinkage, referred to as rescue and catastrophe respectively (Mitchison and Kirschner, 1984; Walker et al., 1988; Dent and Gertler, 2003; Gardner et al., 2013; Brouhard, 2015). This dynamic instability allows the microtubule to probe the intracellular environment prior to its stabilization (Sabry et al., 1991; Holy and Leibler, 1994). Microtubule dynamics are regulated by an array of inherent properties and regulatory proteins. As such, microtubules are regulated by post-translational modifications, including tyrosination/detyrosination, acetylation, polyglutamylation and polyglycylation (Luduena, 1998; Fukushima et al., 2009; Janke and Bulinski, 2011). Although these modifications were shown to have little effect on the stabilization of the microtubules (Khawaja et al., 1988; Webster et al., 1990), they regulate the binding of microtubule-associated proteins and the interaction with other cytoskeletal components (Boucher et al., 1994; Gurland and Gundersen, 1995; Larcher et al., 1996; Kreitzer et al., 1999). Furthermore, several microtubule-associated proteins regulate microtubule dynamics via their stabilization, by acting as motors, or by binding the plus- or minus-end (Dent and Gertler, 2003; Kevenaar and Hoogenraad, 2015).

1.1.1.3 Intermediate filaments

Intermediate filaments are structural proteins that are conserved across eukaryotic cells, but their expression is developmentally regulated and cell type-dependent (Lasek et al., 1985; Laser-Azogui et al., 2015; Kirkcaldie and Dwyer, 2017). In PNS neurons and myelinated CNS neurons, those include nestin, vimentin, the three isoforms of neurofilaments (NFL, NFM, NFH), peripherin in PNS neurons and α-internexin in CNS neurons (Cochard and Paulin, 1984; Trojanowski et al., 1986; Lendahl et al., 1990; Nixon and Shea, 1992; Parlakian et al., 2016; Luo et al., 2017). Nestin and vimentin are detected in the earlier stages of embryogenesis, while neurofilaments, peripherin, and α-internexin are expressed in the later stages (Cochard and Paulin, 1984; Lendahl et al., 1990; Nixon and Shea, 1992; Laser-Azogui et al., 2015; Luo et al., 2017). Despite their diversity, intermediate filaments share a common structure composed of a central α-helical rod domain flanked by a highly variable non-α-helical amino-terminal head and a carboxy-terminal tail. These rods assemble as anti-parallel dimers to form protofilaments that further assemble as filaments (Willard and Simon, 1981; Lasek et al., 1985; Nixon and Shea, 1992; Perrot and Eyer, 2013; Laser-Azogui et al., 2015). In neurons, the main functions of intermediate filaments lie in the establishment and maintenance of the caliber of the axon, which indirectly affects conduction velocity, and enables myelination as well as plasticity (Friede and Samorajski, 1970; de Waegh et al., 1992; Xu et al., 1996; Elder et al., 1998; Garcia et al., 2003). Furthermore, they serve as intermediaries in the communication between microtubules, cell surface molecules and various organelles (Rao et al., 2011; Perrot and Eyer, 2013; Kirkcaldie and Collins, 2016). The functions and interactions of intermediate filaments are regulated by post-translational modifications such as phosphorylation, glycosylation and transglutamination (Perrot and Eyer, 2013; Snider and Omary, 2014).

1.1.2 Physiological organization and functions of the cytoskeleton

The intracellular segregation of the different cytoskeletal components, including microtubules, F-actin and intermediate filaments, confers the characteristic star-like morphology observed in neurons (Coles and Bradke, 2015; Kevenaar and Hoogenraad, 2015). Moreover, their organization as elaborate structures and networks within the neurons underlies specific cellular functions (summarized in Fig. 1.1). Some of these structures, like the actin/spectrin membrane-associated subplasmalemmal skeleton and the microtubule tracts, are fairly stable, consequently

providing mechanical and structural support to the neuronal processes (Xu et al., 2013; Zhong et al., 2014; D'Este et al., 2015; Kapitein and Hoogenraad, 2015; Kevenaar and Hoogenraad, 2015; Dubey et al., 2018). However, some others can rapidly rearrange themselves in response to various stimuli and consequently, they underlie dynamic functions, like synaptic plasticity, neurite elongation or axon guidance (Dent and Gertler, 2003; Sankaranarayanan et al., 2003; Dillon and Goda, 2005; Cingolani and Goda, 2008; Korobova and Svitkina, 2010; Bradke et al., 2012; Vitriol and Zheng, 2012; Nelson et al., 2013; Spira and Erez, 2013; Bury and Sabo, 2014; Rust and Maritzen, 2015; Wolf et al., 2015). Thus, the organization and properties of the different cytoskeletal components underlies many physiological processes that are essential to neuronal structure and functions.

1.1.3 Reorganization of the cytoskeleton in response to neuronal injury

The intricate organization of the neuronal cytoskeleton can become altered in response to various types of insults and the re-organization of the different cytoskeletal components is key to several cellular processes that drive recovery.

1.1.3.1 Neuronal response to PNS injury

An ideal situation is observed in the mammalian PNS, as neurons initiate a stereotypical response that promotes regeneration following injury and recovery occurs to a certain extent (Huebner and Strittmatter, 2009; Mokarram and Bellamkonda, 2011; Mietto et al., 2015). However, this situation remains less than optimal, as long-distance regeneration is slow and impaired by many factors, thus resulting in the partial or complete loss of sensory and/or motor functions (Mokarram and Bellamkonda, 2011). The response to injury observed in mammalian PNS neurons is dependent on a number of intrinsic and extrinsic factors. Intrinsically, damaged PNS neurons can revert to a growth-promoting state that supports regeneration following injury, via cytoskeletal reorganization. This favorable response is initiated by the disruption of the integrity of the cell membrane in response to injury, which triggers membrane depolarization as well as a rise in the intracellular concentration of calcium and the subsequent activation of calpain, a calcium-dependent cysteine protease (Spira et al., 1993; Ziv and Spira, 1993, 1995; Spira et al., 2001). Once activated, calpain promotes the disassembly of the cytoskeleton via the degradation of different cytoskeletal-associated proteins and consequently, favors the collapse

and resealing of the damaged cytoplasmic membrane (Fishman and Bittner, 2003; Yoo et al., 2003; Sahly et al., 2006; Erez et al., 2007; Kamber et al., 2009). In the axon segments still connected to the cell body, microtubule-based plus-end traps are assembled near the ruptured end to accumulate anterogradely-transported Golgi-derived vesicles, which are essential to the repair of the broken cytoplasmic membrane (Erez et al., 2007; Erez and Spira, 2008; Kamber et al., 2009). Additionally, the restructuring of the cytoskeleton combined with local protein translation support the formation of a new growth cone that will drive axon extension (Spira et al., 1993; Spira et al., 2001; Verma et al., 2005; Kamber et al., 2009; Vogelaar et al., 2009; Yan et al., 2009; Ghosh-Roy et al., 2010; Hur et al., 2011; Bradke et al., 2012; Hur et al., 2012; Spira and Erez, 2013). Furthermore, retrograde signals mediate the upregulation of various regeneration-associated genes (RAGs) and the complementary downregulation of pro-apoptotic and pro-degenerative genes, which sustain axon regeneration over long distance (Cavalli et al., 2005; Michaelevski et al., 2010; Xiong et al., 2010; Ben-Yaakov et al., 2012; Shin et al., 2012a).

Also, as part of the regenerative response, the axon segments disconnected from their cell body undergo Wallerian degeneration (Waller, 1850). This process is initiated by the breakage of the membrane, which favors the rise of the intracellular concentration of calcium that triggers many signaling pathways that promote the disassembly of the cytoskeleton and consequently, axon fragmentation (Ziv and Spira, 1993; Kerschensteiner et al., 2005; Osterloh et al., 2012; Yang et al., 2013; Zhang et al., 2016b; Neukomm et al., 2017; Unsain et al., 2018).

Interestingly, regeneration and degeneration are intrinsically linked, as regeneration can be impaired by delayed degeneration (Bisby and Chen, 1990; Brown et al., 1992; Brown et al., 1994). These processes share several signaling pathways, many of which regulate the cytoskeleton, and targeting these simultaneously could potentially improve neuronal regeneration and functional recovery (Girouard et al., 2018).

Furthermore, the regeneration of mammalian PNS neurons is supported by different extrinsic factors. As such, Schwann cells de-differentiate and reorganize as bands of Büngner, which guide and support the growth of the regenerating neurons (Terenghi et al., 1998; Clements et al., 2017; Gomez-Sanchez et al., 2017; Hyung et al., 2018). These non-neuronal cells, combined

with macrophages and neutrophils, also phagocytose the axonal debris generated by the injury and subsequent axonal degeneration, thus creating a growth-promoting environment (Venezie et al., 1995; Kuhlmann et al., 2002; Brosius Lutz et al., 2017; Lindborg et al., 2017). Also, several inhibitory cues including chondroitin sulfate proteoglycans (CSPGs) and myelin-associated glycoproteins, are released in the environment upon injury. These inhibitory cues contribute favorably to neuronal regeneration by limiting hyperinnervation and promoting target reinnervation (Hiraga et al., 2006; Tomita et al., 2007; Cheng et al., 2008; Huelsenbeck et al., 2012). Thus, the intrinsic growth potential of the neurons combined with several external factors contribute to the regeneration of PNS neurons upon injury.

Despite this favorable response to injury, PNS regeneration is still challenging and the recovery of sensory and/or motor functions is often incomplete. These challenges come from multiple sources, several of which stem from the neurons themselves (Verdu et al., 2000; Hoke and Brushart, 2010). For example, the rate of axonal regeneration is highly dependent on the age of the animals, as older subjects experience an age-related decline in several neuronal, glial and immune functions (Verdu et al., 2000). Furthermore, the different subpopulations of PNS neurons possess diverging intrinsic regenerative potential. For example, motor and sensory neurons possess different regenerative abilities, although some controversies remain as to which subpopulation regenerates better (da Silva et al., 1985; Madison et al., 1988; Madorsky et al., 1998; Kawasaki et al., 2000; Moldovan et al., 2006; Allodi et al., 2011; Jianping et al., 2012; Tong et al., 2015; Cheah et al., 2017). Similarly, different subtypes of sensory neurons exhibit heterogeneous response to injury, even if all subtypes upregulate RAGs (Hu and McLachlan, 2003; Welin et al., 2008; Hu et al., 2016). For example, in addition to RAGs, the small-diameter non-peptidergic neurons upregulate several genes associated with cell death and pain, which renders them more vulnerable to cell death and favors the development of neuropathy following injury (Tandrup et al., 2000; Hoitsma et al., 2004; Huang and Song, 2008; Hovaguimian and Gibbons, 2011; Hu et al., 2016).

1.1.3.2 Neuronal response to CNS injury

Unlike their PNS counterparts, neurons located in the CNS fail to mount a regenerative response that would support the growth of damaged axons. The tips of these axons often re-assemble as

dystrophic retraction bulbs, which possess a disorganized microtubule network that is not conductive to axon extension (Li and Raisman, 1995; Hill et al., 2001; Erturk et al., 2007). The formation of these retraction bulbs is due to a combination of extrinsic growth-inhibitory cues and a deficient intrinsic response that fails to upregulate certain genes associated with microtubule polymerization and stabilization (Erturk et al., 2007). Extrinsic cues, including chondroitin sulfate proteoglycans (CSPGs) and myelin-associated inhibitors (MAIs) such as myelin-associated glycoproteins (MAG), oligodendrocyte-myelin glycoprotein (OMgp) and Nogo, are released in the environment upon injury (McKerracher et al., 1994; Mukhopadhyay et al., 1994; Chen et al., 2000; GrandPre et al., 2000; Kottis et al., 2002; Morgenstern et al., 2002; Wang et al., 2002; Filbin, 2003; He and Koprivica, 2004; Mimura et al., 2006; Yiu and He, 2006). These inhibitory cues trigger various signaling pathways that converge onto the small GTPase RhoA (Fig. 1.2; (Hiraga et al., 2006; Mimura et al., 2006; Yiu and He, 2006; Alabed et al., 2007; Cheng et al., 2008)). Once activated, RhoA, via its downstream effectors, inhibits axon extension and promotes growth cone collapse (McKerracher et al., 1994; Mukhopadhyay et al., 1994; Arimura et al., 2000; Chen et al., 2000; GrandPre et al., 2000; Kottis et al., 2002; Wang et al., 2002; Hiraga et al., 2006; Mimura et al., 2006; Yiu and He, 2006; Cheng et al., 2008; Quarta et al., 2017). Consequently, a combination of intrinsic and extrinsic factors contributes to the inhibition of neuronal regeneration in the CNS.

1.1.3.3 Therapeutic strategies to improve the neuronal response to injury

The intricacy of the different players involved in the neuronal response to PNS and CNS injury complicates the development of therapeutic strategies to promote functional recovery. However, despite these limitations, several therapeutic strategies have been developed over the years. Many of these seek to provide a growth-permissive environment to the regenerating neurons by degrading CSPGs via the use of chondroitinase ABC (ChABC) (Zuo et al., 1998; Zuo et al., 2002; Caggiano et al., 2005; Groves et al., 2005; Barritt et al., 2006; Huang et al., 2006; Galtrey and Fawcett, 2007; Graham et al., 2007; Sabatier et al., 2012; Cheng et al., 2015), grafts and/or conduits (Richardson et al., 1980; Aguayo et al., 1987; Campbell et al., 1992; Dam-Hieu et al., 2002; Lee et al., 2012; Zhao et al., 2013a; Wang et al., 2014; Kriebel et al., 2017; Kornfeld et al., 2018; Sarker et al., 2018a; Sarker et al., 2018b), or the sequestration of inhibitory cues such as Nogo-66 (Merkler et al., 2001; GrandPre et al., 2002; Li and Strittmatter, 2003; Liebscher et al.,

2005; Freund et al., 2007; Cao et al., 2008; Steward et al., 2008; Kucher et al., 2018). Alternatively, in the CNS, strategies have been elaborated to alleviate the inhibitory effects of the growth-inhibitory cues via the inhibition of RhoA or of its downstream effectors (Lehmann et al., 1999; Winton et al., 2002; Chan et al., 2005; Lingor et al., 2007; Sagawa et al., 2007; Fehlings et al., 2011). Finally, in the PNS, electrical stimulation and physical activity have also been used to facilitate axon extension and promote functional recovery (Doyle and Roberts, 2006; Goldshmit et al., 2008; Sabatier et al., 2008; Vivo et al., 2008; English et al., 2009; Udina et al., 2011; Boeltz et al., 2013; Cobianchi et al., 2013; Huang et al., 2013; Xu et al., 2014; Elzinga et al., 2015; Gordon and English, 2016; Hayashibe et al., 2016; Jung et al., 2016; Goganau et al., 2018). Interestingly, the success of these strategies is improved when combined, but functional recovery remains challenging (Houle et al., 2006; Asensio-Pinilla et al., 2009; Beaumont et al., 2009; Zhao et al., 2013b; Shinozaki et al., 2016; Chen et al., 2017; Sivak et al., 2017). Thus, the need of efficient therapeutic strategies is still present and as such, it is critical to investigate the signaling pathways underlying the neuronal response to injury to identify targets that could be harnessed to promote neuronal regeneration The laboratory of Alyson Fournier identified that RhoA and CRMP4 interact in the presence of inhibitory cues, and this interaction is key to the inhibition of regeneration observed in response to CNS injury (Alabed et al., 2007). Thus, in this thesis, I further our knowledge about the functions and regulation of these two cytoskeletal regulators in the cellular response to various insults, as this could yield novel targets that could be harnessed to promote neuronal regeneration.

1.2 Collapsin response mediator proteins (CRMPs)

CRMPs were originally identified as homologues of unc-33, a protein that regulates neurite outgrowth and guidance in the developing nervous system of *Caenorhabditis elegans* (Hedgecock et al., 1985; Siddiqui, 1990; Goshima et al., 1995; Minturn et al., 1995a; Byk et al., 1996; Gaetano et al., 1997; Fukada et al., 2000; Inatome et al., 2000; Ricard et al., 2001). Since this initial discovery, a total of 5 family members have been identified in vertebrates. In addition to being homologous to unc-33, these share a high degree of homology between each other, with approximately 70-75% between CRMP1-4, and 50% between CRMP5 and the other CRMPs (Wang and Strittmatter, 1996; Fukada et al., 2000; Inatome et al., 2000; Ricard et al., 2001). Since the 5 CRMP family members were discovered in different organisms, they have been

referred to by different names (summarized in table 1.1), including TOAD-64 (turn on after division, 64 kDa) (Minturn et al., 1995a), CRMP-62 (Goshima et al., 1995), DRP (dihydropyrimidinase-related protein) (Hamajima et al., 1996), Ulip (unc-33-like phospho-protein) (Byk et al., 1996; Gaetano et al., 1997; Byk et al., 1998; Quach et al., 2000; Ricard et al., 2001), CRAM (CRMP3-associated molecule) (Inatome et al., 2000), TUC (TOAD/Ulip/CRMP) (Quinn et al., 1999) or CRMP (Wang and Strittmatter, 1997; Fukada et al., 2000; Ricard et al., 2001). In this thesis, I will refer to them as CRMP1-5. Furthermore, CRMP1-4, but not CRMP5, are found as two splice isoforms, a short and a long isoform referred to as "S" or "L" respectively (Leung et al., 2002; Quinn et al., 2003; Yuasa-Kawada et al., 2003).

1.2.1 Expression pattern

The 5 CRMP family members are cytosolic phospho-proteins that are developmentally regulated in the nervous system, with their expression levels increasing during the late embryonic stages, peaking in the first post-natal week, and decreasing in adulthood (Goshima et al., 1995; Minturn et al., 1995a; Minturn et al., 1995b; Byk et al., 1996; Geschwind et al., 1996; Wang and Strittmatter, 1996; Byk et al., 1998; Fukada et al., 2000; Horiuchi et al., 2000; Inagaki et al., 2000; Inatome et al., 2000; Quach et al., 2000; Ricard et al., 2001; Quinn et al., 2003; Veyrac et al., 2005; McLaughlin et al., 2008; Tsutiya and Ohtani-Kaneko, 2012). Interestingly, in the adult nervous system, their expression pattern correlates with areas where neurogenesis is maintained, such as the granule layer and dentate gyrus of the hippocampus, the olfactory bulb, the subventricular zone and the rostral migratory stream, or regions that correlate with high synaptic plasticity, including the cerebellum, the cerebral cortex, the olfactory glomeruli, the hypothalamus and the visual cortex (Wang and Strittmatter, 1996; Nacher et al., 2000; Bretin et al., 2005; Veyrac et al., 2005; Cnops et al., 2006; Tsutiya and Ohtani-Kaneko, 2012).

1.2.2 Structure, oligomerization and regulation

Structurally, each CRMP are highly homologous (Wang and Strittmatter, 1996; Fukada et al., 2000; Inatome et al., 2000; Ricard et al., 2001). They share a core structure with a dihydropyrimidinase domain that is very similar to the liver dihydropyrimidinase, but not functional (Wang and Strittmatter, 1997; Inatome et al., 2000; Stenmark et al., 2007; Ponnusamy and Lohkamp, 2013; Myllykoski et al., 2017). Additionally, many CRMPs, including CRMP2

and CRMP4, possess domains that allow them to interact with actin and tubulin heterodimers (Kimura et al., 2005; Chae et al., 2009; Brot et al., 2010; Khazaei et al., 2014; Niwa et al., 2017). Furthermore, they share an interaction domain that allows them to form homo- and hetero-oligomers, with the formation of the latter being preferred *in vivo* (Wang and Strittmatter, 1997; Fukada et al., 2000; Stenmark et al., 2007; Majava et al., 2008; Ponnusamy and Lohkamp, 2013). They can also interact with each other, except CRMP5 and CRMP1 (Wang and Strittmatter, 1997; Fukada et al., 2000; Tan et al., 2015). However, the functions associated with their interactions and oligomerization remain poorly characterized.

Additionally, each CRMP has an unstructured carboxy-terminal tail that contains multiple phosphorylation sites (Minturn et al., 1995a; Byk et al., 1996; Byk et al., 1998; Fukada et al., 2000; Inatome et al., 2000; Stenmark et al., 2007; Ponnusamy and Lohkamp, 2013; Myllykoski et al., 2017). *In vitro* studies confirmed that CRMP1, 2 and 4 are phosphorylated by various kinases, including cyclin-dependent kinase 5 (Cdk5), glycogen synthase kinase 3 (GSK3β), Fyn, Rho-associated kinase (ROCK) and dual tyrosine-regulated kinase (DYRK) (Arimura et al., 2000; Cole et al., 2004; Arimura et al., 2005; Uchida et al., 2005; Yoshimura et al., 2005; Cole et al., 2006; Yamashita et al., 2007; Cole et al., 2008; Alabed et al., 2010; Buel et al., 2010; Yao et al., 2016). Phosphorylation promotes the inactivation of the different CRMPs by altering their ability to interact with the different cytoskeletal elements, consequently impairing neuronal functions such as neurite outgrowth and guidance, dendritic arborization and spine development (Arimura et al., 2000; Cole et al., 2004; Arimura et al., 2005; Uchida et al., 2005; Yoshimura et al., 2005; Cole et al., 2006; Yoshimura et al., 2006; Yamashita et al., 2007; Cole et al., 2008; Alabed et al., 2010; Buel et al., 2010; Tan et al., 2013; Nakamura et al., 2014; Yao et al., 2016).

Furthermore, the carboxy-terminal tail of the different CRMP family members contains multiple consensus sites for calpain-mediated cleavage (Kowara et al., 2005; Zhang et al., 2007; Liu et al., 2009). CRMPs are proteolytically processed by calpain to generate truncated versions of approximately 54 kDa in response to various insults, including excitotoxic and ischemic conditions (Kowara et al., 2005; Bretin et al., 2006; Hou et al., 2006; Kowara et al., 2006; Jiang et al., 2007; Zhang et al., 2007; Liu et al., 2009,Touma, 2007 #12; Shinkai-Ouchi et al., 2010; Yang et al., 2016). This cleavage disrupts the ability of CRMP2 and CRMP4 to interact with

actin, tubulin heterodimers and microtubules (Kowara et al., 2005; Rogemond et al., 2008; Khazaei et al., 2014). Consequently, this impairs neurite outgrowth and promotes neuronal degeneration (Kowara et al., 2006; Touma et al., 2007; Rogemond et al., 2008; Aylsworth et al., 2009; Liu et al., 2009). Additionally, the truncated CRMP3 and CRMP4 are retrogradely transported to the cell body of the damaged neurons where they translocate to the nucleus and promote apoptosis (Hou et al., 2006; Aylsworth et al., 2009; Liu et al., 2009; Hou et al., 2013).

1.2.3 Mechanism of action

In developing neurons, CRMPs co-localize and interact with the actin and tubulin networks (Tan et al., 2015; Yang et al., 2015). Thus, the cellular functions of these proteins, which include neuronal extension, guidance and synaptic plasticity, could potentially occur via cytoskeletal regulation.

1.2.3.1 Microtubule assembly and disassembly

Different CRMP family members exhibit differing functions on microtubules, despite their similar ability to interact with tubulin heterodimers (Fukata et al., 2002; Tan et al., 2015; Yang et al., 2015). CRMP2 and CRMP4 facilitate microtubule assembly, while CRMP3 and CRMP5 limit this function (Fukata et al., 2002; Aylsworth et al., 2009; Chae et al., 2009; Brot et al., 2010; Khazaei et al., 2014; Tan et al., 2015; Niwa et al., 2017). More specifically, CRMP2 promotes the elongation of microtubules in the axon by favoring the anterograde transport of tubulin heterodimers towards the growth cone, promoting their addition onto the plus-end of the microtubules and stabilizing the assembled filaments (Fukata et al., 2002; Kimura et al., 2005; Chae et al., 2009; Maniar et al., 2011; Niwa et al., 2017). Interestingly, the activity of CRMP2 towards microtubules is necessary but not sufficient for axonal elongation, which suggests that other CRMPs contribute to this process (Fukata et al., 2002). CRMP4 promotes neurite outgrowth via its interaction with CRMP2 as well as via its ability to promote the assembly of tubulin heterodimers into microtubules (Fukata et al., 2002; Khazaei et al., 2014; Tan et al., 2015). Microtubule assembly is also negatively regulated within neurons to limit neurite outgrowth. CRMP2 and CRMP4 are downregulated by an auto-inhibition domain, which limits their microtubule assembly properties (Chae et al., 2009; Khazaei et al., 2014). Furthermore, CRMP3 and CRMP5 directly interact with tubulin *in vitro* to inhibit their polymerization (Fukata

et al., 2002; Aylsworth et al., 2009; Brot et al., 2010; Tan et al., 2015). In the axon, CRMP5 antagonizes CRMP2 by competing for the binding of tubulin heterodimers, while in the dendrites, it forms a complex with MAP2 and the tubulin heterodimers (Brot et al., 2010). These interactions prevent microtubule polymerization and impair neurite outgrowth, but as CRMP5 is temporally and spatially expressed in the developing neurons, it also regulate the timing of axogenesis and dendritogenesis (Brot et al., 2010). Thus, the coordination of the activity of the different CRMP family members towards tubulin heterodimers and microtubules contributes to neurite extension.

1.2.3.2 F-actin polymerization

CRMPs directly bind to actin *in vitro*, but this binding does not necessarily correlate with functionality (Tan et al., 2015; Yang et al., 2015). CRMP2 has little direct effect on the actin network in neurons (Gu and Ihara, 2000; Yuasa-Kawada et al., 2003). However, it does indirectly regulate actin dynamics through its interaction with CRMP4 (Tan et al., 2015). CRMP4 promotes actin polymerization by interacting with the heterodimers (Rosslenbroich et al., 2005; Khazaei et al., 2014). Additionally, it cross-links with actin filaments at their minus-end to interfere with their depolymerization (Rosslenbroich et al., 2005). Thus, CRMP4, through the regulation of actin dynamics, promotes dendritic and axonal growth (Rosslenbroich et al., 2005; Khazaei et al., 2014; Cha et al., 2016). CRMP1 also accelerates actin polymerization on the barbed-end of the F-actin filament, but it does so in an indirect manner via the interaction with Arp2/3 and Ena/WASP (Yu-Kemp and Brieher, 2016; Yu-Kemp et al., 2017). The functions of CRMP3 and CRMP5 towards the actin cytoskeleton remain to be characterized.

1.2.3.3 Coordination of actin and microtubule dynamics

Cytoskeletal dynamics, including the coordination of microtubule assembly and actin dynamics in the growth cone, is critical for neurite outgrowth. CRMP2 and CRMP4 were found to regulate this process as both localize to the transition zone of the growth cone where they interact with different cytoskeletal components (Fukata et al., 2002; Rosslenbroich et al., 2005; Khazaei et al., 2014; Tan et al., 2015). CRMP2 regulates axonal development via the CRMP4-mediated interactions with actin, while CRMP4 regulates cytoskeletal remodeling via CRMP2-mediated

interactions with tubulin (Tan et al., 2015). Whether other CRMPs have similar functions remains to be investigated.

1.2.4 Functions in the nervous system

Several laboratories sought to investigate the functions of CRMPs using knockout mouse models. Interestingly, these mice did not show gross morphological defects, other than the mice lacking CRMP2 exhibiting enlarged ventricles in the brain (Charrier et al., 2006; Su et al., 2007; Quach et al., 2008; Khazaei et al., 2014; Nakamura et al., 2016; Zhang et al., 2016a). However, many of these mouse lines displayed impairments in spatial learning, memory, olfactory functions and social behaviours (Su et al., 2007; Yamashita et al., 2013; Tsutiya et al., 2015; Nakamura et al., 2016; Zhang et al., 2016a). These behavioural deficits could stem from defects in the finer architecture of the neuronal network.

1.2.4.1 Roles of CRMPs during development

Early studies of the *C. elegans* unc-33 revealed that this protein is instrumental to the regulation of neurite outgrowth and axon guidance (Hedgecock et al., 1985; Siddiqui, 1990). It was hypothesized that these functions could potentially be conserved by CRMPs in the mammalian system, due to their expression in the developing nervous system (Goshima et al., 1995; Minturn et al., 1995a; Minturn et al., 1995b; Byk et al., 1996; Geschwind et al., 1996; Wang and Strittmatter, 1996; Byk et al., 1998; Fukada et al., 2000; Horiuchi et al., 2000; Inagaki et al., 2000; Inatome et al., 2000; Quach et al., 2000; Ricard et al., 2001; Quinn et al., 2003; Veyrac et al., 2005; McLaughlin et al., 2008; Tsutiya and Ohtani-Kaneko, 2012). As such, defects in any of the steps underlying neuronal development could lead to functional and behavioural deficits such as those observed in the CRMP knockout mouse models (Su et al., 2007; Yamashita et al., 2013; Tsutiya et al., 2015; Nakamura et al., 2016; Zhang et al., 2016a).

Several lines of investigation suggested that CRMPs are involved in neurite outgrowth, a process driven by the growth cone, a dynamic structure at the tip of the neurites. CRMPs are spatially expressed within this structure, suggesting that they underlie specific aspects of growth cone dynamics. As such, CRMP1, 4 and 5 are expressed in the peripheral domain of the growth cone, where they regulate the maintenance of the lammelipodia and the dynamics of the filopodia,

while CRMP2 is found in the central domain, where it regulates microtubule dynamics (Fukata et al., 2002; Hotta et al., 2005; Rosslenbroich et al., 2005; Bork et al., 2010; Higurashi et al., 2012; Tan et al., 2013; Ji et al., 2014; Khazaei et al., 2014; Gong et al., 2016; Niwa et al., 2017). Combined, CRMPs regulate cytoskeletal dynamics within the growth cone, which allows them to regulate neurite extension. Furthermore, the local inactivation of CRMPs underlies the turning of the growth cone in response to various inhibitory cues, like Sema3A and EphrinA5, and permissive proteins, including neurotrophin-3 (NT3) and brain-derived neurotrophic factor (BDNF) (Arimura et al., 2000; Brown et al., 2004; Arimura et al., 2005; Hotta et al., 2005; Uchida et al., 2005; Yoshimura et al., 2005; Nakamura et al., 2014; Yao et al., 2016). In addition to their segregation within the growth cone, the different CRMP family members are spatially expressed in the developing neurons, and this pattern contributes to neuronal polarization (Byk et al., 1996; Inagaki et al., 2001; Fukata et al., 2002; Yamashita et al., 2007; Brot et al., 2010; Quach et al., 2013; Tan et al., 2013; Khazaei et al., 2014). CRMP2 and CRMP4 promote axogenesis, while CRMP1-5 regulate dendritogenesis (Fukata et al., 2002; Kawano et al., 2005; Kimura et al., 2005; Quach et al., 2008; Brot et al., 2010; Maniar et al., 2011; Yamashita et al., 2011; Niisato et al., 2012; Niisato et al., 2013; Quach et al., 2013; Tan et al., 2015; Cha et al., 2016; Makihara et al., 2016; Tsutiya et al., 2016; Niwa et al., 2017; Takaya et al., 2017). Furthermore, CRMP1, 2 and 4 regulate the migration and positioning of developing neurons within the nervous system, such as neural crest cells, caudal primary motor neurons and Rohon-Beard neurons in the spinal cord of zebrafishes, as well as that of neurons in the rodent's cerebral cortex (Yamashita et al., 2006; Ip et al., 2011; Tanaka et al., 2012; Morimura et al., 2013). Thus, CRMPs regulate many steps underlying neuronal development, including neurite outgrowth, neuronal polarization and migration.

1.2.4.2 Roles of CRMPs during synaptogenesis

The neurological impairments observed in the CRMP knockout mouse models could also stem from deficits in synaptic plasticity. CRMPs are expressed at the synaptosome, with CRMP2 being more abundant than the other family members (Brittain et al., 2009). There, CRMPs have been shown to regulate several functions, including the regulation of spine density and morphology, neurotransmitter release, as well as trafficking and recycling of synaptic vesicles (Su et al., 2007; Yamashita et al., 2007; Quach et al., 2008; Brittain et al., 2009; Chi et al., 2009;

Quach et al., 2011; Quach et al., 2013; Zhang et al., 2016a; Zhang et al., 2018). Consequently, deficits in the functions of the CRMPs can impair long-term potentiation (LTP) and depression (LTD), thus leading to behavioral defects (Su et al., 2007; Yamashita et al., 2007; Quach et al., 2008; Yamashita et al., 2011).

1.2.4.3 Roles of CRMPs in adulthood

Intriguingly, CRMPs also possess functions in the adult nervous system. In the PNS, CRMP1, 2, 4 and 5 were found to be upregulated following nerve injury (Minturn et al., 1995a; Suzuki et al., 2003; Jang et al., 2010; Yao et al., 2016). CRMP2 promotes axon extension, consequently improving PNS regeneration (Suzuki et al., 2003). The roles of CRMP4 in this context remain to be investigated, but these have been explored in the context of CNS injury. Intriguingly, in the adult CNS, CRMP4 exhibits a paradoxal shift in its functions as it is growth-promoting during development and growth-inhibitory following CNS injury (Nagai et al., 2015; Nagai et al., 2016). Following spinal cord injury, the activity of CRMP4 is downregulated via phosphorylation and calpain-mediated cleavage (Hou et al., 2006; Liu et al., 2009; Alabed et al., 2010; Hou et al., 2013; Nagai et al., 2016). This regulation, combined with the upregulation of cytotoxic CRMP4 isoforms impairs axon extension and promotes apoptosis (Alabed et al., 2007; Liu et al., 2009; Alabed et al., 2010; Nagai et al., 2016). Consequently, the removal of CRMP4 is beneficial to recovery following spinal cord injury, as mice lacking this protein exhibit improved neuronal regeneration and functional recovery (Nagai et al., 2015; Nagai et al., 2016). Thus, several CRMP family members were shown to contribute to the neuronal response to injury, but the specific functions of each family member remain to be further explored.

1.3 The small GTPase RhoA

The family of Rho GTPases is part of the superfamily of Ras-related small GTPases with approximately 20 identified members in mammals, including the extensively studied RhoA (ras homologous member A), Rac (Ras-related C3 botulinum toxin substrate) and Cdc42 (cell division control, protein 42) (Jaffe and Hall, 2005; Narumiya and Thumkeo, 2018). Rho family members share a high degree of homology in their amino acid sequence and tertiary structure (Wei et al., 1997; Ihara et al., 1998; Schaefer et al., 2014). However, these often possess diverging effects and act in a complementary manner to regulate various cellular functions. Early

studies from the laboratory of Alan Hall revealed that, in fibroblasts, RhoA promotes the formation of stress fibres and focal adhesions while Rac works cooperatively to promote membrane ruffling at the edge of the cell (Ridley and Hall, 1992; Ridley et al., 1992). In the following years, this complementarity between the different Rho GTPases was observed in many other cell types. For example, the spatial activation of different Rho GTPases in the growth cone of developing neurons underlies growth cone turning and navigation. There, the combined activity of Rac and Cdc42 favors filopodial extension and lamellipodial protrusion on one side of the growth cone in response to attractive guidance cues, while RhoA promotes growth cone collapse on the opposite side of the growth cone (Yuan et al., 2003; Jin et al., 2005; Sakumura et al., 2005). Despite this interesting crosstalk between the different Rho GTPases, this thesis will focus on the signaling mechanisms and functions of RhoA specifically.

1.3.1 Structure

RhoA, like most Rho GTPases, is a molecular switch that cycles between an inactive guanine nucleotide diphosphate (GDP)-bound state and an active guanine nucleotide triphosphate (GTP)-bound state (Jaffe and Hall, 2005; Heasman and Ridley, 2008; Schaefer et al., 2014). Structurally, it is composed of 193 amino acids, which assemble as a core G domain of six-stranded β-sheet surrounded by 5 α-helices and connected loops (Wei et al., 1997; Ihara et al., 1998; Longenecker et al., 2003; Schaefer et al., 2014). Upon GTP binding, the conformation of its tertiary structure shifts and effector-binding sites located within the Switch I (amino acids 27-43) and Switch II (amino acids 57-68) domains become exposed (Wei et al., 1997; Ihara et al., 1998; Schaefer et al., 2014). Interestingly, the many downstream effectors of RhoA are categorized according to their Rho-binding motifs. With the exception of Rho-associated coiled-coil kinase 1/2 (ROCK1/2), they interact preferentially with effector-binding sites located in a specific Switch domain (Fujisawa et al., 1998).

1.3.2 Mechanism of action

RhoA-dependent signaling pathways are critical to numerous cellular functions during neuronal development, including growth cone turning and neurite retraction, as well as in the neuronal response to injury in adulthood, where it limits neurite branching in the PNS and inhibits axon regeneration in the CNS (McKerracher et al., 1994; Sebok et al., 1999; Chen et al., 2000;

GrandPre et al., 2000; Wahl et al., 2000; Kottis et al., 2002; Morgenstern et al., 2002; Swiercz et al., 2002; Yuan et al., 2003; Jin et al., 2005; Sakumura et al., 2005; Gallo, 2006; Hiraga et al., 2006; Mimura et al., 2006; Tomita et al., 2007; Cheng et al., 2008; Huelsenbeck et al., 2012; Iseppon et al., 2015; Ohtake et al., 2016; Quarta et al., 2017). These neuronal functions are highly dependent on cytoskeletal regulation and as such, RhoA was characterized as a cytoskeletal regulator early following its discovery. It was initially shown to promote the assembly of actin-myosin filaments and actin stress fibres (Ridley and Hall, 1992; Hall, 1998). In the following years, it was also found to stabilize the microtubule cytoskeleton by inhibiting its dynamic nature and by capping the extremity of these filaments (Palazzo et al., 2001; Grigoriev et al., 2006; Bartolini et al., 2008).

Being a molecular switch, RhoA needs to transition to its active GTP-bound state before it can regulate the cytoskeleton. In the situations mentioned in the previous paragraph, RhoA is activated in response to inhibitory cues, such as EphrinA5, sphingosine-1-phosphate, CSPGs, and myelin-associated inhibitors (MAIs) (Fig. 1.2; (Arimura et al., 2000; Arimura et al., 2005; Mimura et al., 2006; Ohtake et al., 2016; Quarta et al., 2017)). Active RhoA, via its numerous downstream effectors, regulates cytoskeletal remodeling, which results in growth cone collapse and neurite retraction (Katoh et al., 1998; Sebok et al., 1999; Wu et al., 2005; Tan et al., 2011; Quarta et al., 2017).

1.3.2.1 Assembly of acto-myosin filaments

RhoA regulates different properties of the actin cytoskeleton via its numerous downstream effectors. RhoA, via ROCK 1/2, regulates the contractibility and stability of acto-myosin fibers. As such, this GTPase activates ROCK, which in turn phosphorylates a number of downstream effectors, including myosin light chain phosphatase and LIM kinase (LIMK) (Maekawa et al., 1999; Ohashi et al., 2000). The phosphorylation of these enzymes has an immediate effect on the levels of phosphorylated myosin light chain, a protein that contributes to the contractibility of acto-myosin filaments (Riento et al., 2003). LIMK also regulates the phospho-regulation of ADF/cofilin, key regulators of actin turnover (Carlier et al., 1997; Maekawa et al., 1999; Ohashi et al., 2000; Ghosh et al., 2004; Zigmond, 2004; Andrianantoandro and Pollard, 2006; Bamburg et al., 2010). The phosphorylation of cofilin inhibits its actin-binding properties, which

consequently stabilizes acto-myosin filaments (Bernstein and Bamburg, 2010; Spiering and Hodgson, 2011). Additionally, RhoA favors F-actin extension via the formin family of proteins. Formin homology 1 (FH1) delivers profilin-bound actin monomers to the formin homology domain 2 (FH2), which is located at the barbed end of the extending F-actin where it promotes the incorporation of actin monomers into the growing filaments (Zigmond et al., 2003; Romero et al., 2004; Zigmond, 2004; Goode and Eck, 2007; Shemesh and Kozlov, 2007). Overall, these signaling mechanisms underlie the formation of actin stress fibres, filopodial actin cables and cytokinetic actin rings (Goode and Eck, 2007; Spiering and Hodgson, 2011).

1.3.2.2 Stabilization of microtubules

RhoA regulates microtubule dynamics independently of its effects on the actin cytoskeleton (Palazzo et al., 2001; Bartolini et al., 2008). This occurs via its downstream effectors mammalian diaphanous-related formin (mDia) and CRMPs (Palazzo et al., 2001; Palazzo et al., 2004; Grigoriev et al., 2006; Mimura et al., 2006; Ohtake et al., 2016; Quarta et al., 2017). mDia, via its interaction with APC and EB1, regulates the stabilization of microtubules by contributing to their capping (Palazzo et al., 2001; Palazzo et al., 2004; Wen et al., 2004; Bartolini et al., 2008). Additionally, RhoA, via ROCK, regulates different CRMP family members. For example, in response to inhibitory cues, including lysophosphatidic acid (LPA), Ephrin-A5, CSPGs and MAIs, the RhoA/ROCK signaling axis can phosphorylate CRMP2, which results in its inactivation (Arimura et al., 2000; Arimura et al., 2005; Mimura et al., 2006; Ohtake et al., 2016; Quarta et al., 2017). This renders CRMP2 unable to bind to tubulin heterodimers, microtubules and Numb, although its ability to interact with actin is conserved (Arimura et al., 2000; Arimura et al., 2005). This disrupts microtubule organization and dynamics, and consequently favors growth cone collapse and neurite outgrowth inhibition (Arimura et al., 2000; Arimura et al., 2005; Mimura et al., 2006; Quarta et al., 2017). Furthermore, in response to MAIs, RhoA interacts with unphosphorylated CRMP4 and this interaction underlies neurite outgrowth inhibition (Alabed et al., 2007; Alabed et al., 2010).

1.3.3 Regulation

Due to its ubiquitous expression and its involvement in many cellular functions, the spatio-temporal activity of the small GTPase RhoA is tightly controlled by several regulatory

mechanisms (Jaffe and Hall, 2005; Heasman and Ridley, 2008; Schaefer et al., 2014; Narumiya and Thumkeo, 2018). As a molecular switch, RhoA cycles between an inactive GDP-bound state and an active GTP-bound state (Jaffe and Hall, 2005; Heasman and Ridley, 2008; Schaefer et al., 2014). Despite its high affinity for guanine nucleotides, RhoA possesses a slow intrinsic GTP hydrolysis and GDP/GTP exchange rates (Schaefer et al., 2014). As these characteristics are not conducive to fast on/off rates, several proteins complement these intrinsic properties and contribute to the spatio-temporal regulation of RhoA signaling. Guanine nucleotide exchange factors (GEFs) catalyze the exchange of GDP for GTP, thus activating RhoA (Schmidt and Hall, 2002; Rossman et al., 2005), while GTPase-activating proteins (GAPs) accelerates the hydrolysis of GTP to inactivate RhoA (Peck et al., 2002; Tcherkezian and Lamarche-Vane, 2007). Finally, guanine nucleotide dissociation inhibitors (GDIs) inhibit the spontaneous activation of RhoA by extracting it from the membrane and sequestering it in the cytosol (Olofsson, 1999; Garcia-Mata et al., 2011; Tnimov et al., 2012). Together, these regulatory proteins regulate the intracellular localization and the activation state of RhoA, consequently affecting the spatio-temporal effect of this GTPase on the cytoskeleton.

The intracellular localization of RhoA is also regulated by prenylation, a post-translational modification (Kranenburg et al., 1997; Allal et al., 2000; Michaelson et al., 2001; Schaefer et al., 2014). This modification involves the addition of a geranylgeranyl methyl ester onto the carboxy-terminal cysteine-containing CAAX sequence (where C, A and X are respectively a cysteine, an aliphatic amino acid and any amino acid) (Zhang and Casey, 1996; Roberts et al., 2008). This modification increases the hydrophobicity of GTP- and GDP-bound RhoA, thus facilitating their insertion into the plasma membrane and indirectly favoring their interaction with numerous GEFs for the subsequent activation of this GTPase (Allal et al., 2000; Michaelson et al., 2001; Roberts et al., 2008).

Additionally, RhoA is regulated via the ubiquitin-proteasome system, a mechanism that targets protein for degradation. Smurf1, an HECT domain E3 ubiquitin ligase, was the first enzyme identified to target RhoA for ubiquitination (Wang et al., 2003), but subsequently, members of the Cullin family of proteins, including Cullin-3 (Cul3) and SCF^{FBXL19} (Skp1-Cul1-F-box (SCF) FBXL19 E3 ubiquitin ligase), were found to have similar functions (Chen et al., 2009; Wei et al.,

2013). These ligases target RhoA for degradation, consequently regulating its spatio-temporal activity (Wang et al., 2003; Bryan et al., 2005; Chen et al., 2009; Wei et al., 2013).

The activity of RhoA is also regulated by different kinases, including ERK, cAMP- and cGMP-dependent kinases (PKA and PKG), which phosphorylate RhoA and regulate its activity (Lang et al., 1996; Sauzeau et al., 2000; Sawada et al., 2001; Forget et al., 2002; Ellerbroek et al., 2003; Rolli-Derkinderen et al., 2005; Tong et al., 2016). Phosphorylation mediates the interaction of RhoA with its different downstream effectors, such as ROCK but not Rhotekin, mDia1 or PKN (Nusser et al., 2006; Tong et al., 2016). Furthermore, it promotes the formation of RhoA-RhoGDI complexes, which consequently sequesters RhoA in the cytosol, and downregulates RhoA activity (Lang et al., 1996; Sauzeau et al., 2000; Sawada et al., 2001; Forget et al., 2002; Ellerbroek et al., 2003). Additionally, phosphorylated RhoA is protected against ubiquitin-mediated proteasomal degradation (Rolli-Derkinderen et al., 2005).

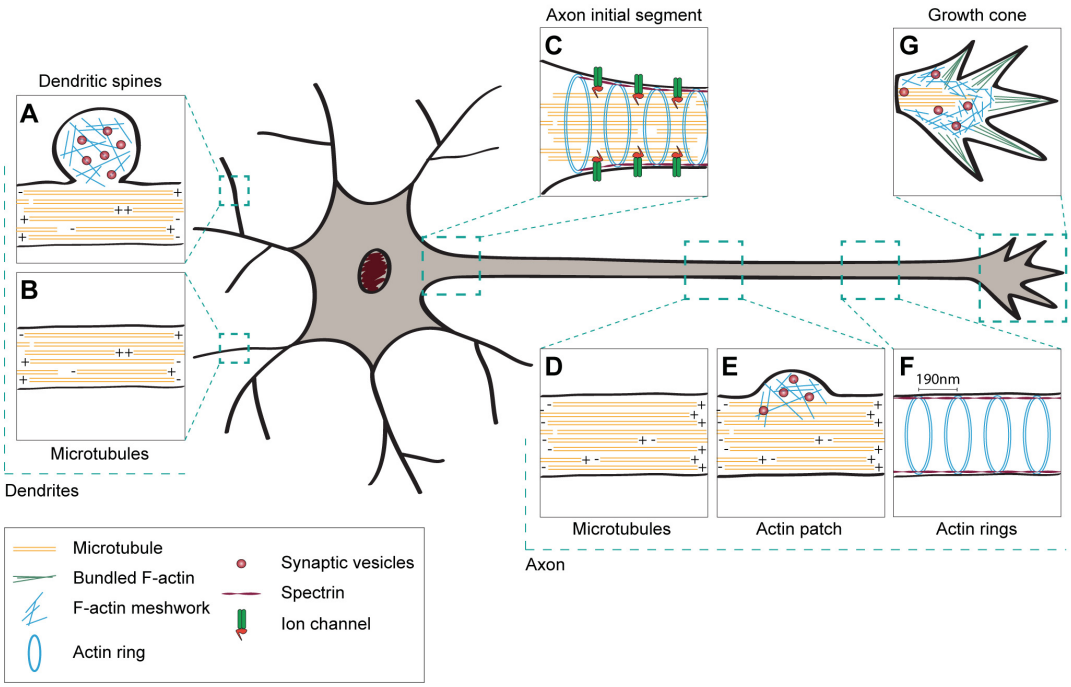
Finally, RhoA is proteolytically cleaved by μ-calpain in an integrin-dependent manner near focal adhesion complexes (Kulkarni et al., 1999; Kulkarni et al., 2002). This generates a dominant-negative protein that inhibits the formation of actin stress fibres and reduces cell spreading (Kulkarni et al., 2002). This proteolytic processing is promoted when Rac1 is active, suggesting that calpain-mediated cleavage of RhoA is a process that mediates crosstalk between the signaling pathways regulated by the different Rho GTPases (Kulkarni et al., 2002).

1.3.4 Functions

Due to its ubiquitous expression, RhoA is implicated in a myriad of cellular functions, playing a central role in many signaling pathways (Jaffe and Hall, 2005; Heasman and Ridley, 2008; Schaefer et al., 2014; Narumiya and Thumkeo, 2018). These include migration (Pertz et al., 2006; Vega et al., 2011), cell morphogenesis (Vega et al., 2011), phagocytosis (Chimini and Chavrier, 2000), cell cycle progression and cytokinesis (Kaibuchi et al., 1999; Miller and Bement, 2009; Melendez et al., 2011), gene expression (Hill et al., 1995; Perona et al., 1997; Montaner et al., 1998), assembly of focal adhesion complexes (Ridley and Hall, 1992; Hall, 1998), and several membrane transport mechanisms (Ridley, 2006).

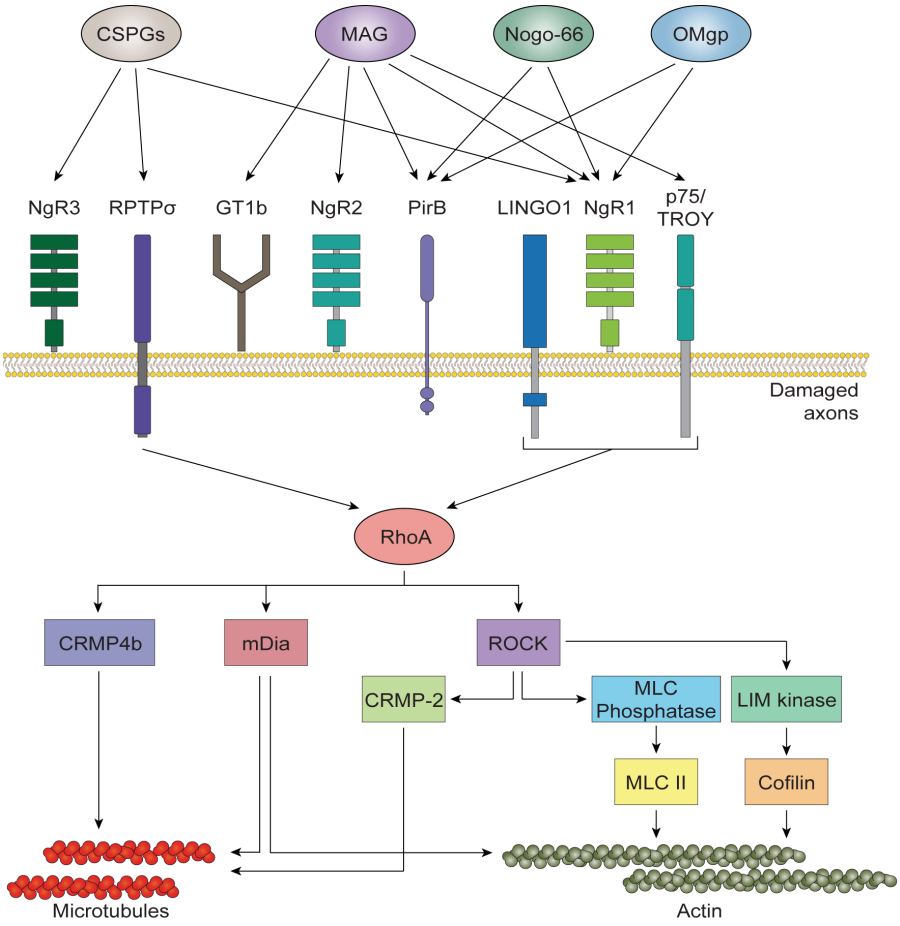
In the neurons more specifically, RhoA is implicated in many aspects of neuronal development. For example, the localized activation and inactivation of RhoA dictates neuronal polarity, as it determines axonal and dendritic branching and growth (Threadgill et al., 1997; Ruchhoeft et al., 1999; Lee et al., 2000; Li et al., 2000; Nakayama et al., 2000; Billuart et al., 2001; Wang et al., 2003; Ahnert-Hilger et al., 2004; Rico et al., 2004; Spillane et al., 2013). Furthermore, it limits neurite outgrowth by promoting neurite retraction and growth cone collapse (Jalink et al., 1994; Tigyi et al., 1996; Kozma et al., 1997; Kranenburg et al., 1999). This feature is key to neurite guidance, as the local activation of RhoA in response to different growth-inhibitory cues such as Ephrin-A5, Sema4D and Sema3A promotes growth cone collapse and favors turning towards the opposite direction (Wahl et al., 2000; Swiercz et al., 2002; Yuan et al., 2003; Jin et al., 2005; Sakumura et al., 2005; Gallo, 2006; Iseppon et al., 2015). Interestingly, these functions are not exclusive to the developing neurons, as they are also observed in injured neurons. Growth-inhibitory proteins released following injury, including CSPGs and myelin-associated glycoproteins (MAG) in the PNS or CSPGs and MAIs in the CNS, activate RhoA via their respective receptors. Active RhoA then inhibits axon extension and consequently, neurite regeneration in both the PNS and CNS, in addition to limiting hyper-innervation in the PNS (McKerracher et al., 1994; Chen et al., 2000; GrandPre et al., 2000; Kottis et al., 2002; Morgenstern et al., 2002; Hiraga et al., 2006; Mimura et al., 2006; Tomita et al., 2007; Cheng et al., 2008; Huelsenbeck et al., 2012; Ohtake et al., 2016; Quarta et al., 2017). Finally, RhoA regulate synapse formation, morphogenesis and plasticity during development and into adulthood (Nakayama et al., 2000; Tashiro et al., 2000; Tashiro and Yuste, 2004; McMullan et al., 2006; Devaux et al., 2017; Mulherkar et al., 2017).

Figure 1.1: Cytoskeletal structures in neurons.



The neuron possesses highly complex cytoskeletal structures, which underlie specific neuronal functions. **A)** The dendrites have synaptic spines, which contains an extensive actin network that traps synaptic vesicles, and mediates their release and re-uptake, consequently regulating synaptic transmission. **B)** Furthermore, in the dendrites, the microtubules exhibit a bi-directional orientation and serve as tracts for motor proteins that transport various proteins and organelles. **C)** The axon initial segment assembles where the axon emerges from the cell body. There, the actin network is organized as a complex network that traps various ion channels that are critical to the initiation of action potentials. **D)** Microtubules are also found in the axon, but unlike those located in the dendrites, they adopt a unipolar orientation. These serve as tracts for anterograde and retrograde transport. **E)** Along the developing axon, the actin periodically accumulates as actin-rich patches, which arise as the axon initiates branching or as synaptic terminals assemble. **F)** Furthermore, in the axon, actin filaments assemble with βIV-spectrin and adductin to form the actin/spectrin membrane-associated skeleton also referred to as F-actin rings. These have a periodicity of 190nm along the length of the axon and play a structural role. **G)** Finally, a growth cone at the tip of the axon drives axon elongation via the remodeling of its cytoskeleton.

Figure 1.2: RhoA signaling pathway in response to neuronal injury.



In response to injury, inhibitory cues, including CSPGs and MAIs (MAG, Nogo-66, OMgp), are released in the environment. These proteins signal to their respective receptor on the membrane of the damaged axons, which triggers intracellular signaling pathways that converge onto the activation of the small GTPase RhoA. RhoA activates ROCK, which in turn regulates the stabilization of acto-myosin filaments via LIM kinase and myosin light chain II (MLC II). ROCK also decreases microtubule dynamics via its interaction with CRMP2. Furthermore, RhoA, via mDia, promotes the stabilization of microtubules and the assembly of F-actin. Finally, RhoA interacts with CRMP4 to regulate cytoskeletal dynamics. Combined, the effects towards the cytoskeleton of the downstream effectors of RhoA promote growth cone collapse, inhibit neurite outgrowth and, consequently, prevent neuronal regeneration.

Table 1.1: Nomenclature of the CRMPs

CRMP1	CRMP2	CRMP3	CRMP4	CRMP5
TUC-1	TUC-2	TUC-3	TUC-4	CRAM
Ulip3	Ulip2	Ulip4	Ulip1	Ulip6
DRP-1	DRP-2	DRP-4	DRP-3	
	TOAD-64			
	CRMP-62			

TUC: TOAD/Ulip/CRMP; Ulip: Unc-33-like phospho-protein; DRP: Dihydropyrimidinase-related proteins; TOAD-64: Turn On After Division, 64 kDa; CRMP: Collapsin Response Mediator Protein; CRAM: CRMP3-Associated Molecule.

THESIS RATIONALE AND OBJECTIVES

In eukaryotes, the cellular morphology stems from the tridimensional arrangement of the cytoskeleton, a complex framework that provides mechanical support and facilitates communication between different regions of the cell. Despite its structural roles, this framework is very dynamic and, in response to various intrinsic and extrinsic cues, it can be reorganized to alter the morphology of the cell. However, in response to various insults, the cytoskeleton can become damaged and, in order to recover, cells need to re-organize each cytoskeleton component, including actin filaments, microtubules and intermediate filaments. Several proteins regulate their assembly, disassembly and/or stabilization, and understanding their functions can provide invaluable knowledge that can be harnessed to modify specific cellular responses, such as initiating neuronal regeneration following injury.

The small GTPase RhoA is a key player in the failure of regeneration observed following CNS injury, as it promotes growth cone collapse while inhibiting neurite outgrowth and hyperinnervation (McKerracher et al., 1994; Chen et al., 2000; GrandPre et al., 2000; Kottis et al., 2002; Morgenstern et al., 2002; Hiraga et al., 2006; Mimura et al., 2006; Tomita et al., 2007; Cheng et al., 2008; Huelsenbeck et al., 2012; Ohtake et al., 2016; Quarta et al., 2017). Its functions are mediated via numerous downstream effectors, including ROCK, CRMP2, and mDia (Fig. 1.2; (Arimura et al., 2000; Palazzo et al., 2001; Palazzo et al., 2004; Arimura et al., 2005; Hiraga et al., 2006; Mimura et al., 2006; Alabed et al., 2007; Bartolini et al., 2008; Cheng et al., 2008; Spiering and Hodgson, 2011; Quarta et al., 2017)). In addition to these signaling pathways, the laboratory of Alyson Fournier identified that, in neurons isolated from the dorsal root ganglions, RhoA interacts with CRMP4 in response to inhibitory cues such as Nogo and myelin (Alabed et al., 2007). This interaction limits neurite outgrowth on inhibitory substrates and interestingly, disrupting it is sufficient to revert its effect (Alabed et al., 2007; Khazaei et al., 2015). Despite these promising results, several aspects of the functions and regulation of RhoA and CRMP4 remain poorly characterized. Thus, in this thesis, I seek to further our knowledge of

these two cytoskeletal regulators, as this could help the identification of novel targets that could be used to promote a favorable response to neuronal damage.

During development, CRMP4 possesses growth-promoting functions, as it regulates growth cone dynamics, axon elongation and dendrite branching (Rosslenbroich et al., 2005; Niisato et al., 2012; Khazaei et al., 2014; Cha et al., 2016). However, in mature CNS neurons, they become growth-inhibitory and contribute to the failure of axons to repair following injury (Alabed et al., 2007; Nagai et al., 2015; Nagai et al., 2016). Intriguingly, despite its well-characterized functions in the mature CNS, its roles in the PNS remain poorly understood, although its expression is upregulated following sciatic nerve injury (Jang et al., 2010). Thus, in chapter 2, I sought to improve our understanding of CRMP4 in the neuronal response to peripheral nerve injury by characterizing the roles of this protein in PNS regeneration and Wallerian degeneration.

RhoA is central to many signaling pathways, and thus, it is involved in an array of functions in many different cell types due to its ubiquitous expression (Jaffe and Hall, 2005; Heasman and Ridley, 2008; Schaefer et al., 2014; Narumiya and Thumkeo, 2018). Thus, its activity is tightly regulated via multiple mechanisms (Lang et al., 1996; Allal et al., 2000; Sauzeau et al., 2000; Michaelson et al., 2001; Sawada et al., 2001; Forget et al., 2002; Kulkarni et al., 2002; Ellerbroek et al., 2003; Wang et al., 2003; Bryan et al., 2005; Jaffe and Hall, 2005; Rolli-Derkinderen et al., 2005; Heasman and Ridley, 2008; Roberts et al., 2008; Chen et al., 2009; Wei et al., 2013; Schaefer et al., 2014; Tong et al., 2016). In chapter 3, I characterized a novel proteolytic event that regulates the activity of RhoA towards the actin cytoskeleton.

CHAPTER 2

Collapsin Response Mediator Protein 4 (CRMP4) facilitates Wallerian degeneration and axon regeneration following sciatic nerve injury

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Abbreviated title: CRMP4 facilitates degeneration and regeneration

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2.1 Preface

In the adult nervous system, the functions of CRMPs are limited due to their low basal expression levels. However, their expression can become upregulated in the response to injury (Minturn et al., 1995a; Suzuki et al., 2003; Jang et al., 2010; Yao et al., 2016). These functions can be beneficial to neuronal recovery. As such, the upregulation of CRMP2 following hypoglossal nerve crush promotes neuronal regeneration of the motor neurons in a manner that is very reminiscent of its growth-promoting roles observed during development (Inagaki et al., 2001; Suzuki et al., 2003; Yuasa-Kawada et al., 2003; Chae et al., 2009; Higurashi et al., 2012; Niisato et al., 2013; Makihara et al., 2016). However, the functions of CRMPs in the injured nervous system can also be nefarious. For example, CRMP4 mediates the response to inhibitory cues released in the environment following CNS injury, thus limiting neuronal regeneration (Alabed et al., 2007; Alabed et al., 2010; Nagai et al., 2015; Nagai et al., 2016). Interestingly, this contrasts with its growth-promoting functions characterized during development (Quinn et al., 2003; Alabed et al., 2007; Niisato et al., 2012; Khazaei et al., 2014; Nagai et al., 2015; Tan et al., 2015; Cha et al., 2016; Nagai et al., 2016; Takaya et al., 2017).

PNS and CNS neurons have contrasting responses to injury, as the former revert to a developmental-like state and regenerate while the later does not. In chapter 2, I sought to investigate the roles of CRMP4 in the neuronal response to PNS injury. With this, I aimed to determine whether those differ from the growth-inhibitory functions previously characterized in the CNS or whether they are comparable to the growth-promoting effects characterized during development (Quinn et al., 2003; Alabed et al., 2007; Alabed et al., 2010; Niisato et al., 2012; Khazaei et al., 2014; Nagai et al., 2015; Tan et al., 2015; Cha et al., 2016; Nagai et al., 2016; Takaya et al., 2017). Using the sciatic nerve injury as a model, I found CRMP4 to be spatially and temporally regulated following PNS injury. This expression pattern correlated with functionality, as the full-length CRMP4 protein was associated with neuronal regeneration while its calpain-mediated cleavage promoted Wallerian degeneration. Intriguingly, these functions differ from those previously characterized in the CNS. Thus, differences in the expression and functions of CRMP4 could partly underlie the differing regenerative potential of PNS versus CNS neurons.

2.2 Abstract

In contrast to neurons in the central nervous system (CNS), damaged neurons from the peripheral nervous system (PNS) regenerate, but this process can be slow and imperfect. Successful regeneration is orchestrated by cytoskeletal reorganization at the tip of the proximal axon segment and cytoskeletal disassembly of the distal segment. Collapsin response mediator protein 4 (CRMP4) is a cytosolic phospho-protein that regulates the actin and microtubule cytoskeleton. During development, CRMP4 promotes growth cone formation and dendrite development. Paradoxically, in the adult CNS, CRMP4 impedes axon regeneration. Here, we investigated the involvement of CRMP4 in response to peripheral injury using *Crmp4*$^{-/-}$ mice that received sciatic nerve injury to determine the effects of CRMP4 on axon regeneration in the regeneration-competent adult PNS. We find that both sensory axon regeneration and Wallerian degeneration are impaired in *Crmp4*$^{-/-}$ mice following sciatic nerve injury. *In vitro* analysis of dissociated dorsal root ganglion (DRG) neurons from *Crmp4*$^{-/-}$ mice revealed that, in the proximal axon segments, CRMP4 promoted the regrowth of severed DRG neurons whereas in the distal axon segment, it facilitated Wallerian degeneration through the calpain-dependent generation of harmful CRMP4 fragments. These findings reveal an interesting dual role for CRMP4 in proximal and distal axon segments of injured sensory neurons that coordinately facilitate PNS axon regeneration.

2.3 Significance statement

PNS neurons spontaneously regenerate after injury; however, functional deficits often arise as a result of slow or misguided repair. Regrowth of the proximal axon segments coordinated with efficient Wallerian degeneration is important for optimal recovery. CRMP4 is a cytoskeletal regulator with growth-promoting functions in the developing nervous system and growth-inhibitory roles in damaged adult CNS neurons. Here, we identify a new pro-regenerative role for CRMP4 in peripheral nerve regeneration through the coordinated regulation of both axon regrowth and Wallerian degeneration.

2.4 Introduction

When confronted with an injury, different types of neurons respond differently based on their intrinsic potential and the extracellular environment at the lesion site. Regeneration of axotomized peripheral nervous system (PNS) neurons is supported by the expression of regeneration-associated genes (RAGs) and a growth-permissive environment, whereas injured central nervous system (CNS) neurons fail to re-express RAGs and encounter glial-derived inhibitors that impede regeneration (Huebner and Strittmatter, 2009; Mokarram and Bellamkonda, 2011; Mietto et al., 2015). Furthermore, in both cases, long-distance regeneration is slow and recovery is often incomplete, resulting in the partial or complete loss of sensory and/or motor functions (Mokarram and Bellamkonda, 2011).

A stereotypical sequence of events is initiated upon PNS injury. The proximal axon segment reforms a growth cone that drives axon extension and regeneration while the detached distal axon segment undergoes Wallerian degeneration and subsequent fragmentation (Waller, 1850; Erez and Spira, 2008; Kamber et al., 2009; Ghosh-Roy et al., 2010; Bradke et al., 2012). Phagocytosis of cellular debris contributes to a growth-permissive environment (Bruck, 1997; Lewis and Kucenas, 2014; Brosius Lutz et al., 2017). Proper coordination of proximal axon repair and distal axon degeneration is key to optimal regeneration, as injured sensory and motor neurons in slow degenerating *Wld*^{S} mice displayed impaired regeneration (Bisby and Chen, 1990; Brown et al., 1992; Brown et al., 1994). Thus, proteins regulating both processes could represent therapeutic targets for promoting recovery following PNS injury.

Collapsin response mediator proteins (CRMPs) are a family of cytosolic phospho-proteins that regulate cytoskeletal dynamics during development and after injury (Alabed et al., 2007; Khazaei et al., 2014; Nagai et al., 2015; Tan et al., 2015; Nagai et al., 2016). More specifically, CRMP4, which exists as two transiently-expressed splice isoforms, is an important neuro-developmental molecule promoting axonal extension and dendrite branching (Quinn et al., 2003; Niisato et al., 2012; Khazaei et al., 2014; Tan et al., 2015; Cha et al., 2016). However, in the adult CNS, CRMP4 adopts a nefarious role, as its deletion enhanced neuronal regeneration and reduced inflammation following spinal cord injury (Nagai et al., 2015; Nagai et al., 2016). This growth-inhibitory effect partly stems from the CRMP4L splice isoform limiting neurite outgrowth in

response to growth-inhibitory myelin-associated inhibitors (MAIs) and chondroitin sulfate proteoglycans (CSPGs) (Alabed et al., 2007; Alabed et al., 2010; Nagai et al., 2012; Nagai et al., 2016). In the adult mammalian PNS, CRMP4 is upregulated following sciatic nerve injury, but its function has not been investigated (Jang et al., 2010).

Here, we investigated the function of CRMP4 in response to PNS injury. We found that *Crmp4* deletion impaired the regeneration of sensory PNS neurons and delayed Wallerian degeneration of the distal processes *in vitro* and *in vivo*. Degeneration of the distal processes was facilitated by the calpain-mediated generation of toxic CRMP4 fragments. We concluded that, in contrast to its role in the injured adult CNS, CRMP4 facilitates PNS axon regeneration by coordinately regulating the regrowth of the injured axons and Wallerian degeneration of the disconnected process.

2.5 Material & Methods

2.5.1 Animals

Animal procedures were performed in accordance with the Canadian Council on Animal Care Guidelines, and approved by the McGill University Animal Care and Use Committee. *Crmp4*$^{+/-}$ mice were generated and maintained in a C57BL/6J background as described previously (Fig. 2.S1; (Khazaei et al., 2014)). *Crmp4*$^{-/-}$ mice and *Crmp4*$^{+/+}$ littermate controls were generated by inter-crossing *Crmp4*$^{+/-}$ mice. *Caspase-3*$^{-/-}$ were obtained from Jackson Laboratory (strain B6.129S1-Casp3tm1Flv/J). Embryonic day 15-16 (E15-16) and postnatal day 4-7 (P4-7) C57BL/6 wild-type mice and Sprague-Dawley rats were provided by Charles River Laboratories.

2.5.2 Antibodies

The following antibodies were used for immunostaining and Western immunoblots: rabbit anti-stathmin-2/STMN2 (Novus Biologicals; catalog #NBP1-49461; RRID: AB_10011569), mouse anti-tubulin β3 (TUBB3) (clone TUJ1; Biolegend; catalog #801202; RRID: AB_10063408), mouse anti-tubulin β3 (TUBB3) (clone TUJ1; Millipore; catalog #AB9354; RRID: AB_570918), purified rabbit anti-tubulin β3 (TUBB3) (clone Poly18020; Biolegend; catalog #802001; RRID: AB_2564645), mouse alpha-tubulin (Sigma-Aldrich; catalog #T9026; RRID: AB_477593), rabbit CRMP4 a/b (prepared in-house; (Alabed et al., 2007)), mouse α-fodrin (clone AA6; Enzo

Life Sciences; catalog #BML-FG6090; RRID: AB_10554860), mouse anti-His antibody (QIAGEN; catalog #34670; RRID: AB_2571551), Alexa-Fluor 488-conjugated goat anti-mouse antibody (Thermo Fisher Scientific; catalog #A11001; RRID: AB_2534069), Fluorescein-conjugated goat anti-rabbit antibody (Thermo Fisher Scientific; catalog #F2765; RRID: AB_2536525), Alexa-Fluor 568-conjugated goat anti-rabbit antibody (Thermo Fisher Scientific; catalog #A11011; RRID: AB_143157), Alexa-Fluor 568-conjugated goat anti-mouse antibody (Thermo Fisher Scientific; catalog #A11031; RRID: AB_144696), horse-radish peroxidase (HRP)-conjugated anti-mouse IgG antibody (Jackson ImmunoResearch Labs; catalog #115-035-003; RRID: AB_10015289), and HRP-conjugated anti-rabbit IgG antibody (Jackson ImmunoResearch Labs; catalog #111-035-003; RRID: AB_2313567).

2.5.3 Plasmids and mutagenesis

Cloning of pcDNA3 CRMP4S-WT, and pET TAT v1 TAT-RFP were previously described (Alabed et al., 2007; Khazaei et al., 2015). To generate a pcDNA3 CRMP4S-T524A construct, the T524A mutation was introduced in pcDNA3 CRMP4S-WT using the Quik Change II XL site-directed mutagenesis kit (Agilent Technologies). To create a DNA construct encoding TAT-CRMP4 NTF and CTF, the nucleotide sequence corresponding to amino acids 1-520 or 521-570 of pcDNA3 CRMP4S-WT respectively was amplified by polymerase chain reaction (PCR). The resulting sequences were introduced in the pET TAT v1 vector via the HindIII and XhoI restriction sites.

2.5.4 Purification of TAT peptides

TAT-RFP peptides were produced from Chinese hamster ovary (CHO) cells as described previously (Khazaei et al., 2015). To generate TAT-CRMP4 NTF and CTF peptides, BL21 bacterial cultures expressing pET Tat v1 CRMP4-NTF or -CTF were induced overnight with 1mM IPTG. The induced cultures were centrifuged and the pellet was resuspended in ice-cold buffer A (10 mM Tris-HCl pH 7.5, 600 mM NaCl, 20 mM imidazole, 1X complete protease inhibitors). The cell lysate was sonicated and cleared by ultracentrifugation. The cleared supernatant was applied to a Ni-NTA column equilibrated with buffer A. The column was washed with buffer A and the proteins were eluted with buffer B (20 mM Tris-HCl pH 7.5, 1 M NaCl, 250 mM imidazole). The buffer in the eluate was exchanged to buffer C (20 mM $NaKPO_4$

pH 6.8, 600 mM NaCl, 5% glycerol) using a PD-10 column (GE Healthcare). The eluate was then applied to a 30S IEX column equilibrated with buffer D (10 mM $NaKPO_4$ pH 6.8, 300 mM NaCl, 2.5% glycerol). Finally, the column was washed with buffer D and eluted with buffer E (10 mM $NaKPO_4$ pH 6.8, 1.5 M NaCl, 2.5% glycerol). The eluate was concentrated on Amicon Ultra-4 3K or 10K Centricon column (EMD Millipore), aliquoted and stored at -80°C.

2.5.5 Culture of dissociated dorsal root ganglion (DRG) neurons

E15-16 and P4-7 rodent DRGs were dissected in ice-cold Leibovitz (L-15) medium (Thermo Fisher Scientific). The DRGs were dissociated in 0.25% Trypsin-EDTA (Thermo Fisher Scientific) at 37°C, gently triturated with a P1000 pipette tip, and resuspended in DRG media (Neurobasal (Thermo Fisher Scientific), 1% B27 (Thermo Fisher Scientific; catalog #17504-044), 1% N2 (Thermo Fisher Scientific; catalog #17502-048), 1% penicillin-streptomycin (Life Technologies; catalog #15140-122), 2 mM L-glutamine (Life Technologies; catalog #25030-081), 10 µM 5-fluoro-2'-deoxyuridine (FDU; Sigma-Aldrich; catalog #F0503)) supplemented with 50 $ng.ml^{-1}$ nerve growth factor (NGF; Cedarlane; catalog #CLMCNET-001). The dissociated DRG neurons were seeded onto culture plates or in microfluidic devices pre-coated with 100 $\mu g.ml^{-1}$ poly-L-lysine (PLL; Sigma-Aldrich; catalog #P1399) and 5-10 $\mu g.ml^{-1}$ laminin (Corning; catalog #354232).

2.5.6 Preparation of Nogo-22 and treatment of DRG cultures

Production of Nogo-22 kDa protein was done as described previously (Huebner et al., 2011). Dissociated P4-7 rat DRG neurons were treated for 5 h with 600 ng GST-Nogo-22 or GST as a control. Lysates were prepared by collecting the treated DRGs in RIPA lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 1 mM Na_3VO_4, 5 mM NaF, 1X complete protease inhibitors). The protein concentration in the lysates was normalized prior to analysis by Western blot.

2.5.7 *In vitro* regeneration of DRG axons in microfluidic devices

Dissociated E15-16 mouse DRG neurons were seeded at a density of 10000-15000 neurons in microfluidic neuro devices (ANANDA devices) adhered to 3.5 cm imaging dishes (MatTek) pre-coated with 100 $\mu g.ml^{-1}$ PLL and 5 $\mu g.ml^{-1}$ laminin. At 4 days *in vitro* (DIV), the axons

projecting to the bottom compartment were stained with 4 µl of 10 µM Mitotracker Green FM (Invitrogen; catalog #M7514) for 30 min. They were then axotomized by increasing the flow of medium in the bottom compartment of the microfluidic device using simultaneous vacuum aspiration and medium replacement to shear the axons. At 24 h following the axotomy, the neurons were fixed and stained with anti-tubulin β3 (TUBB3) antibody (1:1000) and Alexa-Fluor 568-conjugated secondary antibody (1:1000). Fluorescent images of the samples were acquired using an Axiovert1 microscope (Zeiss) with a 20X objective (Plan-APOCHROMAT Pln Apo 20X/0.8; Zeiss). Regrowth of the axons after axotomy was measured by manually tracing the axons with the NeuronJ plugin for ImageJ (Meijering et al., 2004). The extent of regeneration was measured by dividing the total neuronal regrowth in the bottom compartment of the microfluidic device by the number of channels with growing axons.

2.5.8 Treatment of DRG neurons grown in microfluidic devices with TAT-CRMP4 peptides

Dissociated E15-16 mouse DRG neurons were seeded at a density of 17000-25000 neurons in microfluidic neuro devices (ANANDA devices) adhered to a 3.5 cm imaging dish (MatTek) pre-coated with 100 $\mu g.ml^{-1}$ PLL and 5 $\mu g.ml^{-1}$ laminin. At 3 DIV, the axons projecting to the bottom compartment of the microfluidic devices were treated for 8 h with 2.5 µM TAT-CRMP4-His-V5 peptides, or with TAT-RFP as a control. The neurons were then fixed, and stained with anti-tubulin β3 (TUBB3) rabbit antibody (1:1000), anti-His mouse antibody (1:500), Alexa-Fluor 568-conjugated goat anti-mouse and Fluorescein-conjugated goat anti-rabbit secondary antibodies (1:1000). Fluorescent images of the samples were acquired using an Axiovert1 microscope (Zeiss) with a 20X objective (Plan-Apochromat Pln Apo 20X/0.8; Zeiss). The extent of degeneration was measured in at least 3 representative regions of interest (400 µm X 400 µm) per explant by calculating the index of degeneration, which corresponds to the area covered by the axonal fragments with a circularity above 0.9 divided by the total area covered by the axons (Kilinc et al., 2011).

2.5.9 Axotomy-induced degeneration of E12.5 DRG explants

Axotomy-induced degeneration of E12.5 mouse DRG explants was performed as previously described (Unsain et al., 2014). Briefly, the DRG explants were seeded onto cell-filter inserts coated with 1 $mg.ml^{-1}$ PLL, 10 $ug.ml^{-1}$ laminin and 0.1 $mg.ml^{-1}$ collagen, filled with DRG media

with the bottom compartment supplemented with 15 ng.ml^{-1} NGF. After approximately 60 h of growth, the explants on the upper side of the cell-filter inserts were detached with a cell scraper, and the axotomized axons were left to degenerate for 3 h after which they were collected in Laemmli sample buffer for subsequent analysis by Western blot. In some experiments, the DRG explants were treated with 5-15 μM ALLN (EMD Millipore; catalog #208719) prior to the axotomy. Alternatively, the DRG explants were grown on coated cell culture plates containing DRG media supplemented with 10 ng.ml^{-1} NGF for approximately 60 h. The axons of the DRG explants were sectioned with a scalpel blade, and the axotomized axons were allowed to degenerate for 5 h to 5.5 h. The DRG explants were then fixed and stained with anti-tubulin β3 (TUBB3) antibody (1:1000) and Alexa-Fluor 568-conjugated secondary antibody (1:1000). The axotomized DRG explants were imaged with an Axiovert 200M microscope (Zeiss) using a 10X objective (CP-ACHROMAT 10X/0.25 Ph1; Zeiss). The extent of degeneration was measured in at least 3 representative regions of interest per explant (400 μm X 400 μm) by calculating the index of degeneration, which corresponds to the area covered by the axonal fragments with a circularity above 0.9 divided by the total area covered by the axons (Kilinc et al., 2011).

2.5.10 NGF withdrawal from E12.5 DRG explants

This experiment was conducted as previously described using E12.5 mouse explants (Unsain et al., 2014). Briefly, the E12.5 DRG explants were seeded into 6-well plates pre-coated with 1 mg.ml^{-1} PLL, 10 ug.ml^{-1} laminin and 0.1 mg.ml^{-1} collagen in DRG media supplemented with 10 ng.ml^{-1} NGF. After approximately 2 days of growth, the media was exchanged to NGF-free DRG media supplemented with 1 ug.ml^{-1} anti-NGF antibodies. NGF withdrawal-induced degeneration was allowed to proceed for 24 h, after which the NGF-deprived axons were fixed and stained with anti-tubulin β3 (TUBB3) antibody (1:10000) and Alexa-Fluor 568-conjugated antibody (1:5000). The axons were imaged at a 5X magnification using a Zeiss Axioscope 2 microscope. The quantification of the area covered by the axons was done with Axoquant 2.0 in R Studio as previously described (Johnstone et al., 2018).

2.5.11 Immunostaining of dissociated DRG neurons and DRG explants

The dissociated DRG neurons or DRG explants were fixed for 30 min with 4% paraformaldehyde (PFA) diluted in phosphate-buffered saline (PBS) then thoroughly washed

with PBS. The neurons were then permeabilized for 5 min with 0.2% Triton X-100/PBS prior to blocking in 5% bovine serum albumin (BSA) diluted in PBS for 1 h at room temperature. The neurons were then stained with primary antibody diluted in 5% BSA/PBS overnight at 4°C. The next day, the samples were washed in PBS, incubated in secondary antibody diluted in 5% BSA/PBS for 2 h at room temperature, and washed again in PBS.

2.5.12 Sciatic nerve injury

Male and female mice aged 3-4 months old were used for the sciatic nerve injuries. Following anesthesia with isoflurane, the sciatic nerve was exposed with a mid-thigh incision and crushed with a smooth-jaw hemostat (0.6 mm tip; Fine Science Tools) fully closed for 30 s at approximately 1 cm distal to the sciatic notch. The injury site was labeled by attaching a 9-0 non-absorbable silk suture (Ethicon) to the epineurium. Alternatively, the sciatic nerve was transected with straight semi-fine scissor (Fine Science Tools) and the nerve ends were pulled apart to prevent regeneration. Analgesia was managed by injecting buprenorphine (0.10 $mg.kg^{-1}$) subcutaneously and providing carprofen-containing MediGel CPF (ClearH_2O) *ad libitum*. Animals were also given enrofloxacin (5 $mg.kg^{-1}$) subcutaneously pre- and post-operatively to prevent infection.

2.5.13 Optic nerve injury

Male and female mice aged 2-3 months old were used for optic nerve transection. Following anesthesia with isoflurane, the optic nerve was exposed with an incision above the ocular orbit and the extra-ocular muscles were resected. The optic nerve was transected with semi-fine scissors (Fine Science Tools) at 0.5-1.0 mm from the optic nerve head. Care was taken to avoid damage to the ophthalmic artery and the vascular integrity of the retina was assessed by fundus examination. Analgesia was provided by subcutaneous injections of buprenorphine (0.05 $mg.kg^{-1}$).

2.5.14 *In vivo* analysis of neuronal regeneration in whole-mount stained sciatic nerves

At 3 days post-injury (DPI), mice were euthanized with isoflurane and CO_2 inhalation followed by cervical dislocation. The injured and contralateral intact sciatic nerves were harvested and fixed in 4% PFA/PBS for 5 h at 4°C. Following fixation, the tissues were washed in PBS, then

dehydrated in sequential washes of 50%, 80%, and 100% methanol (Sigma-Aldrich) for 1 h each at room temperature, before quenching the endogenous peroxidase activity overnight at 4°C with ice-cold H_2O_2 diluted in 20% DMSO/methanol (1 vol 30% H_2O_2, 1 vol DMSO, 4 vol methanol). The following day, the tissues were rehydrated in a reversed gradient of methanol followed by two washes of 1 h in PBS. Blocking was done overnight at 4°C in blocking buffer (10% normal goat serum (NGS), 10% DMSO (Sigma-Aldrich), 0.2% Triton X-100 (Sigma-Aldrich), PBS). The sciatic nerves were then stained sequentially with anti-stathmin-2 (STMN2; also referred to as superior cervical ganglion 10 (SCG10; 1:200)) primary antibody and Alexa-Fluor 568-conjugated goat anti-rabbit secondary antibody (1:200) diluted in antibody dilution buffer (3% NGS, 5% DMSO, 0.2% Triton X-100, 10 ug.ml^{-1} heparin, PBS) for 7 days at 37°C to label the regenerating sensory axon front as the anti-STMN2/SCG10 antibody preferentially detects regenerating sensory and not motor axons (Shin et al., 2014). Following each incubation, the nerves were thoroughly washed in 0.2% Triton X-100/PBS for 7 h at room temperature, changing the wash buffer every hour. A final overnight wash was done in PBS before tissue clarification. Prior to imaging, the stained nerves were cleared following an adapted 3DISCO protocol (Erturk et al., 2012). Briefly, the nerves were dehydrated in a stepwise gradient of 50%, 80% and 100% tetrahydrofuran (THF; Sigma-Aldrich) diluted in distilled water. The nerves were then cleared by immersion in dibenzyl ether (DBE; Sigma-Aldrich). The whole nerves were imaged with a SP8 confocal laser-scanning microscope (Leica) using a 10X objective (HC PL APO 1.x/0.40 CS). The distance of regeneration was measured as described previously (Leon et al., 2000). Briefly, the number of SCG10+ neurons located at 500 µm increments from the injury site were counted in alternating optical sections. The resulting number was divided by the diameter of the nerve (mm) to calculate the number of axons per mm for each section counted. The number of axons/mm was then averaged over all the sections. Finally, the total number of axons $\sum a_d$ extending to a distance d was estimated by summing all sections: $\sum a_d = \pi r^2 \cdot \left[average\ axons/mm \right] / t$, where r is the radius of the nerve and t is the optical thickness of the sections.

2.5.15 *In vivo* analysis of axonal degeneration in sciatic nerves

The mice were euthanized by intracardial perfusion with ice-cold 4% PFA. The nerve stump distal to the injury site and the corresponding contralateral nerve were harvested and post-fixed

in 2.5% gluteraldehyde in 0.1 M PBS. Post-processing of the nerve samples was conducted by the Facility for Electron Microscopy Research (FEMR) of McGill University. Briefly, the fixed sciatic nerves were stained with 1% osmium tetroxide, dehydrated in sequential washes with a gradient of ethanol and embedded in epoxy embedding medium (Electron Microscopy Sciences). Semi-thin transverse nerve sections with a thickness of 0.5 μm were prepared using an ultramicrotome (Reichert) and mounted with a coverslip using Permount mounting medium (Fisher Scientific; catalog #SP15-100). Imaging was done with an Axiovert 200M epifluorescence microscope (Zeiss) using a 63X objective (Plan-Neofluar 1.25 Oil; Zeiss). To evaluate the extent of degeneration, the number of intact axons per μm^2 was counted.

2.5.16 Tissue homogenization

At selected time points following nerve transection, the mice were euthanized with isoflurane and CO_2 inhalation followed by cervical dislocation. Sciatic nerve, optic nerve, L4-6 DRGs and retina samples were collected, washed briefly in ice-cold PBS, and manually homogenized in RIPA lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 1 mM Na_3VO_4, 5 mM NaF, 1X complete protease inhibitors). The protein concentration in the resulting sonicated and cleared lysates was measured and normalized between samples prior to analysis by Western immunoblot.

2.5.17 Overexpression of CRMP4 in HEK 293T cells and *in vitro* digestion with recombinant calpain

HEK 293T cells maintained in DMEM medium supplemented with 10% fetal bovine serum (FBS) were transfected using Lipofectamine 2000 (Invitrogen) following manufacturer's instruction. The cells were washed briefly in ice-cold PBS and lysed with RIPA lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 1 mM Na_3VO_4, 5 mM NaF, 1X complete protease inhibitors). The protein concentration in the sonicated and cleared lysates was normalized. The lysates were digested *in vitro* with 1 unit of recombinant calpain-1 (EMD Millipore; catalog #208713) and 2 mM $MgCl_2$ for 30 min at 37°C. The digested proteins were then analyzed by Western blot. In some experiments, the calpain inhibitor 5-15 μM ALLN (EMD Millipore; catalog #208719) was added to the reaction.

2.5.18 Western blots

The protein content of tissue and cell lysates was analyzed by SDS-Page gel separation followed by Western immunoblot. PVDF membranes were blocked with 5% milk diluted in Tris-buffered saline (TBS) supplemented with 0.05% Triton X-100 for 1 h at room temperature, probed with primary antibody overnight at 4ºC and with secondary antibodies for 1 h at room temperature. The antibodies used were: rabbit CRMP4 a/b (1:7500), mouse α-fodrin (1:500), mouse anti-tubulin beta3 (TUBB3) (1:5000), mouse alpha-tubulin (1:5000), and horse-radish peroxidase (HRP)-conjugated anti-mouse and anti-rabbit IgG antibodies (1:10000). The signal was revealed with Western Lightning Plus ECL (PerkinElmer).

2.5.18 Statistical analysis

Statistical analyses were performed with the GraphPad Prism 8 software. Two-tailed Student t-test were used when directly comparing 2 conditions, while one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests were used when more than 3 conditions were compared. For experiments with more than one variable parameters such as the *in vivo* regeneration and degeneration experiments, a two-way ANOVA followed by Bonferroni's multiple comparison tests were used. Statistical details, p values and experimental details are indicated in the corresponding figure legends.

2.6 Results

2.6.1 *Crmp4* deletion impairs regeneration of sensory neurons following sciatic nerve injury

Paradoxically, CRMP4 facilitates growth during development, but impedes regeneration of injured CNS neurons (Quinn et al., 2003; Alabed et al., 2007; Nagai et al., 2012; Niisato et al., 2012; Khazaei et al., 2014; Nagai et al., 2015; Tan et al., 2015; Nagai et al., 2016). Thus, we sought to investigate the functions of CRMP4 in regeneration-competent PNS neurons. Sciatic nerve crush injuries were performed on adult *Crmp4*$^{-/-}$ mice and littermate *Crmp4*$^{+/+}$ controls. Regenerating fibres were stained for SCG10, a protein that is preferentially upregulated in regenerating sensory neurons and their growth was evaluated at 3 days post-injury (DPI) (Mason et al., 2002; Shin et al., 2014). In both *Crmp4*$^{+/+}$ and *Crmp4*$^{-/-}$ sciatic nerves, SCG10+ neurons grew spontaneously across the injury site following sciatic nerve crush injury (Fig. 2.1 A)

However, quantification of the number of regenerating fibres at progressive distances from the lesion site revealed that sensory neurons in *Crmp4*$^{+/+}$ mice extend further than those in *Crmp4*$^{-/-}$ mice (Fig. 2.1 B). This suggests a pro-regenerative function for CRMP4 in the PNS, which contrasts to its inhibitory role in the CNS (Alabed et al., 2007; Nagai et al., 2012; Nagai et al., 2015; Nagai et al., 2016).

2.6.2 CRMP4 expression is differentially regulated in the CNS and PNS following injury

Next, we sought to determine whether the distinct regenerative phenotypes observed in the PNS and CNS could stem from differences in the expression profile of CRMP4. Thus, CRMP4 expression was assessed by Western blot analysis of lysates prepared from PNS and CNS cell bodies and axons. For analysis of the PNS, lumbar dorsal root ganglions (DRGs) containing the cell bodies of sensory neurons and sciatic nerves containing their axons were collected at 3 days following sciatic nerve transection (Fig. 2.2 A). For analysis of the CNS, the retina that contain retinal ganglion cell (RGC) bodies and optic nerves, which contain their axons, were analyzed following optic nerve transection (Fig. 2. 2A). CRMP4 was robustly expressed in intact and injured DRGs with little change in expression following injury (Fig. 2.2 B). In the sciatic nerve, CRMP4 expression was markedly reduced in the nerve distal to the lesion site where axons undergo Wallerian degeneration, and bands representing the long isoform of CRMP4 (CRMP4L) and a truncated 55 kDa CRMP4 cleavage fragment (tCRMP4) became apparent (Fig. 2.2 C). In the proximal segment of sciatic nerve where growth cones of regenerating neurons reform, CRMP4S expression remained constant and CRMP4L was modestly upregulated (Fig. 2.2 C). In the CNS, CRMP4 expression was unaltered in the retina at 3 days following optic nerve transection, which is similar to the CRMP4 expression pattern observed in DRG cell bodies (Fig. 2.2 D). In the optic nerve, CRMP4 regulation was notably different from that of the sciatic nerve (Fig. 2.2 E). In the optic nerve segments, truncated CRMP4 was apparent in the distal segment following optic nerve injury, but CRMP4S and CRMP4L expression was largely retained. In the proximal segment of the damaged optic nerve, CRMP4 was strongly downregulated with the appearance of tCRMP4 (Fig. 2.2 E). Intriguingly, this reveals a distinct regulation of CRMP4 following injury to PNS versus CNS neurons. In the regenerating PNS, CRMP4 expression is retained in the proximal segment of sciatic nerve correlating with the regeneration of severed axons, whereas CRMP4 is downregulated in the proximal segment of optic nerve where axons

fail to regrow. Further, in the distal sciatic nerve, CRMP4 is cleaved and downregulated, which coincide with the onset of Wallerian degeneration, while CRMP4 expression is largely retained at the same time point in the distal optic nerve. The distinct expression pattern of CRMP4 in injured PNS and CNS neurons leads us to speculate that full-length CRMP4 in the proximal segment of injured PNS neurons supports their regrowth whereas CRMP4 cleavage contributes to Wallerian degeneration in the distal segment of sciatic nerve while impeding regrowth of CNS neurons.

2.6.3 CRMP4 enhances regrowth of injured sensory neurons *in vitro*

To test whether CRMP4 expression in the proximal segment of injured sensory neurons promotes axon extension after injury, we conducted a more extensive time course analysis of CRMP4 expression following sciatic nerve injury. Following sciatic nerve transection, CRMP4 expression is retained in the proximal nerve segment for up to 14 DPI (Fig. 2.3 A). To test the cell-autonomous contribution of CRMP4 to the regrowth of damaged axons, we measured the extent of regeneration of axotomized DRG neurons *in vitro*. DRGs were isolated from E15-16 wild-type or *Crmp4*$^{-/-}$ mouse embryos and the dissociated neurons were plated in microfluidic devices for 4 days until the axons projected to the opposite compartment (Jones et al., 2006; Frey et al., 2015; Dubovy et al., 2018). Axotomy was then performed by increasing the flow of medium in the axonal compartment to shear the axons, and the damaged neurons were allowed to regenerate for 24 h. Following axotomy, *Crmp4*$^{-/-}$ DRG neurons regrew significantly worse than their wild-type counterparts, exhibiting 43% less regrowth (Fig. 2.3 B, C). The regenerating CRMP4$^{-/-}$ neurites also adopted a curled phenotype, often looping back towards the channels, which differs from the straight outgrowth profile of wild-type neurons (Fig. 2.3 B). This finding demonstrates that CRMP4 enhances the regrowth of the proximal tip of axotomized sensory neurons in a cell-autonomous manner.

2.6.4 *Crmp4* deletion delays Wallerian degeneration in the PNS

We next investigated whether CRMP4 might be involved in the regulation of Wallerian degeneration in the sciatic nerve. Previous studies have demonstrated that axon regeneration is impaired in mice with delayed Wallerian degeneration, suggesting that clearance of axon distal segments facilitate axon regeneration (Bisby and Chen, 1990; Brown et al., 1992; Brown et al.,

1994). We first assessed Wallerian degeneration in the transected sciatic nerve by quantifying the number of intact myelinated fibres in semi-thin cross-sections of the nerve segment located distal to the injury site. This revealed a higher number of myelinated axons in the sciatic nerve collected from *Crmp4*$^{-/-}$ mice compared to the *Crmp4*$^{+/+}$ controls at 36HPI, which became statistically significant at 3 DPI (Fig. 2.4 A, B). By 7 DPI, degeneration was extensive in both *Crmp4*$^{+/+}$ and *Crmp4*$^{-/-}$ sciatic nerves (Fig. 2.4 A, B). These results indicate that *Crmp4* deletion delays Wallerian degeneration following sciatic nerve injury.

To better understand the function of CRMP4 in Wallerian degeneration, we examined axon degeneration in an *in vitro* axotomy model of embryonic DRG neurons (Gerdts et al., 2013; Gamage et al., 2017). E12.5 *Crmp4*$^{+/+}$ and *Crmp4*$^{-/-}$ DRG explants were grown for 60 h, prior to the neurites being sectioned with a blade and allowed to degenerate for 5 to 5.5 h. The index of degeneration of these neurites was then calculated by dividing the area covered by particles with a circularity above 0.9, which corresponds to the axon fragments, by the total area covered by the neurites (Kilinc et al., 2011). The quantification revealed that *Crmp4*$^{-/-}$ neurons have a lower index of degeneration compared to the *Crmp4*$^{+/+}$ neurons, illustrating a cell-autonomous role for CRMP4 in Wallerian degeneration (Fig. 2.4 C, D). Interestingly the pro-degenerative function of CRMP4 following axotomy is not conserved in another model of degeneration: trophic factor deprivation. When E12.5 *Crmp4*$^{-/-}$ and *Crmp4*$^{+/+}$ DRG explants were deprived of nerve growth factor (NGF) and treated with anti-NGF antibody, they exhibited typical signs of degeneration, such as the appearance of axon swellings and fragments (Fig. 2.S2). The area covered by the neurites following degeneration was not significantly different between the NGF-deprived *Crmp4*$^{-/-}$ and *Crmp4*$^{+/+}$ DRG explants (Fig. 2.S2), indicating that the effect of CRMP4 on Wallerian degeneration is somewhat selective.

2.6.5 CRMP4 is cleaved by calpain in axotomized sensory neurons

We found that the expression of CRMP4 was downregulated in degenerating axons located distal to a sciatic nerve injury, coincident with the appearance of a truncated CRMP4 species. We thus sought to investigate the mechanism underlying the cleavage of CRMP4 and its contribution to Wallerian degeneration. CRMP family members including CRMP4 have been described as calpain substrates in response to ischemic and excitotoxic stimuli (Kowara et al., 2005; Kowara

et al., 2006; Jiang et al., 2007; Zhang et al., 2007; Liu et al., 2009). To determine if calpain is active in the injured sciatic nerve, we investigated the cleavage of fodrin, a calpain substrate that is alternatively referred to as αII-spectrin and that is often used as a marker of calpain activity (Wang, 2000). Following injury, 145 kDa and 150 kDa spectrin breakdown products characteristic of calpain-mediated cleavage were elevated in the distal sciatic nerve segment, but largely unregulated in the proximal segment (Fig. 2.5 A; (Wang, 2000)). The fragments were detected at 1, 3 and 5 DPI and resolved by 14 DPI consistent with the time course of CRMP4 cleavage *in vivo* (Fig. 2.3 A). Analysis of proximal and distal segments of optic nerve following transection revealed fodrin cleavage in both the proximal and distal nerve segments, consistent with the appearance of a CRMP4 cleavage product in proximal and distal optic nerve (Fig. 2.2 E). This data suggests that calpain could potentially cleave CRMP4 to generate tCRMP4 in response to axotomy. To directly test this, P4-7 DRG neurons were treated with recombinant calpain, which did indeed lead to the generation of a 55 kDa tCRMP4 cleavage fragment (Fig. 2.5 C), supporting the idea that CRMP4 cleavage is a calpain-dependent process. We also asked whether generation of the CRMP4 cleavage product could be efficiently blocked by a calpain inhibitor. To achieve this, DRG explants were grown on cell-filter inserts and axotomized by removing the cell bodies with a cell scraper. The axons on the underside of the filter were then collected and lysed for Western immunoblot. Severed axons revealed the presence of tCRMP4 and generation of this fragment was efficiently blocked with the calpain inhibitor ALLN (Fig. 2.5 D). Because calpains can activate other enzymes which also contribute to degeneration such as caspases, we repeated the experiment in DRG explants isolated from *Caspase 3*$^{-/-}$ mice (Geden and Deshmukh, 2016). Similar to the wild-type DRG explants, axotomy-dependent cleavage of CRMP4 occurred in the axotomized *Caspase 3*$^{-/-}$ axons and this was prevented by treatment with ALLN (Fig. 2.5 E). These findings illustrate that axotomy is sufficient to drive the process of calpain-dependent CRMP4 cleavage.

Further, to address how CRMP4 may be cleaved proximal and distal to the lesion site in optic nerve while being restricted to the distal portion of the sciatic nerve, we asked if CNS myelin-associated proteins, which are released upon injury, affect CRMP4 cleavage. We found that treatment of P4-P7 DRG neurons with Nogo-22, an outgrowth inhibitory fragment of the CNS myelin-associated inhibitor Nogo-A, was sufficient to induce CRMP4 cleavage (Fig. 2.5 F)

(Huebner et al., 2011). This is consistent with the finding that Nogo promotes calcium influx in neurons, which leads to the activation of calpain (Bandtlow and Loschinger, 1997). This raises the possibility that calpain activation and CRMP4 cleavage may occur both proximally and distally following optic nerve injury in response to Nogo while it may be more spatially restricted in the PNS environment.

2.6.6 CRMP4 cleavage fragments promote axon degeneration

To study the effects of CRMP4 cleavage fragments on neurons, we mapped the calpain cleavage site. Through sequential alanine substitutions of candidate residues in the carboxy-terminal tail of CRMP4, we localized the specific cleavage site to residue T524 and demonstrated that a CRMP4 construct containing a T524A mutation was resistant to *in vitro* calpain digestion (Fig. 2.6 A). We then generated amino- and carboxy-terminal fragments of CRMP4 (CRMP4-NTF and CRMP4-CTF, respectively) as TAT peptides to mediate peptide internalization across the cell membrane (Fig. 2.6 B; (Torchilin, 2008; Khazaei et al., 2015)). Dissociated wild-type E15-16 DRG neurons were grown in microfluidic devices and the axons were treated for 8 h with TAT-CRMP4 peptides or with TAT-RFP as a control. Treatment with either CRMP4-NTF or CRMP4-CTF resulted in significant axonal degeneration as characterized by the appearance of axonal swellings and beading (Fig. 2.6 C). The extent of degeneration was assessed by calculating the index of degeneration (Kilinc et al., 2011). This quantification reveals a significant effect of both CRMP4 fragments on axon degeneration compared to TAT-RFP (Fig. 2.6 C, D), indicating that CRMP4 cleavage is sufficient to promote axonal degeneration.

2.7 Discussion

Following injury, peripheral neurons mount a regenerative response whereby a newly-formed growth cone at the tip of the broken axon drives axon outgrowth, while the disconnected axon segments undergo Wallerian degeneration (Waller, 1850; Kamber et al., 2009; Ghosh-Roy et al., 2010; Bradke et al., 2012; Spira and Erez, 2013). Neuronal regeneration and degeneration are highly inter-dependent, and as such, they share several signaling pathways (Bisby and Chen, 1990; Brown et al., 1992; Brown et al., 1994; Girouard et al., 2018). This is advantageous as both processes can be simultaneously targeted to promote an optimal regenerative response. Here, we characterized CRMP4 as a protein that contributes to both regeneration and

degeneration following axotomy. CRMP4 plays a dual role in the neuronal response to PNS injury, as loss of CRMP4 impairs sensory axon regeneration by limiting axon regrowth and delaying Wallerian degeneration. These findings ascribe to a novel pro-regenerative role to CRMP4 in the PNS, contrasting to its role in the CNS, where it contributes to failed regeneration. Interestingly, these functions are very reminiscent of other proteins that regulate cytoskeletal dynamics and energy supply (Girouard et al., 2018). For example, SCG10/STMN2 accumulates in the regenerating axon segment of sensory neurons following peripheral axotomy to promote microtubule dynamics and axon regrowth (Shin et al., 2014). Conversely, it is degraded in the degenerating axon segment to accelerate axon fragmentation (Shin et al., 2012b).

Here, we find that, in the regenerating neurons, CRMP4 supports axon regrowth, while its calpain-mediated cleavage in the distal degenerating fibres facilitates Wallerian degeneration (Fig. 2.7). It is notable that the relative ratio between full-length and truncated CRMP4 seems to be critical to its function. In the injured PNS, full-length CRMP4 is exclusively expressed in the proximal segment, correlating with axon regeneration (Fig. 2.7 B). In the distal sciatic nerve, massive downregulation of full-length CRMP4 coinciding with the appearance of cleavage fragments correlates with Wallerian degeneration (Fig. 2.7 B). In the transected optic nerve, CRMP4 expression in the proximal nerve segment mirrors the profile of the distal sciatic nerve and this correlates with the formation of retraction bulbs and failed regenerative growth (Fig. 2.7 C). While CRMP4 cleavage also occurs in the distal optic nerve, full-length CRMP4 is retained and may dimerize with tCRMP4 to buffer its neurotoxic activity. This is consistent with the idea that overexpression of non-phosphorylated CRMP2 can protect axons from Wallerian degeneration (Wakatsuki et al., 2011).

Efficient neuronal regeneration requires the assembly of a functional growth cone that will drive axon extension (Bradke et al., 2012). This process is highly dependent on cytoskeletal remodeling and consequently, several cytoskeletal regulators are implicated in this process. Here, we find that CRMP4 contributes favorably to PNS regeneration and this effect potentially stems from its regulatory functions towards the cytoskeleton. To support this hypothesis, hippocampal neurons lacking CRMP4 exhibit poorly elaborated growth cones with defects in their cytoskeletal organization (Khazaei et al., 2014; Tan et al., 2015). Thus, CRMP4 could potentially

facilitate the cytoskeletal rearrangements required for the reformation of the growth cone, either via its direct interaction with actin and tubulin to promote their assembly into filaments or microtubules respectively, or via its interaction with CRMP2 (Rosslenbroich et al., 2005; Khazaei et al., 2014; Tan et al., 2015). Additionally, CRMP4L can interact with intersectin, an adaptor protein that regulates various cellular processes including endocytosis and exocytosis (Acheson et al., 1991; Yamabhai et al., 1998; Okamoto et al., 1999; Simpson et al., 1999; Hussain et al., 2001; Quinn et al., 2003). Thus, CRMP4L could play important roles in providing membrane and cell surface molecules important for axon regrowth. Consequently, CRMP4 could facilitate growth cone reformation in regenerating PNS axons, but also subsequent axon extension.

Conversely, in degenerating PNS axons, we observe the calpain-mediated cleavage of CRMP4 and concomitant down-regulation of CRMP4S. Intriguingly, the expression pattern of CRMP4S and tCRMP4 observed in the sciatic nerve located distal to the injury site is very reminiscent of the CRMP2 expression profile following injury (Touma et al., 2007; Lin et al., 2011; Wakatsuki et al., 2011; Zhang et al., 2016b). In the case of CRMP2, its inactivation either by phosphorylation or calpain-mediated cleavage impairs its microtubule stabilizing effects. Thus, it is likely that CRMP4 cleavage is mediating both a toxic gain-of-function in response to the accumulation of NTF and CTF fragments, as well as a loss of function phenotype (Touma et al., 2007; Lin et al., 2011; Wakatsuki et al., 2011; Zhang et al., 2016b). Axon transport is disrupted in degenerating neurons, and overexpression of CRMP2 in axotomized cortical neurons rescues this defect and restores microtubule organization (Touma et al., 2007; Zhang et al., 2016b). CRMP2 couples with tubulin heterodimers, proteins and organelles to kinesin, allowing their anterograde transport (Kawano et al., 2005; Kimura et al., 2005; Arimura et al., 2009). As CRMP4 is also downregulated in degenerating neurons, this protein could also be involved in the maintenance of neuronal integrity, via the regulation of either microtubule stabilization, axonal transport or other functions that remained to be further explored. Additionally, calpain-mediated cleavage of CRMP4 leads to the generation of tCRMP4 fragments that are sufficient to trigger axonal degeneration in wild-type DRG neurons, which express endogenous levels of full-length CRMP4. CRMP4 and other CRMP family members assemble as hetero-tetramers to mediate their functions (Wang and Strittmatter, 1997). While it is likely that an excess of full-length

CRMP4 expression would buffer against the deleterious effects of tCRMP4, it is also possible that tCRMP4 fragments could integrate in hetero-tetramers compromising their functionality. For example, CRMP2 and CRMP4 assemble as a complex, which coordinate actin and microtubule dynamics in the developing neurons (Tan et al., 2015). The integration of CRMP4 cleavage fragments into this complex might alter cytoskeletal dynamics and stability, consequently promoting axonal fragmentation.

Different kinases and upstream regulators regulate the activity of cytoskeletal regulators in the regenerating and degenerating neurons (Girouard et al., 2018). Interestingly, these regulators also exhibit differential roles in the proximal and distal axon segments. MAP3K dual leucine zipper kinase 1 (DLK-1) functions through JNK and p38 MAP kinase to support transcriptional and translational events required for axon regeneration, while it regulates microtubule dynamics and energetics to favor degeneration of the distal axons (Miller et al., 2009; Watkins et al., 2013; Geden and Deshmukh, 2016). Additionally, nicotinamide mononucleotide acetyltransferase 1 (NMNAT1) is required for axonal integrity and as such, in regenerating neurons, NMNAT1 preserves mitochondria to promote neuroprotection and support regeneration whereas, in degenerating neurons, it limits degeneration by blocking NAD^+ depletion (Chen et al., 2016; Sasaki et al., 2016). Thus, CRMP4, similarly to SCG10/STMN2 and CRMP2, could potentially act downstream of DLK-1 and NMNAT1, but this hypothesis will need to be further explored.

An interesting aspect of this study is the dichotomy between CRMP4 functions in the PNS compared to the CNS. CRMP4 plays an important role in the transduction of growth-inhibitory signals like MAIs and CSPGs (Alabed et al., 2007; Alabed et al., 2010; Nagai et al., 2012; Nagai et al., 2016). Following spinal cord injury, sensory neurons lacking CRMP4 exhibited enhanced growth in the dorsal horn, consistent with desensitization to MAIs and CSPGs (Nagai et al., 2016). Thus, the presence of myelin-associated inhibitory proteins and inhibitory glial scar components in the CNS and their absence in the PNS likely explains the differential regulation of CRMP4 in the two systems. It is also apparent that, following spinal cord injury, CRMP4 is upregulated in the astrocytes surrounding the glial scar, whereas following peripheral nerve injury, CRMP4 is predominantly localized to damaged axons (Jang et al., 2010; Nagai et al.,

2015). This diverging expression pattern suggests that, in the CNS, CRMP4 might possess additional functions in non-neuronal cells that would contribute to the regeneration failure.

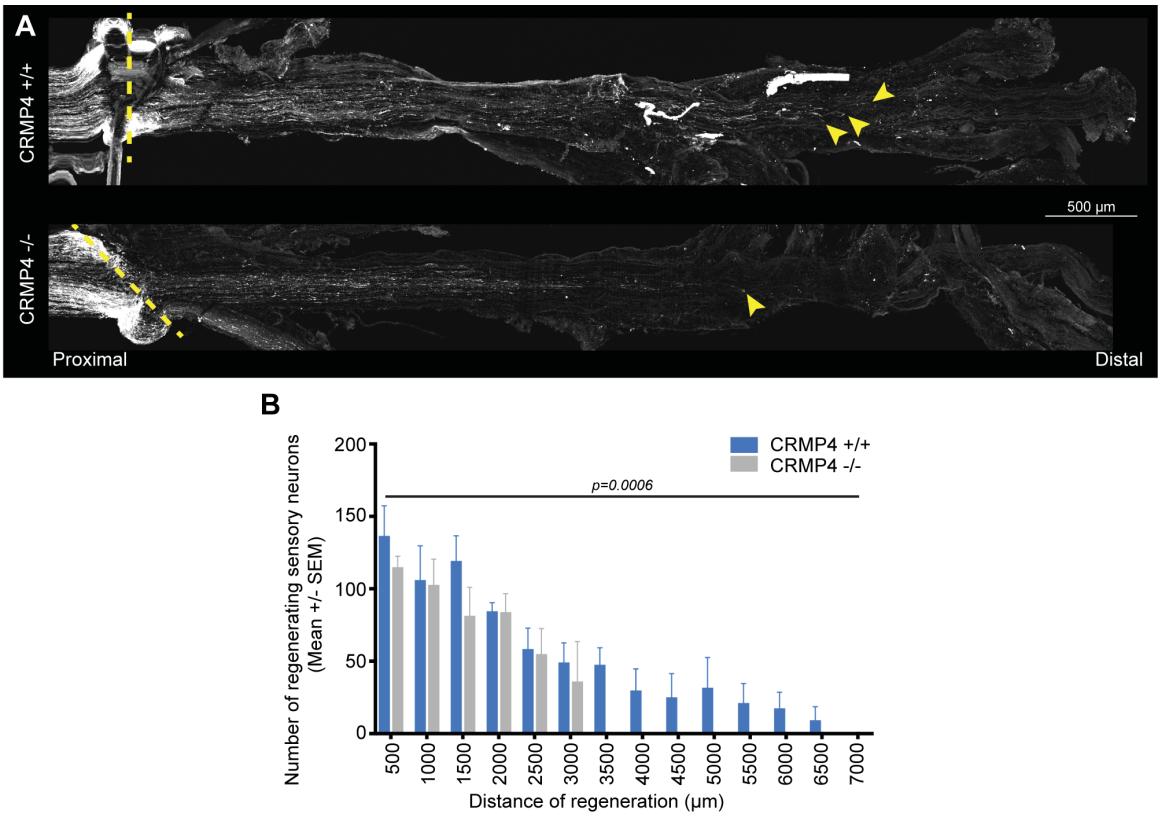
In conclusion, we characterized a dual role for the cytoskeletal regulator CRMP4 in the neuronal response to PNS injury, as it favors neuronal regeneration by promoting axonal regrowth and by facilitating Wallerian degeneration. Interestingly, these functions are very reminiscent of the growth-promoting effects of CRMP4 characterized in developing neurons, but differ from its growth-inhibitory action in the adult CNS. Understanding these differing functions is critical to the elaboration of novel strategies to promote functional recovery following neuronal injuries.

2.8 Acknowledgements

We thank Jeannie Mui and Lee Ann Monaghan from the Facility for Electron Microscopy Research (FEMR) of McGill University for their help with the preparation of semi-thin sciatic nerve sections. We also thank Andrés de Léon, Ricardo Alchini, Camille Juzwik and Tristan Simas for technical advices throughout this research project. This work was supported by grants from the Canadian Institutes of Health Research (CIHR) and the McGill Training Program in Neuroengineering.

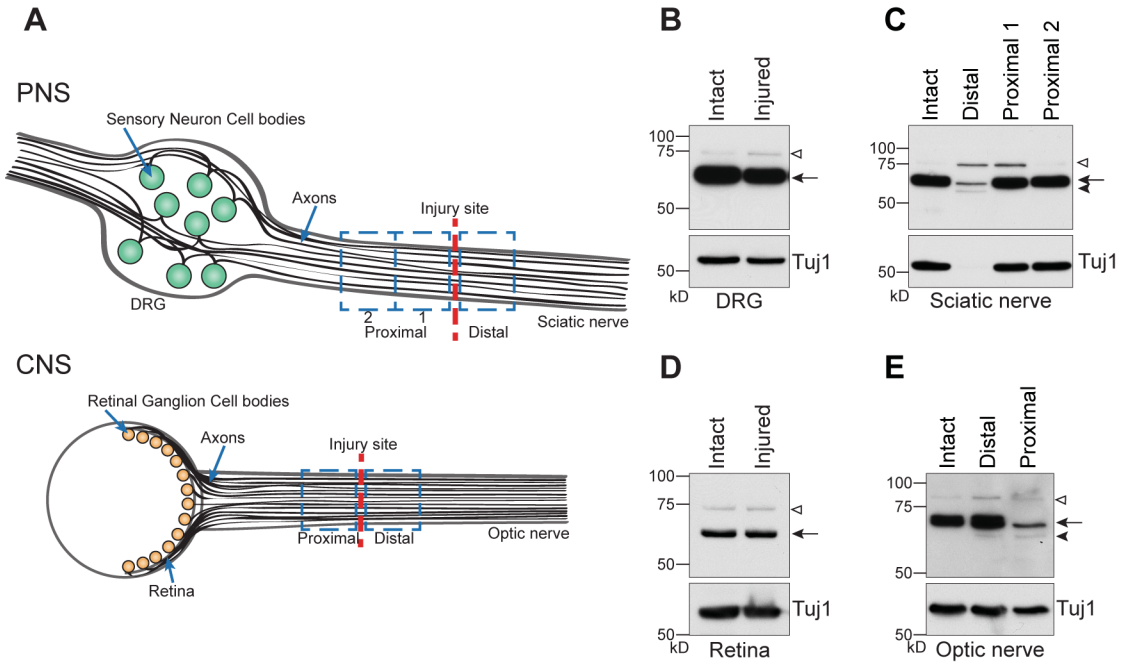
2.9 Figures

Figure 2.1: *Crmp4* deletion impairs the regeneration of sensory neurons following sciatic nerve injury.



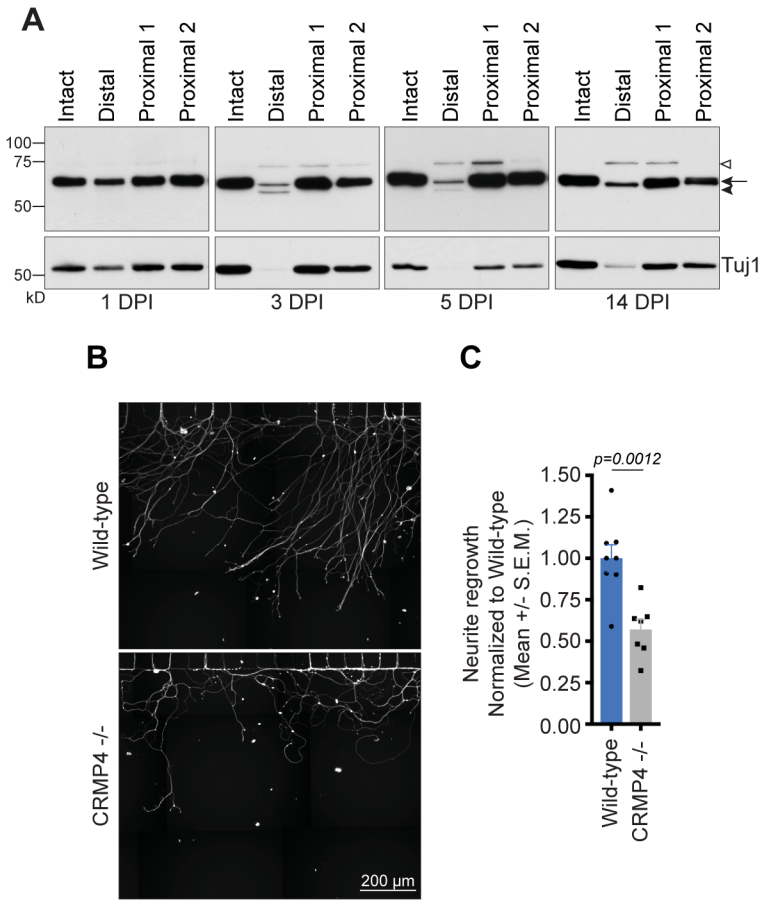
A) Maximal intensity projections of straightened *Crmp4*$^{+/+}$ *or Crmp4* $^{-/-}$ sciatic nerves at 3 DPI following crush injury stained with SCG10, a marker of the regenerating sensory neurons. The injury site is labeled by a dotted line and the extremity of the regenerating fibres is indicated by arrowheads. Scale bar, 500 µm. **B)** Quantification of the distance of regeneration of the SCG10+ sensory neurons at 3 DPI in *Crmp4*$^{+/+}$ and *Crmp4*$^{-/-}$ mice. Data is represented as the mean number of neurons per mm^2 +/- S.E.M. The difference between the genotypes is statistically significant (n=5-6; two-way ANOVA, p=0.0006).

Figure 2.2: The expression of CRMP4 is differentially regulated following CNS and PNS injury.



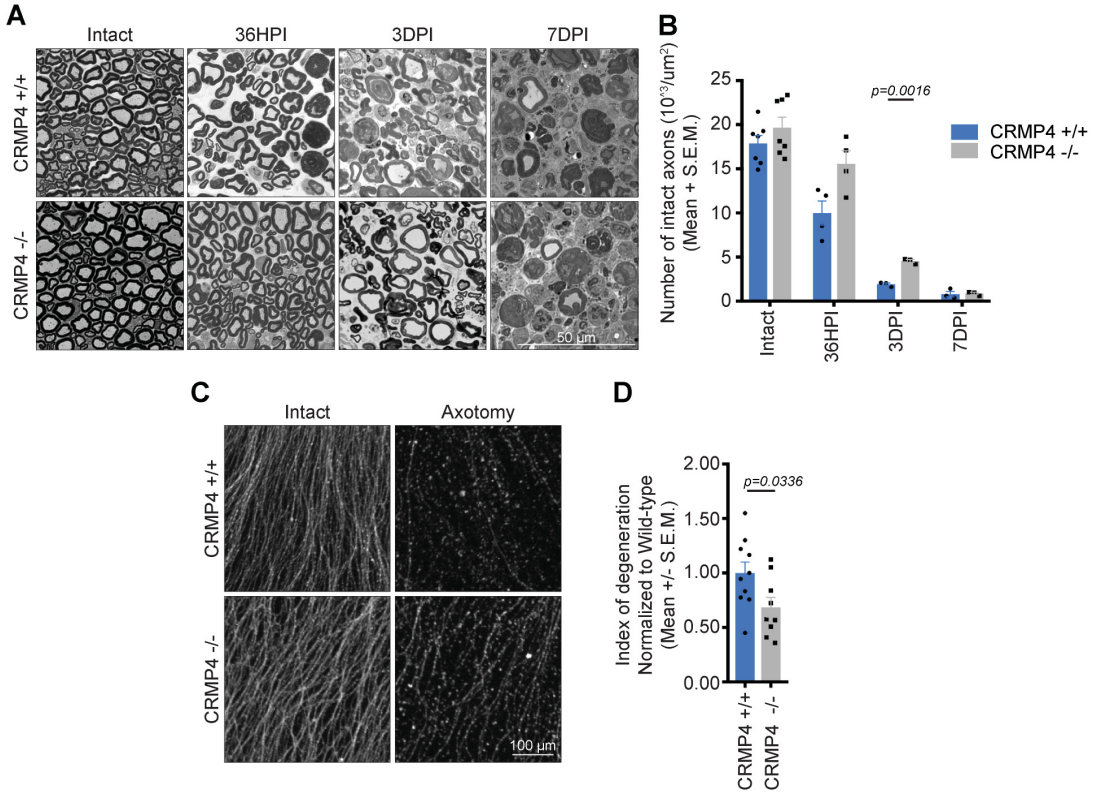
A) Illustration of the PNS and CNS models utilized to examine the response to axotomy. **B, C)** Immunoblot analysis of the CRMP4 expression pattern in DRG **(B)** and sciatic nerve **(C)** lysates from wild-type mice at 3 days following sciatic nerve transection. The nerve samples collected span either 0 to 3 mm distal, 0 to 3 mm proximal (proximal 1), or 3 to 6 mm proximal (proximal 2) to the injury site. The data is representative of results obtained from 7 mice. **D, E)** Immunoblot analysis of CRMP4 expression in retina **(D)** and optic nerve **(E)** lysates at 3 days following optic nerve transection. The nerve portions analyzed span from either 0 to 2 mm distal or 0 to 2 mm proximal to the injury site in the optic nerves. The data is representative of 4 independent replicates. Open arrowhead: long CRMP4 isoform (CRMP4L; 75 kDa); arrow: short CRMP4 isoform (CRMP4S; 65 kDa); solid arrowhead: CRMP4 cleavage product (tCRMP4; 55 kDa).

Figure 2.3: CRMP4 promotes the growth of injured sensory neurons.



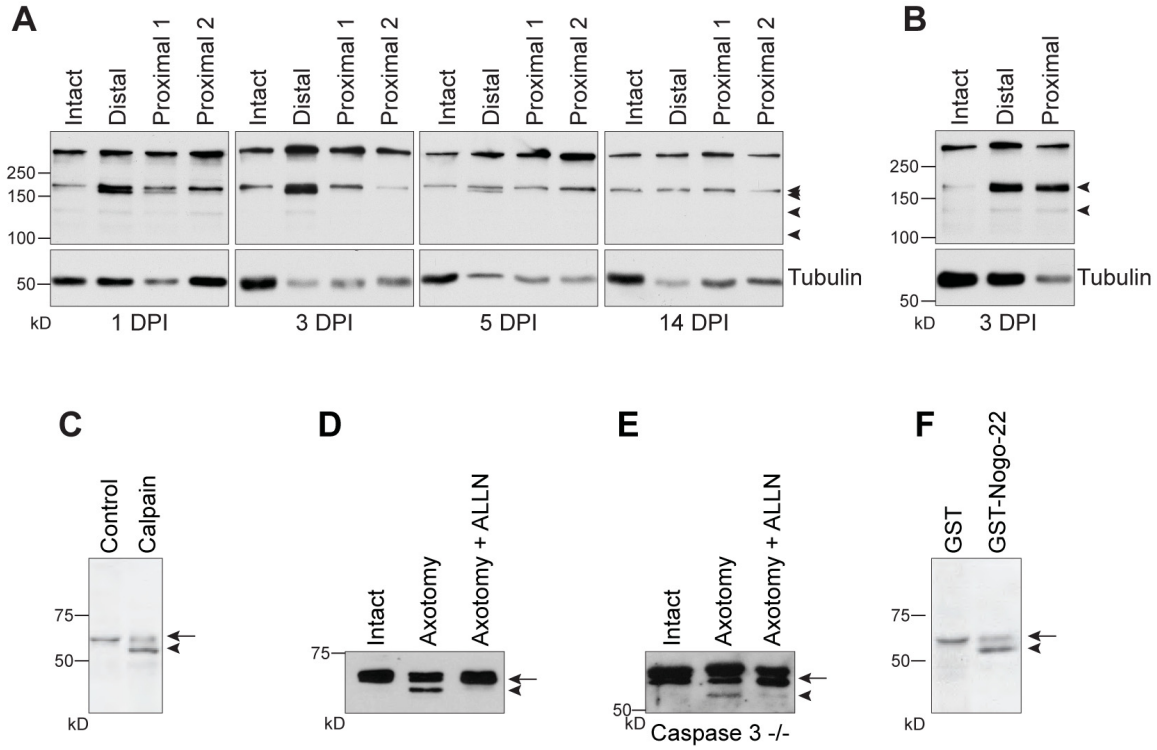
A) Immunoblot analysis of CRMP4 expression in sciatic nerve lysates of wild-type mice at different times following sciatic nerve transection. Samples collected after sciatic nerve transection span either 0 to 3 mm distal, 0 to 3 mm proximal (proximal 1), or 3 to 6 mm proximal (proximal 2) to the injury site. Open arrowhead: CRMP4L (75 kDa); arrow: CRMP4S (65 kDa); solid arrowhead: tCRMP4 (55 kDa). **B, C)** Representative pictures **(B)** and quantification **(C)** of the regrowth of dissociated E15-16 wild-type or *Crmp4*$^{-/-}$ DRG neurons plated in microfluidic devices at 24 h after axotomy indicated an impaired regrowth after axotomy in the *Crmp4*$^{-/-}$ DRG neurons. Data is represented as the mean neurite outgrowth per channel +/- S.E.M. (n=7-8, two-tailed Student t-test). Scale bar, 200 µm.

Figure 2.4: *Crmp4* deletion delays Wallerian degeneration *in vivo* and *in vitro*.



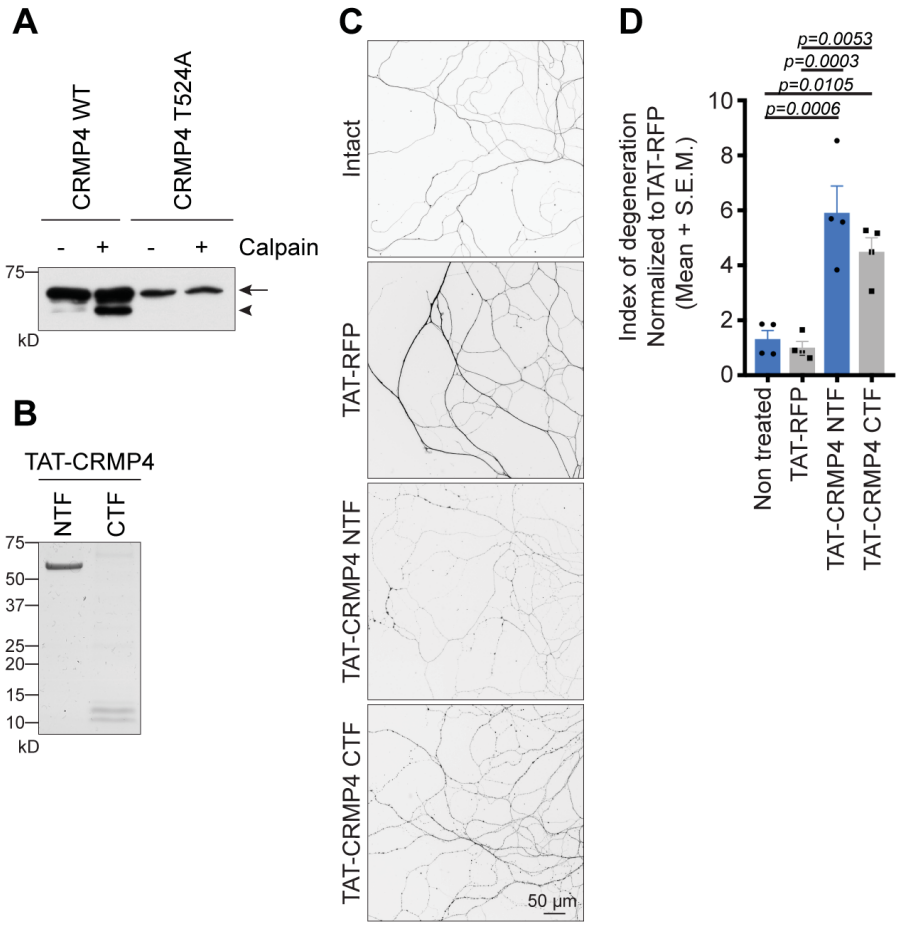
A) Semi-thin cross-sections of the degenerating sciatic nerve at 36 HPI, 3 DPI or 7 DPI following sciatic nerve transection, or of the intact contralateral nerve, in *Crmp4*$^{+/+}$ or *Crmp4*$^{-/-}$ mice. Scale bar, 50 µm. **B)** Quantification of the number of intact myelinated axons in the *Crmp4*$^{-/-}$ sciatic nerves compared to the *Crmp4*$^{+/+}$ controls displayed in **(A)**. Data is represented as the mean of intact myelinated axons per µm^2 +/- S.E.M. (n=3-7; two-way ANOVA with Bonferroni's multiple comparison test). **C, D)** Representative pictures of the axons extending from DRG explants at a distance of 500 µm from the center of the explants stained with Tuj1 **(C)** and quantification **(D)** of the extent of degeneration in E12.5 *Crmp4*$^{+/+}$ or *Crmp4*$^{-/-}$ DRG explants at 5 h after axotomy. Data in the graph is represented as the mean index of degeneration +/- S.E.M. (n=9-10; two-tailed Student t-test). Scale bar, 100 µm.

Figure 2.5: Calpain-dependent cleavage of CRMP4 occurs in the degenerating axons following injury.



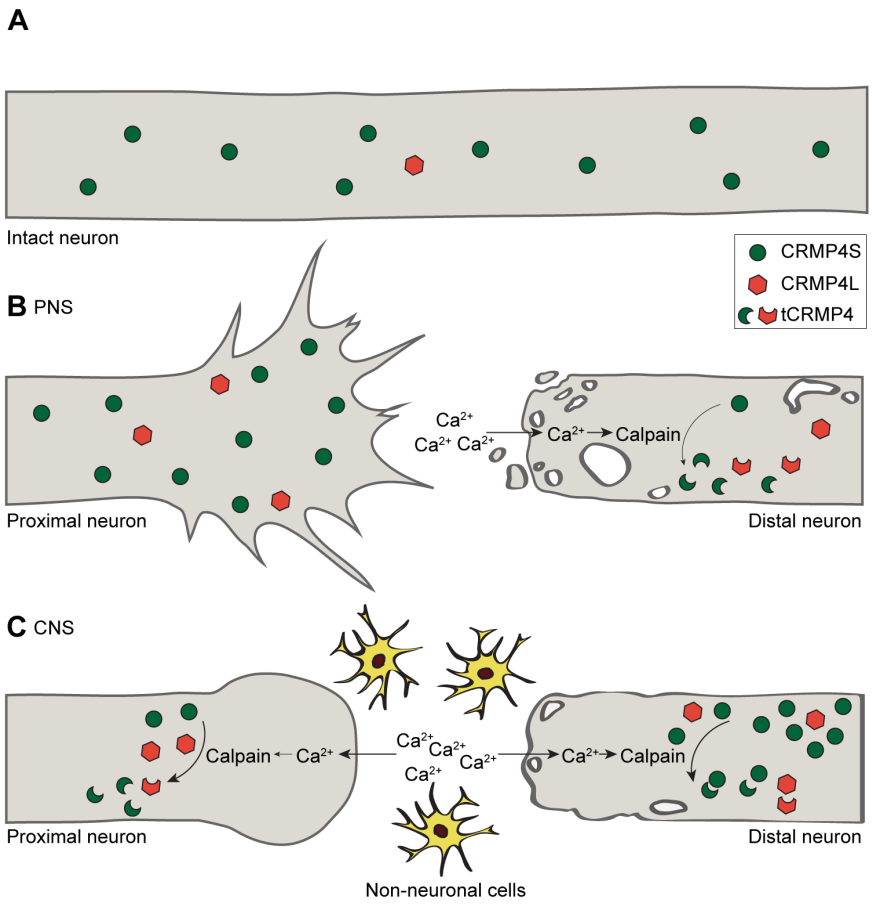
A, B) Immunoblot analysis of the calpain substrate fodrin in sciatic nerve **(A)** and optic nerve **(B)** lysates collected at the indicated days post-injury (DPI). The arrowheads indicate the presence of fodrin breakdown products, which are present when calpain is active. **C)** Western immunoblot analysis of CRMP4 expression in lysates prepared from P4-7 rat DRG neurons and treated with calpain *in vitro*. **D, E)** Western immunoblot analysis of CRMP4 expression in axonal lysates prepared from wild-type **(D)** or *Caspase-3*$^{-/-}$ **(E)** E12.5 DRG explants grown on cell-filter inserts collected at 3 h after axotomy. DRG explants were treated with ALLN to inhibit calpain. **F)** Western immunoblot analysis of CRMP4 expression in lysates prepared from P4-7 rat DRG neurons that were treated for 5 h with either GST or GST-Nogo-22. Arrow: CRMP4S (65 kDa); solid arrowhead: tCRMP4 (55 kDa).

Figure 2.6: CRMP4 cleavage fragments promote axonal degeneration.



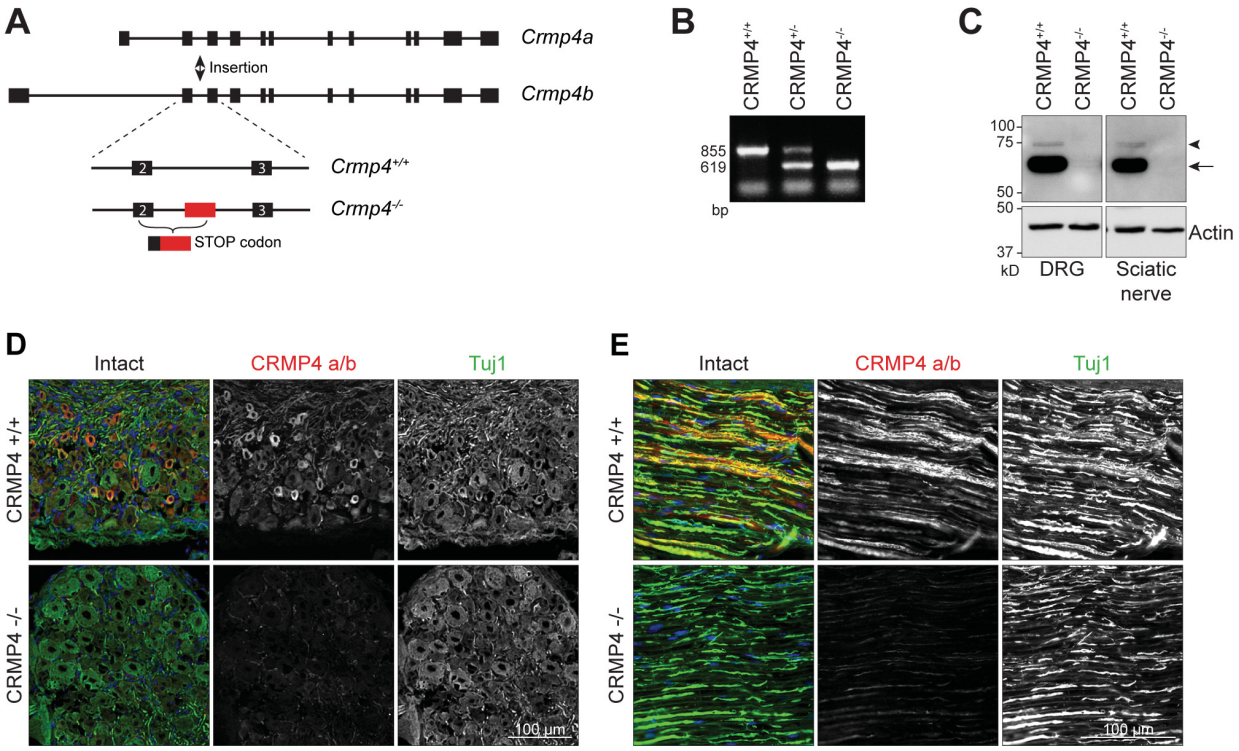
A) Western immunoblot analysis of WT-CRMP4S or CRMP4S-T524A in the presence or absence of calpain. Arrow: CRMP4S (65 kDa); solid arrowhead: tCRMP4 (55 kDa). **B)** Coomassie staining of the TAT-CRMP4 peptides corresponding to the amino- (NTF) or carboxy- (CTF) terminal CRMP4 cleavage fragments. TAT-CRMP4-NTF and -CTF generate peptides of approximately 60 kDa and 10-12 kDa respectively. **C, D)** Representative pictures **(C)** and quantification of the index of degeneration **(D)** following treatment of the axons for 8 h with 2.5 µM TAT-CRMP4 peptides or with TAT-RFP as a control. The data in the graph represents the mean index of degeneration normalized to TAT-RFP +/- S.E.M. (n=4; one-way ANOVA, Tukey's multiple comparison test). Scale bar, 50 µM.

Figure 2.7: Schematic model illustrating the expression profile of CRMP4 following PNS and CNS injury.



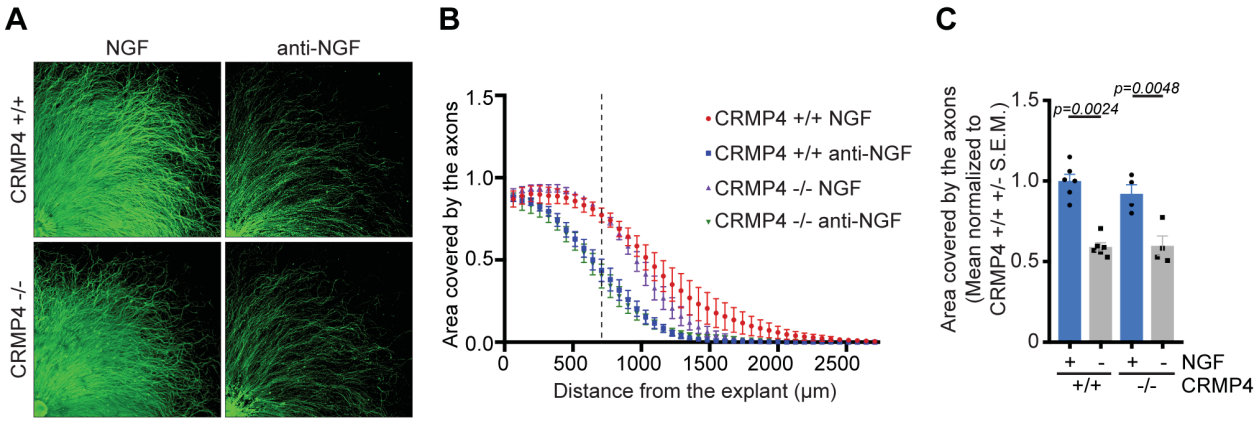
A) In the intact sciatic and optic nerves, CRMP4S is strongly expressed, while levels of CRMP4L are very low. **B)** In the sciatic nerve fibres located proximal to the injury site, the expression of CRMP4S is maintained, while CRMP4L is modestly upregulated. This expression pattern correlates with regeneration and contributes to axon extension. In the axon segments located distal to the injury site, entry of calcium promotes the calpain-dependent cleavage of CRMP4, leading to the formation of tCRMP4 and downregulation of full-length CRMP4, which promotes Wallerian degeneration. **C)** In the optic nerve, CRMP4S is downregulated, while tCRMP4 is upregulated in the axons proximal to the lesion site, correlating with the formation of retraction bulbs and the inhibition of axon regeneration. In the axon fragments located distal to the injury site, the presence of tCRMP4 is accompanied by a sustained expression of CRMP4S.

Supplementary Figure 2.S1: Generation and validation of the *Crmp4*$^{-/-}$ mouse line.



A) Structure of the *Dpysl3* (CRMP4) gene, illustrating the 14 exons of the gene on chromosome 18 that encode the CRMP4S and CRMP4L proteins. The Omnibank 76 trapping vector and a splice acceptor sequence were inserted between exons 2 and 3, a region shared by both isoforms (arrow). This insertion results in incorrect alternative splicing of the CRMP4 mRNA and leads to exons 3-14 to not be expressed. **B)** Genotyping of CRMP4 mice by PCR amplification of DNA isolated from tail samples. The wild-type and mutant alleles yield fragments of 855 bp and 619 bp respectively. **C)** Western immunoblot analysis of adult DRG and sciatic nerve samples using a CRMP4 a/b antibody to validate the loss of the CRMP4 protein. **D, E)** Adult DRG cross-sections **(D)** and longitudinal adult sciatic nerve sections **(E)** stained for CRMP4 a/b (red), the neuronal marker Tuj1 (green) and the nuclear marker Hoechst (blue). Scale bar, 100 µm.

Supplementary Figure 2.S2: *Crmp4* deletion does not alter degeneration induced by NGF withdrawal.



A) Representative images of intact E12.5 DRG explants or at 24 h following NGF withdrawal (anti-NGF). **B)** Curves of the area covered by axons of *Crmp4*$^{+/+}$ and *Crmp4*$^{-/-}$ DRG explants in the presence (NGF) or absence (anti-NGF) of NGF. The curves represent the mean +/- S.E.M. **C)** Mean +/- S.E.M. of the area covered by the axons at a distance of 700 µm from the center of the explants (as indicated by a dotted line in **(B)**) in the presence or absence of NGF. Data is normalized to *Crmp4*$^{+/+}$ grown in the presence of NGF (n=3; one-way ANOVA, Tukey's multiple comparison test).

CHAPTER 3

RhoA Proteolysis Regulates the Actin Cytoskeleton in Response to Oxidative Stress

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Keywords: Ras homolog gene family member A (RhoA), Actin cytoskeleton, Calpain, Caspase, Oxidative stress, Serine protease

Conflict of interest: The authors declare no competing financial interests.

3.1 Preface

The small GTPase RhoA is a ubiquitously expressed protein that plays a central role in many cellular functions, including phagocytosis, cytokinesis, migration and adhesion (Jaffe and Hall, 2005; Heasman and Ridley, 2008; Schaefer et al., 2014; Narumiya and Thumkeo, 2018). In addition to its physiological functions, RhoA is central to many signaling pathways triggered in response to neuronal damage. These include those underlying the inhibition of neuronal regeneration in the CNS and the inhibition of hyperinnervation in the PNS (McKerracher et al., 1994; Mukhopadhyay et al., 1994; Sebok et al., 1999; Arimura et al., 2000; Chen et al., 2000; GrandPre et al., 2000; Wahl et al., 2000; Kottis et al., 2002; Swiercz et al., 2002; Wang et al., 2002; Gallo, 2006; Hiraga et al., 2006; Mimura et al., 2006; Yiu and He, 2006; Tomita et al., 2007; Cheng et al., 2008; Quarta et al., 2017). In those contexts, various inhibitory cues are released in the extracellular milieu upon neuronal damage (McKerracher et al., 1994; Mukhopadhyay et al., 1994; Chen et al., 2000; GrandPre et al., 2000; Kottis et al., 2002; Morgenstern et al., 2002; Wang et al., 2002; Filbin, 2003; He and Koprivica, 2004; Mimura et al., 2006; Yiu and He, 2006). These activate RhoA, which then regulate the actin filaments and microtubules via different downstream effectors including ROCK, mDia, CRMP2 and CRMP4 (Riento et al., 2003; Alabed et al., 2007; Narumiya et al., 2009; Spiering and Hodgson, 2011). The activation of RhoA and consequent effects towards the cytoskeleton are tightly regulated by an array of mechanisms (Jaffe and Hall, 2005; Heasman and Ridley, 2008; Schaefer et al., 2014; Narumiya and Thumkeo, 2018). In chapter 3, I describe a novel regulatory mechanism of RhoA that is promoted upon cell stress. This mechanism leads to the proteolysis of RhoA, generating fragments that regulate the actin cytoskeleton by promoting the assembly of disorganized actin stress fibres and nuclear actin rods, and by disrupting the activity of full-length RhoA. Thus, our findings provide some insights in a novel regulatory mechanism of RhoA that is triggered in response to cell stress.

3.2 Abstract

The small GTPase RhoA regulates the actin cytoskeleton to affect multiple cellular processes including endocytosis, migration and adhesion. RhoA activity is tightly regulated through several mechanisms including GDP/GTP cycling, phosphorylation, glycosylation and prenylation. Previous reports have also reported that cleavage of the carboxy-terminus inactivates RhoA. Here, we describe a novel mechanism of RhoA proteolysis that generates a stable amino-terminal RhoA fragment (RhoA-NTF). RhoA-NTF is detectable in healthy cells and tissues and is upregulated following cell stress. Overexpression of either RhoA-NTF or the carboxy-terminal RhoA cleavage fragment (RhoA-CTF) induces the formation of disorganized actin stress fibres. RhoA-CTF also promotes the formation of disorganized actin stress fibres and nuclear actin rods. Both fragments disrupt the organization of actin stress fibres formed by endogenous RhoA. Together, our findings describe a novel RhoA regulatory mechanism.

3.3 Significance statement

The small RhoA GTPases plays a central role in many signaling pathways, including those underlying migration, process extension and guidance. There, it regulates the cytoskeleton via multiple downstream effectors, such as ROCK, mDia and CRMP2. Due to its ubiquitous expression and its involvement in several cellular functions, the activity of RhoA is tightly regulated via prenylation, phosphorylation and ubiquitination. Here, we characterize a novel regulatory mechanism that occurs via RhoA proteolysis. This cleavage occurs preferentially when RhoA is active and is promoted in response to oxidative stress. It leads to the generation of RhoA fragments that disorganize actin stress fibres and promote to assembly of nuclear actin rods. Thus, this novel regulatory mechanism alters the organization of the actin cytoskeleton.

3.4 Introduction

RhoA is a ubiquitously expressed member of the Ras-related family of GTPases with important roles in the regulation of the cytoskeleton through the assembly of actin stress fibres (Ridley and Hall, 1992; Hall, 1998) and the stabilization of microtubules (Palazzo et al., 2001; Grigoriev et al., 2006). These effects on the cytoskeleton underlie important roles for RhoA in many cellular functions such as cytokinesis (Miller and Bement, 2009), adhesion (Ridley and Hall, 1992; Hall, 1998), and migration (Pertz et al., 2006; Vega et al., 2011). The activation of RhoA is tightly regulated by several mechanisms. Cycling of RhoA between its inactive GDP-bound and active GTP-bound state is coordinated by GTPase activating proteins (GAPs) and guanine nucleotide exchange factors (GEFs) (Schmidt and Hall, 2002; Bernards and Settleman, 2004). In addition, guanine nucleotide dissociation inhibitors (GDIs) sequester RhoA in the cytosol and maintain it in its inactive GDP-bound state by interacting with the lipophilic moiety added post-translationally to the carboxy-terminal end of RhoA in a process known as prenylation (Hori et al., 1991; DerMardirossian and Bokoch, 2005; Roberts et al., 2008). Upon activation, RhoA is released from the GDIs and is able to translocate to the plasma membrane where it interacts with its numerous downstream effectors (Roberts et al., 2008). Additionally, RhoA is negatively regulated by phosphorylation of residue S188 by both cAMP- and cGMP-dependent kinases (Lang et al., 1996; Sawada et al., 2001; Forget et al., 2002; Ellerbroek et al., 2003) as well as by tyrosine glycosylation of residue Y34 (Jank et al., 2015). Thus, the combined effects of regulatory proteins and post-translational modifications control the activation of RhoA and, consequently, its functions.

The temporal and spatial functions of RhoA are also dependent on its stability. Thus, dynamic regulation through proteasomal degradation is critical for cell migration and the establishment of polarity. In protrusions, the Smurf1 E3 ubiquitin ligase controls the local level of RhoA by targeting it for proteasomal degradation (Wang et al., 2003). The Skp1-Cullin1-F-box protein (SCF)-like E3 ubiquitin ligase complex acts similarly to regulate the formation of actin stress fibres and cell morphology (Chen et al., 2009; Wei et al., 2013). Additionally, during cytokinesis, active GTP-bound RhoA is degraded in autophagosomes at the cleavage furrow to limit its functions (Belaid et al., 2013). Moreover, proteolysis is another mechanism resulting in RhoA downregulation. The mammalian cysteine protease μ-calpain cleaves the carboxy-terminal

end of RhoA, generating a dominant-negative protein that inhibits integrin-dependent cell spreading and actin stress fibre formation (Kulkarni et al., 2002). Similarly, upon infection by Yersinia enterocolitica, YopT (Yersinia outer protein), a cysteine protease expressed in host cells, inactivates RhoA by releasing it from the membrane and preventing its interaction with downstream effectors (Sorg et al., 2001; Shao and Dixon, 2003). Thus, several mechanisms contribute to the localized activity of RhoA.

Here, we describe a novel cleavage of RhoA that generates a stable amino-terminal cleavage fragment (RhoA-NTF). Cleavage occurs between the Switch I and Switch II regions and is regulated by the activity of serine proteases, calpain and caspases. The cleavage is upregulated in response to oxidative stress. High levels of RhoA-NTF or carboxy-terminal fragments (RhoA-CTF), such as those potentially found in pathological conditions, induce the formation of actin stress fibres in the cytoplasm and RhoA-CTF also induces formation of small nuclear actin rods. Thus, we describe a novel mechanism of RhoA proteolysis that regulates the actin cytoskeleton in response to oxidative stress.

3.5 Materials & Methods

3.5.1 Plasmids & Cloning

Full-length untagged (clone ID: RHO0A00000) and 2Xmyc-tagged (clone ID: RHO0A0MN00) wild-type (WT) RhoA constructs were obtained from the cDNA Resource Center (cDNA.org). To generate amino-terminal Flag-tagged constructs, the appropriate DNA was subcloned by PCR into a pcDNA3-Flag vector provided by Peter McPherson (Montreal Neurological Institute, Montréal, QC, Canada). A similar cloning was done to generate pGEX 4T-1 GST-tagged WT-RhoA. Other mutations (C190A, A56G/Q63L, A56V/Q63L, L57A/Q63L, W58A/Q63L, D59A/Q63L, Q63L/C83A, LWD-AAA/Q63L, Q63L/C190A, T19N/C190A) were generated by site-directed mutagenesis using the Quik Change II XL Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA). The coding sequences corresponding to the amino- and carboxy-terminal cleavage fragments (amino acids sequence 1-56, 1-83 and 57-193) were amplified by PCR and subcloned into the pcDNA3-Flag vector. A dually tagged Flag-RhoA-V5-His was also created by introducing a V5-His tag internal to the prenylation sequence at the carboxy-terminal end by PCR.

3.5.2 Transfection and Western Blotting

COS-7 cells were transiently transfected for 24 h with the different pcDNA3 Flag-RhoA plasmids using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. In some experiments, the transfected COS-7 cells were treated for 3 h with the calpain inhibitor calpeptin (EMD Millipore/Calbiochem, La Jolla, CA) or with the serine protease inhibitor AEBSF (EMD Millipore/Calbiochem, La Jolla, CA), or for 24 h with the pan-caspase inhibitor z-VAD-fmk (R&D Systems, Minneapolis, MN) or the proteasome inhibitors MG132 (Sigma-Aldrich, St-Louis, MO) and epoxomicin (Sigma-Aldrich, St-Louis, MO). For induction of oxidative stress, transfected cells were treated for 1 h with increasing doses of hydrogen peroxide (H_2O_2) (Thermo Fisher Scientific, Waltham, MA), 24 h prior to lysis. Following treatments, the cells were lysed in lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 mM Na_3VO_4, 5 mM NaF, 1X complete protease inhibitors). Sonicated and cleared lysates were loaded in equal amount of proteins onto SDS-PAGE gels and western blotting was performed with the following antibodies: mouse anti-Flag M2 (Sigma-Aldrich, St-Louis, MO; Catalog #F3165; 1:5000), mouse anti-actin clone C4 (MP Biomedicals, Santa Ana, CA; Catalog #69100; 1:3000), mouse anti-GAPDH (Abcam, Cambridge, UK; Clone 6C5; Catalog #ab8245; 1:2000), mouse anti-c-myc (Sigma-Aldrich, St-Louis, MO; Clone 9E10; Catalog #M5546; 1:2000), rabbit anti-Rho (Abcam, Cambridge, UK; Clone Y486; Catalog #ab32046; 1:500), anti-V5 (Invitrogen, Carlsbad,CA; Catalog #R960-25; 1:5000), and horse-radish peroxidase (HRP)-conjugated anti-mouse and anti-rabbit IgG antibodies (Jackson ImmunoResearch, West Grove, PA; Catalog #111-035-003 and #115-035-003; 1:10000).

3.5.3 Proteomics

RhoA-NTF and FL-RhoA were immunoprecipitated using Flag M2 agarose beads (Sigma-Aldrich, St-Louis, MO) from lysates prepared from COS-7 cells transiently transfected with pcDNA3 Flag-RhoA Q63L as described above. The proteins were separated by SDS-PAGE and stained with Coomassie Brilliant Blue dye. The bands corresponding to FL-RhoA and -NTF were excised from the gel, digested with either chymotrypsin or trypsin, and submitted to the McGill University and Génome Québec Innovation Centre (Montréal, QC, Canada) for

proteomics analysis. The sequences of the peptides generated from these digestions were determined by tandem mass spectrometry and the data analyzed using the Scaffold3 software. To localize the cleavage site, the peptides generated upon trypsin or chymotrypsin digestion and identified by mass spectrometry with 95% probability were aligned onto the theoretical full-length protein sequence of Q63L-RhoA. The regions adjoining but not covered by these peptides and localized in the amino-terminal end of the protein were deemed likely to contain the RhoA cleavage site.

3.5.4 Protein Purification and Calpain Digestion

Lysates of COS-7 cells transiently transfected with either 2Xmyc WT-RhoA or Flag-RhoA 1-56 were collected in calpain digestion buffer (50 mM Tris-HCl pH 7.4, 100 mM KCl, 1 mM EDTA, 5 mM DTT) without protease inhibitors. Immunoprecipitation was performed with either Flag M2 agarose beads (Sigma-Aldrich, St-Louis, MO) or EZview red anti-c-myc affinity gel (Sigma-Aldrich, St-Louis, MO). Flag-tagged proteins were eluted with 5 µg ml^{-1} Flag peptide (Sigma-Aldrich, St-Louis, MO) in calpain digestion buffer. For in vitro digestion, 9 units of recombinant µ-calpain from human erythrocytes (EMD Millipore/Calbiochem, La Jolla, CA) were added to the purified lysates for 45 min at room temperature in the presence of 2 mM $CaCl_2$ and the reaction was stopped by the addition of Laemmli sample buffer. As a negative control, 14 µM calpeptin (EMD Millipore/Calbiochem, La Jolla, CA) was added to the samples to inhibit calpain activity.

3.5.5 Purification of recombinant WT-RhoA

A BL21 E. coli bacterial culture expressing GST-tagged WT-RhoA was induced overnight at room temperature with 100 µM IPTG. The induced bacteria were pelleted, resuspended in lysis buffer (50 mM Tris-HCl pH 7.4, 50 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 1X protease inhibitors), and sonicated. The cleared lysate was then incubated with washed glutathione sepharose resin (GE Healthcare, Mississauga, ON) for 2 h at 4°C. The beads were washed thoroughly and sequentially with wash buffer I (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT) and wash buffer II (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$, 2.5 mM $CaCl_2$, 1 mM DTT). The beads resuspended in wash buffer II were incubated with 50 units thrombin (Sigma-Aldrich, St-Louis, MO; Catalog #T4648-1kU) overnight at 4°C to elute

WT-RhoA without the GST tag. The thrombin-cleaved WT-RhoA was collected in wash buffer III (10 mM Tris-HCl pH 7.4, 2 mM $MgCl_2$, 1 mM DTT). The thrombin was removed from the eluate by incubating it with p-aminobenzamidine (PAB) agarose beads (Sigma-Aldrich, St-Louis, MO) at 4°C for 1 h. Finally, the eluate was concentrated using Amicon Ultra-4 centrifugal filter units with Ultracel-10 membrane (Millipore, La Jolla, CA). A dose curve of recombinant WT-RhoA was used to quantify the amount of FL-RhoA and RhoA-NTF in some lysates by densitometry with Adobe Photoshop CS5.1. Statistical analysis was done by one-way ANOVA on the data collected from 3 independent experiments.

3.5.6 Immunoprecipitation of endogenous RhoA-NTF

All studies using rodents were approved by the McGill University Animal Care and Use Committee. Adult Sprague Dawley rats were euthanized by CO_2 inhalation and cervical dislocation. The brain, heart and lungs were dissected and homogenized in RIPA lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 1 mM Na_3VO_4, 5 mM NaF, 1X complete protease inhibitors). Alternatively, untransfected COS-7 cells were treated for 1 h with 5000 µM H_2O_2 and lysed in RIPA lysis buffer 24 h following treatment. Cell debris were removed by high-speed centrifugation. The lysates were pre-cleared by incubating them with protein A/G Plus-Agarose beads (SantaCruz Biotechnologies, Santa Cruz, CA) for 1 h at 4°C. The endogenous FL-RhoA and -NTF were immunoprecipated from the pre-cleared lysate by incubating it with either 2 µg ml^{-1} anti-Rho antibody (Abcam, Cambridge, UK; Clone Y486; Catalog #ab32046) or anti-GFP antibody (Sigma-Aldrich, St-Louis, MO; Catalog #G1544) as a negative control coupled to A/G agarose beads overnight at 4°C on a rotator. The beads were thoroughly washed in RIPA lysis buffer before eluting the protein by boiling in 2X Laemmli buffer.

3.5.7 Rhotekin-binding-domain (RBD) pull-down

A culture of pGEX2T-RBD *E. coli* was induced with 1 mM IPTG for 4 h at 30°C after which it was pelleted and resuspended in buffer A (20 mM HEPES pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 1X complete protease inhibitors). The bacterial lysate was freeze/thawed and sonicated (6 X 5 sec at 20% amplitude with 30 s rest). Triton X-100 was added to a final concentration of 0.1% and the lysate was mixed thoroughly before clarification by high-speed

centrifugation. The cleared lysate was incubated with glutathione-sepharose beads (GE Healthcare, Mississauga, ON) for 1 h at 4°C. The collected GST-RBD beads were washed 3 times with wash buffer (Buffer A containing 0.1% Triton X-100). Beads were freshly prepared before each experiment. In parallel, COS-7 cells were transfected with various pcDNA3 Flag-RhoA mutants using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) following manufacturer's instructions. The cells were lysed in Mg^{2+} lysis buffer (25 mM HEPES pH 7.5, 150 mM NaCl, 1% NP-40, 10 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, 1X complete protease inhibitors, 25 mM NaF, 1 mM Na_3VO_4). The collected cells were triturated using 25G needles and centrifugated to remove the cell debris. As a positive control for the RBD pull-down, lysate was incubated with GTPγS (Millipore, La Jolla, CA) at 30°C for 30 min and adding 0.1 M $MgCl_2$ ended the reaction. The cell lysates were incubated with the GST-RBD beads for 1 h at 4°C. The beads were washed 2 times with Mg^{2+} lysis buffer and eluted using 2X Laemmli buffer.

3.5.8 Formation of actin stress fibres

Swiss 3T3 fibroblast cells provided by Nathalie Lamarche-Vane (McGill University, Montréal, QC, Canada) were seeded at a density of 75000 cells per well on uncoated 18-mm glass coverslips (Thermo Fisher Scientific, Waltham, MA). After 3 days, Swiss 3T3 cells were serum-starved for 16 h and transfected using Lipofectamine LTX and Plus reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. To activate both the endogenous and the transfected RhoA proteins, DMEM supplemented with 10% FBS was added to the cells 30 min prior to fixation to induce the formation of actin stress fibres. The cells were fixed with 4% paraformaldehyde (PFA) diluted in phosphate-buffered saline (PBS), permeabilized with 0.2% Triton X-100/PBS and stained with anti-Flag M2 antibody (Sigma-Aldrich, St-Louis, MO; Catalog #F3165; 1:1000) revealed with anti-mouse Alexa-Fluor 488 (Life Technologies/Thermo Fisher Scientific, Waltham, MA; Catalog #A11001; 1:500), Alexa-Fluor 546 Phalloidin (Life Technologies/Thermo Fisher Scientific, Waltham, MA; Catalog #12380; 1:500), and Hoechst dye. The cells were imaged using a Zeiss Axiovert 200M epifluorescence microscope or a Zeiss LSM 710 confocal microscope. Counts were performed to determine the number of transfected cells containing either actin stress fibres (presence of well-defined F-actin filaments within the cytosol) or nuclear actin rods (presence of small actin filaments in the nuclear area) and statistical significance was evaluated using the Chi-square test. In the transfected cells containing

stress fibres, the actin accumulation corresponding to fibres was evaluated by measuring the ratio of the actin-covered area divided by the total area of the cell using ImageJ and statistical significance was determined by one-way ANOVA. In those same cells, the orientation of the actin stress fibres was measured using the AngleJ plugin of ImageJ (Günther, 2015). The data was then organized based on the deviation of the angles from the mode, which corresponds to the angle with the highest number of actin segments. The values were plotted as a frequency distribution and the area under the curve was calculated for a 20° deviation from the mode (0°) to determine the proportion of fibres that exhibited the highest degree of organization.

3.6 Results

3.6.1 Cleavage of the amino-terminus of RhoA

Previous reports have shown that calpain and YopT cleave RhoA near its carboxy-terminus to generate a dominant-negative protein (Sorg et al., 2001; Kulkarni et al., 2002; Shao et al., 2003). We found that overexpression of wild-type (WT) RhoA with a Flag tag on the amino-terminus generated a 22 kDa protein corresponding to full-length RhoA (FL) as well as a 10 kDa proteolytic fragment generated from the amino-terminal end of the protein (RhoA-NTF; Fig. 3.1 A). WT-RhoA with a 2Xmyc epitope tag generated a similar fragment, indicating that the cleavage is not a function of the epitope tag (Fig. 3.1 A). RhoA-NTF was stabilized in cells treated with the proteasome inhibitors MG132 and epoxomicin (Fig. 3.1 B, C).

3.6.2 Prenylated active GTP-bound RhoA undergoes enhanced proteolysis

RhoA cycles between an inactive GDP-bound and an active GTP-bound state that interacts with effector proteins to remodel the cytoskeleton (Ridley and Hall, 1992; Hall, 1998; Grigoriev et al., 2006). To determine whether RhoA proteolysis is dependent on the activation state of the protein, the levels of RhoA-NTF were evaluated in lysates collected from COS-7 cells overexpressing wild-type (WT), constitutively active or dominant-negative RhoA mutants. Constitutively active RhoA (Q63L and G14V) generated higher levels of RhoA-NTF compared to WT-RhoA, whereas these levels were lower with the dominant-negative (T19N) mutant, suggesting that active RhoA is preferentially cleaved (Fig. 3.1 D). In addition to the GDP/GTP cycling, RhoA activity is regulated by prenylation, a post-translational modification essential to its translocation to the plasma membrane (Hori et al., 1991; Roberts et al., 2008). Non-prenylated

WT, active and dominant-negative RhoA (C190A-, Q63L/C190A- and T19N/C190A-RhoA, respectively) were weakly proteolysed compared to WT-RhoA (Fig. 3.1 D). Together, this illustrates that the proteolytic processing of RhoA occurs preferentially when RhoA is in its prenylated active GTP-bound state

3.6.3 Oxidative stress promotes RhoA proteolysis

Previous studies have shown that RhoA can be activated in response to a number of apoptotic and toxic stimuli (Subauste et al., 2000; Aghajanian et al., 2009; Zhu et al., 2013; Li et al., 2014). To assess whether RhoA proteolysis is altered in response to cell stress, we examined the levels of RhoA-NTF in response to hydrogen peroxide (H_2O_2) treatment. Exposure of COS-7 cells expressing either WT- or T19N-RhoA to H_2O_2 led to a significant accumulation of RhoA-NTF (Fig. 3.2 A, B). T19N-RhoA was still subject to H_2O_2-dependent proteolysis, indicating that this mechanism is not solely a function of RhoA activation (Fig. 3.2 B). To confirm that endogenous RhoA is also subject to proteolytic cleavage, we screened commercially available RhoA antibodies for their ability to detect the RhoA-NTF proteolytic fragment. A single commercial RhoA antibody (clone Y486; Abcam) that was generated against an epitope in the amino-terminal end of RhoA, immunoprecipitates and detects RhoA-NTF from COS-7 cells overexpressing Q63L-RhoA (Fig. 3.2 C). The antibody is weak compared to the Flag M2 antibody, as it was able to detect RhoA in the immunoprecipitate but only weakly in the cell lysate (Fig. 3.2 C). To determine whether RhoA proteolysis occurs endogenously, we analyzed RhoA immunoprecipitates from untransfected COS-7 cells and detected a weak endogenous RhoA fragment in H_2O_2-treated cells (Fig. 3.2 D). RhoA-NTF was also primarily detected in lung tissue with weaker bands observed in the heart and brain following immunoprecipitation with the Rho Y486 antibody (Fig. 3.2 D). We conclude that endogenous RhoA is proteolytically cleaved but this novel cleavage is difficult to detect because of the weak immunoreactivity of commercial RhoA antibodies.

Using the RhoA Y486 antibody, we quantified the proportion of RhoA-NTF generated in COS-7 cells expressing Flag-tagged WT-RhoA upon H_2O_2 treatment. Using densitometry, the intensity of FL-RhoA and RhoA-NTF bands were determined and compared to a dose curve of recombinant WT-RhoA (Fig. 3.2 E). In untreated COS-7 cells transfected with WT-RhoA,

RhoA-NTF represented on average 5.9% of FL-RhoA, whereas upon treatment with 1000 μM H_2O_2, this ratio increased to 17.1% (Fig. 3.2 F). Correspondingly, a reduction of 39% in the levels of FL-RhoA was also observed (Fig. 3.2 F). We conclude that very little RhoA-NTF is generated in healthy cells and that these levels are appreciably upregulated under conditions of cell stress.

3.6.4 RhoA proteolysis is regulated by serine proteases, calpain and caspases

To identify the protease responsible for the generation of RhoA-NTF, COS-7 cells were transiently transfected with Flag-tagged WT-RhoA and treated with a panel of protease inhibitors. Cells treated with the serine protease inhibitor AEBSF exhibited a dose-dependent decrease in the levels of RhoA-NTF, suggesting that a serine protease is cleaving RhoA (Fig. 3.3 A, B). Further, a pan-caspase inhibitor (z-VAD-fmk) and a calpain inhibitor (calpeptin) increased the levels of RhoA-NTF in COS-7 cells transfected with WT-RhoA (Fig. 3.3 C-F). These results support a role of calpain and caspases either in the degradation of RhoA-NTF or in the inactivation of the protease responsible for the cleavage. The inability of caspases to directly cleave RhoA supports a model in which caspase regulates a serine protease able to cleave RhoA (Tu and Cerione, 2001). In contrast, we find that recombinant μ-calpain directly degrades RhoA-NTF. Treatment of immunoprecipitated 2Xmyc-tagged WT-RhoA or a Flag-tagged amino-terminal fragment of RhoA (1-56) with μ-calpain resulted in the degradation of RhoA-NTF (Fig. 3.3 G, H). Together, our data suggests that RhoA is cleaved by a serine protease that is targeted and inhibited by caspases, and that RhoA-NTF is directly degraded by calpain (Fig. 3.3 I).

3.6.5 RhoA is cleaved between the Switch I and Switch II regions

To further our understanding of RhoA proteolysis, we sought to map the cleavage site leading to the generation of RhoA-NTF. Flag-tagged Q63L-RhoA was overexpressed in COS-7 cells, and RhoA-NTF was purified from the cell lysate by immunoprecipitation followed by SDS-PAGE separation (Fig. 3.4 A). Samples were digested with trypsin or chymotrypsin, and analyzed by tandem mass spectrometry. The peptides obtained with each enzyme were mapped onto the theoretical full-length sequence of Q63L-RhoA. Uncovered regions in the amino-terminal portion of RhoA adjoining detected peptides would be indicative of a potential cleavage site. Unexpectedly, some peptides were identified from the C-terminal region of RhoA and this can

likely be ascribed to smear of full length RhoA in the gel. In the amino-terminal region of the protein, potential cleavage sites were identified at residues 56-59 and at residue C83 (Fig. 3.4 B). To further investigate the cleavage site, we substituted candidate cleavage residues to Alanine, or to Glycine and Valine in the case of A56 in the Q63L-RhoA background and assessed the sensitivity of mutated constructs to proteolysis. Mutating A56 or C83 had no effect on RhoA proteolysis while mutation at residue L57, W58 or D59 significantly diminished the levels of RhoA-NTF and a combined mutation of these last residues eliminated it (Fig. 3.4 C). L57A/Q63L-RhoA is the only mutant with reduced levels of RhoA-NTF that remained active within the cells, as shown by a rhotekin-binding-domain (RBD) pull-down (Fig. 3.4 D), suggesting that this construct retains its function within the cell. To characterize the functions of the RhoA cleavage fragments, we generated cDNA constructs encoding residues 1-56 and 57-193, which approximate RhoA-NTF and -CTF, respectively (Fig. 3.4 E). Transfection of COS-7 cells with RhoA 1-56 produced a fragment that co-migrated with the one generated upon proteolysis of Flag-tagged WT-RhoA while one encoding residues 1-83 generated a peptide with a molecular weight higher than 10 kDa (Fig. 3.4 F). The peptide corresponding to residues 57-193, the predicted carboxy-terminal fragment, generated a 17 kDa protein (Fig. 3.4 F). Transfection of a Flag-Q63L-RhoA construct with a V5 tag inserted at the carboxy-terminus preceding the GPI anchor (Flag Q63L-RhoA V5-His) results in expression of a weak V5-positive band at 17 kDa (Fig. 3.4 F). The carboxy-terminal RhoA fragment co-migrates with RhoA 57-193, further confirming the proteolytic cleavage site. Weak expression of the carboxy-terminal fragment in cells transfected with full length RhoA suggests that the fragment is unstable in baseline conditions (Fig. 3.4 G). These findings support a cleavage site encompassing residues 57-59 localized between the hydrophobic Switch I and Switch II regions (Ihara et al., 1998).

3.6.6 RhoA-NTF induces mild stress fibre formation while RhoA-CTF leads to the assembly of nuclear actin rods

To investigate the functions of the RhoA fragments generated upon proteolysis, Flag-tagged RhoA 1-56 or 57-193, which correspond to RhoA-NTF and -CTF respectively, was overexpressed in serum-starved Swiss 3T3 fibroblasts and their effects on filamentous actin stress fibres were analyzed. Introduction of either WT- or constitutively active Q63L-RhoA led to the formation of transcytoplasmic actin stress fibres in 31.9% and 63.8% of the transfected

cells, respectively (Fig. 3.5 A, B). This is reflected by a significant increase in the ratio of actin-covered area in the cells containing stress fibres when compared to the cells expressing pcDNA3 (Fig. 3.5 C). Flag-tagged RhoA 1-56 and 57-193 also induced the formation of stress fibres in 46.4% and 38.4% of the transfected cells, respectively (Fig. 3.5 A, B). In both cases, the ratio of actin-covered area was comparable to WT-RhoA, suggesting that a similar amount of actin accumulation occurs in the transfected cells containing stress fibres. Intriguingly, in many cells expressing RhoA 57-193 (38.4%), small actin rods were observed near the nucleus (Fig. 3.5 A, B). Confocal microscopy revealed that RhoA 57-193 is expressed in close apposition to the actin rods in the proximity of the nucleus (Fig. 3.5 D). This suggests that RhoA-CTF could translocate to the nucleus to promote the formation of actin rods and have a potential role in the organization of the nucleoskeleton. Overall, this data indicates that both RhoA-NTF and -CTF retain some activity towards the cytoskeleton by inducing stress fibre formation in cytoplasmic and nuclear regions of the cell.

3.6.7 RhoA cleavage fragments disrupt the orientation of actin stress fibres induced by endogenous full-length RhoA

We next determined whether either RhoA-NTF or -CTF could interfere with the function of endogenous RhoA on actin cytoskeleton remodeling. Swiss 3T3 cells transfected with Flag-tagged RhoA fragments were treated with fetal bovine serum (FBS) to activate RhoA and promote actin stress fibre formation (Ridley and Hall, 1992). Cells treated with FBS exhibited long parallel transcytoplasmic actin stress fibres (Fig. 3.6 A-C). In FBS-treated cells, the extent of stress fibre formation was diminished in RhoA 1-56- and RhoA 57-193-transfected cells (Fig. 3.6 A-C). Notably, FBS-treated cells transfected with RhoA 1-56 or RhoA 57-193 were characterized by less organized stress fibres. To quantify this phenotype, we quantified the angle of deviation from the mode for all actin segments using AngleJ. The data obtained was then plotted as a frequency distribution with the origin of the graph corresponding to the mode, where the mode represents the orientation that is most common within a given distribution (Fig. 3.6 D). Control transfected cells treated with FBS were highly organized with 60% of all actin segments deviating less than 20° from the mode. Cells transfected with RhoA 1-56 or RhoA 57-193 exhibited a more disorganized actin structure with under 40% of transfected cells deviating less

than 20° from the mode. We conclude that an effect of the RhoA fragments appears to be a disruption of stress fibre organization upon RhoA activation.

3.6.8 Proteolysis is not essential to the functions of RhoA towards the actin cytoskeleton

To determine the role of proteolysis in the physiological functions of RhoA towards actin stress fibre formation, we examined the activity of RhoA mutated at residue L57, which is a cleavage-resistant version of this protein that retains RhoA activity (Fig. 3.4 C, D). This construct was overexpressed in serum-starved Swiss 3T3 fibroblasts to analyze the induction of actin stress fibres. Our data revealed that 43.9% of the transfected cells exhibited stress fibre formation to a level comparable to the one obtained with either WT- or Q63L-RhoA as shown with the ratio of actin-covered area within those cells (Fig. 3.5 A-C). Furthermore, the cleavage-resistant RhoA construct did not interfere with the activity of endogenous WT-RhoA upon activation as 91.5% of transfected cells have long parallel actin stress fibres extending across the cell (Fig. 3.6 A, B). However, the cells expressing this construct had a reduced ratio of actin-covered compared to the control cells transfected with pcDNA3 and treated with FBS (Fig. 3.6 B). The organization of the stress fibres was also impaired (Fig. 3.6 D). Thus, cleavage of RhoA is not essential to the induction of actin stress fibres although these are disrupted in their abundance and organization.

3.7 Discussion

Here, we report a novel mechanism of RhoA proteolysis that occurs in the amino-terminal end of the protein to generate a stable 10 kDa fragment. Low levels of endogenous RhoA-NTF are detected in healthy cells and tissues, but these are increased in response to oxidative stress. The mechanism underlying this processing is dependent on a complex interplay of a serine protease, calpain and caspases. RhoA proteolysis is not essential for its activity on the actin cytoskeleton; however RhoA cleavage fragments disrupt the formation of organized stress fibres. RhoA-CTF also promoted the formation of small actin rods near the nuclear area.

3.7.1 Mechanism underlying RhoA proteolysis

RhoA is a GTPase that acts as a molecular switch, cycling between an inactive GDP-bound and an active GTP-bound state (Schmidt and Hall, 2002; Bernards and Settleman, 2004). When transitioning between these states, RhoA undergoes a conformational change in both Switch

domains, which exposes the hydrophobic patches necessary to the interaction with downstream effectors (Ihara et al., 1998). Our findings revealed that RhoA proteolysis occurs preferentially for the active GTP-bound RhoA whereas both inactive and non-prenylated RhoA are poorly cleaved. Additionally, the cleavage site characterized here encompasses residues L57 to D59, which are located next to the Switch II domain. These residues are not displaced by the conformational change, but transitioning to the GTP-bound state brings the Switch I domain in close proximity (Ihara et al., 1998). This could enhance the accessibility of the cleavage site to proteases and explain the increased amount of RhoA-NTF obtained with the constitutively active RhoA. Thus, RhoA proteolysis could be part of a regulation loop that prevents the persistent activation of RhoA.

Furthermore, GTP binding and prenylation favor the translocation of RhoA to the plasma membrane (Adamson et al., 1992; Solski et al., 2002), and these, combined with the action of several proteases, regulate RhoA proteolysis. Our data suggests a mechanism where, upon oxidative stress, a serine protease would become activated while caspases would be downregulated, leading to the generation of a stable RhoA-NTF and an unstable RhoA-CTF. Additionally, the proteasome system and calpain would further degrade RhoA-NTF generated upon proteolysis to regulate its stability. The specific serine protease involved in RhoA proteolysis remains to be identified and as such, it is difficult to determine whether it is mediating direct cleavage of RhoA or if it is involved upstream in the regulation of FL-RhoA. Previous reports have documented roles for such proteases in the regulation of RhoA, but these need to be validated for RhoA proteolysis. Potential candidates include the tissue kallikreins-related peptidase (KRK)-5, which reduces the levels of active RhoA via the regulation of isoprenoid synthesis (Pampalakis et al., 2014), and thrombin since it increases RhoA activity (Greenberg et al., 2003). Additionally, the serine protease Htr2A is upregulated in response to cell stress but its relation to RhoA has not been documented (Gray et al., 2000). Similar uncertainties remain concerning the involvement of calpain in RhoA proteolysis as calpeptin, in addition to inhibiting calpain, also promotes RhoA activity by inhibiting a tyrosine phosphatase upstream of this GTPase (Schoenwaelder and Burridge, 1999). Our data support that calpain might be involved in the degradation of RhoA-NTF as it directly cleaves this fragment. However, this does not exclude the activation of RhoA by calpeptin that could lead to the

upregulation of RhoA proteolysis. Thus, further experiments are required to characterize the exact role of each protease in RhoA proteolysis.

The constitutive activation of proteases including serine proteases, calpain and caspases is minimal in healthy cells, which could explain the low amount of endogenous RhoA found in healthy cells and tissues. However, oxidative stress increases levels of RhoA-NTF, suggesting that it could either activate RhoA or promote its proteolysis. Previous reports have shown that lower doses of H_2O_2 induce apoptosis via caspase activation and activate RhoA, whereas higher doses promote necrosis in a caspase-independent fashion (Teramoto et al., 1999; Saito et al., 2006; Aghajanian et al., 2009). According to our data, the increased levels of RhoA-NTF are observed with the higher doses of H_2O_2 associated with necrosis. Furthermore, H_2O_2 also promotes proteolysis of a dominant-negative RhoA, suggesting that oxidative stress predominately activates the pathway underlying RhoA cleavage.

3.7.2 Functions of the RhoA proteolytic fragments

RhoA plays a major role in the reorganization of the actin cytoskeleton by mediating the assembly of stress fibres (Ridley and Hall, 1992) and this role was confirmed by overexpressing FL-RhoA in Swiss 3T3 fibroblasts. A similar phenotype was observed with the cleavage-resistant RhoA construct although the stress fibres generated were slightly disorganized, but this suggests that proteolysis is not essential for RhoA functions towards the actin cytoskeleton. We next sought to investigate the effects of high levels of RhoA proteolytic fragments. Both RhoA-NTF and RhoA-CTF induce the formation of actin stress fibres, suggesting that these could retain some functions towards the actin cytoskeleton. RhoA effectors are classified into three groups based on their RhoA binding motifs. Class I (eg. rhotekin, PKN, Dia1) and Class III (eg. citron) effectors bind RhoA at a site that partially overlaps with the Switch II and Switch I domains respectively, while Class II (eg. ROCK) effectors interact with RhoA at multiple sites overlapping with both Switch regions (Fujisawa et al., 1998). As the cleavage site is located between the Switch regions, each resulting fragment contains at least one effector-binding domain and should have the ability to interact with RhoA effectors provided that the fragments maintain the appropriate tridimensional conformation. RhoA-NTF contains the binding site for class III effectors and one of the binding sites for class II effectors, whereas RhoA-CTF contains

the binding site for class I effectors and two of the three binding sites for class II effectors. Previous reports have shown that both ROCK and Dia1 play a role in actin cytoskeleton remodeling (Watanabe et al., 1999), and the ability of the fragments to induce actin stress fibre formation is probably due to the partial interaction with those effectors, but this will need to be confirmed experimentally. Furthermore, both RhoA-NTF and -CTF disrupt the abundance and organization of the actin stress fibres generated by the activation of endogenous RhoA, indicating that they are able to interfere with the functions of full-length RhoA without having a dominant-negative effect. Interestingly, oxidative stress also induces the formation of actin stress fibres, although the mechanism is still poorly characterized (Huot et al., 1997), but it would be interesting to determine whether RhoA proteolysis is involved in this process.

A striking phenotype was the formation of a network of actin rods and the localization of RhoA-CTF near the nuclear membrane upon its overexpression in fibroblasts. This suggests that this fragment could potentially regulate the nuclear actin cytoskeleton. Under cell stress, actin rods are formed within the nucleus, which could be part of a protective response aiming to minimize the impact of an accumulation of actin within the cytoplasm (Ono et al., 1993; Domazetovska et al., 2007; Munsie et al., 2012). However, if these nuclear actin rods are maintained, they can impair cellular functions and disrupt nuclear morphology. The presence of nuclear actin rods has been observed in several diseases such as nemaline myopathy (Hutchinson et al., 2006) and Huntington's disease (Munsie et al., 2011). Thus, the generation of RhoA-CTF and its nuclear translocation could either be a protective response to cell stress or a disruption in the nucleoskeleton that could lead to cell death. Further studies are required to investigate the role of RhoA proteolysis in oxidative stress and its link to the formation of nuclear actin rods.

3.7.3 Proteolysis of other Rho GTPases

The Rho GTPases, including RhoA, Rac and Cdc42, have a high degree of homology in their protein sequences with most of the variations located in the carboxy-terminal end of the proteins (Ihara et al., 1998). Interestingly, residues L57, W58 and D59 constituting the RhoA cleavage site are conserved between RhoA, RhoB, RhoC, Rac1, and Rac2, while in Cdc42, only residues L57 and D59 are conserved (Ihara et al., 1998). Thus, it is possible that RhoB, RhoC, Rac1 and Rac2 are also cleaved at this position if the recognition sites for the protease are conserved. As it

is well known that crosstalk between the different Rho GTPases occurs during remodeling of the actin cytoskeleton (Hall, 1998; Stultiens et al., 2012), it will be interesting to determine whether regulation through proteolytic cleavage is a mechanism common to multiple Rho GTPases and whether it is partially responsible for this crosstalk. As Rho GTPases are major regulators of the actin cytoskeleton, this novel processing mechanism could potentially be harnessed to regulate cell migration and division, two processes that are essential for normal development as well as central to the progression of cancer.

3.8 Acknowledgements

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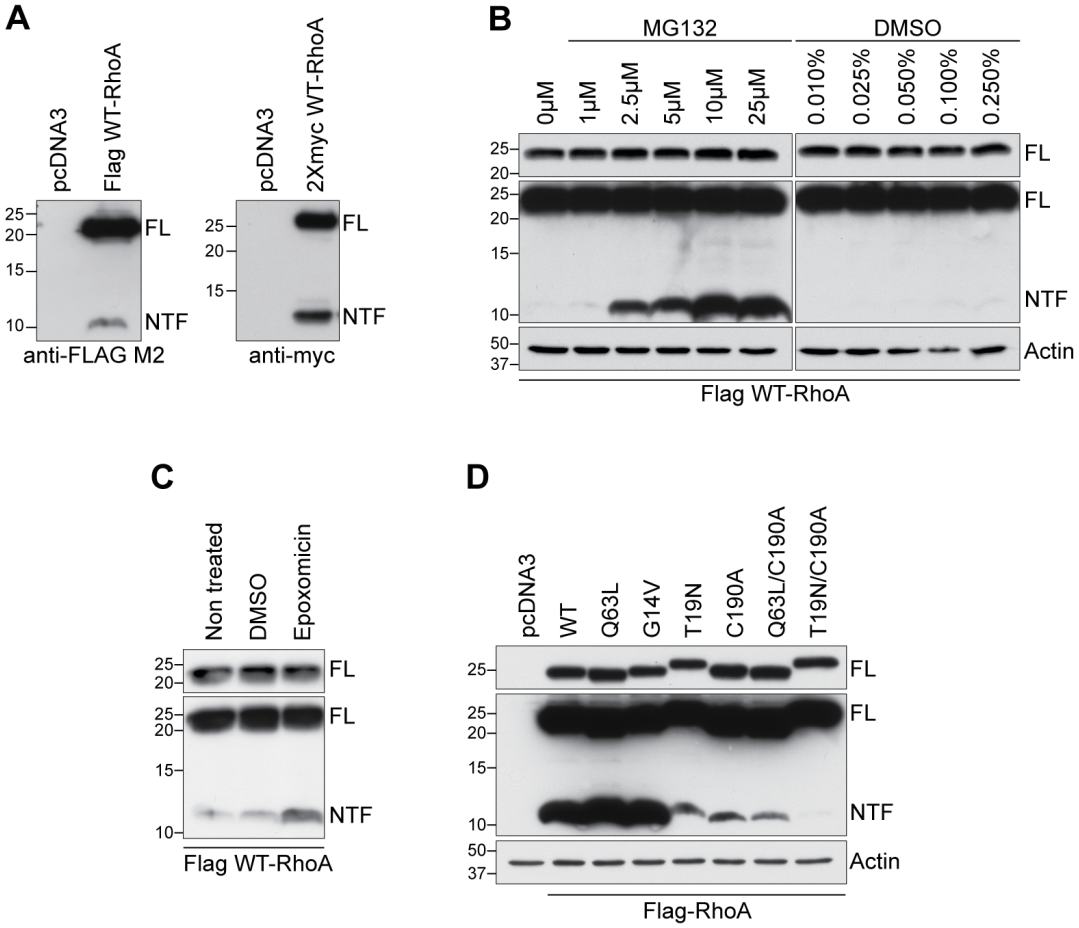
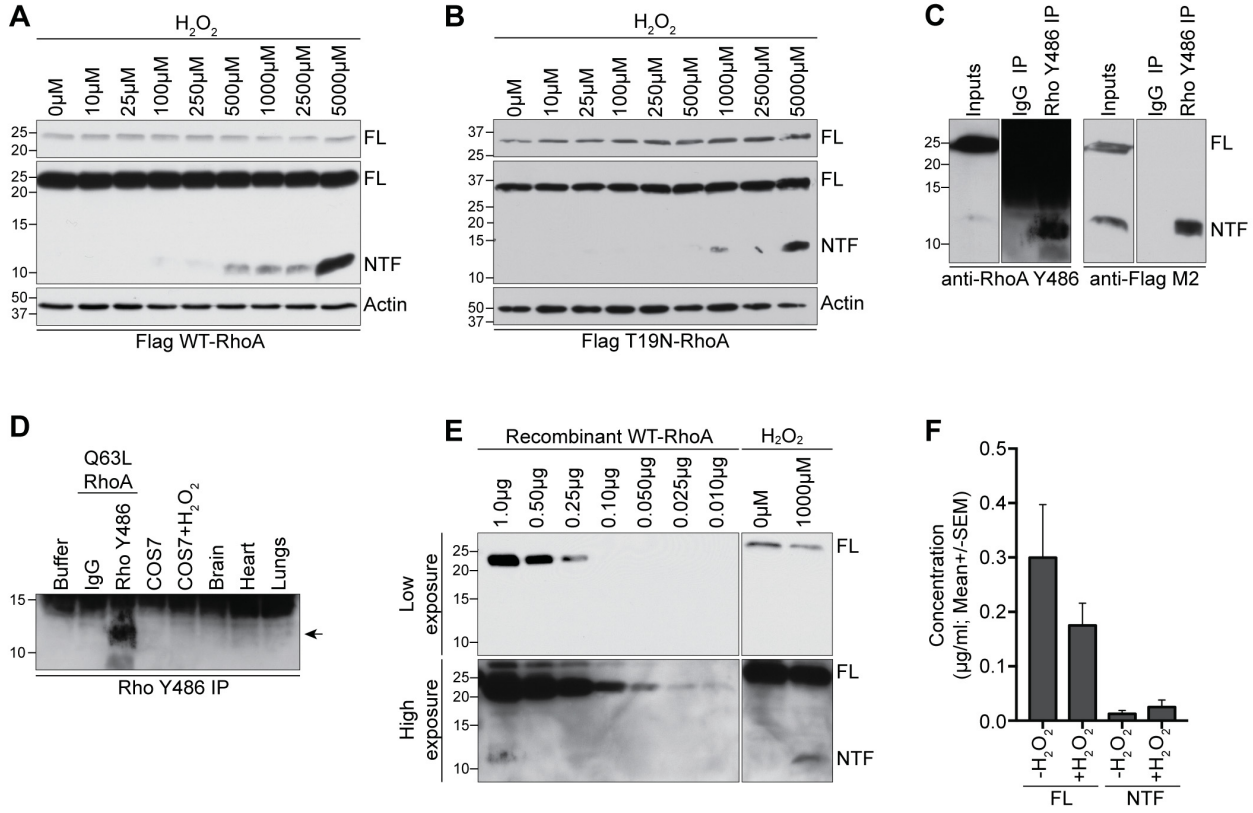
3.9 Figures

Figure 3.1: Identification of a 10 kDa amino-terminal RhoA fragment.



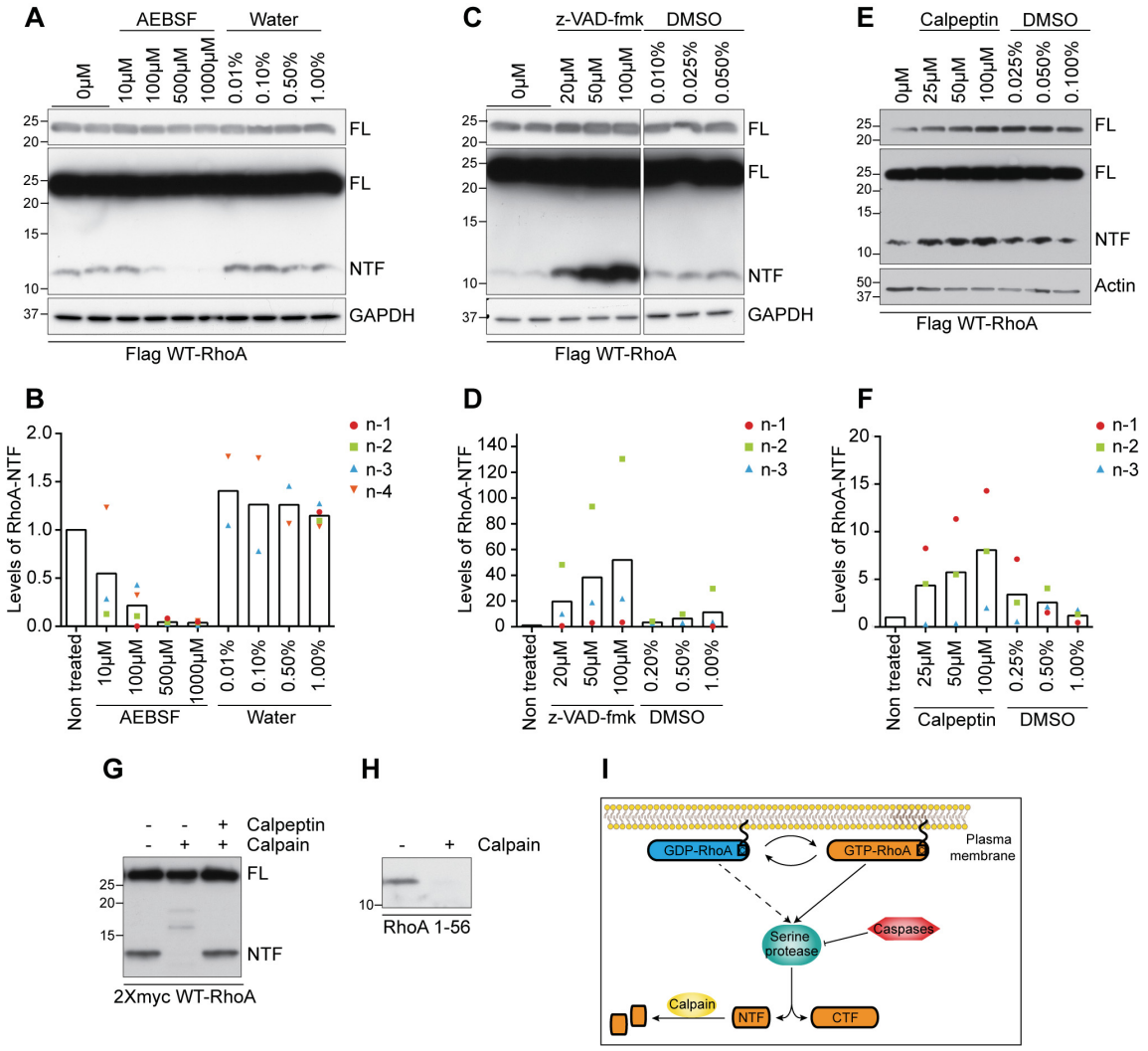
A) Western blot analysis of COS-7 cell lysates transfected with Flag-tagged WT-RhoA or with 2Xmyc-tagged WT-RhoA using an anti-Flag M2 or anti-c-myc antibody reveals the presence of FL-RhoA and RhoA-NTF bands. **B, C)** Western blot of cell lysates following treatment with the proteosome inhibitors MG132 **(B)** or epoxomicin **(C)**. **D)** Expression of wild-type (WT), constitutively active (Q63L and G14V), dominant-negative (T19N) as well as non-prenylated wild-type (C190A), active (Q63L/C190A) and inactive (T19N/C190A) RhoA constructs in COS-7 cells analyzed by western blot using the Flag M2 antibody. Western blot panels illustrating FL-RhoA only are exposed to evaluate loading of full-length protein while panels with FL-RhoA and RhoA-NTF are a longer exposure of the same blot to visualize RhoA-NTF.

Figure 3.2: Endogenous RhoA proteolysis is enhanced by oxidative stress.



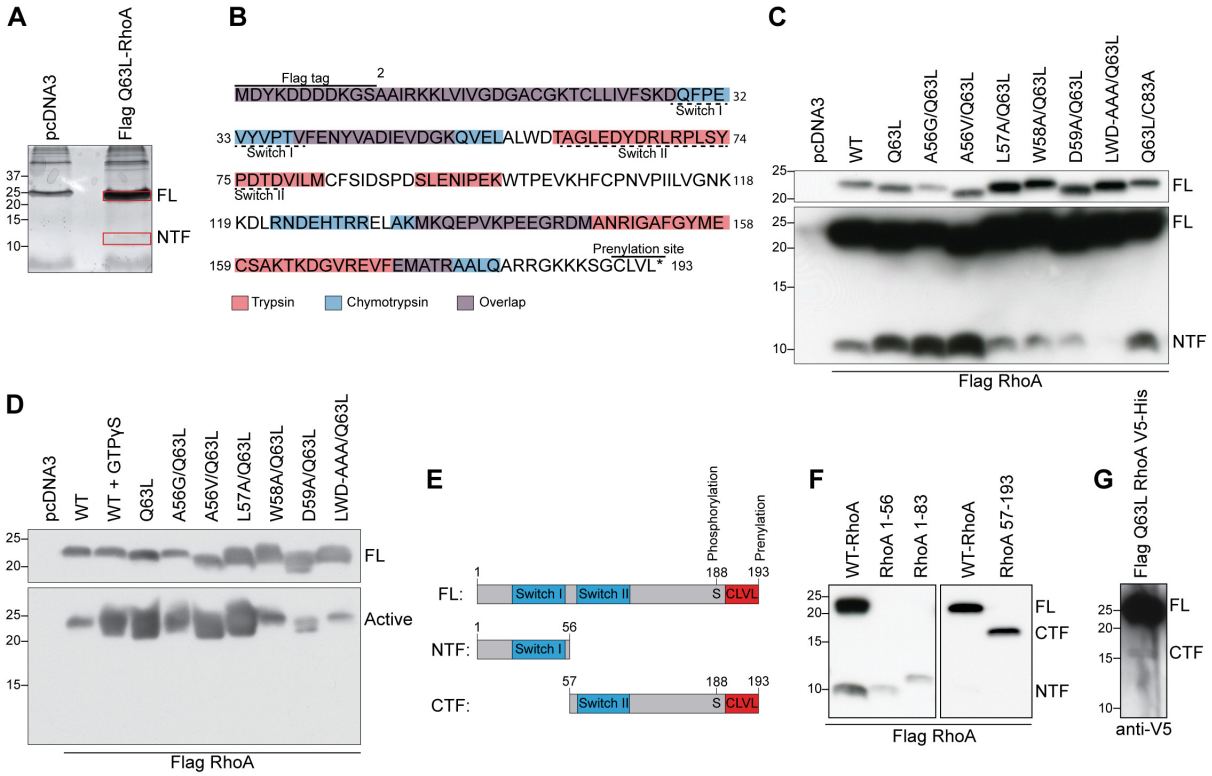
A, B) Lysates from COS-7 cells were transfected with Flag-tagged WT-RhoA **(A)** or T19N-RhoA **(B)** and western blotted with anti-Flag M2 antibody. Cells were exposed to H_2O_2 for 1 hour, 24 hours prior to lysis. **C)** COS-7 cells transfected with Flag-tagged WT-RhoA were immunoprecipitated with the Rho Y486 antibody from Abcam. Lysates and immunoprecipitates were probed with anti-RhoA Y486 or anti-FLAG M2 antibodies. **D)** Immunoprecipitation of RhoA-NTF from untransfected COS-7 cells treated with H_2O_2 for 1 hour at 24 hour prior to lysis or from various healthy adult rat tissues, including the heart, brain and lungs, using the Rho Y486 antibody. Arrow: RhoA-NTF. **E, F)** Analysis of the relative abundance of FL-RhoA and RhoA-NTF in cell lysates compared to a dose curve of recombinant WT-RhoA by Western blot analysis with the Rho Y486 antibody. The graph in **F** quantifies the average concentration of FL-RhoA as well as RhoA-NTF upon treatment with 1000 µM H_2O_2.

Figure 3.3: Serine proteases, caspases and calpain regulate RhoA proteolysis.



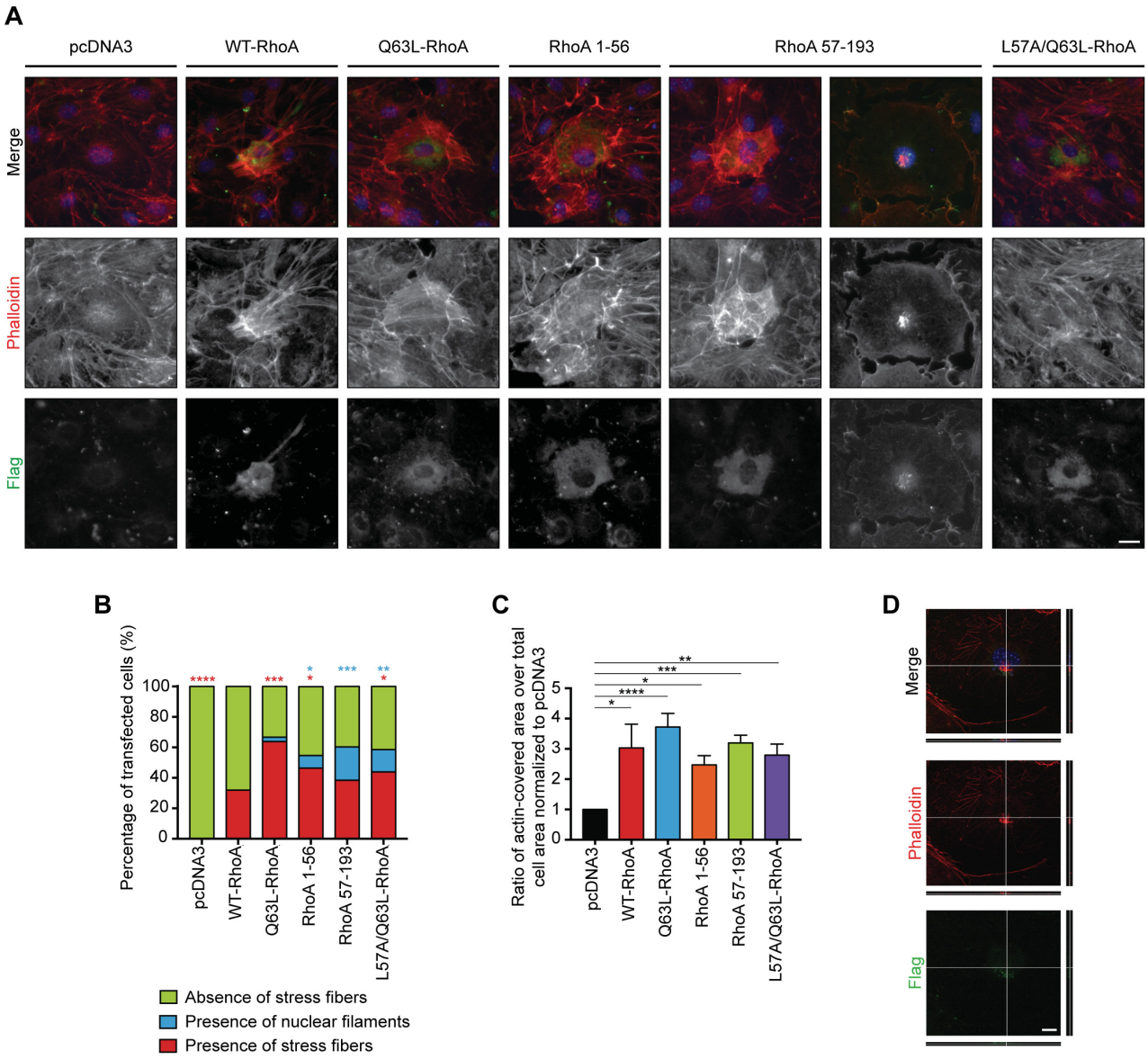
A-F) Lysates from COS-7 cells transfected with Flag-tagged WT-RhoA were analyzed by western blot with anti-Flag M2 antibody following treatment with protease inhibitors. Transfected cells were treated for 3 h with the serine protease inhibitor AEBSF **(A)**, 24 h with the pan-caspase inhibitor z-VAD-fmk **(C)**, or 3 h with the calpain inhibitor calpeptin **(E)** and the levels of RhoA-NTF were quantified by densitometry **(B, D, F)**. Water and DMSO were vehicle controls. **G, H)** 2Xmyc WT-RhoA **(G)** or Flag RhoA 1-56 **(H)** were immunoprecipitated from transfected COS-7 cells and treated for 45 min with recombinant µ-calpain in the presence or absence of 14 µM calpeptin. **I)** Diagram illustrating the mechanism underlying RhoA proteolysis.

Figure 3.4: Mapping the RhoA cleavage site.



A) Coomassie brilliant blue-stained SDS-Page gel of FL-RhoA and RhoA-NTF showing the bands excised (boxes) for tandem mass spectrometry. **B)** Alignment of the peptides obtained by mass spectrometry of RhoA-NTF following trypsin (red) or chymotrypsin (blue) digest onto the theoretical sequence of Q63L-RhoA. **C)** Mutations were introduced in Q63L-RhoA to localize the cleavage site. **D)** Rhotekin-binding-domain (RBD) pull-down of COS-7 cell lysates transfected with the various Q63L-RhoA mutants evaluated the activation of each construct. **E)** Diagram of the FL-RhoA as well as both amino (NTF)- and carboxy (CTF)-terminal RhoA fragments. **F)** Expression of the constructs encoding the Flag-tagged RhoA fragments illustrated in **E** by western blot with Flag M2 antibody. **G)** Western blot analysis of COS-7 cells lysates transfected with the dual tagged Flag Q63L-RhoA V5-His to evaluate the presence of RhoA-CTF using anti-V5 antibody.

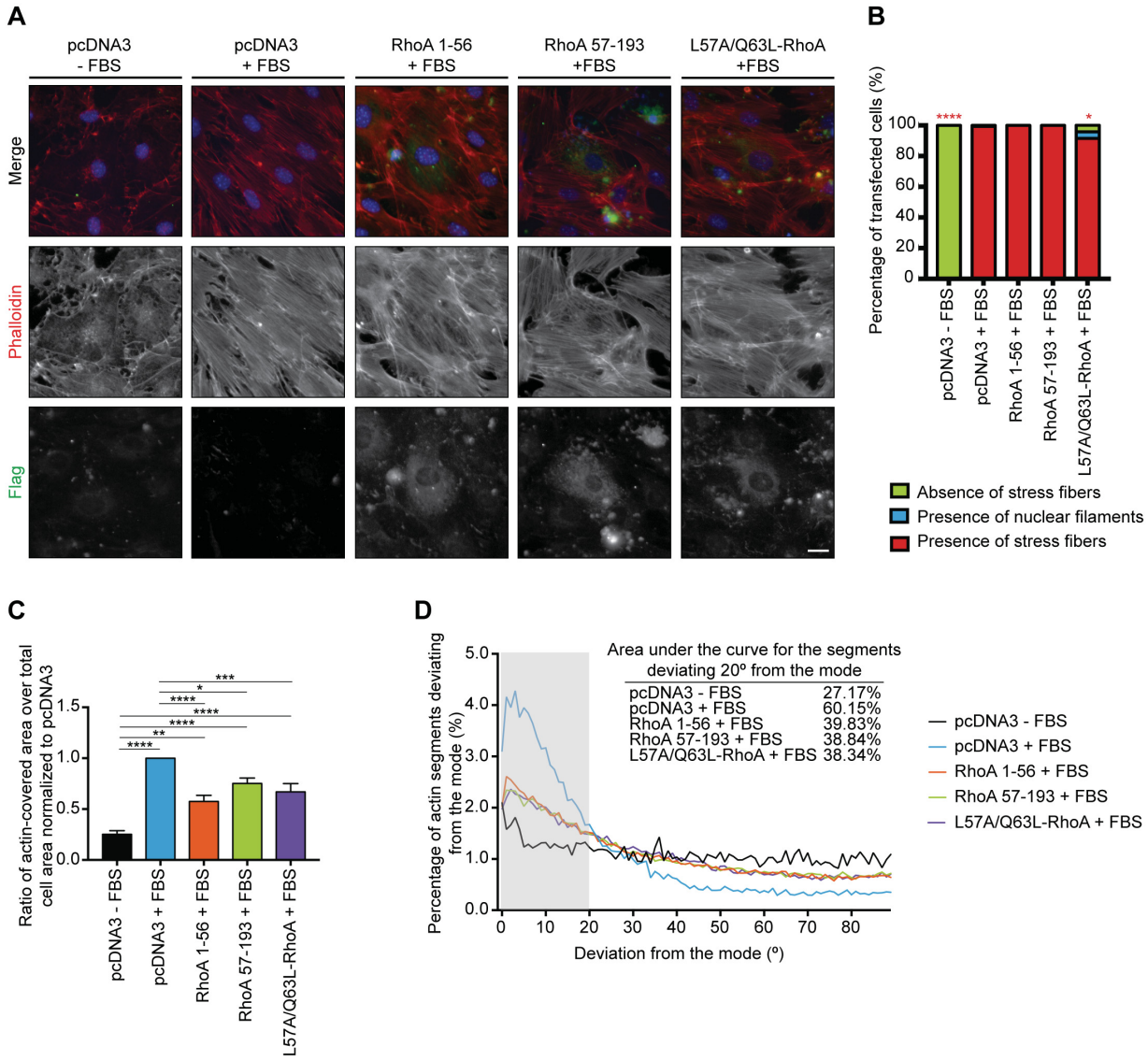
Figure 3.5: RhoA fragments and cleavage-resistant promote the formation of actin stress fibres.



A) Serum-starved Swiss 3T3 fibroblasts were transfected with Flag-tagged RhoA 1-56 and 57-193, which correspond to RhoA-NTF and -CTF respectively, as well as WT-, Q63L- and the cleavage-resistant L57A/Q63L-RhoA. Cells were stained with anti-Flag M2 antibody (green), Alexa-Fluor 546 phalloidin (red) and Hoechst (blue) to label RhoA, the actin stress fibres and the nucleus respectively. Scale bar, 20 µm. **B)** Classification of the actin phenotype in transfected Swiss 3T3 cells. Significance was determined by the Chi-square test. *, $p < 0.05$; ** $p < 0.005$;

***, $p < 0.0005$; ****, $p < 0.0001$. $n > 40$ cells from 7 independent experiments. **C)** Quantification of the ratio of actin-covered area divided by the total surface of the transfected cells represented as the mean +/- S.E.M. Significance was established by one-way ANOVA from $n > 40$ cells collected from 7 independent experiments. *, $p < 0.05$; ** $p < 0.005$; ***, $p < 0.0005$; ****, $p < 0.0001$. **D)** Representative side view of a z-stack of a Swiss 3T3 cell overexpressing RhoA-CTF showing the localization of nuclear actin rods (red) relative to the nucleus (blue) and RhoA 57-193 (green). Scale bar, 10 µm.

Figure 3.6: Both RhoA fragments, but not cleavage-resistant RhoA, interfere with the organization of actin stress fibres induced by activated endogenous full-length RhoA.



A) Serum-starved Swiss 3T3 fibroblasts were transfected with the Flag-tagged RhoA 1-56 and 57-193 fragments as well as WT-, Q63L- and L57A/Q63L-RhoA. The endogenous full-length RhoA was activated by treating the cells with 10% FBS for 30 min prior to fixation. The cells were stained with anti-Flag M2 antibody (green), Alexa-Fluor 546 phalloidin (red) and Hoechst (blue) to label RhoA, the actin stress fibres and the nucleus respectively. Scale bar, 20 µm. **B)** Classification of the actin phenotype in the transfected Swiss 3T3 cells in **A**. n > 20 cells from 3 independent experiments. Significance was determined by the Chi-square test. *, $p < 0.1$; ****, p

< 0.0001. **C)** Quantification of the ratio of the actin-covered area divided by the total area of the transfected cell in **A** represented as the mean +/- S.E.M. Significance was established by one-way ANOVA from n > 20 cells from 3 independent experiments. *, $p < 0.05$; ** $p < 0.005$; ***, $p < 0.0005$; ****, $p < 0.0001$. **D)** Frequency distribution of the angles' deviation from the mode of the actin stress fibres present in the transfected Swiss 3T3 cells as measured with AngleJ. The area under the curve contained within 20° of the mode is representative of the proportion of segments with a high degree of organization.

DISCUSSION AND CONCLUSION

The cytoskeleton is an essential structural component of eukaryotic cells that can be rapidly reorganized in response to various intrinsic and extrinsic stimuli during development and adulthood. In response to injury, damage to this intricate network can often be incurred as a result of the physical breakage of the cellular architecture and/or of the disturbance of signaling pathways. The repair of the cytoskeletal architecture in the damaged cell is key to functional recovery. In neurons, the reorganization of the cytoskeleton following injury is impaired by a number of intrinsic and extrinsic factors that collaborate to limit regeneration and prevent functional recovery. A central player in those growth-inhibitory signaling pathways is RhoA, which regulate the actin filaments and microtubules via its numerous downstream effectors (Arimura et al., 2000; Palazzo et al., 2001; Palazzo et al., 2004; Arimura et al., 2005; Hiraga et al., 2006; Mimura et al., 2006; Yiu and He, 2006; Alabed et al., 2007; Bartolini et al., 2008; Cheng et al., 2008; Bamburg and Bernstein, 2010; Spiering and Hodgson, 2011; Quarta et al., 2017). In response to inhibitory cues such as Nogo and myelin, RhoA interacts with CRMP4, a cytoskeletal regulator (Alabed et al., 2007). This interaction contributes to the deficient neuronal regeneration in the damaged CNS (Alabed et al., 2007; Khazaei et al., 2015).

Whether it occurs during development, adulthood or in response to injury, the remodeling of the cytoskeleton is highly dependent on numerous regulatory proteins. Understanding these regulatory mechanisms is key to designing therapeutic strategies to promote recovery following injury. For example, preventing the interaction between RhoA and CRMP4 limits the growth-inhibitory effects of myelin (Alabed et al., 2007; Khazaei et al., 2015). Despite these promising results, several unknowns remain towards the functions and regulation of RhoA and CRMP4. In this thesis, I furthered our understanding of these two cytoskeletal regulators in the cellular response to various insults. As such, I characterized the roles of CRMP4 in the neuronal response to PNS injury (chapter 2) and identified a novel regulatory mechanism of RhoA that occurs via its proteolysis in response to cell stress (chapter 3). As RhoA and CRMP4 are involved in numerous functions, the findings of this thesis could be extrapolated to numerous injury contexts, including PNS and CNS injury as well as neurodegenerative diseases.

4.1 CRMP4 regulates the neuronal response to damage

CRMP4 is a cytoskeletal regulator that is developmentally expressed in the nervous system (Wang and Strittmatter, 1996; Nacher et al., 2000; Rosslenbroich et al., 2005; Khazaei et al., 2014; Tan et al., 2015; Cha et al., 2016). It possesses several growth-promoting functions in the developing neurons, while it becomes growth-inhibitory in the injured CNS (Alabed et al., 2007; Alabed et al., 2010; Nagai et al., 2012; Niisato et al., 2012; Khazaei et al., 2014; Nagai et al., 2015; Tan et al., 2015; Cha et al., 2016; Nagai et al., 2016). Despite this knowledge, its roles in the PNS remained unclear.

4.1.1 Functions of CRMP4 following PNS injury

Upon injury, neurons located in the PNS initiate a stereotypical response in which the neuron segments still attached to their cell bodies regenerate while the disconnected ones degenerate. Both processes are highly dependent on cytoskeletal rearrangements that occur in response to damage. In chapter 2, I found that CRMP4 plays a dual role in this process, as the full-length protein supports regeneration of the regenerating sensory axons, while its calpain-mediated cleavage underlies Wallerian degeneration.

4.1.1.1 Signaling pathways involving CRMP4

In regenerating PNS axons, CRMP4 favors regeneration, potentially by promoting the re-formation of a functional growth cone at the tip of the damaged axons and by supporting axon extension. As such, CRMP4S and CRMP4L could facilitate the cytoskeletal rearrangements that are critical to the formation of a newly-formed growth cone, as both splice isoforms regulate actin and microtubule dynamics (Rosslenbroich et al., 2005; Khazaei et al., 2014; Tan et al., 2015). Additionally, CRMP4L could be involved in the trafficking of membrane and cell surface proteins by its interaction with intersectin, a protein that facilitate endocytosis and exocytosis (Acheson et al., 1991; Yamabhai et al., 1998; Okamoto et al., 1999; Simpson et al., 1999; Hussain et al., 2001; Quinn et al., 2003). Furthermore, CRMP4 could support axon extension, as it favors the polymerization of F-actin and the assembly of microtubules (Rosslenbroich et al., 2005; Khazaei et al., 2014; Tan et al., 2015). Further experiments will seek to characterize the specific functions of CRMP4S and CRMP4L in the regulation of the cytoskeleton in the

regenerating PNS neurons. To achieve this, several *in vitro* approaches could be used. For example, dissociated cultures of DRG neurons could be transduced with herpes simplex virus (HSV) encoding the DNA corresponding to each CRMP4 isoforms. Following this, specific features of the transduced neurons, including neurite outgrowth, dendritic branching and growth cone morphology, could be assessed to evaluate the roles of each isoforms in sensory neurons. Alternatively, to determine whether these isoforms alter neurite regrowth after axotomy, dissociated cultures of DRG neurons could be grown in microfluidic devices, transduced with HSV encoding the DNA for each CRMP4 isoform, and axotomized as done previously in chapter 2. These findings could then be confirmed *in vivo* by electroporating or injecting DNA constructs encoding each CRMP4 isoform in the lumbar L4-6 DRGs of adult *Crmp4*$^{-/-}$ mice prior to sciatic nerve crush (Saijilafu et al., 2014; Li et al., 2018). Thus, these experiments would deepen our understanding of the functions of the different CRMP4 isoforms in PNS regeneration.

Oppositely, in degenerating neurons, the calpain-mediated cleavage of CRMP4 promotes axonal fragmentation and Wallerian degeneration. The truncated proteins generated upon this cleavage could potential act as dominant-negative proteins by inserting themselves in the functional CRMP tetramers, consequently impairing their effects within the neurons (Wang and Strittmatter, 1997). Additionally, this regulatory mechanism leads to the depletion of the full-length CRMP4, which could compromise the integrity of the axon's cytoskeleton. Interestingly, other CRMP family members are cleaved in a calpain-dependent manner in response to excitotoxicity and ischemia (Jiang et al., 2007; Zhang et al., 2007; Liu et al., 2009), suggesting that there might be some redundancy in the functions of the different CRMPs in Wallerian degeneration. To address this, we should determine whether other CRMPs are cleaved in response to peripheral nerve injury. Additionally, selective knockdown of each family member would help us determine the relative contribution of each to Wallerian degeneration.

The various cytoskeletal regulators involved in regeneration and degeneration, such as MAP1, SCG10 and CRMP2, are regulated by various upstream kinases and proteins, such as DLK/JNK and AKT/GSK3β (Touma et al., 2007; Hammarlund et al., 2009; Miller et al., 2009; Gilley and Coleman, 2010; Wakatsuki et al., 2011; Fang et al., 2012; Shin et al., 2012b; Xiong et al., 2012; Watkins et al., 2013; Gobrecht et al., 2014; Liz et al., 2014; Shin et al., 2014; Chen et al., 2016;

Zhang et al., 2016b; Leibinger et al., 2017). These could potentially also regulate CRMP4 in response to PNS injury. CRMP4 was already found to be phosphorylated by GSK3β in a myelin-dependent manner (Alabed et al., 2010). Such a regulatory mechanism could potentially be conserved between the PNS and CNS. Interestingly, the roles of GSK3β in the neuronal response to injury remain controversial. As such, in regenerating PNS axons, the inactivation of GSK3β observed following peripheral nerve injury allows CRMP2 to retain its polymerizing action towards the microtubules, consequently promoting axon extension (Liz et al., 2014). Oppositely, activation of GSK3β also promotes neuronal regeneration, but this effect is likely mediated via MAP1B rather than CRMP2 (Gobrecht et al., 2014). Thus, it would be interesting to determine whether GSK3β or other kinases phosphorylate CRMP4 following sciatic nerve injury.

4.1.1.2 Functions of CRMP4 in the regeneration of sensory neurons

The PNS is composed of an amalgam of different neuronal subpopulations, each with its own development stages and properties (Lawson and Biscoe, 1979; Phillips and Armanini, 1996; Ma et al., 1999; Catala and Kubis, 2013). Sensory neurons are able to upregulate RAGs in response to injury, but despite this occurring in all subtypes, the regenerative response it still heterogeneous (Hu and McLachlan, 2003; Welin et al., 2008; Hu et al., 2016). This suggests that the different subtypes of sensory neurons do not regenerate at the same rate. In our experiments, we discounted the possibility that the different regenerative potential of the sensory neurons affect the extent of regeneration following sciatic nerve crush injury in the *Crmp4*$^{-/-}$ mice compared to the *Crmp4*$^{+/+}$ littermate controls. To achieve this, we counted the number of regenerating fibres at 500 μm before the injury site, which where positively stained for SCG10, a marker of the regenerating sensory neurons (Shin et al., 2012b; Shin et al., 2014). This number was not statistically different in the *Crmp4*$^{-/-}$ and *Crmp4*$^{+/+}$ mice (Fig. 2.1 B), which suggests that the same number of sensory neurons is regenerating at 3 DPI. However, this does not exclude the possibility of variations occurring at later time points.

The loss of CRMP4 did not lead to gross morphological defects in the nervous system, but previous studies have revealed alterations in the finer architecture of neurons located in the brain (Niisato et al., 2012; Khazaei et al., 2014). The PNS could similarly be affected by the loss of CRMP4, but this remains to be evaluated. Thus, we are currently characterizing the peripheral

sensory system of the *Crmp4*$^{-/-}$ mouse model compared to *Crmp4*$^{+/+}$ littermate controls to determine if it possesses any defects that might affect neuronal regeneration in the adult animals. We first characterized the expression pattern of CRMP4 in the lumbar L4 DRG of adult wild-type mice. This revealed that, although the expression of CRMP4 is ubiquitous to all neuronal subtypes in the DRG, its expression levels are higher in the small-diameter neurons, some of which are positive for IB4 and CGRP markers (Fig. A1). To follow-up on this experiment, we sought to investigate the effect of the deletion of *Crmp4* on the different neuronal subpopulations. Preliminary results revealed a reduction in the total number of Tuj1+ neurons in the L4 lumbar DRG of adult *Crmp4*$^{-/-}$ mice compared to the *Crmp4*$^{+/+}$ littermate controls (Fig. A2). As the small-diameter neurons highly express CRMP4, this neuronal subpopulation might be more vulnerable to the loss of this protein. Furthermore, as these neurons are involved in the transmission of various stimuli such as thermoception, mechanical pressure and nociception (Krames, 2014; Le Pichon and Chesler, 2014; Usoskin et al., 2015), the sensory functions of adult *Crmp4*$^{-/-}$ mice might be impaired. We are currently conducting additional experiments to determine the full extent of the consequences of the loss of CRMP4 on the peripheral sensory system.

4.1.1.3 Functions of CRMP4 in the regeneration of motor neurons

The sciatic nerve contains both sensory and motor neurons, each possessing different regenerative abilities (Schmalbruch, 1986; Swett et al., 1986; Swett et al., 1991; Cheah et al., 2017). In chapter 2 of this thesis, we focused on the characterization of the roles of CRMP4 in the sensory neurons specifically. However, the relative contribution of CRMP4 to the neuronal response to injury might differ in motor neurons. As such, our laboratory and others found CRMP4 mRNAs and proteins to be upregulated in the DRGs and peripheral neurons after sciatic nerve injury (Fig. 2.2 C; (Jang et al., 2010)). However, its expression levels are only slightly increased in the hypoglossal nerve, which is composed exclusively of motor neurons, whereas that of CRMP1, CRMP2 and CRMP5 is significantly upregulated (O'Reilly and FitzGerald, 1990; Suzuki et al., 2003). Suzuki et al. further showed that the upregulation of CRMP2 improved regeneration in motor neurons, but they did not investigate the contribution of CRMP4 or other family members in this injury model (Suzuki et al., 2003). Based on these observations, we could hypothesize that the contribution of CRMP4 to PNS regeneration might vary in

different types of neurons. Thus, we should investigate the roles of CRMP4 in the regeneration of motor neurons. To achieve this, we could investigate the extent of regeneration in *Crmp4*$^{-/-}$ mice and *Crmp4*$^{+/+}$ littermate controls following hypoglossal nerve crush. Alternatively, we could immunostain the motor neurons in the crushed sciatic nerve with using ChAT and GAP-43 antibodies to respectively label motor neurons and regenerating neurons. Both approaches would reveal the contribution of CRMP4 to the regeneration of motor neurons specifically, and these functions could then be compared to those characterized in the sensory neurons (chapter 2) to determine whether the functions of CRMP4 are conserved across different types of regenerating PNS neurons.

4.1.2 Functions of CRMP4 following CNS injury

Unlike their PNS counterparts, CNS neurons fail to spontaneously regenerate following injury (Huebner and Strittmatter, 2009). This failure is partly explained by the presence of growth-inhibitory cues that are released in the environment, including myelin-associated inhibitors (MAIs) and chondroitin sulfate proteoglycans (CSPGs) (McKerracher et al., 1994; Chen et al., 2000; GrandPre et al., 2000; Kottis et al., 2002; Morgenstern et al., 2002), as well as the failure to initiate intrinsic growth-promoting signaling pathways (Fernandes et al., 1999; Neumann et al., 2002; Qiu et al., 2002; Marklund et al., 2006; He, 2010; Sun and He, 2010; He and Jin, 2016), or the timely Wallerian degeneration of the disconnected neuronal segments (Vargas and Barres, 2007). These mechanisms contribute to the assembly of retraction bulbs at the tip of the regenerating axons(Li and Raisman, 1995; Hill et al., 2001; Erturk et al., 2007).

Our findings in chapter 2 regarding the roles of CRMP4 in PNS regeneration differ from those previously reported in the CNS. As such, where CRMP4 promotes regeneration in the PNS, it impairs it in the CNS (Nagai et al., 2015; Nagai et al., 2016). In CNS neurons, CRMP4 mediates the growth-inhibitory response to various cues that are released following injury, including MAIs and CSPGs (Alabed et al., 2007; Alabed et al., 2010; Nagai et al., 2012; Nagai et al., 2016). Thus, the loss of CRMP4 in CNS neurons is conductive to neuronal regeneration and functional recovery following spinal cord injury (Nagai et al., 2015; Nagai et al., 2016). Intrigued by these diverging functions, we compared the expression pattern of the different CRMP4 isoforms following optic and sciatic nerve injury to determine if these were differentially regulated in

response to injury. Surprisingly, we found that the expression pattern of this protein differs in the CNS compared to the PNS. The truncated CRMP4 protein, which favors the progression of Wallerian degeneration in the PNS, is present in the axonal segments located proximal to the injury in the optic nerve. This truncated CRMP4 isoform possesses the same molecular weight as the one detected following spinal cord injury (Nagai et al., 2015) and excitotoxic stimuli (Kowara et al., 2005; Kowara et al., 2006; Jiang et al., 2007; Zhang et al., 2007; Liu et al., 2009). Our laboratory and others found that the truncation of CRMP4 occurs in a calpain-dependent manner, that likely results from a calcium influx in response to various insults (Kowara et al., 2005; Kowara et al., 2006; Jiang et al., 2007; Zhang et al., 2007; Liu et al., 2009; Zhang et al., 2016b). Thus, we hypothesize that the calcium influx occurring in response to the neuronal damage incurred following CNS injury promotes the cleavage of CRMP4, among that of other proteins. The truncated protein generated could potentially mediate acute axonal degeneration, neurite retraction and apoptosis. However, these functions as well as the effect of tCRMP4 on the cytoskeleton will need to be further validated.

Furthermore, following CNS injury, Nagai et al. observed an upregulation of CRMP4 in reactive astrocytes surrounding the injury site following spinal cord injury (Nagai et al., 2015). This suggests that, in addition to its inhibitory roles in the damaged CNS neurons, CRMP4 could contribute to the non-neuronal response to injury, which includes glial scar formation. To support this hypothesis, Nagai et al. reported that loss of CRMP4 limits glial scar formation following spinal cord injury (Nagai et al., 2015). This differs from the PNS, where the expression of CRMP4 is altered primarily in neurons.

4.1.3 CRMP4 in Wallerian degeneration and neurodegenerative diseases

In addition to its roles in PNS and CNS regeneration, CRMP4 is also involved in neurodegeneration. In chapter 2, we found that, in the PNS, calpain-mediated cleavage of CRMP4 facilitates Wallerian degeneration in the axon segments disconnected from their cell bodies. We hypothesize that this could occur via tCRMP4 promoting the disassembly of the cytoskeleton, thus leading to axonal fragmentation. This truncated protein was also detected in the regenerating, but not the degenerating, CNS neurons following optic nerve injury, as well as in the injured spinal cord (chapter 2; (Nagai et al., 2015)). The absence of tCRMP4 in the

degenerating neurons could potentially be attributed to the slow degeneration rate of CNS neurons following axotomy (Vargas and Barres, 2007). Interestingly, this proteolytic event could potentially occur in neurodegenerative diseases, as phosphorylation and cleavage of CRMPs have already been described in those pathologies (Taghian et al., 2012). Thus, it would be interesting to determine whether CRMP4 is also cleaved in a calpain-dependent manner in neurons from these pathologies. Calpain was previously shown to favor the progression of degeneration via the disassembly of the cytoskeleton in several neurodegenerative diseases including Alzheimer's disease (Nixon et al., 1994; Ferreira, 2012; Kurbatskaya et al., 2016; Mahaman et al., 2018), Huntington's disease (Gafni and Ellerby, 2002; Lee and Kim, 2006), multiple sclerosis (Shields et al., 1999; Das et al., 2008; Trager et al., 2014), and Parkinson's disease (Crocker et al., 2003; Samantaray et al., 2013; Diepenbroek et al., 2014). Part of the neurodegenerative events could be mediated via the cleavage and subsequent inactivation of different cytoskeletal regulators including CRMP4.

Additionally, CRMP4 was identified as a risk factor for amyotrophic lateral sclerosis (ALS), a neurodegenerative disease characterized by the progressive loss of motorneurons (Duplan et al., 2010; Blasco et al., 2013). As such, a rare missense mutation in the DPYSL3 gene occurs with a higher frequency among French ALS patients (Blasco et al., 2013). This mutation has a detrimental effect on axonal growth, consequently shortening motor neuron survival (Blasco et al., 2013). Furthermore, as ALS progress from pre-symptomatic to early-onset stages, an increased number of motorneurons display increasing levels of CRMP4S (Duplan et al., 2010). This upregulation of CRMP4S renders the motorneurons more vulnerable to axonal degeneration and cell death (Duplan et al., 2010). Whether CRMP4 is an effector of neurodegeneration in other neurodegenerative disease remains to be determined.

4.2 RhoA in the neuronal response to injury

As a regulator of cytoskeletal remodeling, the ubiquitously expressed small GTPase RhoA is involved in many cellular functions during development and in response to cellular insults (Jaffe and Hall, 2005; Heasman and Ridley, 2008; Schaefer et al., 2014; Narumiya and Thumkeo, 2018). Due to its broad involvement, the activity of RhoA is tightly regulated by several mechanisms (Lang et al., 1996; Zhang and Casey, 1996; Kranenburg et al., 1997; Allal et al.,

2000; Sauzeau et al., 2000; Michaelson et al., 2001; Sawada et al., 2001; Forget et al., 2002; Kulkarni et al., 2002; Ellerbroek et al., 2003; Wang et al., 2003; Bryan et al., 2005; Rolli-Derkinderen et al., 2005; Nusser et al., 2006; Roberts et al., 2008; Chen et al., 2009; Wei et al., 2013; Tong et al., 2016).

4.2.1 Mechanism and functions of RhoA proteolysis on the cytoskeleton

In chapter 3, we identified a novel regulatory mechanism that occurs via the proteolytic processing of RhoA. The characterization of the underlying signaling pathway revealed that this mechanism occurred preferentially when RhoA is in its prenylated active GTP-bound form and is promoted in response to oxidative stress. Although some work is still required to fully characterize the underlying signaling pathways, we found that RhoA proteolysis requires serine proteases to promote the cleavage of RhoA near its amino-terminal domain. The specific protease and its relation to RhoA remain to be identified, but potential candidates include kallikreins-related peptidase (KRK)-5 (Pampalakis et al., 2014), thrombin (Greenberg et al., 2003), and HtrA2 (Gray et al., 2000). Additionally, we found caspases and calpain to have regulatory functions, either by down-regulating RhoA proteolysis or preventing the accumulation of the RhoA cleavage fragments when they are activated. However, their relationship to the protease cleaving RhoA remains to be determined once the latter is identified.

RhoA regulates the assembly of acto-myosin fibres and the stabilization of microtubules, via its numerous downstream effectors (Ridley and Hall, 1992; Arimura et al., 2000; Palazzo et al., 2001; Riento et al., 2003; Palazzo et al., 2004; Arimura et al., 2005; Mimura et al., 2006; Narumiya et al., 2009; Spiering and Hodgson, 2011; Morgan-Fisher et al., 2013). We found that pathologic expression levels of both RhoA-NTF and -CTF promotes the assembly of disorganized actin stress fibres and interferes to a certain extent with the activity of the endogenous full-length RhoA. Additionally, RhoA-CTF promotes the assembly of phalloidin-positive actin rods near the nuclear region in some transfected cells. Thus, the accumulation of RhoA fragments within the cell disrupts the organization of the actin cytoskeleton. To further our understanding of the functions of RhoA proteolysis on the cell's cytoskeleton, we should next determine the effect of the RhoA fragments on microtubules, as the active full-length RhoA was found to stabilize these cytoskeletal filaments via mDia and CRMP2 (Arimura et al., 2000;

Palazzo et al., 2001; Palazzo et al., 2004; Arimura et al., 2005; Mimura et al., 2006; Morgan-Fisher et al., 2013). Next, we should investigate the signaling pathways underlying the functions of the RhoA fragments towards the cell's cytoskeleton. In chapter 3, we localized the cleavage site to an amino acid sequence located between the Switch I and Switch II domains, which contain the effector binding sites. As such, the fragments generated upon RhoA proteolysis could potentially interact with some downstream effectors, such as ROCK, mDia and CRMP2, but these interactions remains to be investigated. This would further our understanding of the effects of RhoA proteolysis on the cytoskeleton.

4.2.2 Functions of RhoA following PNS injury

Despite the growth-promoting potential of PNS neurons, regeneration is still a slow process that is impaired by various factors. These include the release of CSPGs and myelin-associated glycoprotein (MAG) in the extracellular milieu following injury, which leads to the activation of RhoA, a protein that inhibits neuronal regeneration (Hiraga et al., 2006; Tomita et al., 2007; Cheng et al., 2008; Huelsenbeck et al., 2012). Several studies have shown that the inhibition of RhoA or of its downstream effector ROCK partially improves regeneration and functional recovery following PNS injury (Hiraga et al., 2006; Cheng et al., 2008; Huelsenbeck et al., 2012). However, RhoA was also found to favorably contribute to PNS regeneration by limiting dendritic branching, which consequently reduces hyperinnervation, promotes target reinnervation and enhances the overall quality of regeneration (Tomita et al., 2007). Thus, locally regulating the activity of this GTPase might be a viable strategy to ameliorate the neuronal response to injury and promote PNS regeneration. In chapter 3, I describe a novel regulatory mechanism of RhoA that preferentially cleaves the active GTP-bound form. The cleavage fragments generated upon this proteolytic event then promote the assembly of disorganized actin stress fibres and partially interfere with the activity of the full-length RhoA when they accumulate within the fibroblast cells. As RhoA is activated upon PNS injury (Hiraga et al., 2006; Cheng et al., 2008; Huelsenbeck et al., 2012), this regulatory mechanism could occur as part of the neuronal response to injury and potentially be harnessed to alter PNS regeneration. Thus, we should determine whether RhoA proteolysis is promoted in damaged PNS neurons and whether the RhoA fragments accumulate to a level sufficient to regulate the cytoskeleton. Next, we should investigate whether this regulatory mechanism impact neurite outgrowth, dendritic branching,

growth cone dynamics, or neuronal degeneration. These experiments will provide a better understanding of whether RhoA proteolysis might be involved in the signaling mechanisms underlying PNS regeneration in response to injury and whether this regulatory mechanism could be harnessed to promote functional recovery.

4.2.3 Functions of RhoA following CNS injury

In the CNS, the signaling pathways limiting regeneration are slightly different than those characterized in the PNS. Various growth-inhibitory cues, including CSPGs and MAIs, are released in the environment upon neuronal damage and these promote the formation of a retraction bulb that fails to drive axon extension (Filbin, 2003; Yiu and He, 2006; Erturk et al., 2007; He, 2010; Kaplan et al., 2015; He and Jin, 2016). The signaling pathways initiated by the growth-inhibitory proteins released upon CNS injury converge onto RhoA, which promote neurite retraction and growth cone collapse via its numerous downstream effectors (Filbin, 2003; Yiu and He, 2006). As the proteolytic processing of RhoA that we characterized in chapter 3 occurs preferentially with the GTP-bound active RhoA, we should determine whether this mechanism is promoted in damaged CNS neurons and whether the RhoA fragments accumulate in the cells. Furthermore, we should investigate the functions of the RhoA fragments on the cytoskeletal architecture of the neurons, including in neurite outgrowth and growth cone collapse. This would provide insights into whether RhoA proteolysis could be used to promote regeneration in response to CNS injury.

4.2.4 RhoA in neurodegenerative diseases

Intriguingly, RhoA proteolysis might also be promoted in neurodegenerative diseases, as several of them have an oxidative stress component that could promote this regulatory mechanism (Liu et al., 2017). In chapter 3, we found that the accumulation of RhoA fragments generated upon this cleavage promotes the formation of disorganized stress fibres, but also of nuclear actin rods in the nuclear area. A similar assembly of actin rods has been characterized in several pathological, including Alzheimer's disease, Huntington's disease and spinal muscular atrophy (Bamburg et al., 2010; Rademacher et al., 2017). In these diseases, actin rods are assembled as a neuroprotective mechanism to prevent the actin-mediated ATP decline (Bernstein et al., 2006). However, under continuous stress, the persistent presence of these rods often correlates with

neurodegeneration (Minamide et al., 2000; Bernstein et al., 2006; Cichon et al., 2012; Bamburg and Bernstein, 2016). Furthermore, in SMA specifically, Rademacher et al. found that truncated PlexinD1 associated with the cofilin-positive actin rods, and their presence combined with a switch in the response to Sema3E disrupts circuit formation (Rademacher et al., 2017). This signaling mechanism is very reminiscent of the proteolytic event that we characterized in chapter 3 for RhoA and as such, we hypothesize that RhoA proteolysis could potentially underlie the formation of actin rods observed in various neurodegenerative diseases. Thus, we should determine whether RhoA proteolysis is promoted in degenerating neurons isolated from tissues of rodent models or human patients with Alzheimer's disease, Huntington's disease or SMA. If this regulatory pathway is promoted, we should then determine whether altering this mechanism affects the formation of actin rods, and consequently alter the progression of neuronal degeneration. These findings will lead to the characterization of additional signaling pathways that underlie pathological neurodegeneration and might reveal interesting targets to either delay the progression of degeneration or promote recovery.

4.3 Development of future therapeutic strategies for neuronal damage

Neuronal injuries often induce damages to the cytoskeletal architecture and, to recover, the neurons need to reorganize their cytoskeleton in order to promote regeneration. The importance of cytoskeletal rearrangements in response to injury is well illustrated in a series of experiments conducted by Erturk et al. This research group found that treatment of injured CNS neurons with taxol, a microtubule stabilizer, limits the formation of retraction bulb, while treatment of PNS growth cones with nocodazole, an inducer of microtubule destabilization, is sufficient to generate retraction bulb-like structures and prevent axon outgrowth (Erturk et al., 2007). Additional studies revealed that, in damaged neurons, multiple signaling pathways regulate cytoskeletal dynamics and thus, contribute to the regenerative potential (Filbin, 2003; Yiu and He, 2006; Erturk et al., 2007; He, 2010; Kaplan et al., 2015; He and Jin, 2016). Unfortunately, despite our growing knowledge of these underlying signaling pathways, treatment options to promote recovery remain limited (Kaplan et al., 2015). Thus, the identification of additional targets remains critical to the development of therapeutic strategies to promote neuronal regeneration.

4.3.1 Therapeutic therapies for PNS injury

In the PNS, several strategies have been developed to promote regeneration, including the use of electrical stimulation and/or physical exercise (Gordon et al., 2009; English et al., 2014; Gordon and English, 2016). Additionally, some therapies target components of the underlying intracellular signaling pathways. For example, the upregulation of CRMP2 in the hypoglossal nerve improves the extent of regeneration following injury (Suzuki et al., 2003). The upregulation of CRMP4 in the sensory neurons of the sciatic nerve could have a similar effect and improve recovery (chapter 2; (Alabed et al., 2007)). Thus, this could potentially become a therapeutic strategy to promote a favorable outcome for the PNS sensory neurons in response to injury that could be extrapolated to motor neurons. Maintaining the expression of CRMP4 could promote regeneration of the damaged axons. Alternatively, downregulating the activity of RhoA by promoting its proteolysis limits its growth-inhibitory effects in the regeneration of PNS neurons. Thus, these studies revealed novel targets that could be used for the development of therapies to promote functional recovery following PNS injury.

4.3.2 Therapeutic therapies for CNS injury

In the CNS, therapeutic strategies seek to improve the intrinsic growth potential of neurons or limit the effect of inhibitory cues present in the extracellular environment. For example, the use of MAIs antagonists and/or chondroitinase ABC, an enzyme degrading CSPGs, promotes neuronal regeneration and improves functional recovery after CNS injury (Bradbury et al., 2002; GrandPre et al., 2002; Li and Strittmatter, 2003; Wiessner et al., 2003; Li et al., 2004; Li et al., 2005; Seymour et al., 2005; Barritt et al., 2006; Massey et al., 2006; Cafferty et al., 2007; Shields et al., 2008). Furthermore, a function-blocking Nogo-A antibody as well as an inhibitor of RhoA known as Cethrin are currently in the earlier stages of clinical trials as treatment for neuronal injuries (Zorner and Schwab, 2010; Fehlings et al., 2011; McKerracher and Anderson, 2013; McKerracher and Guertin, 2013). CSPGs and MAIs initiate signaling pathways that lead to cytoskeletal remodeling that inhibit regeneration (Filbin, 2003; Yiu and He, 2006). Thus, targeting specific components downstream of these proteins could promote recovery. Interestingly, these signaling pathways possess convergence points between RhoA and CRMPs. As such, in response to inhibitory cues, RhoA becomes activated and regulates cytoskeletal

remodeling, thus promoting neurite retraction and growth cone collapse (Filbin, 2003; Yiu and He, 2006). These effects could partly be mediated via the interaction of RhoA with CRMP4L (Alabed et al., 2007). Thus, our laboratory developed a TAT-C4RIP peptide that prevents this interaction, and consequently attenuates myelin inhibition *in vitro* (Khazaei et al., 2015). Interestingly, a previous study found that CRMP4L is upregulated following spinal cord injury (Nagai et al., 2015), and, in chapter 2, we observed a similar trend following optic nerve injury. As such, the growth-inhibitory effect of the RhoA-CRMP4L interaction might be favored following CNS injury. It remains to be determined whether the application of TAT-C4RIP would be sufficient promote neuronal regeneration. This strategy could be combined with the promotion of RhoA proteolysis, which could potentially reduce the abundance and functions of active RhoA within the damaged neurons, although the functions of this signaling pathway in neurons remain to be elucidated.

Alternatively, calpain has predominant roles in the neuronal response to CNS injury (Ray and Banik, 2003; Ma, 2013). Previous studies discovered that inhibiting calpain using either the endogenous inhibitor calpastatin or a drug such as leupeptin is neuroprotective, as it limits degeneration and the progression of secondary injury following spinal cord injury and traumatic brain injury, and improves regeneration (Ray and Banik, 2003; Wingrave et al., 2004; Schoch et al., 2012; Ribas et al., 2017). These effects could partly be mediated by the inhibition of the calpain-mediated cleavage of CRMP4 but also that of CRMP2, whose cleavage drives acute axonal degeneration (Zhang et al., 2016b). Other signaling pathways could mediate the neuroprotection observed upon calpain inhibition, including the limitation of the activation of c-Jun (Ribas et al., 2017) and the maintenance of axonal transport (Ma et al., 2012). Additionally, as calpain also promotes the degradation of RhoA-NTF (chapter 3), its inhibition could potentially lead to the accumulation of this RhoA fragments within the damaged neurons where RhoA proteolysis is promoted in response to injury. This would disrupt the organization of the actin cytoskeleton and the activity of the full-length RhoA.

4.3.3 Therapeutic therapies for neurodegenerative diseases

In addition to providing novel targets to promote neuronal regeneration in the PNS and CNS, the findings of this thesis could potentially be utilized in the development of therapeutic strategies

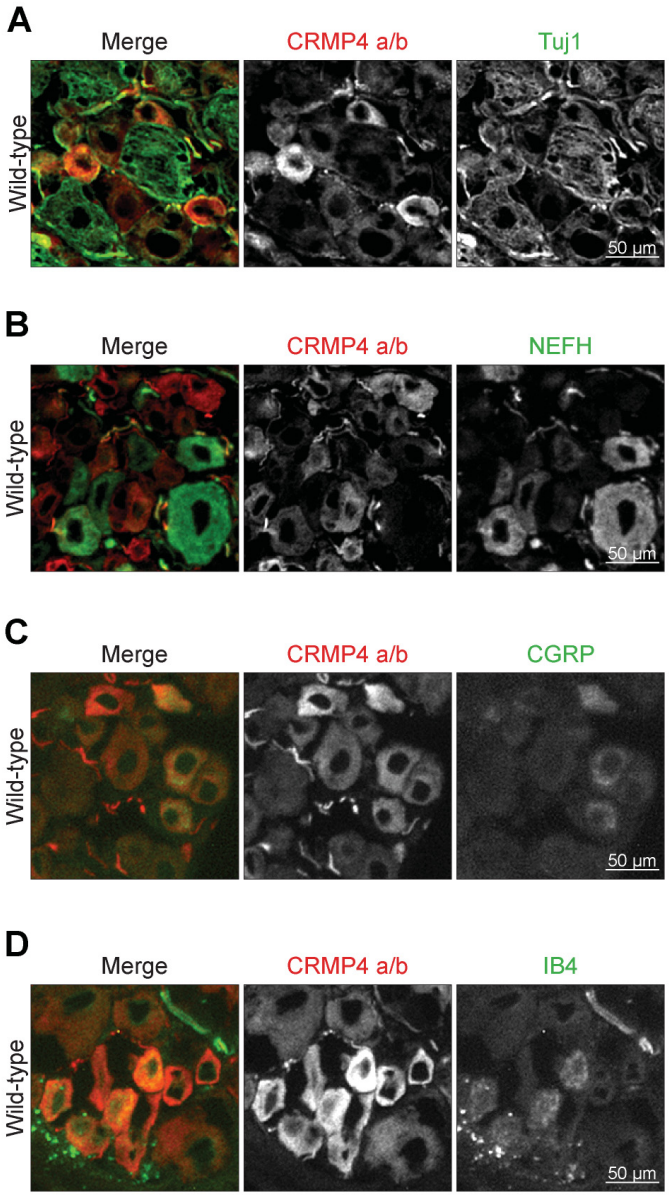
for neurodegenerative diseases. For example, in ALS, the expression of CRMP4 is upregulated in motorneurons, and this correlated with axonal degeneration and cell death (Duplan et al., 2010; Blasco et al., 2013). Additionally, in various neurodegenerative diseases, calpain and the cleavage of CRMP4 were found to favor the progression of axonal degeneration (Nixon et al., 1994; Shields et al., 1999; Gafni and Ellerby, 2002; Crocker et al., 2003; Lee and Kim, 2006; Das et al., 2008; Ferreira, 2012; Taghian et al., 2012; Samantaray et al., 2013; Diepenbroek et al., 2014; Trager et al., 2014; Kurbatskaya et al., 2016; Mahaman et al., 2018). Thus, the downregulation of the expression of CRMP4 and inhibition of its calpain-mediated cleavage could potentially delay the rate of axonal degeneration observed in various neurodegenerative diseases. Similarly, the formation of actin rods is observed in many neurodegenerative diseases such as Alzheimer's disease and Huntington's disease (Bernstein et al., 2006; Bamburg et al., 2010; Rademacher et al., 2017). As the cleavage of RhoA correlates with the appearance of these rods and axonal fragmentation, preventing this processing mechanism could potentially delay the progression of neurodegenerative diseases.

4.4 Conclusion

The intricate cytoskeletal network is a key component of neurons, as it provides structural and mechanical support in addition to the plasticity required to respond to various stimuli. Physical and chemical damage often disrupts its integrity and in order to achieve functional recovery, it is critical that the cytoskeleton reorganizes itself appropriately. In this thesis, I furthered our understanding of the involvement of two cytoskeletal regulators, CRMP4 and RhoA, in the cellular response to two different insults, PNS injury and oxidative stress. This led to a deeper understanding of the regulation of RhoA and the functions of CRMP4. Continuing our investigation of the signaling pathways mediated by RhoA and CRMP4 might reveal additional insights about their involvement in the neuronal response to injury and neurodegenerative diseases, and consequently lead to the identification of potential targets that could be used to promote neuronal recovery following various insults.

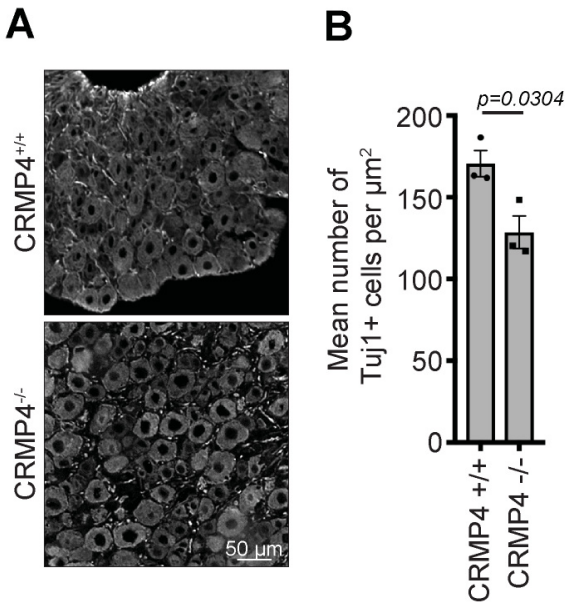
APPENDICES

Figure A1: Expression of CRMP4 in different subpopulations of DRG sensory neurons.



Representative images of lumbar (L4) DRG cross-sections from wild-type mice stained with CRMP4 a/b antibody (red) and Tuj1 (green; **A**), NEFH (green; **B**), CGRP (green; **C**) or IB4 (green; **D**). Scale bar, 50 µm.

Figure A2: *Crmp4* deletion reduces the total number of neurons in the L4 lumbar DRG of adult mice.



Representative pictures **(A)** and quantification **(B)** of the number of Tuj1+ neurons in L4 DRG cross-sections from *Crmp4*$^{+/+}$ and *Crmp4*$^{-/-}$ mice stained with Tuj1 (green). The data, which is representative of 3 mice, is graphed as the mean number of Tuj1+ cells per μm^2 +/- S.E.M. Statistical significance was determined with an unpaired *t*-test. Scale bar, 50 μm.

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