The role of RhoA interacting proteins in the Nogo signalling pathway of axon outgrowth inhibition

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ABSTRACT

Regrowth in the lesioned central nervous system is impeded by inhibitory molecules including myelin-associated inhibitors (MAIs) and chondroitin sulfate proteoglycans (CSPGs). Inhibitory molecules engage neuronal cell surface receptors and activate the small GTPase RhoA in injured neurons to mediate neurite outgrowth inhibition through targeted modifications to the cytoskeleton. Inhibition of RhoA with the ribosyltransferase C3 attenuates neurite outgrowth inhibition *in vitro* and *in vivo* but the ubiquitous expression and multifunctionality of RhoA may limit the specificity of therapeutic RhoA antagonists. **The hypothesis of the thesis is that molecules that functionally interact with RhoA to mediate myelin-dependent inhibition may represent more specific targets for therapeutic intervention.** We have explored the contribution of two RhoA interacting proteins to the neurite outgrowth inhibitory effects of MAIs. In Chapter 2 we describe the contribution of the rho effector, Rho kinase (ROCK) to MAI responses in neurons. In Chapter 3 we identify the cytosolic phosphoprotein CRMP4b (Collapsin Response Mediator Protein 4b) as a novel RhoA binding partner that mediates neuronal responses to CNS inhibitors. By structure function analysis we have developed a molecular antagonist of CRMP4b-RhoA binding that promotes neurite outgrowth on inhibitory substrates *in vitro* and has the potential to be a potent and specific molecular therapeutic for spinal cord injury. In Chapter 4 we identify glycogen sythase kinase 3b (GSK3b) as an important kinase in the MAI pathway that regulates protein interactions with RhoA. This thesis provides insights into the signal transduction machinery that is engaged in response to CNS inhibitors and suggests several novel therapeutic targets to promote axon regeneration following CNS injury.

RESUME

La croissance dans le système nerveux central (SNC) lesé est bloquée par la presence de molécules inhibitrices telles que les inhibiteurs associés à la myeline (IAM) et les proteoglycanes chondroitine sulfate (PGCS). Ce milieu inhibiteur stimule des recepteurs cellulaires des neurones et active la GTPase RhoA pour promouvoir l'inhibition de la croissance des neurites à travers des modifications spécifiques du cytoskelette.

L'inhibition de RhoA avec la ribosyltransferase C3 atténue l'inhibition de la croissance des neurites *in vitro* et *in vivo*, mais l'espression ubiquitaire de RhoA ainsi que sa multifonctionalité pourraient limiter la spécificité des antagonistes therapeutiques de RhoA. **L'hypothèse principale de la thèse est que l'identification de molécules nouvelles qui interagissent spécifiquement avec RhoA pour l'inhibition induite par la myeline, constituerait des cibles plus spécifiques pour l'intervention therapeutique.** Nous avons investigué la contribution de deux proteines qui interagissent avec RhoA dans le contexte de l'inhibition de la croissance de neurites induite par les IAM. Dans le deuxième chapitre, nous avons decrit la contribution de Rho kinase (ROCK), une proteine partenaire de RhoA, à la reponse des neurones aux IAM. Dans le troisième chapitre, nous avons identifié la phosphoproteine cytosolique CRMP4b (Collapsin Response Mediator Protein 4b) en tant qu'une nouvelle proteine partenaire de RhoA qui promouvoie la reponse des neurones aux inhibiteurs du SNC. Par une étude de structure et de fonction, nous avons développé un antagoniste moléculaire de l'interaction entre CRMP4b et RhoA qui a pour fonction de stimuler la croissance des neurites sur des substrats inhibiteurs *in vitro* et qui a aussi le potentiel d'être un agent therapeutique puissant et spécifique contre les lesions de la moelle épiniere. Dans le quatrième chapitre,

nous avons identifié dans la voie des IAM, l'enzyme glycogene synthase kinase 3β (GSK3β) en tant que kinase importante qui régule les interactions des proteines avec RhoA. Cette thèse fournit un apercu de la machinerie des voie de transduction qui est activée en réponse aux inhibiteurs du SNC et suggère plusieurs nouvelles cibles therapeutique pour promouvoir la régéneration axonale après une lesion du système nerveux central.

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LIST OF ABBREVIATIONS

ADF	Actin Depolymerization Factor
BBB	Basso, Beattie, and Bresnahan
BDA	Biotin Dextran Amine
BDNF	Brain Derived Neurotrophic Factor
BSA	Bovine Serum Albumin
C4RIP	CRMP4b RhoA Inhibitory Peptide
Ca^{2+}	Calcium
cAMP	cyclic Adenosine Monophosphate
CGN	Cerebellar granule neurons
ChABC	Chondroitinase ABC
CNS	Central Nervous System
CRD	Cysteine Rich Domain
CRMP	Collapsin Response Mediator protein
CSPG	Chondroitin Sulfate Proteoglycan
CST	Corticospinal Tract
DHPase	Dihydropyrimidinase
DMEM	Dulbecco's Modified Eagle Medium
DN	Dominant Negative
DRG	Dorsal Root Ganglion
DRP	Dihydropyrimidinase-Related Protein
DYRK	Dual tyrosine Regulated Kinase
E15	Embryonic day 15
ECM	Extracellular Matrix
ER	Endoplasmic Reticulum
EGFR	Epidermal Growth Factor Receptor
FBS	Fetal Bovine Serum
FGF	Fibroblast Growth Factor
GAG	Glycoaminoglycan
GAP	GTPase Activating Protein
GC	Growth Cone

GDNF	Glial cell line Derived Neurotrophic Factor
GDP	Guanosine Diphosphate
GEF	Guanine Exchange Factor
GFP	Green Fluroescent Protein
GPI	Glycosyl Phosphatidyl Inositol
GSK	Glycogen Synthase Kinase
GST	Glutathione S transferase
GTP	Guanosine Triphosphate
GTPase	Guanosine Triphosphatase
HBS	Hepes buffered Saline
HSV	Herpes Simplex Virus
IP	Immunoprecipitation
kDa	KiloDalton
LIMK	Lin-11, Isl-1, Mec-3 Kinase
LPA	Lysophosphatidic Acid
LRR	Leucine Rich Repeat
LTP	Long Term Potentiation
MAG	Myelin Associated Glycoprotein
MAI	Myelin Associated Inhibitor
MAP	Microtubule Associated Protein
MLCII	Myosin Light Chain II
NEP1 40	Nogo-66 (1 40) antagonist peptide
NgCAM	Neuron glia-Cell Adhesion Molecule
NGF	Nerve Growth Factor
NgR	Nogo-66 Receptor
NT	Neurotrophin
OMgp	Oligodendrocyte Myelin glycoprotein
$p75^{NTR}$	p75 neurotrophin receptor
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PH	Pleckstrin-Homology Domain

PKA	Protein Kinase A
PKC	Protein Kinase C
PLL	Poly L Lysine
PMSF	Phenylmethylsulphonyl fluoride
PNS	Peripheral Nervous System
PP	Protein Phosphatase
PRK2	Protein kinase C related Kinase
RIPA	Radioimmunoprecipitation assay
RBD	Rho Binding Domain
RGC	Retinal Ganglion Cell
RGN	Retinal Ganglion Neuron
RhoGDI	Rho Guanine Dissociation Inhibitor
RNA	Ribosylnucleic Acid
ROCK	Rho-associated Kinase
RPMI	Roswell Park Memorial Institute
RST	Rubrospinal tract
RTN	Reticulon
SAP	Shrimp Alkaline Phosphatase
SCI	Spinal Cord Injury
SDS	Sodium Dodecyl Sulfate
SEM	Standard Error of Mean
Sema3A	Semaphorin 3A
Ser	Serine
siRNA	Short interfering Ribonucleic Acid
SSH	Slingshot
TBS	Tris Buffered Saline
TGF	Transformation Growth Factor
Thr	Threonine
Tyr	Tyrosine
ULIP	Unc-33 Like protein

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CONTRIBUTIONS OF AUTHORS

First Article "**Neuronal responses to myelin are mediated by ROCK**"

- **Yazan Z. Alabed:** Performed experiments, analysis and revisions pertaining to Figures 3, 4, 5. Writing and editing of manuscript in collaboration with principal investigator **Alyson E. Fournier**.
- **Edith-Grados Munro:** Performed experiments and analysis pertaining to Figure 2 and Figure 4.

Second Article **"Identification of CRMP4 as a convergent regulator of axon outgrowth inhibition"**

- **Yazan Z. Alabed:** Performed experiments, analysis and revisions pertaining to all figures except Figure 3a. Writing and editing of manuscript in collaboration with principal investigator **Alyson E. Fournier**.

Third Article **"GSK3β regulates myelin-dependent axon outgrowth inhibition through CRMP4"**

- **Yazan Z. Alabed:** Perfomed experiments and analysis pertaining to all figures except Figure 3b and 5d. Contributed to text editing and comments.
- **Madeline Pool:** Performed experiments and analysis pertaining to Figures 3b and 5d. Revised the manuscript.
- **Alyson E. Fournier:** Managed hypothesis development, manuscript preparation and editing.

CHAPTER 1

I. GENERAL INTRODUCTION

There are approximately two million people worldwide living with spinal cord injury (SCI). Approximately 250,000 individuals in the United States have spinal cord injuries. Every year, approximately 12,000 new injuries occur. Most of these people are injured in automobile and sports accidents. Furthermore, an estimated 60% of these individuals are 30 years old or younger, and the majority of them are men. The location of the injury determines the degree of neurological deficit. Cervical injuries result in full or partial quadriplegia (52% of spinal cord injured individuals) whereas thoracic injuries or below result in full or partial paraplegia (47%, National Spinal Cord Injury Statistical Center).

Trauma in the adult mammalian central nervous system (CNS) results in devastating clinical consequences due to the failure of injured axons to spontaneously regenerate.

I. 1. The CNS Environment

Classic experiments performed over 20 years ago demonstrated that CNS neurons are capable of long distance regeneration when provided with a growth permissive environment (David and Aguayo, 1981). The failure of severed nerve fibers to spontaneously re-extend to their targets distinguishes the CNS from the peripheral nervous system (PNS) where individual fibers do regenerate to restore function. Since the historical finding that CNS neurons are regeneration competent, the CNS environment has been extensively studied and it is clear that multiple influences at the CNS lesion site

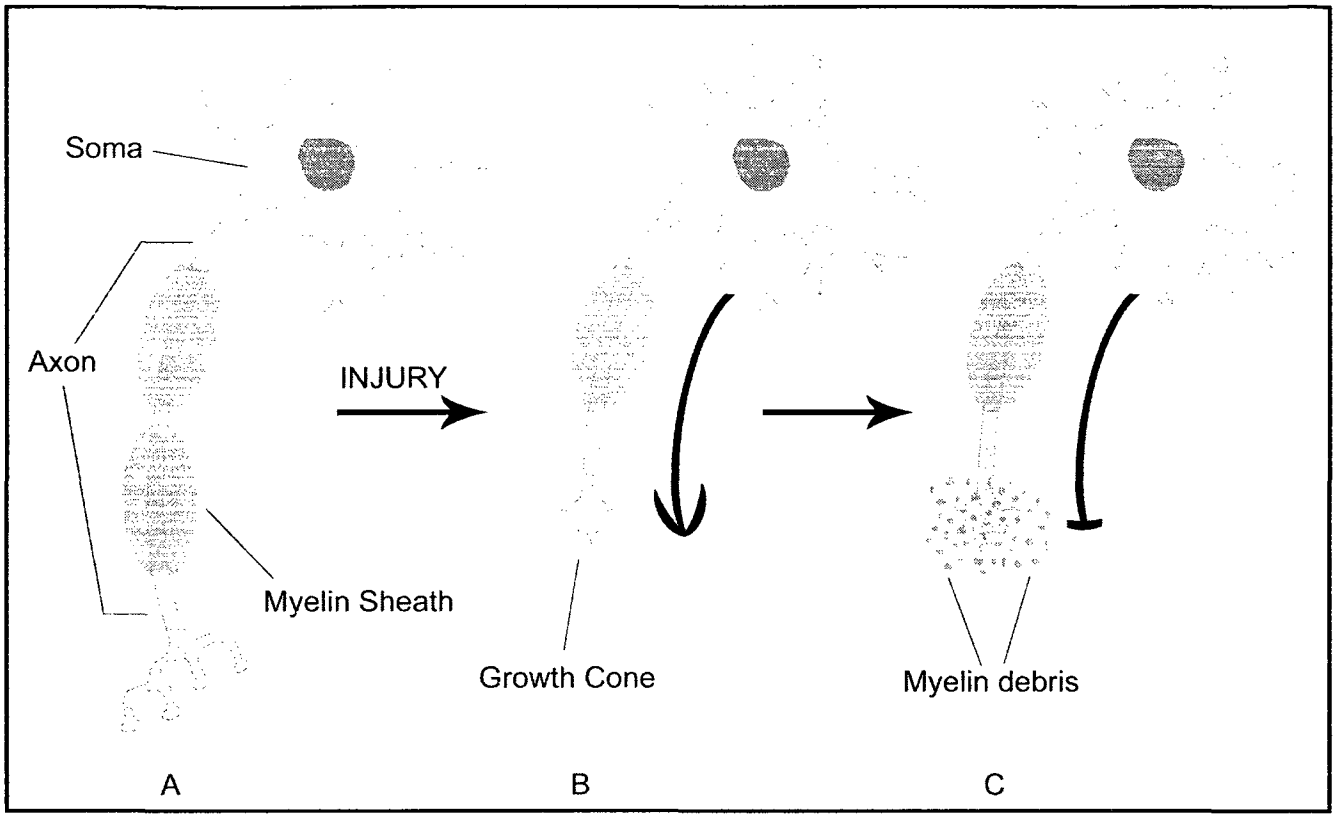

Figure 1. CNS neurons are inhibited by a negative extracellular milieu. In the CNS, oligodendrocytes form the myelin sheath surrounding the axon (A). Following injury, neurons in the CNS reextend neurites (B). The leading edge of the neurite is guided by the growth cone. The myelin debris along with a glial scar that results from the injury creates an impermissive environment for the growing axon. The CNS neurons are therefore unable to reestablish their target connections (C).

inhibit regenerative growth. Both myelin debris (Caroni and Schwab, 1988a, b) and an astroglial scar (McKeon et al., 1991; Davies et al., 1997; Davies et al., 1999) actively inhibit regeneration by challenging the severed axon with inhibitory proteins that signal to the axon and prevent growth (Figure 1). Further there is also evidence of the reexpression of a subset of repulsive axon guidance molecules in the intact and the injured CNS (De Winter et al., 2002; Yaron and Zheng, 2007). Extensive research is therefore being done to identify inhibitory components at the CNS lesion site and to define their mechanism of action.

I. 1. 1. The glial scar

The absence of regeneration in the injured CNS has been attributed to a non permissive environment that does not support extensive axonal extension (Aguayo et al., 1981; Reier et al., 1983). A glial scar forms which is characterized by the proliferation and migration of astrocytes into the lesion site where they deposit extracellular matrix creating a mechanical barrier for regeneration. In addition to this physical barrier, there is a strong inhibitory effect mediated by two types of extracellular matrix (ECM) molecules, tenascins and chondroitin sulphate proteoglycans (CSPG,(Asher et al., 2001)). CSPGs consist of a protein core and long, unbranched polysaccharides (glycosaminoglycans, GAG) including chondroitin sulphate disaccharide unit repeats. In the nervous system, proteoglycans regulate the structural organisation of the extracellular matrix and modulate growth factor activities and cellular adhesive and motility events such as cell migration and axon outgrowth. A number of CSPGs are upregulated following CNS injury including neurocan, versican, brevican, decorin and biglycan (McKeon et al., 1991; Asher et al., 2000; Asher et al., 2001).

The inhibitory activity of CSPGs is mediated mainly by the GAGs. Treatment with chondroitinase ABC (ChABC), an enzyme that cleaves the sugar chains, promotes axon regrowth both *in vitro* and *in vivo* (Moon et al., 2001; Bradbury et al., 2002; Morgenstern et al., 2002). Modest effects on the growth of severed nigrostriatal axons have been observed with hyaluronidase treatment to cleave neurocan, versican and brevican (Moon et al., 2003). Chondroitinase treatment of reactive astrocytes harvested from animals following various injury paradigms (McKeon et al., 1991) and of frozen spinal cord sections (Zuo et al., 1998) improve the capacity of these substrates to support neurite outgrowth in culture. Recent evidence has implicated the Rho pathway in mediating the inhibitory activity of the CSPGs (Dergham et al., 2002). Mueller and colleagues recently showed that CSPG-associated inhibition of RGC axons outgrowth was blocked using C3 transferase or Y27632 suggesting that it is mediated by the Rho/ROCK signaling pathway (Monnier et al., 2003).

Despite all these findings, the role of CSPGs *in vivo* in blocking axon regeneration and the precise molecular machinery by which inhibition is mediated by the CSPGs is still not clear. More work is required before any therapies are designed in targeting these molecules.

I. 1. 2. Myelin-Associated Axon Growth Inhibitors

The inability of CNS neurons to regenerate following injury is also attributed to the presence of growth inhibitors in the CNS myelin sheath. This was originally demonstrated by the fact that CNS neurons fail to grow on white matter (Savio and Schwab, 1989). Biochemical fractionation of CNS myelin led to the identification of an

axon outgrowth inhibitory activity termed NI35/250 (Caroni and Schwab, 1988a, b). A function-blocking monoclonal antibody named IN-1, which recognizes NI35/250, generated great excitement in the regeneration field because it promoted regrowth of injured corticospinal tract (CST) fibers and behavioral recovery when injected or infused into the site of a spinal cord lesion (Caroni and Schwab, 1988b; Schnell and Schwab, 1990). The protein responsible for the inhibitory activity in this myelin fraction was subsequently identified as Nogo (Chen et al., 2000; GrandPre et al., 2000; Prinjha et al., 2000). Further analysis of myelin has identified other inhibitors in the last several years, including Myelin-Associated Glycoprotein (MAG) (McKerracher et al., 1994; Mukhopadhyay et al., 1994) and Oligodendrocyte Myelin Glycoprotein (OMgp) (Kottis et al., 2002; Wang et al., 2002b).

I. 1. 2. 1. Reexpression of repulsive guidance molecules

In addition to the aforementioned MAIs, there is a potential new role for axon guidance molecules as growth inhibitory factors in the injured CNS. Ephrin-B3, a repulsive guidance molecule expressed during CST development, was shown to be expressed by postnatal myelinating oligodendrocytes and mediates neurite outgrowth inhibition in postnatal cortical neurons (Benson et al., 2005). Genetic ablation of EphA4 in mice, the receptor for Ephrin-B3, resulted in significant axonal regeneration and improved functional recovery (Goldshmit et al., 2004). In addition to Ephrin-B3, Sema4D is similarly expressed by postnatal oligodendrocyte with a transient upregulation around the lesion site after CNS injury. Further, Sema4D inhibits postnatal DRG and granule cell axons (Moreau-Fauvarque et al., 2003). Recently, Netrin-1, a well known

axon guidance molecule required for commissural neurons axon guidance at the embryonic spinal cord ventral midline, has been suggested as a potential oligodendrocyte-associated inhibitor of axonal regeneration in the adult spinal cord. Netrin-1 is constitutively expressed by neurons and myelinating oligodendrocytes in the adult rat spinal cord (Manitt et al., 2001). Its repulsion-mediated receptor UNC5H is expressed both before and after spinal cord injury by corticospinal and rubrospinal projections. Disruption of Netrin-1 promotes neurite outgrowth *in vitro* (Manitt et al., 2006; Low et al., 2008). Taken together, this suggests that axon guidance molecules expressed by oligodendrocytes and CNS myelin may contribute to the failure of axon regeneration.

I. 1. 2. 2. Nogo: Inhibitory Molecule for CNS Regeneration

Nogo-A, a member of the Reticulon (RTN) family of proteins, is highly expressed in the ER and Golgi and on the surface of oligodendrocytes in the CNS myelin. Nogo-A induces growth cone collapse and neurite retraction (Chen et al., 2000; GrandPre et al., 2000). There are three major isoforms of Nogo generated by alternative RNA splicing (Nogo-A/B) and promoter usage (Nogo-C). All of these three Nogo isoforms share a common C-terminal domain of 188 amino acids, which includes a 66 amino acid inhibitory domain called Nogo-66. Nogo-66, a potent inhibitory fragment of Nogo, is an extracellular loop region of Nogo separated by two transmembrane domains. Nogo-B and Nogo-C are identified in many tissues and cell types but their functions are still not yet understood. Recent studies on Nogo-B suggest a role in tumor suppression (Qi et al., 2003) and in vascular remodeling (Acevedo et al., 2004; Paszkowiak et al., 2007).

The N-terminal domain of Nogo-A (amino-Nogo) and Nogo-66 are two distinct inhibitory domains that affect neurite outgrowth through discrete mechanisms and through different receptors (GrandPre et al., 2000; Prinjha et al., 2000; Oertle et al., 2003). According to the current topological model, Nogo-66 is presented extracellularly on oligodendrocytes. More than one topology of Nogo-A has been described, as it can be present both intracellularly and extracellularly (Oertle et al., 2003). Following SCI, damaged oligodendrocytes release their contents, thus injured neurons may be exposed to domains of Nogo-A not normally expressed at the cell surface.

The function of Nogo *in vivo* has been studied by examining the phenotype and injury response of Nogo knockout mice however the results of these studies are controversial. Three independent groups have generated Nogo knockout mice. A Nogo-A/B mutant mouse line was studied by Strittmatter and colleagues (Kim et al., 2003). In this line a significant increase in corticospinal sprouting above and below spinal cord dorsal hemilesions was observed. This anatomical recovery was accompanied by enhanced functional recovery as assessed by the Basso, Beattie, and Bresnahan (BBB) scale. This study suggests that Nogo-A plays an important role in the failure of regeneration of adult CNS neurons. Intriguingly this regeneration was restricted to young mice suggesting that other plasticity mechanisms may facilitate regeneration. In a second mouse line generated by Schwab and colleagues (Simonen et al., 2003), Nogo-A expression was disrupted by conventional gene targeting. As compared with the Strittmatter mice, these mice exhibit a compensatory upregulation of Nogo-B expression by oligodendrocytes, and a significant, but less dramatic regenerative response of CST fibers following dorsal hemisections. The more modest phenotype could be attributed to

the compensatory increase in Nogo-B, which contains the Nogo-66 inhibitory domain. Two other Nogo knockout lines were generated by Tessier-Lavigne and colleagues (Zheng et al., 2003). One line lacks Nogo-A and –B (Nogo-A/B mutant) and the second line lacks all three isoforms (Nogo-A/B/C mutant). In these two lines, no improvement in anatomical or functional recovery following spinal cord lesions was found but the Nogo-A/B/C was generated from the same allele as the Nogo-A/B mouse. A follow up study demonstrated that the difference in the penetrance of the Nogo-A mutant phenotype depends on the strain background, with a more pronounced regenerative axon growth of CST in the Sv129 strain as compared to the mixed C57BL/6 (Dimou et al., 2006). Misslabeling of ectopic axons due to ventricular biotin dextran amine (BDA) injection artifact has also been suggested as potential source of variability for the CST regenerative phenotype (Steward et al., 2007), however carefully analysis shows that although artifacts can rarely occur they cannot fully account for the regenerative phenotype observed with the Nogo-A/B mutant mice (Cafferty et al., 2007). In addition it appears that the age after sexual maturity modifies the restrictive effect of Nogo-A/B on axonal growth and on functional recovery with a significant improvement of 8 week old versus 14 week old mice (Cafferty et al., 2007). Finally the same group also noted that when all other parameters are held constant, different mutant Nogo alleles displayed different regenerative and functional recovery phenotypes even though the different mutant alleles equally eliminate detectable Nogo-A protein. Therefore among the factors that might account for the different responses to injury in these Nogo knockout lines are differences in age at the time of injury, mouse strain background and the nature of the mutant allele. Further, Nogo-A/B mutant mice displayed a strong sprouting effect of uninjured CST

fibers into the denervated spinal cord after a unilateral pyramidotomy which correlated with a recovery of fine motor skill in the affected forelimb (Cafferty and Strittmatter, 2006). This suggests that in addition to the aforementioned modifiers, the nature of the CNS lesion determines the penetrance of the mutant background as well.

I. 1. 2. 3. The Nogo receptor (NgR): common mediator of inhibition by Nogo-66, MAG and OMgp.

NgR1 is a GPI-linked, leucine-rich repeat (LRR) protein and it is expressed by multiple neuronal populations within the brain (Fournier et al., 2001). NgR1 is a surface receptor specific to Nogo-66 but not to amino-Nogo. Originally identified as a receptor for Nogo-66, NgR1 was subsequently identified as a functional binding partner for MAG and OMgp, which are structurally different proteins. In contrast with Nogo-66, amino-Nogo does not interact with NgR1 and its receptor has not been specified yet. Two homologues NgR2 and NgR3 were later identified which share 55% identity in the LRR domain of NgR1 (Barton et al., 2003; He et al., 2003; Lauren et al., 2003; Pignot et al., 2003). Nogo-66 does not bind NgR2 and NgR3 (Barton et al., 2003), however MAG binds to NgR2 (Venkatesh et al., 2005).

The functional importance of NgR1 in neuronal regeneration was examined by various approaches. NgREcto, a truncated soluble NgR1 inhibits binding of MAIs to surface bound NgR1, promotes neurite outgrowth on myelin substrate, and enhances CNS regeneration in animal models of SCI (Fournier and Strittmatter, 2002; Li and Strittmatter, 2003). The NgR1 antagonist: NEP1-40, a peptide fragment of Nogo-66 designed to interact with NgR1 without eliciting its downstream activation can also diminish the inhibitory effect after SCI (GrandPre et al., 2002). NgR1 -/- mice reveal a significant

regeneration of rubrospinal and raphespinal axons (Kim et al., 2004), but no regeneration of CST fibers. Despite NgR2's affinity to MAG and its ability to selectively mediate MAG's inhibitory function (Venkatesh et al., 2005), NgR1 -/- mice derived DRG neurons are not inhibited by MAG (Kim et al., 2004). Another NgR1 -/- mouse line yielded neurons that were inhibited by myelin *in vitro* (Zheng et al., 2005) indicating that NgR1 is dispensable for the growth inhibitory effect of MAIs. This apparent discrepancy has been attributed to the finding that NgR1 is required for the acute growth cone collapsing properties of Nogo-66 (Zheng et al., 2005), MAG and OMgp (Chivatakarn et al., 2007) but not for their ability to chronically inhibit outgrowth when presented in a substrate form (Chivatakarn et al., 2007). NgR1 -/- mice were equally analyzed in a different injury paradigm and were found to have no long distance CST regeneration following a unilateral pyramidotomy but a strong CST growth pattern proximal to the injury site which was also accompanied with a recovery of fine motor skill (Cafferty and Strittmatter, 2006).

Analysis of the growth inhibitory property of MAG revealed multiple response patterns between different neuronal populations including cerebellar granular neurons (CGNs), retinal ganglion neurons (RGNs) and DRGs (Venkatesh et al., 2007). For example, NgR1 deficient RGNs were strongly inhibited by MAG. Taken together this suggests that MAG uses distinct and cell type-specific mechanisms to mediate growth inhibition.

It is now obvious that different types of CNS neurons possess very different regenerative capacities and therefore respond differently to experimental treatment strategies *in vivo*.

FIGURE 2

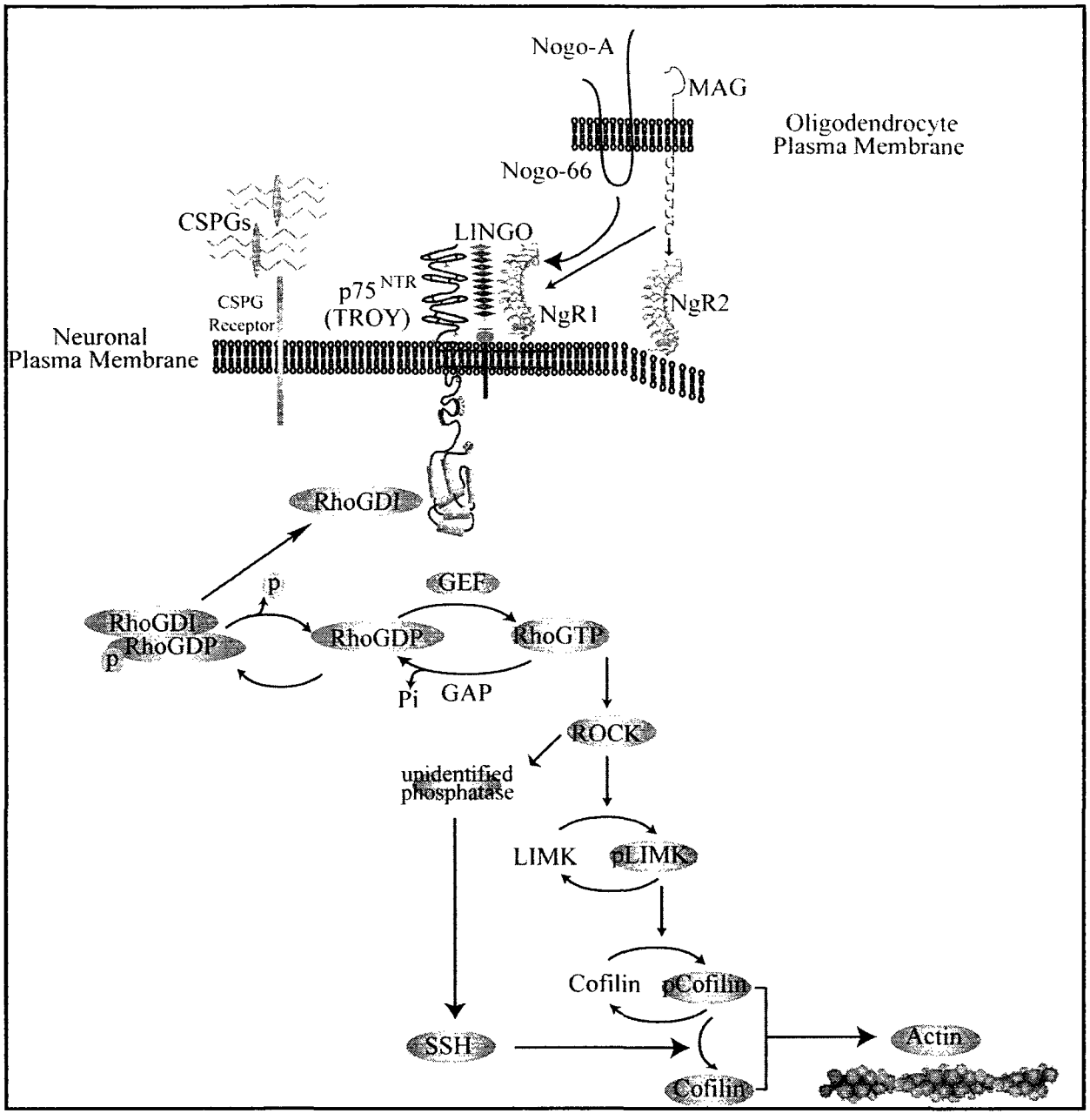
Nogo-A
MAG
Oligodendrocyte
Plasma Membrane
Nogo-66
CSPGs
LINGO
p75 NTR
(TROY)
NgR1
NgR2
CSPG
Receptor
Neuronal
Plasma Membrane
RhoGDI
p
GEF
RhoGDI
RhoGDP
p
RhoGDP
RhoGTP
Pi
GAP
ROCK
unidentified
phosphatase
LIMK
pLIMK
Cofilin
pCofilin
SSH
Actin
Cofilin

Figure 2. Schematic representation of the NgR1 signalling cascade. In the CNS, neurons express at the cell surface a trimeric complex consisting of NgR1/LINGO/$p75^{NTR}$ or alternatively NgR1/LINGO/TROY that responds to myelin associated inhibitors (Nogo-66, MAG, OMgp) stimulation. The inhibitory signals of the the MAIs leads to growth cone collapse and neurite outgrowth inhibition via the activation of RhoA and ROCK. Subsequently cytoskeletal rearrangement ensues via proteins such as cofilin (or actin depolymerizing factor, ADF) via LIMK and SSH (Hsieh et al., 2006). Other neurite outgrowth inhibitors such as CSPGs also converge and lead to the activation of the Rho-ROCK pathway.

Therefore it is suggested that the observed differences in regenerative capacity among different fiber systems are a reflection of their intrinsic ability to elongate axons and their distinct cell surface receptor profiles to respond to the growth inhibitory extracellular milieu.

I. 1. 3. Signal transduction

NgR1 is GPI-linked and therefore has no intracellular signaling domain thus requiring co-receptor/s to transduce an inhibitory signal. Recently, p75 neurotrophin receptor ($p75^{NTR}$), Lingo-1 and TROY were identified as co-receptors to NgR1 (Wang et al., 2002a; Wong et al., 2002; Mi et al., 2004; Shao et al., 2005). In certain neurons, the expression of $p75^{NTR}$ is gradually decreased during development. TROY is a functional homolog of $p75^{NTR}$, which substitutes for $p75^{NTR}$ in different neuronal population (Park et al., 2005; Shao et al., 2005). Nogo-66 signals through the tripartite receptor complex consisting of NgR1/$p75^{NTR}$/Lingo-1 or NgR1/TROY/Lingo-1 (Figure 2). Furthermore, transactivation of epidermal growth factor receptor (EGFR) after NgR1 ligand binding appears to be necessary for the engagement of axonal outgrowth inhibition (Koprivica et al., 2005).

$p75^{NTR}$ affects the activity of the small guanosine triphosphatase (GTPase) Rho, which is known to effect the neuronal cytoskeleton, and mediates axon inhibition via the recruitment of Rho to the $p75^{NTR}$ through Rho-GDI (see below) (Yamashita and Tohyama, 2003). Rho-GDI (Rho Guanine Dissociation Inhibitor) is a binding partner of Rho GTPases that restricts its activation by sequestering it from the cell membrane (Gosser et al., 1997). Kalirin-9, a dual RhoGEF, was recently proposed as a molecular link between the activation of $p75^{NTR}$ and RhoA activation in cerebellar granule neurons

in response to MAG (Harrington et al., 2008). Upon stimulation, RhoA is recruited to $p75^{NTR}$ via Rho-GDI which in turn releases Kalirin-9 to activate RhoA. Activation of the downstream signalling via $p75^{NTR}$ might also require its intramembrane proteolysis by α- and γ-secretases (Domeniconi et al., 2005). Transduction of CSPG- and myelin-based activation of RhoA also seems to depend on the activation of protein kinase C (PKC; (Sivasankaran et al., 2004).

I. 2. RhoGTPases as gatekeepers of cytoskeletal remodeling

Factors regulating neuronal outgrowth and inhibition mediate their effects through the leading edge of the axon, the growth cone. A fine balance between attractive and repulsive forces at the growth cone is required for accurate wiring of the nervous system. These two opposing external signals modulate growth cone motility by inducing changes in cytoskeletal structure. At the growth cone leading edge, a balance between actin polymerization and retrograde flow control growth cone advancement and retraction (Lewis and Bridgman, 1992; Fan and Raper, 1995).

Detailed signaling mechanisms responsible for transducing inhibitory information from NgR to the actin cytoskeleton have not been defined. One group of intracellular proteins that have been implicated in the myelin-dependent inhibitory signaling cascade is the Rho family of small guanosine triphosphatases (GTPases). Rho GTPases play an important role in transducing many extracellular signals to changes in cytoskeletal proteins (Hall, 1998). The best characterized members of the Rho family are Rho, Rac and Cdc42 (Bishop and Hall, 2000). Guanine nucleotide exchange factors (GEF) promote the conversion of GDP-bound Rho (inactive) to a GTP-bound (active) form of Rho.

Oppositely, GTPase activating proteins (GAP) stimulate the GTPase activity of Rho and subsequently lead to the conversion of GTP-bound Rho to an inactive GDP-bound state (Figure 2). This cycle is controlled by over 60 activators (GEFs) and 70 inactivators (GAPs). GTP-bound forms of Rho, Rac and Cdc42 interact with many downstream effectors that have widespread effects on the actin cytoskeleton potentially contributing to growth cone collapse (Narumiya et al., 1997; Cotteret and Chernoff, 2002). Rho activation promotes the formation of focal adhesions and stress fibers; Rac1 and Cdc42 activation result in lamellipodial and filopodial extension respectively. GTP-bound forms of Rho, Rac and Cdc42 interact with many downstream effectors that alter the actin cytoskeleton and dictate cellular responses. Following myelin stimulation, Rho is activated and Rac is inactivated (Fournier et al., 2000a; Niederost et al., 2002; Borisoff et al., 2003; Dubreuil et al., 2003). RhoA phosphorylation downregulates RhoA activity by enhancing it's membrane extraction through binding to RhoGDIs (Ellerbroek et al., 2003a). Recent evidence has implicated LIM kinase (LIMK) and Slingshot phosphatase (SSH) in the regulation of the actin depolymerizing factor Cofilin downstream of Nogo-66 signalling (Hsieh et al., 2006).

Development of antagonists to intracellular targets represents one of the most promising ideas to promote regeneration since convergent targets of multiple inhibitory and growth-promoting pathways are being identified. Rho can be specifically inactivated by C3 transferase (an enzyme found in *Clostridium botulinum*) (Jalink et al., 1994). Treatment with this enzyme promotes neurite outgrowth *in vitro* on inhibitory substances such as myelin or CSPGs (Lehmann et al., 1999; Winton et al., 2002; Fournier et al., 2003; Monnier et al., 2003). Infusion of C3 at the site of dorsal hemisection *in vivo* also

promotes regeneration following spinal cord injury however these effects are dependent on the mode of delivery (Dergham et al., 2002; Fournier et al., 2003).

The levels of cAMP can alter the effects of neurotrophic factors and guidance factors, with low levels of cyclic nucleotides promoting chemorepulsion and high levels supporting chemoattraction. Several studies have described the importance of cAMP levels in the neuronal response to myelin-derived inhibitors of axon growth (reviewed by (Snider et al., 2002). cAMP elevation, by injecting dibutyryl cAMP (db-cAMP), a membrane permeable cAMP analogue, promotes spinal and sensory axon regeneration (Neumann et al., 2002; Qiu et al., 2002). Elevation of cAMP in growth cones can convert repulsion by MAG to attraction (Cai et al., 1999). In addition, cAMP and neurotrophin-mediated increases in neurite outgrowth on myelin *in vitro* requires the expression of Arginase I and the synthesis of polyamines (Cai et al., 2002). It appears that the cAMP mediated effect on regeneration, involves the upregulation of Arginase I, a key enzyme in the synthesis of polyamines such as putrescine, later converted into spermidine and spermine (Seiler et al., 2000). Many studies have shown the role of polyamines in the growth and development of the nervous system as well as axonal regeneration (Slotkin and Bartolome, 1986; Gilad et al., 1996). Targeting this pathway represents therefore a potential avenue for treating nerve injury by increasing the levels of cAMP, via the use of polyamine based molecules, or by increasing the expression of Arginase I.

However, the tradeoff with targeting intracellular signaling substrates such as Rho and cAMP is that they are common to multiple cell types and side effects on other cells will occur at the lesion site. Thus the identification of neuronal-specific intracellular substrates of axon outgrowth inhibitors remains an important pursuit. The aim of this

project is to identify and characterize novel RhoA-interacting proteins with functional relevance to neuronal injury and regeneration.

I. 3. RhoGTPase interacting proteins as potential molecules of axon outgrowth inhibition of the adult CNS

I. 3. 1. Rho Kinase – ROCK

Many effector proteins downstream of Rho modulate actin cytoskeletal dynamics and are reasonable candidates for the transduction of myelin-associated signals, including citron kinase, mDia, rho kinase and protein kinase N (Bishop and Hall, 2000). One of the well characterized Rho downstream effectors of Rho is Rho associated coiled-coil-containing protein kinase (ROCK) (Leung et al., 1995; Ishizaki et al., 1996; Matsui et al., 1996a). Rho kinase (ROCK) is a serine/threonine kinase with two isoforms (p160ROCK/ROCK-I/ROCKβ, and ROCKII/ROKα) which share 65% similarity in their sequence. ROCK has a kinase domain at the amino terminus followed by a coiled-coil domain containing the Rho-binding domain (RBD) and a pleckstrin-homology domain (PH) with an internal cystein-rich domain (CRD). ROCKI and ROCKII are ubiquitously expressed with a preferential expression in brain and muscle tissues for ROCKII, whereas ROCKI is more abundantly expressed in nonneuronal tissues such as liver, spleen (Riento and Ridley, 2003) where they phosphorylate multiple downstream substrates (Riento and Ridley, 2003). Genetic deletion of ROCKII in mice causes embryonic lethality in most cases. These mice exhibited placental dysfunction and intrauterine growth retardation. While ROCKI deletion in mice resulted in eyelid open at birth and omphalocele which affects the survival of the neonates due to cannibalization by the mother. However it

seems that both isoforms might overlap in their biological functions since after overcoming their perinatal problems, both ROCKI and II mice develop normally without any functional abnormalities (Thumkeo et al., 2003; Shimizu et al., 2005). ROCK has been the favored effector candidate due to the ability of the drug Y-27632, an ATP competitive rho kinase antagonist, to promote neuronal outgrowth on MAI substrates *in vitro* and *in vivo* (Dergham et al., 2002; Niederost et al., 2002; Fournier et al., 2003). Once ROCKII is activated by GTP-bound RhoA, ROCKII phosphorylates a number of substrates to control actin-cytoskeleton assembly and cell contractility. In particular, ROCKII phosphorylates collapsin response mediator protein-2 (CRMP-2) (Arimura et al., 2000a), a neuronal protein involved in semaphorin 3A-induced growth cone collapse and in axon outgrowth (Inagaki et al., 2001). In addition, ROCK regulates actomyosin dynamics within the growth cone via phosphorylation of myosin light chain II (MLCII) and via phosphorylation and inhibition of MLC phosphatase (Fujita et al., 2001). Therefore it is reasonable to speculate that rho kinase might mediate myelin-dependent growth cone collapse by virtue of its ability to regulate myosin light chain phosphorylation and consequently actomyosin dynamics within the growth cone. However the evidence involving ROCK is indirectly based on the drug Y-27632 which is an ATP-competitive inhibitor that inhibits a number of kinases. In fact at a concentration of 10 μM, Y-27632 inhibits protein-kinase C related kinase (PRK2/PKNγ), which also binds to Rho and regulates cytoskeletal dynamics (Vincent and Settleman, 1997). Other studies have used Y-27632 at concentrations up to 50 μM *in vitro*, a concentration at which other kinases are inhibited (Davies et al., 2000). Therefore one issue with this drug is its specificity and it is therefore difficult to determine the exact contribution of each

affected kinase to the growth promoting effect observed. The contribution of ROCK to myelin-dependent inhibition is addressed in Chapter 2.

I. 3. 2. The CRMP family of proteins

The CRMP (TOAD-64/Ulip/Drp) family of proteins has been implicated in axon guidance and outgrowth. The CRMP proteins are homologs of UNC-33, a protein involved in axon guidance and extension in *Caenorhabditis elegans* (Hedgecock et al., 1985; Siddiqui and Culotti, 1991). The members of this family, which consist of at least 5 isoforms (Figure 3), have been independently identified as Ulip (UNC-33 like protein), CRMP (Collapsin response mediator protein), TOAD-64 (Turned on after Division, 64 kd) and DRP (dihydropyriminidase related protein) (Wang and Strittmatter, 1997a; Byk et al., 1998; Quinn et al., 1999). Four isoforms (CRMP1 through CRMP4) share 70–80% amino acid identity, while another member CRMP5 (CRAM: CRMP-3 associated molecule), shares only 50% sequence identity. In a screen to identify proteins that functionally interact with RhoA, we identified CRMP4 as a key protein in the myelin signaling cascade (Ch. 3).

Expression pattern analysis indicates an almost exclusive expression of the CRMP proteins in the nervous system (Minturn et al., 1995; Hamajima et al., 1996; Wang and Strittmatter, 1996; Kamata et al., 1998; Nacher et al., 2000; Tsim et al., 2004). CRMPs share significant sequence similarity with dihydropyrimidinase (DHPase), an enzyme involved in pyrimidine catabolism, although no DHPase activity however has been described for CRMPs (Wang and Strittmatter, 1997b).

CRMP2 (Ulip-2/TOAD-64/DRP-2) is the most widely studied member and appears to be crucial for axon outgrowth (Goshima et al., 1995a; Arimura et al., 2004). CRMP2 can bind to tubulin heterodimers and is an important organizer of microtubule assembly for establishing axon-dendrite fate during development (Fukata et al., 2002a). In addition, CRMP2 is phosphorylated at Thr-555, by Rho-kinase during lysophosphatidic acid (LPA) and EphrinA5 induced growth cone collapse in DRGs (Arimura et al., 2000b; Arimura et al., 2005a). CRMP2 is phosphorylated in a ROCK-dependent manner by Nogo or MAG and may contribute to neurite outgrowth inhibition via dysregulated microtubule dynamics (Mimura et al., 2006). CRMP2 promotes outgrowth and collapse in response to active RhoA and Rac1 respectively therefore reversing from a RhoA to a Rac1 morphology in N1E-115 neuroblastoma cells (Hall et al., 2001). CRMP1, 2 and 5 have been shown to act as downstream components of Semaphorin-PlxAs signal transduction pathway (Deo et al., 2004).

More recently, analysis of the UNC-33 gene in *Caenorhabditis elegans*, revealed that multiple transcripts were encoded by the same allele, suggesting splice variants. In chick, a novel subtype for each isoform ("b" isoforms) has been identified; the CRMPb variants represent longer amino terminal variants of the original CRMPa isoforms (Figure 3). CRMP4b identified by Hockfield and colleagues (2003), was found to play a role in neurite outgrowth and associates with vesicles in the growth cone and was shown to interact with intersectin, an endocytic-exocytic adaptor protein, suggesting a potential role in endocytosis (Quinn et al., 2003).

The role of the CRMP proteins in regeneration is still not well addressed. CRMP4a expression was increased in adult sciatic motor neurons that are regrowing axons

following axotomy (Minturn et al., 1995). CRMP1a, CRMP2a and CRMP5a mRNA levels increased after hypoglossal nerve injury. CRMP2a was shown to have a potent neurite elongating effect in nerve regeneration *in vivo* (Suzuki et al., 2003).

The CRMP proteins are extensively phosphorylated during neuronal development, and the phosphorylation states are altered in response to nerve growth factor (NGF) and lysophosphatidic acid (LPA) (Byk et al., 1996; Gu et al., 2000). CRMP2 is a substrate for ROCK but not CRMP4. CRMP2 and CRMP4 were shown to be substrates of glycogen synthase kinase 3 beta (GSK-3β) (Cole et al., 2004c). GSK-3β might regulate neuronal polarity in hippocampal neurons through the phosphorylation of CRMP2 at Thr-514, by decreasing CRMP2 affinity towards tubulin (Yoshimura et al., 2005).

I. 4. Role of Protein Phosphatases and Kinases in regulating neurite outgrowth and GC dynamics

Following injury or during embryonic development the precise connectivity of the neurons and their targets requires a delicate balance of growth cone steering and repulsion. The axonal growth is determined by an interaction between the growth cone and its microenvironment. The complexity of the regulation of growth cone morphology in response to the extracellular environment is determined by the presence of multiple signalling pathways that couple receptor stimulation on the plasma membrane to alteration of the cytoskeletal elements. This signal transduction is mediated via direct protein-protein interaction and via protein phosphorylation which is regulated by protein kinases and protein phosphatases. The regulation of both actin and microtubule dynamics is essential for driving axonal growth. Cofilin is a key regulatory protein of actin

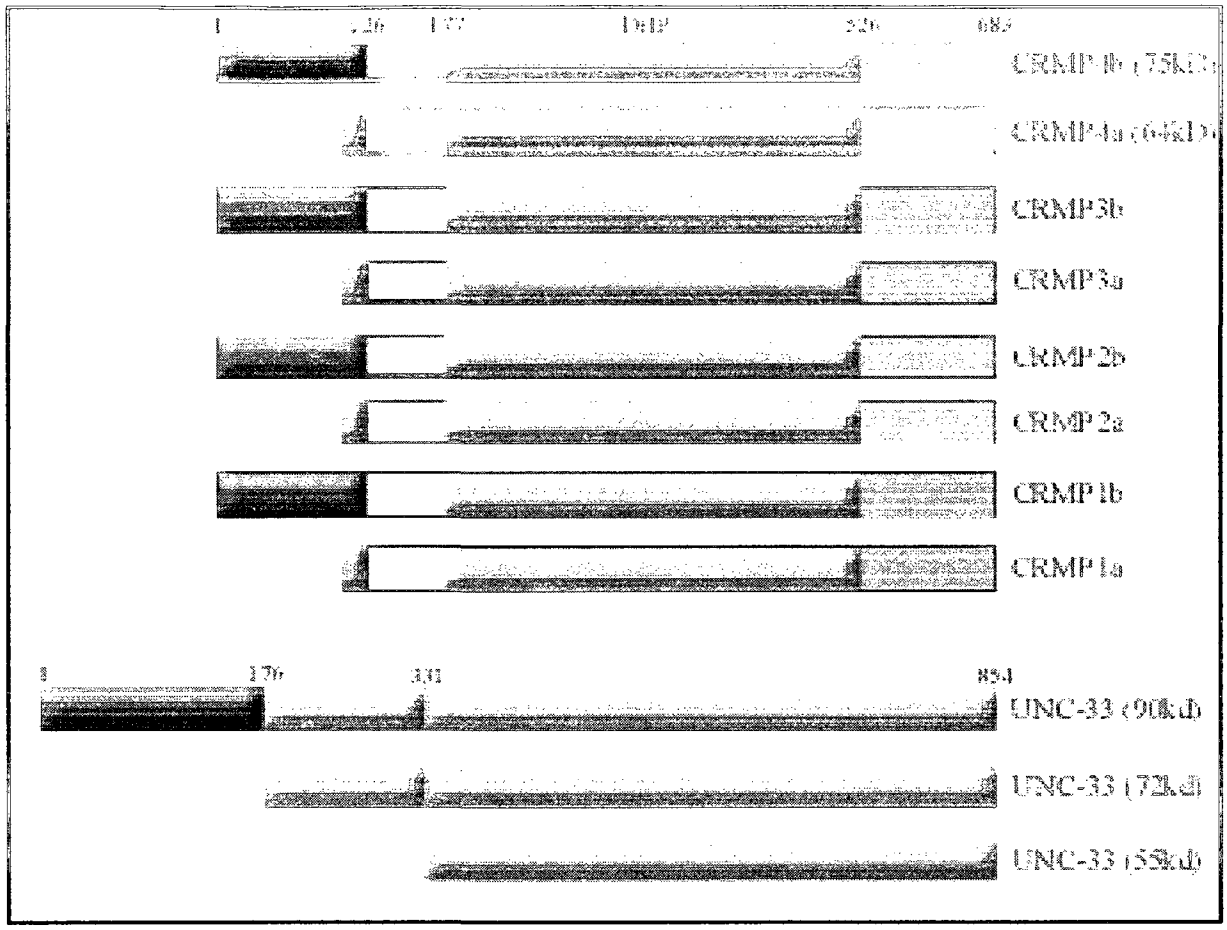

Figure 3. Schematic representation of the CRMP family of proteins. CRMPs are homologues of the UNC-33 protein in C.*elegans*. The gene *unc-33* encodes three transcripts that are translated into proteins of 55, 72, and 90 kDa. The four original members of the CRMP family correspond to the transcript that encodes the 55 kDa isoform of UNC-33. Each CRMP isoform has 2 subtypes: CRMPa (short version) and CRMPb (long version). CRMP4 consists of a 126 residue (CRMP4b) or 13 residue (CRMP4a) unique amino terminal domain followed by 557 common residues.

depolymerization and subsequently in altering growth cone dynamics in response to external cues. Cofilin regulates actin dynamics by increasing the off-rate of actin monomers from the pointed actin filament end and by severing actin filaments (Aizawa et al., 2001; Bamburg and Wiggan, 2002). Several kinases and phosphatases regulate Cofilin phosphorylation and activity including LIM Kinase and Slingshot phosphatase which have been implicated in regulating growth cone motility (Endo et al., 2003).

I. 4. 1. LIM-kinase

The LIM (an acronym for the three gene products Lin-11, Isl-1 and Mec-3) kinase family members (LIMK1 and LIMK2) possess two N-terminal LIM motifs: a PDZ domain and a C-terminal protein kinase domain (Edwards and Gill, 1999). LIMK phosphorylates and inactivates the actin depolymerization factor cofilin (Yang et al., 1998). Previous studies had demonstrated a requirement for LIMK-dependent cofilin phosphorylation in axon repulsion in response to the guidance molecule Semaphorin3A (Aizawa et al., 2001). LIMK1 knockout mice exhibit significant abnormalities in dendritic spine morphology and demonstrate increased hippocampal long-term potentiation; possibly indicative of enhanced plasticity (Meng et al., 2002). LIMK can be threonine phosphorylated by ROCK, and our lab has demonstrated that Nogo-dependent phosphorylation of LIMK is indeed ROCK dependent (Hsieh et al., 2006). Central to this proposal, introduction of a dominant negative (DN) LIMK1 construct attenuates myelin-dependent inhibition in DRG neurons *in vitro*. Further, DNLIMK1 blocked Nogo-66-dependent cofilin phosphorylation. Together this data suggests that cofilin is a critical LIMK substrate mediating myelin-dependent inhibition (Hsieh et al., 2006).

I. 4. 2. Slingshot

Slingshot (SSH) is a protein phosphatase (Niwa et al., 2002), originally identified in *Drosophila*, that has three related homologues in vertebrates (SSH1, SSH2, and SSH3); each has long and short variants. The long isoforms (-L) of SSH family members with a C-terminal F-actin binding domain are abundant in mammalian CNS. Overexpression of wild type SSH increase growth cone motility, whereas the presence of the phosphatase-inactive form of SSH increases cofilin phosphorylation and stabilizes actin filaments in DRG neurons (Niwa et al., 2002; Endo et al., 2003). MAIs were recently linked to induce cofilin dephosphorylation via SSH1 phosphatase activation (Hsieh et al., 2006).

I. 5. Multifaceted role of GSK3β in axon guidance and regeneration

In the CNS, axonal fate seems to be dictated by a panel of extracellular cues including ECM proteins such as neuron-glia cell adhesion molecule (NgCAM) or integrins (Esch et al., 1999) as well as secreted factors such as NT-3 (Neurotrophin-3) and BDNF (Brain Derived Neurotrophic Factor) which enhance axon elongation and branching (Ip et al., 1993; Labelle and Leclerc, 2000; Pfenninger et al., 2003). This complex process requires a precise regulation of several mechanisms including actin filaments reorganization, microtubule assembly, axonal protein trafficking and endocytosis of adhesion molecules (Figure 4). A body of evidence is currently implicating GSK as a key molecule in neuronal polarity and specifying axon-dendrite fate. GSK seems to regulate microtubule dynamics via microtubule-associating proteins.

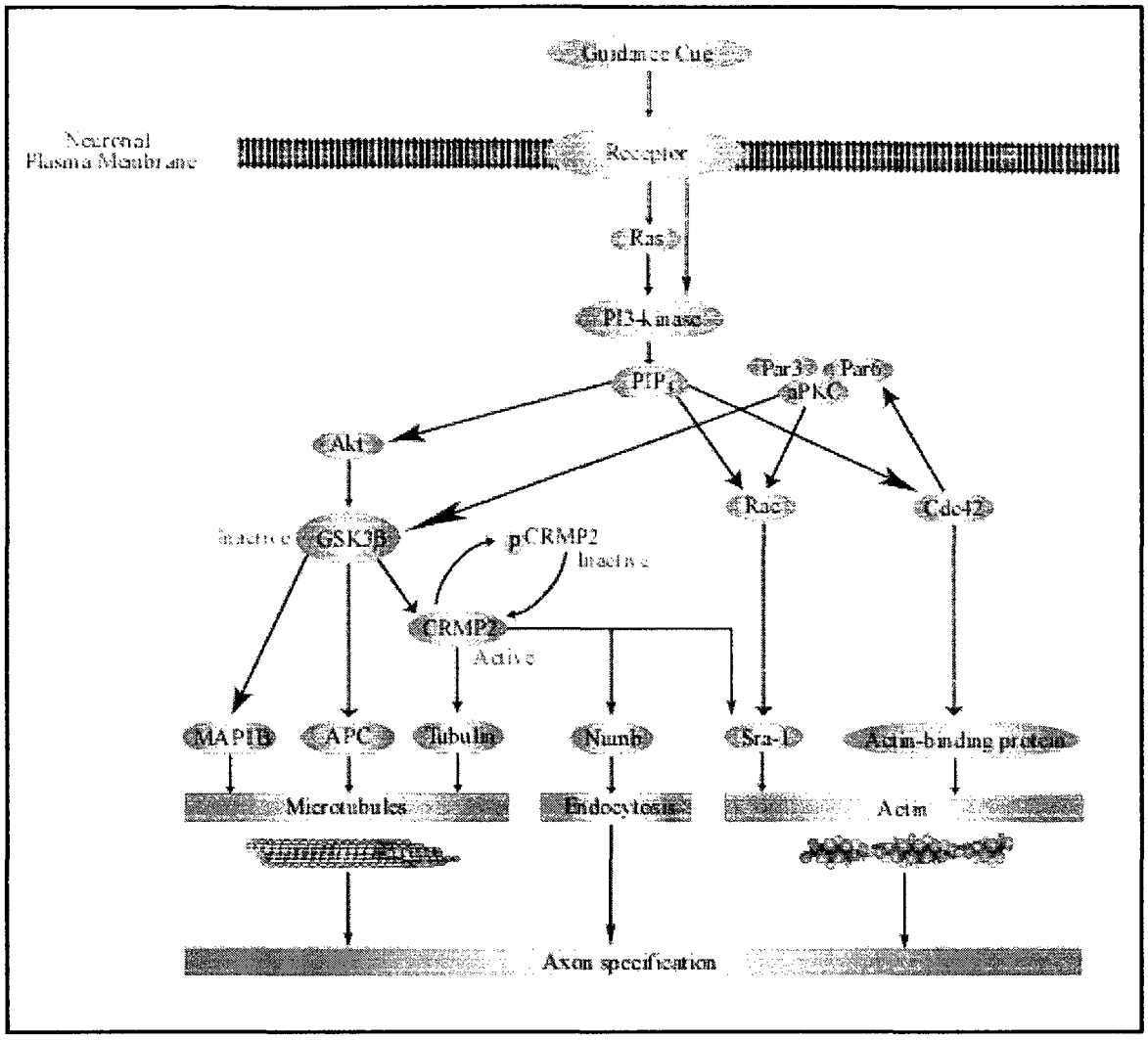

Figure 4. Schematic of the signalling pathways that mediate axon specification. Axon dendrite fate is established in a growing immature neurite in response to a series of extracellular matrix cues and secreted factors (neurotrophins). Engagement of these pathways leads to PI3-kinase activation. 2 majors axis are involved: Akt/GSK-3β/CRMP2 and Cdc42/Par complex/Rac1 which ultimately converge on rearranging the cytoskeleton. GSK-3β is a centerpiece molecule that receives input from multiple cross pathways.

Two major pathways have been identified as key for driving the regulatory cascades necessary for neurite elongation and neuronal polarization: the Akt/GSK-3β/CRMP2 pathway (Nishimura et al., 2003; Arimura et al., 2005b; Yoshimura et al., 2005) and the positive feedback loop composed of cdc42, the PAR complex PAR3/PAR6/aPKC and Rac1 (Nishimura et al., 2004; Shi et al., 2004).

GSK3α and β are ubiquitously expressed, constitutively active, serine/threonine kinases originally identified as regulatory kinases for glycogen synthase and subsequently implicated in signaling cascades downstream of Wnts, NGF, EGF, Semaphorins and Hedgehog (Eickholt et al., 2002; Kockeritz et al., 2006). GSK-3 is regulated at multiple levels by phosphorylation. GSK3α/β activity is negatively regulated by phosphorylation at Ser21/9 and positively regulated by phosphorylation at Tyr279/216 by multiple protein kinases (Jope and Johnson, 2004). GSK regulates the phosphorylation and activation of many microtubule-associated proteins including APC, CRMP2, CRMP4, MAP1b, MAP2, NF, Tau and Kinesin light chain (Cole et al., 2004a; Zhou and Snider, 2005). CRMP4b phosphorylation is sequentially regulated by GSK3β on residues Ser631, Thr627 and Thr622 following a priming phosphorylation event which may be mediated by DYRK2 (Cole et al., 2004b). In Ch. 4 we describe GSK3β as an important intracellular mediator of myelin-dependent inhibition.

I. 6. Thesis rationale and objectives

Spontaneous regeneration of the injured central nervous system (CNS) fails due to the action of potent outgrowth inhibitors that block growth cone extension. Growth inhibitory molecules exert their effects by binding specific cell surface receptors that result in Rho

activation and cytoskeletal rearrangements but the precise signaling mechanisms that link receptor engagement to growth inhibition remain poorly understood. The goal of this thesis is to delineate the intracellular events that link myelin-associated inhibitor (MAI) signals to the cytoskeleton and to use this information to develop strategies to promote regeneration. More specifically we are interested in studying RhoA-interacting proteins and their role in mediating growth inhibition. In Chapter 2 we provide direct evidence for the involvement of the RhoA-ROCK pathway in response to Nogo-66 signalling (Ch.2). In Chapter 3 we identify a novel RhoA interacting protein, CRMP4, as an important component of the MAI pathway with known roles in actin and microtubule cytoskeletal regulation. Through structure function analysis of CRMP4 we describe a potent and specific molecular antagonist of MAI-dependent neurite outgrowth inhibition. In Chapter 4 we find that the novel association between CRMP4 and RhoA is regulated by phosphorylation by GSK3β and further define the role of this kinase in MAI-dependent outgrowth inhibition.

I. 7. REFERENCES

Acevedo L, Yu J, Erdjument-Bromage H, Miao RQ, Kim JE, Fulton D, Tempst P, Strittmatter SM, Sessa WC (2004) A new role for Nogo as a regulator of vascular remodeling.[see comment]. Nature Medicine 10:382-388.

Aguayo AJ, David S, Bray GM (1981) Influences of the glial environment on the elongation of axons after injury: transplantation studies in adult rodents. Journal of Experimental Biology 95:231-240.

Aizawa H, Wakatsuki S, Ishii A, Moriyama K, Sasaki Y, Ohashi K, Sekine-Aizawa Y, Sehara-Fujisawa A, Mizuno K, Goshima Y, Yahara I (2001) Phosphorylation of cofilin by LIM-kinase is necessary for semaphorin 3A- induced growth cone collapse. Nat Neurosci 4:367-373.

Arimura N, Menager C, Fukata Y, Kaibuchi K (2004) Role of CRMP-2 in neuronal polarity. Journal of Neurobiology 58:34-47.

Arimura N, Inagaki N, Chihara K, Menager C, Nakamura N, Amano M, Iwamatsu A, Goshima Y, Kaibuchi K (2000a/b) Phosphorylation of collapsin response mediator protein-2 by Rho-kinase. Evidence for two separate signaling pathways for growth cone collapse. Journal of Biological Chemistry 275:23973-23980.

Arimura N, Menager C, Kawano Y, Yoshimura T, Kawabata S, Hattori A, Fukata Y, Amano M, Goshima Y, Inagaki M, Morone N, Usukura J, Kaibuchi K (2005a/b) Phosphorylation by Rho kinase regulates CRMP-2 activity in growth cones. Molecular & Cellular Biology 25:9973-9984.

Asher RA, Morgenstern DA, Moon LD, Fawcett JW (2001) Chondroitin sulphate proteoglycans: inhibitory components of the glial scar. Prog Brain Res 132:611-619.

Asher RA, Morgenstern DA, Fidler PS, Adcock KH, Oohira A, Braistead JE, Levine JM, Margolis RU, Rogers JH, Fawcett JW (2000) Neurocan is upregulated in injured brain and in cytokine-treated astrocytes. J Neurosci 20:2427-2438.

Bamburg JR, Wiggan OP (2002) ADF/cofilin and actin dynamics in disease. Trends Cell Biol 12:598-605.

Barton WA, Liu BP, Tzvetkova D, Jeffrey PD, Fournier AE, Sah D, Cate R, Strittmatter SM, Nikolov DB (2003) Structure and axon outgrowth inhibitor binding of the Nogo-66 receptor and related proteins. Embo J 22:3291-3302.

Benson MD, Romero MI, Lush ME, Lu QR, Henkemeyer M, Parada LF (2005) Ephrin-B3 is a myelin-based inhibitor of neurite outgrowth. Proceedings of the National Academy of Sciences of the United States of America 102:10694-10699.

Bishop AL, Hall A (2000) Rho GTPases and their effector proteins. Biochem J 348 Pt 2:241-255.

Borisoff JF, Chan CC, Hiebert GW, Oschipok L, Robertson GS, Zamboni R, Steeves JD, Tetzlaff W (2003) Suppression of Rho-kinase activity promotes axonal growth on inhibitory CNS substrates. Mol Cell Neurosci 22:405-416.

Bradbury EJ, Moon LD, Popat RJ, King VR, Bennett GS, Patel PN, Fawcett JW, McMahon SB (2002) Chondroitinase ABC promotes functional recovery after spinal cord injury. Nature 416:636-640.

Byk T, Ozon S, Sobel A (1998) The Ulip family phosphoproteins--common and specific properties. European Journal of Biochemistry 254:14-24.

Byk T, Dobransky T, Cifuentes-Diaz C, Sobel A (1996) Identification and molecular characterization of Unc-33-like phosphoprotein (Ulip), a putative mammalian homolog of the axonal guidance-associated unc-33 gene product. Journal of Neuroscience 16:688-701.

Cafferty WB, Strittmatter SM (2006) The Nogo-Nogo receptor pathway limits a spectrum of adult CNS axonal growth. Journal of Neuroscience 26:12242-12250.

Cafferty WB, Kim JE, Lee JK, Strittmatter SM (2007) Response to correspondence: Kim et al., "axon regeneration in young adult mice lacking Nogo-A/B." Neuron 38, 187-199. Neuron 54:195-199.

Cai D, Shen Y, De Bellard M, Tang S, Filbin MT (1999) Prior exposure to neurotrophins blocks inhibition of axonal regeneration by MAG and myelin via a cAMP-dependent mechanism. Neuron 22:89-101.

Cai D, Deng K, Mellado W, Lee J, Ratan RR, Filbin MT (2002) Arginase I and polyamines act downstream from cyclic AMP in overcoming inhibition of axonal growth MAG and myelin in vitro. Neuron 35:711-719.

Caroni P, Schwab ME (1988a) Antibody against myelin-associated inhibitor of neurite growth neutralizes nonpermissive substrate properties of CNS white matter. Neuron 1:85-96.

Caroni P, Schwab ME (1988b) Two membrane protein fractions from rat central myelin with inhibitory properties for neurite growth and fibroblast spreading. J Cell Biol 106:1281-1288.

Chen MS, Huber AB, van der Haar ME, Frank M, Schnell L, Spillmann AA, Christ F, Schwab ME (2000) Nogo-A is a myelin-associated neurite outgrowth inhibitor and an antigen for monoclonal antibody IN-1. Nature 403:434-439.

Chivatakarn O, Kaneko S, He Z, Tessier-Lavigne M, Giger RJ (2007) The Nogo-66 receptor NgR1 is required only for the acute growth cone-collapsing but not the chronic growth-inhibitory actions of myelin inhibitors.[see comment]. Journal of Neuroscience 27:7117-7124.

Cole A, Frame S, Cohen P (2004a) Further evidence that the tyrosine phosphorylation of glycogen synthase kinase-3 (GSK3) in mammalian cells is an autophosphorylation event. Biochem J 377:249-255.

Cole AR, Knebel A, Morrice NA, Robertson LA, Irving AJ, Connolly CN, Sutherland C (2004b/c) GSK-3 phosphorylation of the Alzheimer epitope within collapsin response mediator proteins regulates axon elongation in primary neurons. J Biol Chem 279:50176-50180.

Cotteret S, Chernoff J (2002) The evolutionary history of effectors downstream of Cdc42 and Rac. Genome Biol 3.

David S, Aguayo AJ (1981) Axonal elongation into peripheral nervous system "bridges" after central nervous system injury in adult rats. Science 214:931-933.

Davies SJ, Goucher DR, Doller C, Silver J (1999) Robust regeneration of adult sensory axons in degenerating white matter of the adult rat spinal cord. J Neurosci 19:5810-5822.

Davies SJ, Fitch MT, Memberg SP, Hall AK, Raisman G, Silver J (1997) Regeneration of adult axons in white matter tracts of the central nervous system. Nature 390:680-683.

Davies SP, Reddy H, Caivano M, Cohen P (2000) Specificity and mechanism of action of some commonly used protein kinase inhibitors. Biochem J 351:95-105.

De Winter F, Oudega M, Lankhorst AJ, Hamers FP, Blits B, Ruitenberg MJ, Pasterkamp RJ, Gispen WH, Verhaagen J (2002) Injury-induced class 3 semaphorin expression in the rat spinal cord. Experimental Neurology 175:61-75.

Deo RC, Schmidt EF, Elhabazi A, Togashi H, Burley SK, Strittmatter SM (2004) Structural bases for CRMP function in plexin-dependent semaphorin3A signaling. Embo J 23:9-22.

Dergham P, Ellezam B, Essagian C, Avedissian H, Lubell WD, McKerracher L (2002) Rho signaling pathway targeted to promote spinal cord repair. J Neurosci 22:6570-6577.

Dimou L, Schnell L, Montani L, Duncan C, Simonen M, Schneider R, Liebscher T, Gullo M, Schwab ME (2006) Nogo-A-deficient mice reveal strain-dependent differences in axonal regeneration. Journal of Neuroscience 26:5591-5603.

Domeniconi M, Zampieri N, Spencer T, Hilaire M, Mellado W, Chao MV, Filbin MT (2005) MAG induces regulated intramembrane proteolysis of the p75 neurotrophin receptor to inhibit neurite outgrowth. Neuron 46:849-855.

Dubreuil CI, Winton MJ, McKerracher L (2003) Rho activation patterns after spinal cord injury and the role of activated Rho in apoptosis in the central nervous system. Journal of Cell Biology 162:233-243.

Edwards DC, Gill GN (1999) Structural features of LIM kinase that control effects on the actin cytoskeleton. J Biol Chem 274:11352-11361.

Eickholt BJ, Walsh FS, Doherty P (2002) An inactive pool of GSK-3 at the leading edge of growth cones is implicated in Semaphorin 3A signaling. J Cell Biol 157:211-217.

Ellerbroek SM, Wennerberg K, Burridge K (2003) Serine phosphorylation negatively regulates RhoA in vivo. Journal of Biological Chemistry 278:19023-19031.

Endo M, Ohashi K, Sasaki Y, Goshima Y, Niwa R, Uemura T, Mizuno K (2003) Control of growth cone motility and morphology by LIM kinase and Slingshot via phosphorylation and dephosphorylation of cofilin. J Neurosci 23:2527-2537.

Esch T, Lemmon V, Banker G (1999) Local presentation of substrate molecules directs axon specification by cultured hippocampal neurons. Journal of Neuroscience 19:6417-6426.

Fan J, Raper JA (1995) Localized collapsing cues can steer growth cones without inducing their full collapse. Neuron 14:263-274.

Fournier AE, Strittmatter SM (2002) Regenerating nerves follow the road more traveled. Nat Neurosci 5:821-822.

Fournier AE, Kalb RG, Strittmatter SM (2000) Rho GTPases and axonal growth cone collapse. Methods Enzymol 325:473-482.

Fournier AE, GrandPre T, Strittmatter SM (2001) Identification of a receptor mediating Nogo-66 inhibition of axonal regeneration. Nature 409:341-346.

Fournier AE, Takizawa BT, Strittmatter SM (2003) Rho Kinase inhibition enhances axonal regeneration in the injured CNS. J Neurosci 23:1416-1423.

Fujita A, Hattori Y, Takeuchi T, Kamata Y, Hata F (2001) NGF induces neurite outgrowth via a decrease in phosphorylation of myosin light chain in PC12 cells. Neuroreport 12:3599-3602.

Fukata Y, Itoh TJ, Kimura T, Menager C, Nishimura T, Shiromizu T, Watanabe H, Inagaki N, Iwamatsu A, Hotani H, Kaibuchi K (2002) CRMP-2 binds to tubulin heterodimers to promote microtubule assembly.[see comment]. Nature Cell Biology 4:583-591.

Gilad VH, Tetzlaff WG, Rabey JM, Gilad GM (1996) Accelerated recovery following polyamines and aminoguanidine treatment after facial nerve injury in rats. Brain Res 724:141-144.

Goldshmit Y, Galea MP, Wise G, Bartlett PF, Turnley AM (2004) Axonal regeneration and lack of astrocytic gliosis in EphA4-deficient mice. Journal of Neuroscience 24:10064-10073.

Goshima Y, Nakamura F, Strittmatter P, Strittmatter SM (1995) Collapsin-induced growth cone collapse mediated by an intracellular protein related to UNC-33. Nature 376:509-514.

Gosser YQ, Nomanbhoy TK, Aghazadeh B, Manor D, Combs C, Cerione RA, Rosen MK (1997) C-terminal binding domain of Rho GDP-dissociation inhibitor directs N-terminal inhibitory peptide to GTPases. Nature 387:814-819.

GrandPre T, Li S, Strittmatter SM (2002) Nogo-66 receptor antagonist peptide promotes axonal regeneration. Nature 417:547-551.

GrandPre T, Nakamura F, Vartanian T, Strittmatter SM (2000) Identification of the Nogo inhibitor of axon regeneration as a Reticulon protein. Nature 403:439-444.

Gu Y, Hamajima N, Ihara Y (2000) Neurofibrillary tangle-associated collapsin response mediator protein-2 (CRMP-2) is highly phosphorylated on Thr-509, Ser-518, and Ser-522. Biochemistry 39:4267-4275.

Hall A (1998) Rho GTPases and the actin cytoskeleton. Science 279:509-514.

Hall C, Brown M, Jacobs T, Ferrari G, Cann N, Teo M, Monfries C, Lim L (2001) Collapsin response mediator protein switches RhoA and Rac1 morphology in N1E-115 neuroblastoma cells and is regulated by Rho kinase. Journal of Biological Chemistry 276:43482-43486.

Hamajima N, Matsuda K, Sakata S, Tamaki N, Sasaki M, Nonaka M (1996) A novel gene family defined by human dihydropyrimidinase and three related proteins with differential tissue distribution. Gene 180:157-163.

Harrington AW, Li QM, Tep C, Park JB, He Z, Yoon SO (2008) The role of Kalirin9 in p75/nogo receptor-mediated RhoA activation in cerebellar granule neurons. Journal of Biological Chemistry 283:24690-24697.

He XL, Bazan JF, McDermott G, Park JB, Wang K, Tessier-Lavigne M, He Z, Garcia KC (2003) Structure of the nogo receptor ectodomain. A recognition module implicated in myelin inhibition. Neuron 38:177-185.

Hedgecock EM, Culotti JG, Thomson JN, Perkins LA (1985) Axonal guidance mutants of Caenorhabditis elegans identified by filling sensory neurons with fluorescein dyes. Developmental Biology 111:158-170.

Hsieh S, Ferraro GB, Fournier AE (2006) Myelin-associated inhibitors regulate cofilin phosphorylation and neuronal inhibition through Lim kinase and Slingshot phosphatase. J Neurosci 26:1006-1015.

Inagaki N, Chihara K, Arimura N, Menager C, Kawano Y, Matsuo N, Nishimura T, Amano M, Kaibuchi K (2001) CRMP-2 induces axons in cultured hippocampal neurons. Nature Neuroscience 4:781-782.

Ip NY, Li Y, Yancopoulos GD, Lindsay RM (1993) Cultured hippocampal neurons show responses to BDNF, NT-3, and NT-4, but not NGF. Journal of Neuroscience 13:3394-3405.

Ishizaki T, Maekawa M, Fujisawa K, Okawa K, Iwamatsu A, Fujita A, Watanabe N, Saito Y, Kakizuka A, Morii N, Narumiya S (1996) The small GTP-binding protein Rho binds to and activates a 160 kDa Ser/Thr protein kinase homologous to myotonic dystrophy kinase. Embo J 15:1885-1893. abs.html.

Jalink K, van Corven EJ, Hengeveld T, Morii N, Narumiya S, Moolenaar WH (1994) Inhibition of lysophosphatidate- and thrombin-induced neurite retraction and neuronal cell rounding by ADP ribosylation of the small GTP-binding protein Rho. J Cell Biol 126:801-810.

Jope RS, Johnson GV (2004) The glamour and gloom of glycogen synthase kinase-3. Trends Biochem Sci 29:95-102.

Kamata T, Subleski M, Hara Y, Yuhki N, Kung H, Copeland NG, Jenkins NA, Yoshimura T, Modi W, Copeland TD (1998) Isolation and characterization of a bovine neural specific protein (CRMP-2) cDNA homologous to unc-33, a C. elegans gene implicated in axonal outgrowth and guidance. Brain Research Molecular Brain Research 54:219-236.

Kim JE, Liu BP, Park JH, Strittmatter SM (2004) Nogo-66 receptor prevents raphespinal and rubrospinal axon regeneration and limits functional recovery from spinal cord injury. Neuron 44:439-451.

Kim JE, Li S, GrandPre T, Qiu D, Strittmatter SM (2003) Axon regeneration in young adult mice lacking nogo-a/b. Neuron 38:187-199.

Kockeritz L, Doble B, Patel S, Woodgett JR (2006) Glycogen synthase kinase-3--an overview of an over-achieving protein kinase. Curr Drug Targets 7:1377-1388.

Koprivica V, Cho KS, Park JB, Yiu G, Atwal J, Gore B, Kim JA, Lin E, Tessier-Lavigne M, Chen DF, He Z (2005) EGFR activation mediates inhibition of axon regeneration by myelin and chondroitin sulfate proteoglycans.[see comment]. Science 310:106-110.

Kottis V, Thibault P, Mikol D, Xiao ZC, Zhang R, Dergham P, Braun PE (2002) Oligodendrocyte-myelin glycoprotein (OMgp) is an inhibitor of neurite outgrowth. J Neurochem 82:1566-1569.

Labelle C, Leclerc N (2000) Exogenous BDNF, NT-3 and NT-4 differentially regulate neurite outgrowth in cultured hippocampal neurons. Brain Research Developmental Brain Research 123:1-11.

Lauren J, Airaksinen MS, Saarma M, Timmusk T (2003) Two novel mammalian Nogo receptor homologs differentially expressed in the central and peripheral nervous systems. Molecular & Cellular Neurosciences 24:581-594.

Lehmann M, Fournier A, Selles-Navarro I, Dergham P, Sebok A, Leclerc N, Tigyi G, McKerracher L (1999) Inactivation of Rho signaling pathway promotes CNS axon regeneration. J Neurosci 19:7537-7547.

Leung T, Manser E, Tan L, Lim L (1995) A novel serine/threonine kinase binding the Ras-related RhoA GTPase which translocates the kinase to peripheral membranes. J Biol Chem 270:29051-29054.

Lewis AK, Bridgman PC (1992) Nerve growth cone lamellipodia contain two populations of actin filaments that differ in organization and polarity. J Cell Biol 119:1219-1243.

Li S, Strittmatter SM (2003) Delayed systemic nogo-66 receptor antagonist promotes recovery from spinal cord injury. J Neurosci 23:4219-4227.

Low K, Culbertson M, Bradke F, Tessier-Lavigne M, Tuszynski MH (2008) Netrin-1 is a novel myelin-associated inhibitor to axon growth. Journal of Neuroscience 28:1099-1108.

Manitt C, Wang D, Kennedy TE, Howland DR (2006) Positioned to inhibit: netrin-1 and netrin receptor expression after spinal cord injury. Journal of Neuroscience Research 84:1808-1820.

Manitt C, Colicos MA, Thompson KM, Rousselle E, Peterson AC, Kennedy TE (2001) Widespread expression of netrin-1 by neurons and oligodendrocytes in the adult mammalian spinal cord. J Neurosci 21:3911-3922.

Matsui T, Amano M, Yamamoto T, Chihara K, Nakafuku M, Ito M, Nakano T, Okawa K, Iwamatsu A, Kaibuchi K (1996) Rho-associated kinase, a novel serine/threonine kinase, as a putative target for small GTP binding protein Rho. Embo J 15:2208-2216. abs.html.

McKeon RJ, Schreiber RC, Rudge JS, Silver J (1991) Reduction of neurite outgrowth in a model of glial scarring following CNS injury is correlated with the expression of inhibitory molecules on reactive astrocytes. Journal of Neuroscience 11:3398-3411.

McKerracher L, David S, Jackson DL, Kottis V, Dunn RJ, Braun PE (1994) Identification of myelin-associated glycoprotein as a major myelin-derived inhibitor of neurite growth. Neuron 13:805-811.

Meng Y, Zhang Y, Tregoubov V, Janus C, Cruz L, Jackson M, Lu WY, MacDonald JF, Wang JY, Falls DL, Jia Z (2002) Abnormal spine morphology and enhanced LTP in LIMK-1 knockout mice. Neuron 35:121-133.

Mi S, Lee X, Shao Z, Thill G, Ji B, Relton J, Levesque M, Allaire N, Perrin S, Sands B, Crowell T, Cate RL, McCoy JM, Pepinsky RB (2004) LINGO-1 is a component of the Nogo-66 receptor/p75 signaling complex. Nat Neurosci 7:221-228.

Mimura F, Yamagishi S, Arimura N, Fujitani M, Kubo T, Kaibuchi K, Yamashita T (2006) Myelin-associated Glycoprotein Inhibits Microtubule Assembly by a Rho-kinase-dependent Mechanism. J Biol Chem 281:15970-15979.

Minturn JE, Fryer HJ, Geschwind DH, Hockfield S (1995) TOAD-64, a gene expressed early in neuronal differentiation in the rat, is related to unc-33, a C. elegans gene involved in axon outgrowth. Journal of Neuroscience 15:6757-6766.

Monnier PP, Sierra A, Schwab JM, Henke-Fahle S, Mueller BK (2003) The Rho/ROCK pathway mediates neurite growth-inhibitory activity associated with the chondroitin sulfate proteoglycans of the CNS glial scar. Mol Cell Neurosci 22:319-330.

Moon LD, Asher RA, Fawcett JW (2003) Limited growth of severed CNS axons after treatment of adult rat brain with hyaluronidase. J Neurosci Res 71:23-37.

Moon LD, Asher RA, Rhodes KE, Fawcett JW (2001) Regeneration of CNS axons back to their target following treatment of adult rat brain with chondroitinase ABC. Nat Neurosci 4:465-466.

Moreau-Fauvarque C, Kumanogoh A, Camand E, Jaillard C, Barbin G, Boquet I, Love C, Jones EY, Kikutani H, Lubetzki C, Dusart I, Chedotal A (2003) The transmembrane semaphorin Sema4D/CD100, an inhibitor of axonal growth, is

expressed on oligodendrocytes and upregulated after CNS lesion. Journal of Neuroscience 23:9229-9239.

Morgenstern DA, Asher RA, Fawcett JW (2002) Chondroitin sulphate proteoglycans in the CNS injury response. Progress in Brain Research 137:313-332.

Mukhopadhyay G, Doherty P, Walsh FS, Crocker PR, Filbin MT (1994) A novel role for myelin-associated glycoprotein as an inhibitor of axonal regeneration. Neuron 13:757-767.

Nacher J, Rosell DR, McEwen BS (2000) Widespread expression of rat collapsin response-mediated protein 4 in the telencephalon and other areas of the adult rat central nervous system. Journal of Comparative Neurology 424:628-639.

Narumiya S, Ishizaki T, Watanabe N (1997) Rho effectors and reorganization of actin cytoskeleton. FEBS Lett 410:68-72.

National Spinal Cord Injury Statistical Center, Birmingham, Alabama http://www.spinalcord.uab.edu/show.asp?durki=116979

Neumann S, Bradke F, Tessier-Lavigne M, Basbaum AI (2002) Regeneration of sensory axons within the injured spinal cord induced by intraganglionic cAMP elevation. Neuron 34:885-893.

Niederost B, Oertle T, Fritsche J, McKinney RA, Bandtlow CE (2002) Nogo-A and myelin-associated glycoprotein mediate neurite growth inhibition by antagonistic regulation of RhoA and Rac1. J Neurosci 22:10368-10376.

Nishimura T, Kato K, Yamaguchi T, Fukata Y, Ohno S, Kaibuchi K (2004) Role of the PAR-3-KIF3 complex in the establishment of neuronal polarity. Nature Cell Biology 6:328-334.

Nishimura T, Fukata Y, Kato K, Yamaguchi T, Matsuura Y, Kamiguchi H, Kaibuchi K (2003) CRMP-2 regulates polarized Numb-mediated endocytosis for axon growth. Nature Cell Biology 5:819-826.

Niwa R, Nagata-Ohashi K, Takeichi M, Mizuno K, Uemura T (2002) Control of actin reorganization by Slingshot, a family of phosphatases that dephosphorylate ADF/cofilin. Cell 108:233-246.

Oertle T, van der Haar ME, Bandtlow CE, Robeva A, Burfeind P, Buss A, Huber AB, Simonen M, Schnell L, Brosamle C, Kaupmann K, Vallon R, Schwab ME (2003) Nogo-A inhibits neurite outgrowth and cell spreading with three discrete regions. J Neurosci 23:5393-5406.

Park JB, Yiu G, Kaneko S, Wang J, Chang J, He Z (2005) A TNF Receptor Family Member, TROY, Is a Coreceptor with Nogo Receptor in Mediating the Inhibitory Activity of Myelin Inhibitors. Neuron 45:345-351.

Paszkowiak JJ, Maloney SP, Kudo FA, Muto A, Teso D, Rutland RC, Westvik TS, Pimiento JM, Tellides G, Sessa WC, Dardik A (2007) Evidence supporting changes in Nogo-B levels as a marker of neointimal expansion but not adaptive arterial remodeling. Vascular Pharmacology 46:293-301.

Pfenninger KH, Laurino L, Peretti D, Wang X, Rosso S, Morfini G, Caceres A, Quiroga S (2003) Regulation of membrane expansion at the nerve growth cone. Journal of Cell Science 116:1209-1217.

Pignot V, Hein AE, Barske C, Wiessner C, Walmsley AR, Kaupmann K, Mayeur H, Sommer B, Mir AK, Frentzel S (2003) Characterization of two novel proteins, NgRH1 and NgRH2, structurally and biochemically homologous to the Nogo-66 receptor. J Neurochem 85:717-728.

Prinjha R, Moore SE, Vinson M, Blake S, Morrow R, Christie G, Michalovich D, Simmons DL, Walsh FS (2000) Inhibitor of neurite outgrowth in humans. Nature 403:383-384.

Qi B, Qi Y, Watari A, Yoshioka N, Inoue H, Minemoto Y, Yamashita K, Sasagawa T, Yutsudo M (2003) Pro-apoptotic ASY/Nogo-B protein associates with ASYIP. Journal of Cellular Physiology 196:312-318.

Qiu J, Cai D, Dai H, McAtee M, Hoffman PN, Bregman BS, Filbin MT (2002) Spinal axon regeneration induced by elevation of cyclic AMP. Neuron 34:895-903.

Quinn CC, Gray GE, Hockfield S (1999) A family of proteins implicated in axon guidance and outgrowth. Journal of Neurobiology 41:158-164.

Quinn CC, Chen E, Kinjo TG, Kelly G, Bell AW, Elliott RC, McPherson PS, Hockfield S (2003) TUC-4b, a novel TUC family variant, regulates neurite outgrowth and associates with vesicles in the growth cone. Journal of Neuroscience 23:2815-2823.

Reier PJ, Perlow MJ, Guth L (1983) Development of embryonic spinal cord transplants in the rat. Brain Research 312:201-219.

Riento K, Ridley AJ (2003) Rocks: multifunctional kinases in cell behaviour. Nat Rev Mol Cell Biol 4:446-456.

Savio T, Schwab ME (1989) Rat CNS white matter, but not gray matter, is nonpermissive for neuronal cell adhesion and fiber outgrowth. J Neurosci 9:1126-1133.

Schnell L, Schwab ME (1990) Axonal regeneration in the rat spinal cord produced by an antibody against myelin-associated neurite growth inhibitors. Nature 343:269-272.

Seiler N, Badolo L, Duranton B, Vincent F, Schneider Y, Gosse F, Raul F (2000) Effect of the polyamine oxidase inactivator MDL 72527 on N(1)-(n-octanesulfonyl)spermine toxicity. International Journal of Biochemistry & Cell Biology 32:1055-1068.

Shao Z, Browning JL, Lee X, Scott ML, Shulga-Morskaya S, Allaire N, Thill G, Levesque M, Sah D, McCoy JM, Murray B, Jung V, Pepinsky RB, Mi S (2005) TAJ/TROY, an Orphan TNF Receptor Family Member, Binds Nogo-66 Receptor 1 and Regulates Axonal Regeneration. Neuron 45:353-359.

Shi SH, Cheng T, Jan LY, Jan YN (2004) APC and GSK-3beta are involved in mPar3 targeting to the nascent axon and establishment of neuronal polarity. Curr Biol 14:2025-2032.

Shimizu Y, Thumkeo D, Keel J, Ishizaki T, Oshima H, Oshima M, Noda Y, Matsumura F, Taketo MM, Narumiya S (2005) ROCK-I regulates closure of the eyelids and ventral body wall by inducing assembly of actomyosin bundles. Journal of Cell Biology 168:941-953.

Siddiqui SS, Culotti JG (1991) Examination of neurons in wild type and mutants of Caenorhabditis elegans using antibodies to horseradish peroxidase. Journal of Neurogenetics 7:193-211.

Simonen M, Pedersen V, Weinmann O, Schnell L, Buss A, Ledermann B, Christ F, Sansig G, van der Putten H, Schwab ME (2003) Systemic deletion of the myelin-associated outgrowth inhibitor nogo-a improves regenerative and plastic responses after spinal cord injury. Neuron 38:201-211.

Sivasankaran R, Pei J, Wang KC, Zhang YP, Shields CB, Xu XM, He Z (2004) PKC mediates inhibitory effects of myelin and chondroitin sulfate proteoglycans on axonal regeneration. Nat Neurosci 7:261-268.

Slotkin TA, Bartolome J (1986) Role of ornithine decarboxylase and the polyamines in nervous system development: a review. Brain Res Bull 17:307-320.

Snider WD, Zhou FQ, Zhong J, Markus A (2002) Signaling the pathway to regeneration. Neuron 35:13-16.

Steward O, Zheng B, Banos K, Yee KM (2007) Response to: Kim et al., "axon regeneration in young adult mice lacking Nogo-A/B." Neuron 38, 187-199.[comment]. Neuron 54:191-195.

Suzuki Y, Nakagomi S, Namikawa K, Kiryu-Seo S, Inagaki N, Kaibuchi K, Aizawa H, Kikuchi K, Kiyama H (2003) Collapsin response mediator protein-2 accelerates axon regeneration of nerve-injured motor neurons of rat. Journal of Neurochemistry 86:1042-1050.

Thumkeo D, Keel J, Ishizaki T, Hirose M, Nonomura K, Oshima H, Oshima M, Taketo MM, Narumiya S (2003) Targeted disruption of the mouse rho-associated kinase 2 gene results in intrauterine growth retardation and fetal death. Molecular & Cellular Biology 23:5043-5055.

Tsim TY, Wong EY, Leung MS, Wong CC (2004) Expression of axon guidance molecules and their related genes during development and sexual differentiation of the olfactory bulb in rats. Neuroscience 123:951-965.

Venkatesh K, Chivatakarn O, Sheu SS, Giger RJ (2007) Molecular dissection of the myelin-associated glycoprotein receptor complex reveals cell type-specific mechanisms for neurite outgrowth inhibition. Journal of Cell Biology 177:393-399.

Venkatesh K, Chivatakarn O, Lee H, Joshi PS, Kantor DB, Newman BA, Mage R, Rader C, Giger RJ (2005) The Nogo-66 receptor homolog NgR2 is a sialic acid-dependent receptor selective for myelin-associated glycoprotein. J Neurosci 25:808-822.

Vincent S, Settleman J (1997) The PRK2 kinase is a potential effector target of both Rho and Rac GTPases and regulates actin cytoskeletal organization. Molecular & Cellular Biology 17:2247-2256.

Wang KC, Kim JA, Sivasankaran R, Segal R, He Z (2002a) P75 interacts with the Nogo receptor as a co-receptor for Nogo, MAG and OMgp. Nature 420:74-78.

Wang KC, Koprivica V, Kim JA, Sivasankaran R, Guo Y, Neve RL, He Z (2002b) Oligodendrocyte-myelin glycoprotein is a Nogo receptor ligand that inhibits neurite outgrowth. Nature 417:941-944.

Wang LH, Strittmatter SM (1996) A family of rat CRMP genes is differentially expressed in the nervous system. J Neurosci 16:6197-6207.

Wang LH, Strittmatter SM (1997a/b) Brain CRMP forms heterotetramers similar to liver dihydropyrimidinase. Journal of Neurochemistry 69:2261-2269.

Winton MJ, Dubreuil CI, Lasko D, Leclerc N, McKerracher L (2002) Characterization of new cell permeable C3-like proteins that inactivate Rho and stimulate neurite outgrowth on inhibitory substrates. J Biol Chem 277:32820-32829.

Wong ST, Henley JR, Kanning KC, Huang KH, Bothwell M, Poo MM (2002) A p75(NTR) and Nogo receptor complex mediates repulsive signaling by myelin-associated glycoprotein. Nat Neurosci 5:1302-1308.

Yamashita T, Tohyama M (2003) The p75 receptor acts as a displacement factor that releases Rho from Rho-GDI. Nat Neurosci 6:461-467.

Yang N, Higuchi O, Ohashi K, Nagata K, Wada A, Kangawa K, Nishida E, Mizuno K (1998) Cofilin phosphorylation by LIM-kinase 1 and its role in Rac-mediated actin reorganization. Nature 393:809-812.

Yaron A, Zheng B (2007) Navigating their way to the clinic: emerging roles for axon guidance molecules in neurological disorders and injury. Developmental Neurobiology 67:1216-1231.

Yoshimura T, Kawano Y, Arimura N, Kawabata S, Kikuchi A, Kaibuchi K (2005) GSK-3beta regulates phosphorylation of CRMP-2 and neuronal polarity. Cell 120:137-149.

Zheng B, Ho C, Li S, Keirstead H, Steward O, Tessier-Lavigne M (2003) Lack of enhanced spinal regeneration in nogo-deficient mice. Neuron 38:213-224.

Zheng B, Atwal J, Ho C, Case L, He XL, Garcia KC, Steward O, Tessier-Lavigne M (2005) Genetic deletion of the Nogo receptor does not reduce neurite inhibition in vitro or promote corticospinal tract regeneration in vivo. Proc Natl Acad Sci U S A 102:1205-1210.

Zhou FQ, Snider WD (2005) Cell biology. GSK-3beta and microtubule assembly in axons. Science 308:211-214.

Zuo J, Neubauer D, Dyess K, Ferguson TA, Muir D (1998) Degradation of chondroitin sulfate proteoglycan enhances the neurite-promoting potential of spinal cord tissue. Exp Neurol 154:654-662.

CHAPTER 2

II. NEURONAL RESPONSES TO MYELIN ARE MEDIATED BY ROCK

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Neuronal responses to myelin are mediated by ROCK

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II.1. PREFACE

As described in Chapter 1 above, MAIs stimulate responsive neurons after CNS injury and activate the small GTPase RhoA to mediate neurite outgrowth inhibition. RhoA-interacting proteins represent potential targets for therapy. The main objective of this chapter was to determine the involvement and the relative contribution of ROCK, a known RhoA effector, in the Nogo-66 signalling pathway.

II.2. ABSTRACT

CNS myelin inhibits axon growth due to the expression of several growth inhibitory proteins, including myelin-associated glycoprotein, oligodendrocyte myelin glycoprotein and Nogo. Myelin associated inhibitory proteins activate Rho GTPase in responsive neurons. Rho kinase has been implicated as a critical rho effector in this pathway due to the ability of the pharmacological inhibitor Y-27632 to circumvent myelin-dependent inhibition. Y-27632, however inhibits the activity of additional kinases. Using three independent approaches, we provide direct evidence that ROCKII is activated in response to the myelin-associated inhibitor Nogo. We demonstrate that Nogo treatment enhances ROCKII translocation to the cellular membrane in PC12 cells and enhances ROCKII kinase activity towards an *in vitro* substrate. In addition, Nogo treatment enhances phosphorylation of myosin light chain II, a known ROCK substrate. Further, we demonstrate that primary dorsal root ganglia (DRG) neurons can be rendered insensitive to the inhibitory effects of myelin via infection with dominant negative ROCKII. Together this data provides direct evidence for a Rho-ROCK-MLC-II signalling cascade in response to myelin-associated inhibitors.

Key Words: CNS regeneration, rho kinase, protein kinase c related kinase, myelin inhibition.

Running Title: Myelin dependent ROCKII activation

II.3. INTRODUCTION

Neurons in the adult mammalian central nervous system (CNS) do not spontaneously regenerate after injury due to the combination of an inhibitory environment and a lack of positive cues. CNS myelin contains several growth inhibitory proteins, including myelin-associated glycoprotein (McKerracher et al., 1994; Mukhopadhyay et al., 1994), oligodendrocyte myelin glycoprotein (Kottis et al., 2002; Wang et al., 2002b), and Nogo (Chen et al., 2000; GrandPre et al., 2000; Prinjha et al., 2000). These myelin-associated inhibitors (MAIs) exert their effects via a common, tripartite neuronal receptor complex consisting of Nogo receptor (NgR) (Fournier et al., 2001), $p75^{NTR}$ (Wang et al., 2002a; Wong et al., 2002), and Lingo1 (Mi et al., 2004).

Intracellularly, Rho GTPase plays a critical role in transducing MAI signals to the actin cytoskeleton. Rho GTPase is activated in response to MAIs (Dergham et al., 2002; Niederost et al., 2002; Fournier et al., 2003), and blockade of Rho function with C3, a Rho-specific ADP ribosyltransferase, partially circumvents myelin-dependent inhibition (Jin and Strittmatter, 1997; Lehmann et al., 1999; Dergham et al., 2002). This pathway has been particularly well studied in response to Nogo-66, a potent inhibitory component of full length Nogo-A. Many effector proteins downstream of Rho modulate actin cytoskeletal dynamics and are reasonable candidates for the transduction of myelin-associated signals, including citron kinase, mDia, rho kinase and protein kinase N (Bishop and Hall, 2000). Rho kinase has been the favored effector candidate due to the ability of the drug Y-27632, a rho kinase antagonist, to promote neuronal outgrowth on MAI substrates *in vitro* and *in vivo* (Dergham et al., 2002; Niederost et al., 2002; Fournier et al., 2003). Rho kinase (ROCK) is a serine/threonine kinase with two isoforms

(p160ROCK/ROCK-I/ROCKβ, and ROCKII/ROKα). ROCKS are ubiquitously expressed in rat tissue(Riento and Ridley, 2003) where they phosphorylate multiple downstream substrates (Riento and Ridley, 2003). It is reasonable to speculate that rho kinase mediates myelin-dependent growth cone collapse by virtue of its ability to regulate myosin light chain phosphorylation and consequently actomyosin dynamics within the growth cone (Amano et al., 1996; Kimura et al., 1996; Dent and Gertler, 2003). However, to date there are no reports directly demonstrating that ROCK is activated in response to MAIs. This is a key issue to the rational design of antagonists to promote axon outgrowth following CNS injury.

The importance of this question is highlighted by several observations. First, treatment with Y-27632 promotes neurite outgrowth on permissive substrates in addition to inhibitory substrates bringing into question the supposition that ROCK is directly activated by MAIs (Fournier et al., 2003). Second, Y-27632 is an ATP-competitive inhibitor which inhibits a number of kinases. The majority of studies published to date have used Y-27632 at a concentration of 10 μM. At this concentration Y-27632 also inhibits protein-kinase C related kinase (PRK2/PKNγ). PRK2 is a serine/threonine kinase which binds to Rho and regulates cytoskeletal dynamics affecting actin stress fiber formation (Vincent and Settleman, 1997) and cell-cell adhesions (Calautti et al., 2002). Other studies have used Y-27632 at concentrations up to 50 μM *in vitro*, a concentration at which other kinases are inhibited (Davies et al., 2000). This is also relevant to *in vivo* studies examining the effects of Y-27632 in spinal cord injury models where it is difficult to accurately estimate the active concentration of the drug (Dergham et al., 2002; Fournier et al., 2003). Finally, the notion that myosin II, the presumptive target of ROCK

in this pathway, provides the critical activity for retrograde actin flow and neurite retraction has recently been challenged (Brown and Bridgman, 2003).

To resolve these ambiguities, we set out to determine if ROCKII is directly activated by MAI signals. Using two independent approaches, a membrane translocation assay and an *in vitro* phosphorylation assay, we find that ROCKII is directly activated in response to Nogo stimulation. In contrast PRK2, although expressed in many types of neurons, is not activated. Further, we demonstrate a ROCK-dependent increase in myosin light chain II (MLCII) phosphorylation in response to MAIs. We find that dominant negative ROCKII, a more specific blocking reagent than Y-27632, blocks myelin-dependent growth cone collapse and neurite outgrowth inhibition. Together, this data provides a direct demonstration of a critical Rho-ROCK-MLCII signalling cascade in response to MAIs.

II.4. MATERIALS AND METHODS

II.4.1. Plasmid construction and recombinant HSV preparation

To construct pHSVmycDNROCK, a dominant negative ROCKII fragment was amplified by polymerase chain reaction (PCR) from pMalC2-Rho-kinase/RB/PH(TT) (a gift from Dr. Kozo Kaibuchi, Nagoya University) using a 5'primer containing a c-Myc epitope sequence. The PCR product was ligated into the *BamHI*- and *EcoRI*-site of pHSVprPUC (provided by Dr. Rachel Neve, Harvard Medical School). The resulting plasmid was transfected into 2-2 Vero cells which were superinfected with 5*dl* 1.2 HSV helper virus 1 day later. Recombinant virus was amplified through three passages and stored at −80°C as previously described (Neve et al., 1997).

II.4.2. Preparation of myelin and recombinant proteins

To purify alkaline-phosphatase-conjugated Nogo-66 (AP-Nogo-66) or alkaline phosphatase (AP), conditioned medium was collected from HEK293A cells stably transfected with pcDNA3.1AP-Nogo66-His (generously provided by Dr. Stephen Strittmatter, Yale University) or HEK293T cells transiently transfected with pcDNA-AP-His. Secreted protein was purified by Ni^{2+} affinity chromatography (Nakamura et al., 1998). For all cellular treatments AP-Nogo66-His (8nM) or AP (8nM) was preaggregated with 100 ng/ml anti-human AP (Niederost et al., 2002). Glutathione *S*-transferase (GST) or GST-Nogo-66 were expressed in Escherichia coli and purified on glutathione-Sepharose (Amersham Pharmacia Biosciences, Baie D'Urfe, Quebec) as previously described (GrandPre et al., 2000). GST-Nogo-66 was added at a final concentration of 50 nM. Myelin extracts were prepared from the bovine brain as previously described (Igarashi et al., 1993). After clarification of the myelin extract by centrifugation at 400,000*g*, the detergent was removed by dialyzing with PBS.

RT PCR

Total RNA was prepared using an RNeasy kit (Qiagen, Mississauga, Ontario). cDNA libraries were made with a Thermoscript Reverse Transcriptase kit (Invitrogen, Burlington, Ontario). Primers for PCR detection were designed to span an intron boundary. ROCKII primers: 5'-AGATCAGTGCAGCGGCTATT-3', 5'-GAATTTGGCTCTCTTCAGC-3'. PRK2 primers: 5'-CTCCATGGTACAGCTCAGCA-3', 5'-CTTGTTCGACTGCGACGG-3'.

II.4.3. Immunofluorescence

Cerebellar neurons were dissected from P8 rat brain, dissociated with trypsin and mechanical trituration and cultured on poly-L-lysine-coated substrates for 24 hours in Sato medium (Dulbecco's Modified Eagle Medium with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, 1% N2 Supplement, 400 ng/mL triiodothyronine and 400 ng/mL tetraiodothyronine). Cultures were fixed with 4% paraformaldehyde, permeabilized in 0.2% triton X-100, and incubated with anti-ROCKII or anti-PRK2 antibody (BD Bioscience, Mississauga, Ontario), followed by a CY2-conjugated secondary antibody.

II.4.4. *In vitro* kinase assay

An S6 Kinase Assay kit (Upstate Biotechnology, Waltham, MA) was used to measure the kinase activity of ROCKII and PRK2 *in vitro*. PC-12 cells were transfected for 24 hours with pCDNA3-Flag-PRK2 (a gift from Dr. Jeff Settleman, Harvard Medical School) or pCAG-myc-mROCK II (a gift from Dr. Shuh Narumiya, Kyoto University) using Lipofectamine 2000 (Invitrogen, Burlington, Ontario). The cells were then differentiated with 50 ng/ml nerve growth factor (Upstate Biotechnology, Waltham, MA) in serum free media for 24 hours (Roswell Park Memorial Institute 1640, 5% BSA, 1% penicillin-streptomycin). Cells were treated with preclustered AP or AP-Nogo-66 for 10 min, washed twice with ice-cold PBS and lysed in 50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% (v/v) Triton X-100, 1 mM sodium fluoride (NaF), 1 mM sodium orthovanadate (Na_3VO_4), 1 mM phenylmethylsulfonyl fluoride (PMSF) and Cømplete protease inhibitors (Roche Diagnostics, Laval PQ). The supernatant fractions containing

2 mg of proteins were precleared with protein A/G-agarose (Santa Cruz Biotechnology, Santa Cruz, CA) for 20 min at 4 °C and then ROCKII and PRK2 were immunoprecipitated with myc-agarose or flag-agarose antibody at 4 °C for 2 hours (Sigma Chemical Co., Oakville, Ontario). After washing three times with 50 mM Tris (pH 7.4), 150 mM NaCl, the immunoprecipitates were analyzed for *in vitro* kinase activity as per manufacturers instructions. As a control, ROCKII and PRK2 kinase activity were blocked with 10 μM Y-27632 (Calbiochem, San Diego, Ca). For the PRK2 assay, the PKC inhibitor, which is included in the kit's assay mixture, was replaced with assay dilution buffer. In parallel with the *in vitro* kinase assay, ROCKII and PRK2 were immunoprecipitated from 1 mg of transfected cell lysates and analyzed by western blotting to ensure equal immunoprecipitation of the relevant kinase for each treatment. After substraction of background from each of the samples, the kinase activity was normalized relative to total ROCKII or PRK2 levels detected by western blotting. The AP-Nogo-66 treatment values were then normalized to AP controls and expressed as fold change. To assess the non-specific *in vitro* kinase activity from the immunoprecipitation, in vitro kinase activity associated with myc-agarose or flag-agarose immunoprecipitations from 2 mg of mock-transfected cell lysates was analyzed. The amounts of incorporated phosphate from mock immunoprecipitation and from control treated samples were compared. Myc-agarose and Flag-agarose immunoprecipitates from mock transfected cells account for 1.5% and 30.9% of the control ROCK2 and PRK2 kinase activity respectively.

II.4.5. PC12 cell fractionation

PC12 cells were grown to sub-confluence on collagen-coated plates then differentiated for 24 hours with 50 ng/mL NGF (Upstate Biotechnology, Waltham, MA). After stimulation with Nogo-66 or the appropriate control ligand, cells were washed twice in ice cold Tris-buffered saline (TBS), and harvested in lysis buffer A (20 mM HEPES, pH 7.3, 150 mM NaCl) with 2 mM Na_3VO_4, 1 mM PMSF and Cømplete protease inhibitors (Roche Products, Laval, Quebec). Cells were sonicated on ice, after which they were centrifuged for 10 minutes at 800 x g, to remove any unlysed cells. Supernatants were then centrifuged at 100,000 x g for 30 minutes. Supernatants containing the cytosolic fraction were removed, the membrane fraction-containing pellets were washed twice with lysis buffer A, and resuspended in RIPA buffer (TBS containing 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS). Protein concentrations were determined with a detergent compatible protein assay (Bio-Rad, Mississauga, Ontario). ROCKII or PRK2 protein content in the membrane fraction was analyzed by separating the lysates by SDS-PAGE on 4-15% gradient gels, transfer to polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Mississauga, Ontario) and probing with anti-ROCKII or anti-PRK2 antibodies. Membrane fractions were analyzed with anti-p75 antibody (generously provided by Dr. Phil Barker, McGill University) as a control for equal protein loading. Bands were analyzed by densitometry using ImageJ analysis software.

II.4.6. Growth cone collapse and neurite outgrowth assays

For growth cone collapse experiments, E13 chick DRG explants were cultured in DRG media (F-12 medium, 10% FBS, 1% penicillin/streptomycin, 1% L-glutamine, 50

ng/ml NGF) on poly-L-lysine- and laminin- coated substrates for 18 hours. For viral infections, recombinant viral preparations were added to the media 1 hour after plating. After 18 hours, cultures were treated and fixed with 4% paraformaldehyde/20% sucrose/ PBS and double stained with rhodamine-phalloidin (Molecular Probes, Eugene, OR) and anti-myc antibody to assess the mycDNROCK infection efficiency. For the HA1077 experiments, cultures were treated with 10 μM of HA1077 (Calbiochem, San Diego, Ca) for 30 minutes prior to myelin treatment.

For neurite outgrowth assays, Nogo or myelin was dried down on poly-L-lysine-coated substrates. Substrates were washed and coated with 10 μg/ml laminin for 1 hr. To examine the effects of HA1077, dissociated E13 chick dorsal root ganglia (DRG) neurons were grown in the presence or absence of 10 μM HA1077 for 4-6 hours, fixed and stained with rhodamine phalloidin. For HSVDNROCK experiments, dissociated E13 chick DRG neurons were grown in the presence of virus for 24 hours and double stained with anti-βIII tubulin and anti-myc antibody. For shorter term outgrowth experiments examining the effects of HA1077, potent inhibition could be obtained with approximately 100 ng of myelin substrate. For 24 hour experiments with the DNROCK virus potent inhibition was achieved with 400 ng of myelin. Neurite outgrowth lengths per cell were assessed using Image J, a public domain JAVA image processing program (http://rsb.info.nih.gov/ij/) as previously described (Fournier et al., 2003).

II.4.7. MLC phosphorylation

PC12 cells were differentiated for 24 hours with 50 ng/ml NGF and treated with pre-clustered AP-Nogo-66. Lysates were analyzed by Western blot using anti-phospho-

MLC (Santa Cruz biotechnology, Santa Cruz, CA) and anti-MLC (Sigma Aldrich, Oakville, ON) antibodies. Bands were analyzed by densitometry using ImageJ analysis software

II.5. RESULTS

II.5.1. HA1077 enhances neurite outgrowth on laminin and myelin substrates

Previous studies have shown that Y-27632 attenuates myelin-dependent neurite outgrowth inhibition (Fournier et al., 2003). Similar to Y-27632, HA1077 antagonizes ROCK and PRK (Davies et al., 2000), however it may have distinct activity towards other targets. To address the functional specificity of this drug, we analyzed the effects of HA1077 on myelin-dependent neuronal responses. We first assessed the effect of HA1077 on myelin-dependent growth cone collapse. DRG explants from E13 chick were pre-treated with 10 μM HA1077 prior to a 1 hour exposure to myelin. DRG explants treated with HA1077 are significantly less sensitive to the growth cone collapse activity of myelin (Fig. 1A, C). We also assessed the effect of HA1077 on the neurite outgrowth inhibitory properties of myelin and of Nogo-66. Dissociated E13 chick DRG neurons were cultured for 4 to 6 hours on inhibitory substrates in the presence or absence of 10 μM HA1077. HA1077-treated DRG neurons grow significantly better on myelin and Nogo substrates (Fig. 1B, D, E). These results together with previous data (Fournier et al., 2003), demonstrate that two different pharmacological inhibitors with common target proteins, ROCK and PRK, both promote outgrowth on myelin substrates. This data suggests that either ROCK or PRK may mediate MAI signalling. However, a confounder of these results, and of previous results with Y-27632 treatment, is that these agents also

promote outgrowth on permissive substrates. A more direct demonstration of the regulated activity of ROCK and PRK is required to fully implicate these proteins in MAI signalling.

II.5.2. ROCKII and PRK2 are expressed in PC12 cells and in the rat nervous system

To begin to assess the relative contribution of ROCK and PRK to MAI signalling we analyzed their expression in neural tissue by RT-PCR and immunofluorescence. mRNA for ROCKII and PRK2 is present in rat brain, cerebellum, DRGs, and in PC12 cells (Fig. 2A). To analyze the distribution of ROCKII and PRK2 in primary neurons, dissociated P8 rat cerebellar neurons were immunostained with anti-ROCKII and anti-PRK2 antibodies. ROCKII and PRK2 are present in both cell bodies and neurites of dissociated cerebellar neurons (Fig. 2B).

II.5.3. Nogo-66 modulates the kinase activity of ROCKII but not of PRK2

Binding to active RhoA enhances the kinase activity of both ROCKII and PRK2 (Leung et al., 1995; Ishizaki et al., 1996; Matsui et al., 1996b). To directly assess the activity of ROCKII and PRK2 in the MAI pathway, we assessed their kinase activities towards S6 kinase substrate *in vitro* (Borisoff et al., 2003) following treatment with Nogo-66, a potent inhibitory fragment of Nogo-A (GrandPre et al., 2000). Nogo-66 was chosen as a potent soluble ligand which contributes significantly to the inhibitory activity of MAIs (GrandPre et al., 2000). Evidence to date suggests that the intracellular signalling cascades engaged by Nogo-66 binding to NgR are similar to those of other MAIs. PC12 cells transfected with myc-ROCKII or flag-PRK2 were treated for 10 min

with AP or AP-Nogo-66. ROCKII and PRK2 were immunoprecipitated from cell lysates using anti-myc-agarose or anti-flag-agarose respectively, and tested for their ability to phosphorylate the S6 substrate using [γ-^{32}P] ATP. Equal immunoprecipitations for control and treated samples were verified by western blot analysis (Fig. 3B). Kinase activity was normalized to control (AP-stimulation) for each experiment. Treatment of PC12 cells with AP-Nogo-66 for 10 minutes causes a 91% increase in ROCKII kinase activity (Fig. 3A). Treatment with Y-27632 blocks the kinase activity of ROCKII by 96% as anticipated. PRK2 is also able to phosphorylate the S6 kinase substrate *in vitro* (Zhu et al., 2004), although less efficiently than ROCKII (data not shown). Treatment of PC12 cells with FBS enhances PRK2 kinase activity, presumably due to lysophosphatidic acid (LPA)- dependent stimulation of Rho (Kranenburg et al., 1999) (Fig. 3A), however Nogo treatment has no direct effect on PRK2 kinase activity (Fig. 3A). PRK2 kinase activity is also inhibited by treatment with Y-27632. Together, this data demonstrates that Nogo-66 stimulates the kinase activity of ROCKII but not PRK2.

II.5.4. Nogo-66 induces ROCKII translocation to the membrane in PC12 cells

To verify that Nogo can activate ROCKII in an independent assay, we examined the ability of Nogo-66 to stimulate ROCKII translocation to the cell membrane. ROCKII has been previously shown to translocate to the membrane in cultured cells transfected with constitutively active RhoAV14 (Leung et al., 1995). Differentiated PC12 cells were stimulated with GST-Nogo-66 for 0.5, 15 min and 60 min. Membrane fractions were isolated and analyzed for ROCKII content by SDS-PAGE and western blotting. GST-Nogo-66 treatment increases the translocation of ROCKII from the cytosol to the

membrane by 30 seconds and ROCKII is retained at the membrane at 60 min (Fig. 4A, B). Control GST treatment has no effect on ROCKII translocation (data not shown). These results demonstrate that Nogo-66 treatment enhances the recruitment of ROCKII to the membrane providing independent evidence that ROCKII is activated by Nogo.

II.5.5. Nogo-66 mediates phosphorylation of myosin light chain

To examine potential downstream targets of ROCKII in the MAI signalling cascade, we examined the effect of Nogo-treatment on myosin light chain II (MLCII) phosphorylation. MLCII is a substrate for ROCKII phosphorylation, which can regulate actomyosin dynamics. Differentiated PC12 cells were stimulated with AP-Nogo-66, and lysates were analyzed by western blotting with anti-phospho-MLCII antibody. MLCII phosphorylation is rapidly (30 seconds) increased following Nogo-66 stimulation and this is maintained at 30 minutes (Fig. 5A, B). The ability of Y-27632 to block the Nogo-dependent increase in MLCII phosphorylation was also assessed. Pre-treatment of cells with 10 μM Y-27632 prior to Nogo stimulation completely blocks the Nogo-dependent increase in MLCII phosphorylation (Fig. 5A, B).

II.5.6. Dominant negative ROCKII protects neurons from myelin inhibition

To directly assess the effect of ROCK antagonism on neuronal responses to myelin, we examined DRG growth cone collapse and neurite outgrowth following neuronal infection with dominant negative ROCK (DNROCK). E13 DRG explants were infected with mycDNROCK recombinant viral preparations for 24 hours prior to myelin stimulation and assessment of growth cone collapse. The efficiency of viral infection was

verified by immunofluorescence for myc (Fig. 6A). We further verified that recombinant mycDNROCK protein is efficiently transported into DRG neurites and growth cones (Fig. 6B). Infection of neurons with DNROCK significantly decreases the percentage of collapsed growth cones in response to myelin (Fig. 6C, E).

To analyze the effects of DNROCK on neurite outgrowth inhibition, dissociated E13 chick DRG neurons were infected with DNROCK or control HSV recombinant viral preparations and tested for their ability to grow on myelin substrates. DRG neurite outgrowth is significantly improved on inhibitory myelin substrates in neurons infected with DNROCK compared to control HSV preparations (Fig. 6D, F). Taken together, these results demonstrate that antagonism of ROCKII is sufficient to enhance neuronal outgrowth on permissive and inhibitory substrates.

II.6. DISCUSSION

Previous data has implicated ROCK proteins in the MAI signalling cascade. By in vitro phosphorylation and membrane translocation assays we provide the first direct evidence that ROCKII is activated in response to Nogo-66 stimulation. Further, we find that the ROCKII effector MLCII is phosphorylated in response to stimulation with Nogo-66. We are unable to detect Nogo-dependent activation of PRK2, a kinase which is equally inhibited by many ROCK small molecule inhibitors such as Y-27632 and HA1077. Functional studies further demonstrate that blockade of ROCKII activity with a previously characterized DNROCK construct circumvents myelin-dependent growth cone collapse and neurite outgrowth inhibition.

Targeting intracellular signalling substrates has proven to be an effective approach to antagonize the inhibitory environment at the CNS lesion site (Dergham et al., 2002; Fournier et al., 2003). This approach may be more advantageous than targeting inhibitory ligands or their neuronal receptors for several reasons. First, the efficacy of blocking individual inhibitors is limited by the presence of multiple ligands in CNS myelin (1-7). Blockade of individual ligands with function blocking antibodies has met with some success (Fouad et al., 2001). However gene knockout studies of individual inhibitory ligands have either proven ineffective in CNS injury models (Bartsch et al., 1995), or difficult to interpret due to different phenotypes in different strains, and age-dependent responses (Kim et al., 2003; Simonen et al., 2003; Zheng et al., 2003). Antagonism of NgR, a common binding partner for all 3 MAIs has also yielded promising effects in a variety of injury models (GrandPre et al., 2002; Lee et al., 2004; Li et al., 2004a; Li et al., 2004b), although positive effects on regeneration in NgR knockout mice are restricted to select neuronal populations (Kim et al., 2004; Zheng et al., 2005). Further, other members of the NgR family (NgR2 and NgR3) have now been identified (Pignot et al., 2003) which may contribute to the transduction of MAI signals (Venkatesh et al., 2005). Similarly, regeneration in $p75^{NTR}$ null mice has been disappointing (Song et al., 2004) and this may be partially attributed to heterogeneity in co-receptor requirements based on neuronal cell types (Park et al., 2005; Shao et al., 2005). A promising aspect of experiments utilizing small molecule inhibitors such as Y-27632 has been their ability to antagonize multiple inhibitory influences including MAIs (Dergham et al., 2002; Fournier et al., 2003) and components of the glial scar (Borisoff et al., 2003). However, the effects of these drugs on multiple physiological processes in many cell

types (Riento and Ridley, 2003) highlights the need to better understand the intracellular signalling substrates with the aim of developing more specific and potent antagonists.

The direct demonstration of Nogo-regulated ROCKII activity in membrane translocation and *in vitro* kinase assays strongly implicates ROCKII as a critical target in this pathway. The ability of a dominant negative ROCKII reagent to circumvent myelin inhibition further suggests that it plays a critical role in myelin-dependent inhibition. However, a ROCKI contribution to the functional effects of MAIs cannot be ruled out due to the ability of the DNROCK construct to also antagonize ROCKI, the less abundant isoform in the CNS (Kobayashi et al., 2004). DNROCK is a truncated protein lacking its kinase domain and containing a double mutation that prevents its binding to Rho (Amano et al., 1997). It is thought to exert its dominant negative effect by binding to and inhibiting the kinase domain of endogenous wild type ROCK. The ability of DNROCK to inhibit wild type ROCK without binding to Rho, suggests that the effects seen on growth cone collapse and neurite outgrowth inhibition are mediated by effectors downstream of ROCK. The ability of DNROCK to promote neurite outgrowth on control substrates is consistent with the neurite outgrowth promoting activity of pharmacological antagonists suggesting that basal ROCK activation also modulates neurite outgrowth under permissive conditions. It is also intriguing that DNROCK is a more efficient antagonist of myelin dependent outgrowth inhibition than HA1077. While it is possible that 10 μm HA1077 does not block ROCKII activity as efficiently as DNROCK, it is also possible that DNROCK may sequester additional target substrates that are critical for myelin-dependent inhibition. The inability of HA1077 or DNROCK to completely circumvent

myelin inhibition also raises the possibility that additional Rho effectors may be involved in this process.

While ROCKII has many substrates (Riento and Ridley, 2003), in neuronal cells the Rho-ROCK-actomyosin pathway leads to growth cone collapse and neurite retraction, and inhibition of this pathway promotes neurite outgrowth (Amano et al., 1998). ROCK's ability to drive actomyosin contractility is largely attributed to its ability to regulate MLCII both by direct phosphorylation and by phosphorylation and inhibition of MLC phosphatase (Fujita et al., 2001). The mechanism by which MLCII promotes neurite outgrowth is not completely understood. While it is clear that blockade of actin retrograde flow with general myosin inhibitors such as BDM (2,3-butanedione monoxime) correlates with growth at the leading edge of *Aplysia* neurons (Cheung et al., 2002), inactivation of myosin isoforms via micro-chromophore-assisted laser inactivation suggests that myosin 1C, not IIB may be the critical isoform for mediating retrograde F-actin flow (Diefenbach et al., 2002). Irrespective of the mechanism, functional data for the critical role of myosinII in neurite outgrowth continues to mount. In one study examining the mechanism of neurite retraction, inhibition of ROCK with Y-27632 or of myosinII with bebbistatin blocked axon retraction following an *in vitro* cut. This type of effect correlates well with the protective effects on growth inhibitory substrates (Gallo, 2004). Together, this data encourages further study of the basic cellular mechanisms of growth cone collapse and neurite retraction in the context of inhibitory influences.

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II.7. REFERENCES

Amano M., Kruse H. J., Kaibuchi K., Weber P. C. and Aepfelbacher M. (1998) Myosin II activation promotes neurite retraction during the action of Rho and Rho-kinase. *Journal of Biological Chemistry* **273,** 21867-21874.

Amano M., Chihara K., Kimura K., Fukata Y., Nakamura N., Matsuura Y. and Kaibuchi K. (1997) Formation of actin stress fibers and focal adhesions enhanced by Rho-kinase. *Science* **275,** 1308-1311.

Amano M., Ito M., Kimura K., Fukata Y., Chihara K., Nakano T., Matsuura Y. and Kaibuchi K. (1996) Phosphorylation and activation of myosin by Rho-associated kinase (Rho- kinase). *J Biol Chem* **271,** 20246-20249.

Bartsch U., Bandtlow C. E., Schnell L., Bartsch S., Spillmann A. A., Rubin B. P., Hillenbrand R., Montag D., Schwab M. E. and Schachner M. (1995) Lack of evidence that myelin-associated glycoprotein is a major inhibitor of axonal regeneration in the CNS. *Neuron* **15,** 1375-1381.

Bishop A. L. and Hall A. (2000) Rho GTPases and their effector proteins. *Biochem J* **348 Pt 2,** 241-255.

Borisoff J. F., Chan C. C., Hiebert G. W., Oschipok L., Robertson G. S., Zamboni R., Steeves J. D. and Tetzlaff W. (2003) Suppression of Rho-kinase activity promotes axonal growth on inhibitory CNS substrates. *Mol Cell Neurosci* **22,** 405-416.

Brown M. E. and Bridgman P. C. (2003) Retrograde flow rate is increased in growth cones from myosin IIB knockout mice. *J Cell Sci* **116,** 1087-1094.

Calautti E., Grossi M., Mammucari C., Aoyama Y., Pirro M., Ono Y., Li J. and Dotto G. P. (2002) Fyn tyrosine kinase is a downstream mediator of Rho/PRK2 function in keratinocyte cell-cell adhesion. *J Cell Biol* **156,** 137-148.

Chen M. S., Huber A. B., van der Haar M. E., Frank M., Schnell L., Spillmann A. A., Christ F. and Schwab M. E. (2000) Nogo-A is a myelin-associated neurite outgrowth inhibitor and an antigen for monoclonal antibody IN-1. *Nature* **403,** 434-439.

Cheung A., Dantzig J. A., Hollingworth S., Baylor S. M., Goldman Y. E., Mitchison T. J. and Straight A. F. (2002) A small-molecule inhibitor of skeletal muscle myosin II. *Nat Cell Biol* **4,** 83-88.

Davies S. P., Reddy H., Caivano M. and Cohen P. (2000) Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochem J* **351,** 95-105.

Dent E. W. and Gertler F. B. (2003) Cytoskeletal dynamics and transport in growth cone motility and axon guidance. *Neuron* **40,** 209-227.

Dergham P., Ellezam B., Essagian C., Avedissian H., Lubell W. D. and McKerracher L. (2002) Rho signaling pathway targeted to promote spinal cord repair. *J Neurosci* **22,** 6570-6577.

Diefenbach T. J., Latham V. M., Yimlamai D., Liu C. A., Herman I. M. and Jay D. G. (2002) Myosin 1c and myosin IIB serve opposing roles in lamellipodial dynamics of the neuronal growth cone. *J Cell Biol* **158,** 1207-1217.

Fouad K., Dietz V. and Schwab M. E. (2001) Improving axonal growth and functional recovery after experimental spinal cord injury by neutralizing myelin associated inhibitors. *Brain Res Brain Res Rev* **36,** 204-212.

Fournier A. E., GrandPre T. and Strittmatter S. M. (2001) Identification of a receptor mediating Nogo-66 inhibition of axonal regeneration. *Nature* **409,** 341-346.

Fournier A. E., Takizawa B. T. and Strittmatter S. M. (2003) Rho Kinase inhibition enhances axonal regeneration in the injured CNS. *J. Neurosci* **23,** 1416-1423.

Fujita A., Hattori Y., Takeuchi T., Kamata Y. and Hata F. (2001) NGF induces neurite outgrowth via a decrease in phosphorylation of myosin light chain in PC12 cells. *Neuroreport* **12,** 3599-3602.

Gallo G. (2004) Myosin II activity is required for severing-induced axon retraction in vitro. *Exp Neurol* **189,** 112-121.

GrandPre T., Li S. and Strittmatter S. M. (2002) Nogo-66 receptor antagonist peptide promotes axonal regeneration. *Nature* **417,** 547-551.

GrandPre T., Nakamura F., Vartanian T. and Strittmatter S. M. (2000) Identification of the Nogo inhibitor of axon regeneration as a Reticulon protein. *Nature* **403,** 439-444.

Igarashi M., Strittmatter S. M., Vartanian T. and Fishman M. C. (1993) Mediation by G proteins of signals that cause collapse of growth cones. *Science* **259,** 77-79.

Ishizaki T., Maekawa M., Fujisawa K., Okawa K., Iwamatsu A., Fujita A., Watanabe N., Saito Y., Kakizuka A., Morii N. and Narumiya S. (1996) The small GTP-binding protein Rho binds to and activates a 160 kDa Ser/Thr protein kinase homologous to myotonic dystrophy kinase. *Embo J* **15,** 1885-1893. abs.html.

Jin Z. and Strittmatter S. M. (1997) Rac1 mediates collapsin-1-induced growth cone collapse. *J Neurosci* **17,** 6256-6263.

Kim J. E., Liu B. P., Park J. H. and Strittmatter S. M. (2004) Nogo-66 receptor prevents raphespinal and rubrospinal axon regeneration and limits functional recovery from spinal cord injury. *Neuron* **44,** 439-451.

Kim J. E., Li S., GrandPre T., Qiu D. and Strittmatter S. M. (2003) Axon regeneration in young adult mice lacking nogo-a/b. *Neuron* **38,** 187-199.

Kimura K., Ito M., Amano M., Chihara K., Fukata Y., Nakafuku M., Yamamori B., Feng J., Nakano T., Okawa K., Iwamatsu A. and Kaibuchi K. (1996) Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho- kinase). *Science* **273,** 245-248.

Kobayashi K., Takahashi M., Matsushita N., Miyazaki J., Koike M., Yaginuma H., Osumi N., Kaibuchi K. and Kobayashi K. (2004) Survival of developing motor neurons mediated by Rho GTPase signaling pathway through Rho-kinase. *J Neurosci* **24,** 3480-3488.

Kottis V., Thibault P., Mikol D., Xiao Z. C., Zhang R., Dergham P. and Braun P. E. (2002) Oligodendrocyte-myelin glycoprotein (OMgp) is an inhibitor of neurite outgrowth. *J Neurochem* **82,** 1566-1569.

Kranenburg O., Poland M., van Horck F. P., Drechsel D., Hall A. and Moolenaar W. H. (1999) Activation of RhoA by lysophosphatidic acid and Galpha12/13 subunits in neuronal cells: induction of neurite retraction. *Mol Biol Cell* **10,** 1851-1857.

Lee J. K., Kim J. E., Sivula M. and Strittmatter S. M. (2004) Nogo receptor antagonism promotes stroke recovery by enhancing axonal plasticity. *J Neurosci* **24,** 6209-6217.

Lehmann M., Fournier A., Selles-Navarro I., Dergham P., Sebok A., Leclerc N., Tigyi G. and McKerracher L. (1999) Inactivation of Rho signaling pathway promotes CNS axon regeneration. *J Neurosci* **19,** 7537-7547.

Leung T., Manser E., Tan L. and Lim L. (1995) A novel serine/threonine kinase binding the Ras-related RhoA GTPase which translocates the kinase to peripheral membranes. *J Biol Chem* **270,** 29051-29054.

Li S., Liu B. P., Budel S., Li M., Ji B., Walus L., Li W., Jirik A., Rabacchi S., Choi E., Worley D., Sah D. W., Pepinsky B., Lee D., Relton J. and Strittmatter S. M. (2004a) Blockade of Nogo-66, myelin-associated glycoprotein, and oligodendrocyte myelin glycoprotein by soluble Nogo-66 receptor promotes axonal sprouting and recovery after spinal injury. *J Neurosci* **24,** 10511-10520.

Li W., Walus L., Rabacchi S. A., Jirik A., Chang E., Schauer J., Zheng B. H., Benedetti N. J., Liu B. P., Choi E., Worley D., Silvian L., Mo W., Mullen C., Yang W., Strittmatter S. M., Sah D. W., Pepinsky B. and Lee D. H. (2004b) A neutralizing

anti-Nogo66 receptor monoclonal antibody reverses inhibition of neurite outgrowth by central nervous system myelin. *J Biol Chem* **279,** 43780-43788.

Matsui T., Amano M., Yamamoto T., Chihara K., Nakafuku M., Ito M., Nakano T., Okawa K., Iwamatsu A., Kaibuchi K. and Ishizaki T. (1996) Rho-associated kinase, a novel serine/threonine kinase, as a putative target for small GTP binding protein Rho

The small GTP-binding protein Rho binds to and activates a 160 kDa Ser/Thr protein kinase homologous to myotonic dystrophy kinase. *EMBO Journal* **15,** 2208-2216.

McKerracher L., David S., Jackson D. L., Kottis V., Dunn R. J. and Braun P. E. (1994) Identification of myelin-associated glycoprotein as a major myelin-derived inhibitor of neurite growth. *Neuron* **13,** 805-811.

Mi S., Lee X., Shao Z., Thill G., Ji B., Relton J., Levesque M., Allaire N., Perrin S., Sands B., Crowell T., Cate R. L., McCoy J. M. and Pepinsky R. B. (2004) LINGO-1 is a component of the Nogo-66 receptor/p75 signaling complex. *Nat Neurosci* **7,** 221-228.

Mukhopadhyay G., Doherty P., Walsh F. S., Crocker P. R. and Filbin M. T. (1994) A novel role for myelin-associated glycoprotein as an inhibitor of axonal regeneration. *Neuron* **13,** 757-767.

Nakamura F., Tanaka M., Takahashi T., Kalb R. G. and Strittmatter S. M. (1998) Neuropilin-1 extracellular domains mediate semaphorin D/III-induced growth cone collapse. *Neuron* **21,** 1093-1100.

Neve R. L., Howe J. R., Hong S. and Kalb R. G. (1997) Introduction of the glutamate receptor subunit 1 into motor neurons in vitro and in vivo using a recombinant herpes simplex virus. *Neuroscience* **79,** 435-447.

Niederost B., Oertle T., Fritsche J., McKinney R. A. and Bandtlow C. E. (2002) Nogo-A and myelin-associated glycoprotein mediate neurite growth inhibition by antagonistic regulation of RhoA and Rac1. *J Neurosci* **22,** 10368-10376.

Park J. B., Yiu G., Kaneko S., Wang J., Chang J. and He Z. (2005) A TNF Receptor Family Member, TROY, Is a Coreceptor with Nogo Receptor in Mediating the Inhibitory Activity of Myelin Inhibitors. *Neuron* **45,** 345-351.

Pignot V., Hein A. E., Barske C., Wiessner C., Walmsley A. R., Kaupmann K., Mayeur H., Sommer B., Mir A. K. and Frentzel S. (2003) Characterization of two novel proteins, NgRH1 and NgRH2, structurally and biochemically homologous to the Nogo-66 receptor. *J Neurochem* **85,** 717-728.

Prinjha R., Moore S. E., Vinson M., Blake S., Morrow R., Christie G., Michalovich D., Simmons D. L. and Walsh F. S. (2000) Inhibitor of neurite outgrowth in humans. *Nature* **403,** 383-384.

Riento K. and Ridley A. J. (2003) Rocks: multifunctional kinases in cell behaviour. *Nat Rev Mol Cell Biol* **4,** 446-456.

Shao Z., Browning J. L., Lee X., Scott M. L., Shulga-Morskaya S., Allaire N., Thill G., Levesque M., Sah D., McCoy J. M., Murray B., Jung V., Pepinsky R. B. and Mi S. (2005) TAJ/TROY, an Orphan TNF Receptor Family Member, Binds Nogo-66 Receptor 1 and Regulates Axonal Regeneration. *Neuron* **45,** 353-359.

Simonen M., Pedersen V., Weinmann O., Schnell L., Buss A., Ledermann B., Christ F., Sansig G., van der Putten H. and Schwab M. E. (2003) Systemic deletion of the myelin-associated outgrowth inhibitor nogo-a improves regenerative and plastic responses after spinal cord injury. *Neuron* **38,** 201-211.

Song X. Y., Zhong J. H., Wang X. and Zhou X. F. (2004) Suppression of p75NTR does not promote regeneration of injured spinal cord in mice. *J Neurosci* **24,** 542-546.

Venkatesh K., Chivatakarn O., Lee H., Joshi P. S., Kantor D. B., Newman B. A., Mage R., Rader C. and Giger R. J. (2005) The Nogo-66 receptor homolog NgR2 is a sialic acid-dependent receptor selective for myelin-associated glycoprotein. *J Neurosci* **25,** 808-822.

Vincent S. and Settleman J. (1997) The PRK2 kinase is a potential effector target of both Rho and Rac GTPases and regulates actin cytoskeletal organization. *Molecular & Cellular Biology* **17,** 2247-2256.

Wang K. C., Kim J. A., Sivasankaran R., Segal R. and He Z. (2002a) P75 interacts with the Nogo receptor as a co-receptor for Nogo, MAG and OMgp. *Nature* **420,** 74-78.

Wang K. C., Koprivica V., Kim J. A., Sivasankaran R., Guo Y., Neve R. L. and He Z. (2002b) Oligodendrocyte-myelin glycoprotein is a Nogo receptor ligand that inhibits neurite outgrowth. *Nature* **417,** 941-944.

Wong S. T., Henley J. R., Kanning K. C., Huang K. H., Bothwell M. and Poo M. M. (2002) A p75(NTR) and Nogo receptor complex mediates repulsive signaling by myelin-associated glycoprotein. *Nat Neurosci* **5,** 1302-1308.

Zheng B., Ho C., Li S., Keirstead H., Steward O. and Tessier-Lavigne M. (2003) Lack of enhanced spinal regeneration in nogo-deficient mice. *Neuron* **38,** 213-224.

Zheng B., Atwal J., Ho C., Case L., He X. L., Garcia K. C., Steward O. and Tessier-Lavigne M. (2005) Genetic deletion of the Nogo receptor does not reduce neurite

inhibition in vitro or promote corticospinal tract regeneration in vivo. *Proc Natl Acad Sci U S A* **102,** 1205-1210.

Zhu Y., Stolz D. B., Guo F., Ross M. A., Watkins S. C., Tan B. J., Qi R. Z., Manser E., Li Q. T., Bay B. H., Teo T. S. and Duan W. (2004) Signaling via a novel integral plasma membrane pool of a serine/threonine protein kinase PRK1 in mammalian cells. *Faseb J* **18,** 1722-1724.

Figure 1. HA1077 protects neurons from myelin-induced growth cone collapse and neurite outgrowth inhibition. *A.* E13 chick DRG explants were treated for 1 hour with soluble myelin following a 30 minute pre-treatment with PBS (control) or 10 μM HA1077. Explants were stained with rhodamine-phalloidin. Arrows, spread growth cones. Arrowheads, collapsed growth cones. Scale bar, 40 μm . *B.* Dissociated chick DRG neurons were cultured on control (PBS), myelin or Nogo-66 substrates in the presence or absence of 10 μm HA1077 and stained with rhodamine-phalloidin. Scale bar, 100 μm. *C-E.* Quantification of growth cone collapse (*C*) or neurite outgrowth per cell (*D, E*). Outgrowth is expressed as a percentage of control ± S.E.M. Determinations are from 4 separate experiments each performed in duplicate. $*p<0.05$ compared with controls by student t-test.

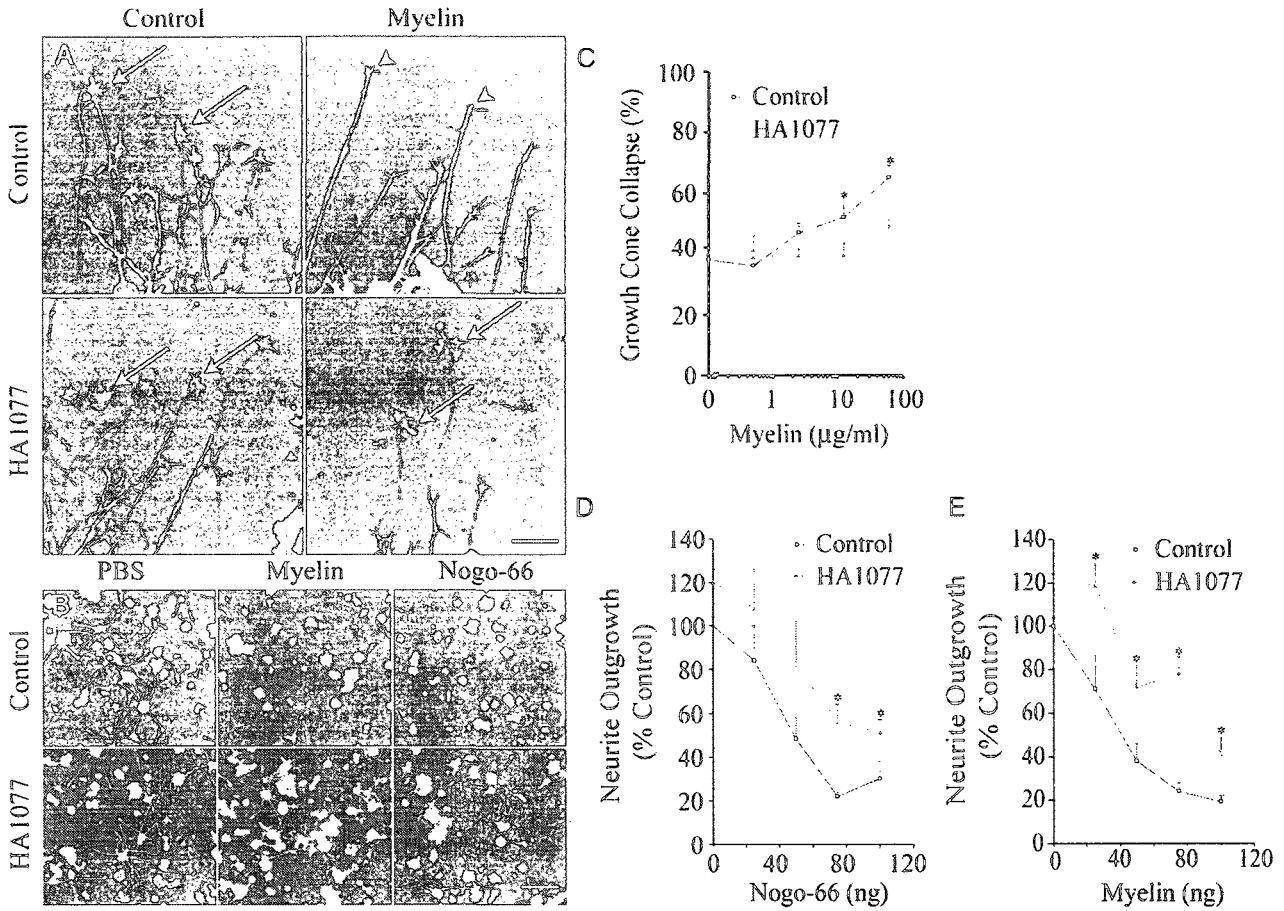

Control
Myelin
A
Control
HA1077
PBS
Myelin
Nogo-66
B
Control
HA1077
C
Control
HA1077
Growth Cone Collapse (%)
100
80
60
40
20
0
0
1
10
100
Myelin (μg/ml)
D
Control
HA1077
Neurite Outgrowth (% Control)
140
120
100
80
60
40
20
0
0
40
80
120
Nogo-66 (ng)
E
Control
HA1077
Neurite Outgrowth (% Control)
140
120
100
80
60
40
20
0
0
40
80
120
Myelin (ng)

Figure 2. Expression of ROCKII and PRK2 in PC12 cells and rat neural tissue. *A.* mRNA detection of ROCKII and PRK2 in various tissues by RT-PCR. GAPDH is detected as a positive control. For the negative control, a parallel RT-PCR reaction was run on brain lysates in the absence of reverse transcriptase. *B.* ROCKII and PRK2 immunostaining of P8 rat cerebellar neurons demonstrating that both proteins are expressed in neuronal cell bodies and neurites. The control culture is stained with Cy-2 conjugated secondary antibody only. Scale bar, 100 μm.

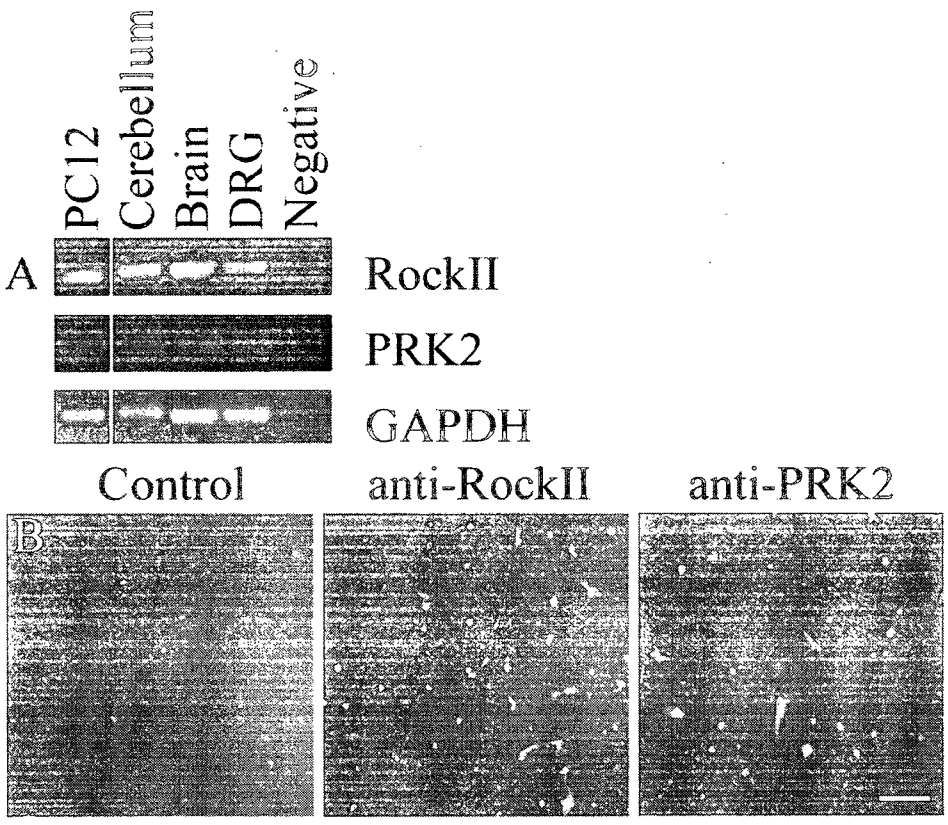

A
PC12
Cerebellum
Brain
DRG
Negative
RockII
PRK2
GAPDH
Control
anti-RockII
anti-PRK2
B

Figure 3. Nogo-66 activates ROCKII kinase activity. *A.* Kinase activity assay demonstrates the ability of Nogo-66 to enhance ROCKII- but not PRK2-dependent incorporation of ^{32}P-labeled-ATP into S6 substrate peptide. Y-27632 blocks the *in vitro* kinase activity of ROCKII and PRK2. *B.* Immunoprecipitation of myc-ROCKII and flag-PRK2 from 30% of the lysates used for the *in vitro* kinase assay demonstrates that equal amounts of kinase were immunoprecipitated for each treatment. Determinations are from three independent experiments each performed in duplicate. *Error bars* represent the SEM. *$p>0.05$.

FIGURE 3

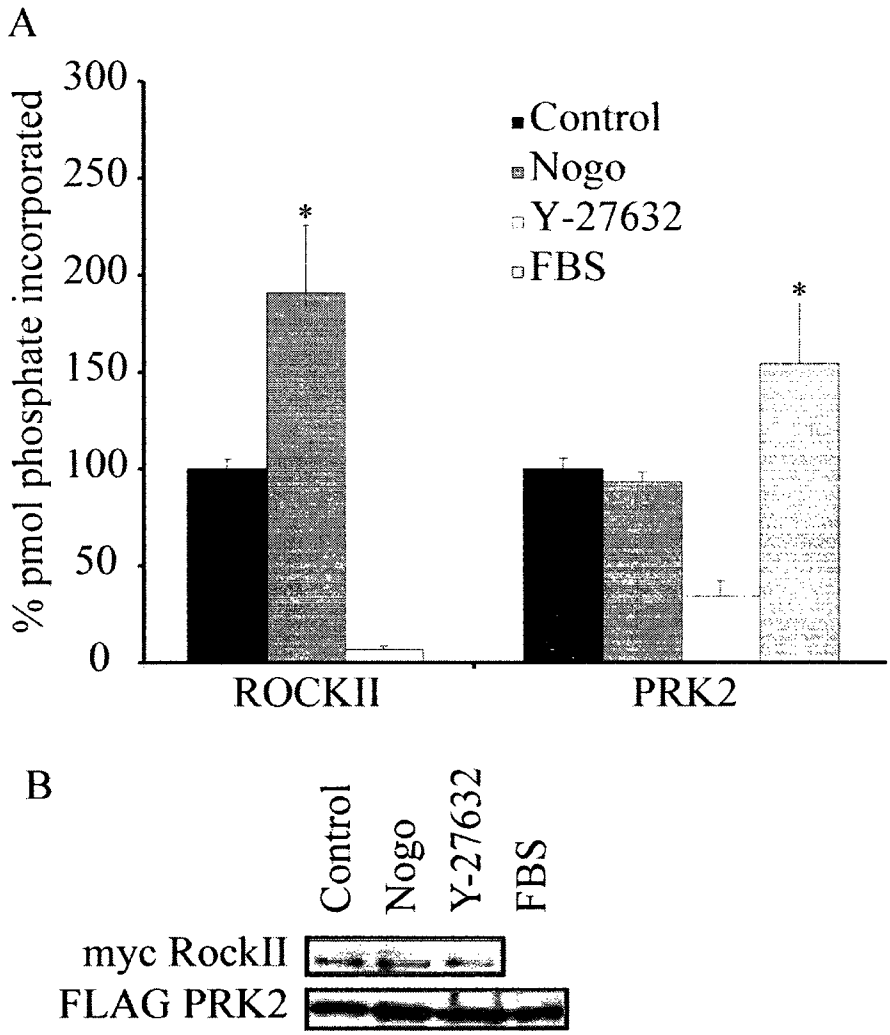

A
% pmol phosphate incorporated
300
250
200
150
100
50
0
Control
Nogo
Y-27632
FBS
*
*
ROCKII
PRK2
B
Control
Nogo
Y-27632
FBS
myc RockII
FLAG PRK2

Figure 4. Nogo-66 induces ROCKII translocation to the membrane in PC12 cells. *A.* PC12 cells were treated with GST-Nogo-66 for 0.5, 15, or 60 min. Crude membrane fractions (Mb) were prepared and analyzed for ROCKII content by Western blotting. Membrane lysates were analyzed for $p75^{NTR}$ content to control for equal membrane protein loading. ROCK levels in the cytosolic fraction (Cyt) were also assessed. *B.* ROCKII levels in the membrane fraction of PC12 cells were analyzed by densitometry. ROCKII levels were normalized to p75 levels and expressed as a percentage of ROCKII levels in non-stimulated PC12 lysates (0) for each experiment.

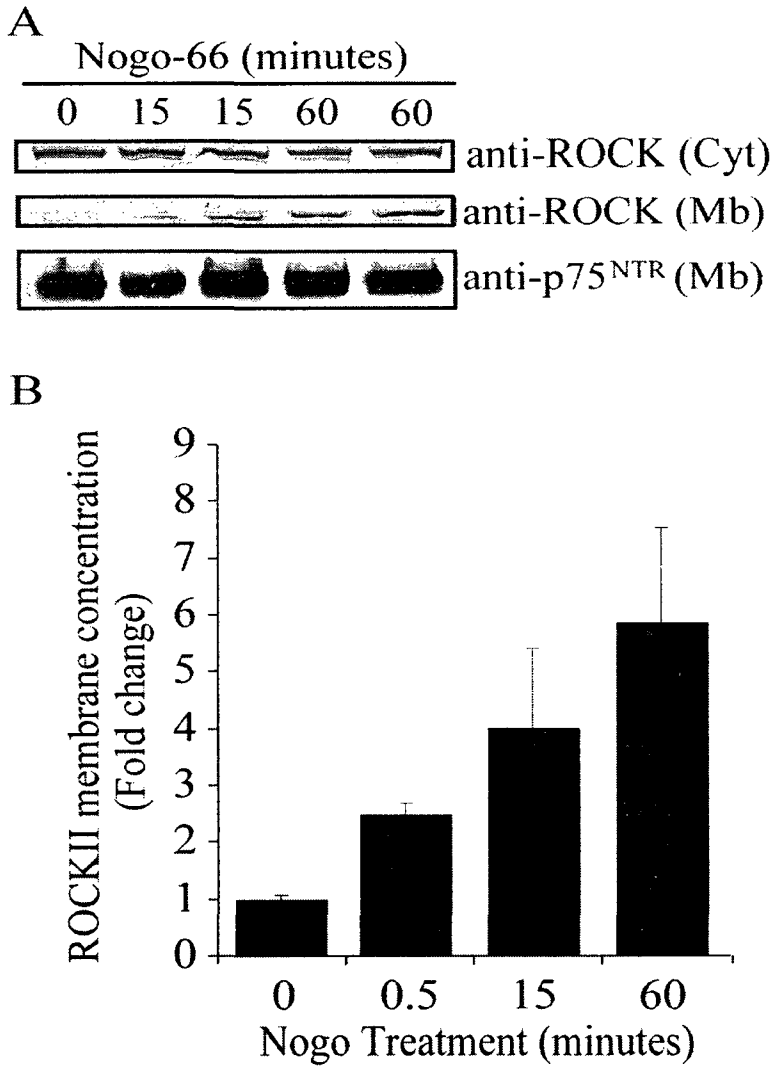

A
Nogo-66 (minutes)
0 15 15 60 60
anti-ROCK (Cyt)
anti-ROCK (Mb)
anti-p75NTR (Mb)
B
ROCKII membrane concentration (Fold change)
0 1 2 3 4 5 6 7 8 9
0 0.5 15 60
Nogo Treatment (minutes)

Figure 5. Nogo-66 increases phosphorylation of MLCII. *A*. PC12 cells were treated with Nogo-66 and lysates were analyzed by western blotting with anti-phospho-MLC or anti-MLC antibody. MLC phosphorylation in Nogo-treated PC12 cells pre-treated with Y-27632 or water (vehicle) was also analyzed. *B*. Phosphorylated MLC in cell lysates was quantified by densitometry. Values were normalized to total MLC levels and to untreated control values for each experiment. In the case of Y-27632 pre-treatment, values following Nogo stimulation were normalized to the Y-27632-pre-treated controls. Values are for at least three independent experiments. *Error bars* represent the S.E.M.; $*p<0.05$.

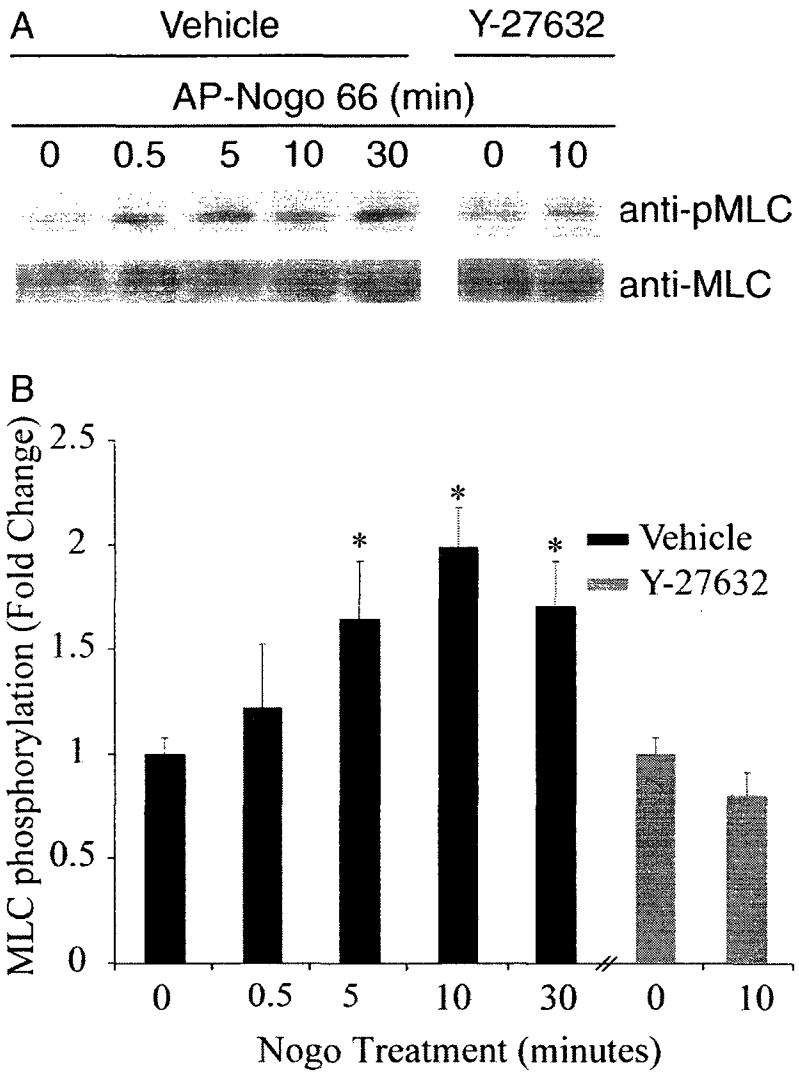
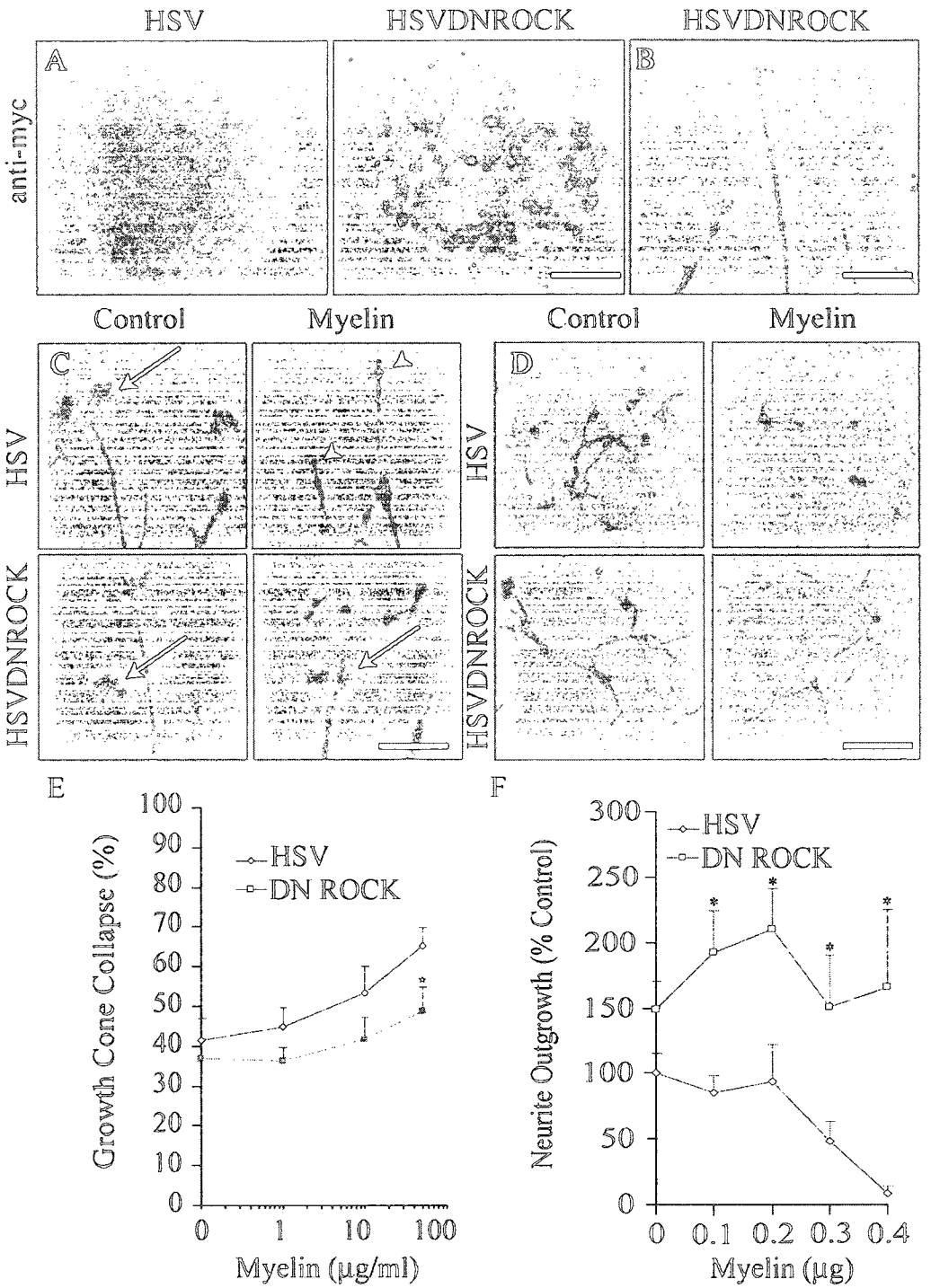

A
Vehicle
Y-27632
AP-Nogo 66 (min)
0 0.5 5 10 30 0 10
anti-pMLC
anti-MLC
B
MLC phosphorylation (Fold Change)
2.5
2
1.5
1
0.5
0
*
*
*
Vehicle
Y-27632
0 0.5 5 10 30 0 10
Nogo Treatment (minutes)

Figure 6. Dominant negative ROCKII rescues neurons from myelin-dependent inhibition. *A.* Low magnification views of HSV- or HSVDNROCK-infected explants immunostained with anti-myc antibody demonstrate the efficiency of viral infection. Scale bar, 100 μm. *B.* High magnification view of an HSVDNROCK-infected growth cone stained with anti-myc antibody demonstrates that the virus is transported into DRG neurites and growth cones. Scale bar, 40 μm. *$p<0.01$. *C.* HSV or HSVDNROCK-infected E13 DRG explants were treated with myelin for one hour and stained with rhodamine phalloidin. Arrows, spread growth cones. Arrowheads, collapsed growth cones. Scale bar, 40 μm. *D.* HSV or HSVDNROCK-infected dissociated E13 DRG neurons were cultured on myelin and stained with anti-βIII tubulin antibody. Scale bar, 100 μm. *E-F.* Quantification of myelin-dependent DRG growth cone collapse (*E*) and neurite outgrowth/cell on myelin substrate (*F*). Determinations are from at least three separate experiments each performed in duplicate. *Error bars* represent the S.E.M.; *$p<0.05$.


HSV
HSVDNROCK
HSVDNROCK
A
B
anti-myc
Control
Myelin
Control
Myelin
C
D
HSV
HSV
HSVDNROCK
HSVDNROCK
E
F
Growth Cone Collapse (%)
HSV
DN ROCK
Myelin (µg/ml)
Neurite Outgrowth (% Control)
HSV
DN ROCK
Myelin (µg)

CHAPTER 3

III. IDENTIFICATION OF CRMP4 AS A CONVERGENT REGULATOR OF AXON OUTGROWTH INHIBITION.

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Identification of CRMP4 as a convergent regulator of axon outgrowth inhibition

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III.1. PREFACE

Engagement of neuronal cell surface receptors for MAIs and CSPGs activate intracellular molecules including RhoA and Rho kinase (ROCK) to block axonal extension through targeted modifications to the cytoskeleton. In the context of CNS injury, RhoA and ROCK represent promising targets for therapeutic intervention; however these molecules are also involved in multiple key cellular processes and signalling pathways, which would limit the specifity of any targeted therapy. The aim of next project was to identify molecules that functionally interact with RhoA to mediate myelin-dependent inhibition and that may represent more specific targets for therapeutic intervention.

III.2. ABSTRACT

Myelin-associated inhibitors (MAIs) and chondroitin sulphate proteoglycans (CSPGs) contribute to failed regeneration following neuronal injury. MAIs and CSPGs stimulate intracellular signals including the activation of RhoA and Rho kinase to block axonal extension through targeted modifications to the cytoskeleton. RhoA and ROCK are promising targets for therapeutic intervention to promote CNS repair; however, their ubiquitous expression will limit the specificity of drugs targeted to these molecules. We have identified the cytosolic phosphoprotein CRMP4b as a protein that physically and functionally interacts with RhoA to mediate neurite outgrowth inhibition. siRNA-mediated knockdown of CRMP4 promotes neurite outgrowth on myelin substrates indicating a critical role for CRMP4 in neurite outgrowth inhibition. Disruption of CRMP4b-RhoA binding with a competitive inhibitor attenuates neurite outgrowth inhibition on myelin and aggrecan substrates. Stimulation of neuronal growth cones with Nogo leads to co-localization of CRMP4b and RhoA at discrete regions within the actin rich central and peripheral domains of the growth cone indicative of a potential function in cytoskeletal rearrangements during neurite outgrowth inhibition. Together these data indicate that a RhoA-CRMP4b complex forms in response to inhibitory challenges in the growth cone environment and regulate cytoskeletal dynamics at distinct sites necessary for axon outgrowth inhibition. Competitive inhibition of CRMP4b-RhoA binding suggests a novel, highly specific therapeutic avenue for promoting regeneration following CNS injury.

Key Words: Nogo, CRMP, TUC, glial scar, regeneration, myelin inhibition, Rho GTPase.

Running Title: CRMP4 and axon outgrowth inhibition

III.3. INTRODUCTION

Trauma in the adult mammalian central nervous system (CNS) results in devastating clinical consequences due to the failure of injured axons to spontaneously regenerate. Chondroitin sulphate proteoglycans (CSPGs) and the myelin-associated inhibitors (MAIs) myelin-associated glycoprotein (MAG) (McKerracher et al., 1994; Mukhopadhyay et al., 1994), Nogo-A (Chen et al., 2000; GrandPre et al., 2000; Prinjha et al., 2000) and Oligodendrocyte-myelin glycoprotein (OMgp) (Kottis et al., 2002; Wang et al., 2002b) bind receptor molecules on injured axons initiating intracellular signaling cascades which block axonal regrowth (Mandemakers and Barres, 2005). In part, CSPGs and MAIs inhibit axon regeneration by disrupting Rho-GTPase-dependent cytoskeletal dynamics. Blockade of RhoA and a downstream effector Rho kinase (ROCK) promotes axon regeneration both *in vitro* and *in vivo* (Dergham et al., 2002; Borisoff et al., 2003; Fournier et al., 2003); however, the ability of RhoA and ROCK to affect multiple physiological processes in many cell types (Riento and Ridley, 2003) highlights the need to identify novel intracellular signaling substrates of neurite outgrowth inhibition to develop more specific and potent therapeutic avenues.

In a screen to identify molecules that functionally interact with RhoA to mediate neurite outgrowth inhibition, we identified collapsin-response mediator protein 4b (CRMP4b) as a molecule that interacts with Rho GTPase in a Nogo-dependent manner. The CRMPs are a family of cytosolic phosphoproteins with five vertebrate family members (CRMP1-5) (Goshima et al., 1995b; Minturn et al., 1995; Byk et al., 1996; Gaetano et al., 1997; Inatome et al., 2000). CRMP1-4 alleles each produce two transcripts, a and b, and CRMPb variants are longer amino terminal variants of the originally

identified CRMPa isoforms (Yuasa-Kawada et al., 2003). Although CRMPs share significant sequence similarity with dihydropyrimidinase (DHPase), an enzyme involved in pyrimidine catabolism, no DHPase activity has been described for CRMPs (Wang and Strittmatter, 1997b). Rather a role for CRMPs in axon growth and pathfinding has been revealed. CRMPs are homologs of UNC-33, a protein that influences axon guidance and extension in *C. elegans* (Hedgecock et al., 1985; Siddiqui and Culotti, 1991). CRMP2 mediates growth cone collapse in response to the repulsive guidance cue Semaphorin3A (Goshima et al., 1995b), and CRMP2 and CRMP4 influence neurite outgrowth (Minturn et al., 1995; Quinn et al., 1999; Quinn et al., 2003; Yoshimura et al., 2005). Mechanistically, CRMP2 can bind to tubulin heterodimers and organizes microtubule assembly to establish axon-dendrite fate during development (Fukata et al., 2002c; Arimura et al., 2005b) and CRMP4 can promote F-actin bundling (Rosslenbroich et al., 2005). Further, a role for CRMP2 in endocytosis has been described (Nishimura et al., 2003) and an association between CRMP4b and intersectin, an endocytic-exocytic adaptor protein, is consistent with an endocytic role for this isoform (Quinn et al., 2003). The role of the CRMP proteins in nervous system injury and regeneration has not been extensively studied; however CRMP2a does have a potent neurite elongating effect in nerve regeneration *in vivo* (Suzuki et al., 2003). CRMP1a, CRMP2a and CRMP5a mRNA levels increase after hypoglossal nerve injury and CRMP4a expression is increased in regenerating adult sciatic motor neurons (Minturn et al., 1995; Suzuki et al., 2003) suggesting a more general role for CRMP proteins in the neuronal response to injury.

We find that CRMP4b interacts with RhoA in a Nogo-dependent manner leading us to investigate the potential role of this complex in the inhibition of neurite outgrowth. We find that antagonism of CRMP4 or of the CRMP4b-RhoA interaction attenuates neurite outgrowth inhibition. This protein-protein interaction represents a novel, specific target for therapeutic intervention following CNS injury.

III.4. METHODS AND MATERIALS

III.4.1. Plasmid construction

To construct CRMP-V5 expression vectors, cDNA was amplified by polymerase chain reaction (PCR) from rat pEGFP-CRMP constructs (generously provided by Dr. Peter McPherson, McGill University). The PCR product was ligated into the *HindIII*- and *XhoI*- sites of pcDNA 3.1 V5-His for CRMP1a, CRMP2a and CRMP3a and into *HindIII*- and *EcoRI*-site of pcDNA 3.1 V5-His for CRMP4a. The cDNA for CRMP4b was amplified from pcDNA 3.1CRMP4bV5-His TOPO (Quinn et al., 2003) and ligated into the *HindIII*- and *EcoRI*-site pcDNA 3.1 V5-His in frame with the V5-His tag. CRMP1b-V5 was constructed by amplifying the coding sequence by PCR from an EST clone (IMAGE:5686818) and ligated into *EcoRI*- and *XhoI*- sites of pcDNA 3.1 V5-His.

pcDNA myc-wild type RhoA (wt) was obtained from UMR cDNA Resource Center (http://www.cdna.org/). pRK5 myc-Rac1 (wt), pRK5 myc-Cdc42 (wt), pRK5 myc-RhoA63L, pRK5 myc-RhoAN19, pRK5 myc-RacQ61L, pRK5 myc-RacN17, pRK5 myc-Cdc42Q61L and pRK5 myc-Cdc42N17 constructs were generously provided by Dr. Nathalie Lamarche-Vane (McGill University). FLAG-RhoA63L was generated by sub-

cloning RhoA63L into the *BamHI-* and *EcoRI-* sites of pcDNA3FLAG. pCAG-myc-mROCK II construct was generously provided by Dr. Shuh Narumiya (Kyoto University).

To generate C4RIP-V5, the unique amino terminal domain of CRMP4b (residues 1-126) was introduced into the *BamHI-* and *EcoRI-* sites of pcDNA 3.1V5-His. pHSVC4RIP was generated by subcloning C4RIP-V5 into the *HindIII-* and *SalI-* sites of pHSVPrPUC. pHSVCRMP4bGFP was generated by cloning CRMP4b into the *HindIII-* and *EcoRI-* sites of pEGFP N2 (Clonetech) and subsequently sub-cloning into the *HindIII-* and *XbaI-* sites of pHSVPrPUC. Chimeric CRMP4bNCRMP2-V5 was constructed by PCR by ligating residues 14-572 of CRMP2a into *EcoRI-* and *XhoI-* sites of C4RIP-V5. CRMP4ΔN consists of CRMP4a with the first 12 residues replaced by a single methionine. CRMP4ΔN was amplified by PCR and ligated into *HindIII-* and *EcoRI-* sites of pcDNA 3.1 V5-His.

III.4.2. Preparation of HSV Viruses

pHSVPrPUC plasmids were transfected into 2-2 Vero cells which were superinfected with 5*dl* 1.2 HSV helper virus 1 day later. Recombinant virus was amplified through three passages and stored at −80°C as described previously (Neve et al., 1997).

III.4.3. Preparation of recombinant proteins

Stimulations to examine inhibitory responses were performed with alkaline-phosphatase-conjugated Nogo-66 (AP-Nogo-66) purified from stably transfected 293 cells or Nogo-P4 peptide or myelin. AP-Nogo-66 or AP was purified by Ni^{2+} affinity chromatography as previously described (Nakamura et al., 1998; Fournier et al., 2001). For all treatments

AP-Nogo66-His (8nM) or AP (8nM) was preaggregated with 100 ng/ml anti-human AP (Niederost et al., 2002). Nogo-P4 (Alpha Diagnostics, San Antonio, TX) is a 25 aa inhibitory peptide sequence (residues 31-55 of Nogo-66) sufficient to mediate the inhibitory properties of Nogo-66, a potent inhibitory component of Nogo-A (GrandPre et al., 2000). Myelin extracts were prepared from bovine brain as described previously (Igarashi et al., 1993; Hsieh et al., 2006).

GST, GST-RhoAWT and GST-RhoA63L (construct generously provided by Dr. Keith Burridge, University of North Carolina, Chapel Hill) were expressed in *Escherichia coli* and purified on glutathione-Sepharose as previously described (Arthur et al., 2002; Wennerberg et al., 2002). For overlay assays, RhoA was cleaved from the GST moiety via thrombin cleavage. Aggrecan was purchased from Sigma Chemical Co. (Oakville, Ontario).

III.4.4. GST-RhoA Pull down assays

PC12 cells were grown to sub-confluence on collagen-coated plates in Roswell Park Memorial Institute Media (RPMI) 1640 supplemented with L-glutamine, and containing 10% horse serum, 5% fetal bovine serum (FBS), and 1% penicillin/streptomycin (Invitrogen, Burlington, Ontario), after which differentiation was induced for 24 hours with RPMI supplemented with 1% bovine serum albumin (BSA) fraction V and 50 ng/mL nerve growth factor (NGF) (Upstate Biotechnology, Waltham, MA). GST-RhoA63L pull downs were performed as described previously (Arthur et al., 2002). Briefly, after stimulation with AP-Nogo-66, cells were washed twice in ice cold Hepes-buffered saline (HBS), harvested in 1 mL of ice-cold lysis buffer containing 20 mM

HEPES, pH 7.3, 150 mM NaCl, 5 mM $MgCl_2$, 1% (v/v) Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), supplemented with Cømplete protease inhibitors (Roche Products, Laval, Quebec). GST-precleared lysates were then mixed with 30 µg of GST-RhoA63L fusion protein bound to sepharose beads for 1 hour at 4°C. Precipitated proteins were eluted with 2X sample buffer and analyzed by SDS-PAGE on a 4-15% gradient gel, followed by silver staining.

III.4.5. CRMP-RhoA Co-Immunoprecipitation Assays

HEK 293T cells were grown to sub-confluence and transfected with Lipofectamine 2000 according to manufacturer instructions (Invitrogen, Burlington, Ontario), washed twice with ice-cold PBS and lysed in lysis buffer A (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% (v/v) Triton X-100, 1 mM Na_3VO_4, 1 mM NaF, 1 mM PMSF and Cømplete protease inhibitors (Roche Diagnostics, Laval, PQ)). Lysates were pre-cleared with protein A/G-agarose (Santa Cruz Biotechnology, Santa Cruz, CA) and subjected to immunoprecipitation with myc-agarose or V5-agarose (Sigma Chemical Co., Oakville, Ontario). After washing 3 times with ice-cold PBS, bound protein was eluted with SDS and immunoblotted with anti-Myc (9E10, 1:1000; Sigma Chemical Co., Oakville, Ontario) or anti-V5 (1:5000; Invitrogen, Burlington, Ontario). For time course experiments, PC12 cells were transfected for 24 hours using Lipofectamine 2000 (Invitrogen, Burlington, Ontario) and differentiated with 50 ng/ml nerve growth factor (NGF; Upstate Biotechnology, Waltham, MA) for 24 hours. Cells were treated with Nogo-P4 peptide for the indicated period of time at 37°C. Cells were then lysed and proteins were immunoprecipitated as described above.

III.4.6. Far Western

Overlay of CRMP4 with RhoA was performed as previously described (McPherson et al., 1994). Briefly, HEK293T cell lysates transfected with CRMP4a-V5, CRMP4b-V5 or empty vector were subjected to immunoprecipitation with V5-agarose (Sigma Chemical Co., Oakville, Ontario). Immunoprecipitates were separated by SDS-PAGE, transferred to PVDF membranes and overlaid with 10 μg/ml of bacterially-purified RhoA for 1 hour at room temperature. RhoA was detected with a rabbit anti-RhoA antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

III.4.7. Neurite outgrowth and Growth Cone Collapse assays

For neurite outgrowth assays, myelin was dried down on poly-L-lysine-coated substrates. Substrates were washed and coated with 10 μg/ml laminin for 1 hr. For outgrowth on aggrecan, poly-L-lysine-coated substrates were coated with aggrecan and 10 μg/ml laminin for 2 hr at 37°C. Dissociated E13 chick DRG neurons were cultured in DRG media (F-12 medium, 10% FBS, 1% penicillin/streptomycin, 1% L-glutamine, 50 ng/ml NGF) in the presence of virus for 24 hours, fixed with 4% paraformaldehyde/20% sucrose in PBS and double stained with anti-βIII tubulin (Covance, Berkeley, CA) and anti-V5 antibody (Sigma Chemical Co., Oakville, Ontario). Neurite outgrowth lengths per cell were assessed using Image J, a public domain JAVA image processing program (http://rsb.info.nih.gov/ij/) as previously described (Fournier et al., 2003).

For growth cone collapse assays, chick E7 DRGs were cultured in DRG media for 18 hours on 4 well glass chamber slides sequentially coated with PLL and laminin as above. Sema3A-AP or AP conditioned media was prepared as described previously

(Takahashi et al., 1998). Explants were stimulated with Sema3A-AP- or AP- conditioned media at indicated concentrations for 20 min and fixed with 4% paraformaldehyde, 20% sucrose and 0.1M $NaPO_4$. Explants were stained with rhodamine phalloidin and assessed for growth cone collapse as described (Luo et al., 1993).

III.4.8. CRMP-4 siRNA

For knockdown of CRMP-4a and CRMP-4b, silencer pre-designed siRNA against rat CRMP-4 was used (siRNA ID: 48833; Ambion, Austin, TX). Controls were with a scrambled siRNA (CAGCAUGGUGGUACGCUUGUAAGCA) for an ineffective CRMP4b-targetted siRNA designed with the BLOCK-IT algorithm (Invitrogen, Burlington, Ontario). siRNAs were validated by co-transfecting siRNAs with CRMP4-V5 in HEK293T cells for 24 hours. Cell lysates were separated by SDS-PAGE and analyzed with an anti-V5 antibody. To validate siRNA efficacy in neurons, siRNA-transfected DRGs were co-infected with HSVCRMP4b-GFP. Twenty-four hours following infection GFP fluorescence was evaluated by fluorescence microscopy. For neurite outgrowth assays, P5 dissociated rat DRGs were prepared and seeded on laminin substrates as described previously (Hsieh et al., 2006). After 4 hours, DRGs were serum starved (in F-12 medium and 50 ng/ml NGF) and transfected with indicated siRNAs using Lipofectamine 2000. After 5 hours of transfection, media was replaced with fresh DRG media. Twenty-four hours following transfection, DRGs were removed from the plate with EDTA and re-seeded on myelin substrates. Neurons were left to grow for an additional 18 hours and processed for neurite outgrowth analysis as described above.

III.4.9. Immunofluorescence

E7-13 chick DRG explants were cultured in DRG media on poly-L-lysine- and laminin-coated substrates for 18 hours. For viral infections, recombinant viral preparations were added to the media 1 hour after plating. After 18 hours, cultures were treated with myelin and fixed with 4% paraformaldehyde/20% sucrose/PBS, permeabilized in 0.2% triton X-100 and double stained with polyclonal anti-RhoA antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-CRMP4b or V5 antibody. For filopodia and branch analysis, GFP and CRMP4bV5-infected growth cones were double stained with rhodamine-phalloidin (Molecular Probes, Eugene, OR) and anti-V5 antibody. Filopodial length per growth cone was assessed using Image J, by measuring total filopodial length per growth cone averaged by the total number of filopodia per growth cone. Neurite branches were quantified by counting total branch buds per neurite.

III.5. RESULTS

III.5.1. A novel Nogo-dependent interaction between CRMP4b and RhoA

To identify molecules that functionally interact with RhoA to mediate neurite outgrowth inhibition, we screened for proteins that have enhanced affinity for a constitutively active, GTPase deficient mutant of RhoA (Khosravi-Far et al., 1994) following treatment with Nogo-66, a potent inhibitory fragment of Nogo-A (GrandPre et al., 2000). Bacterially-purified *glutathione-S-transferase*-RhoA63L (GST-RhoA63L) was used as bait to precipitate proteins from PC12 cells following stimulation with Nogo-66 fused to alkaline phosphatase (AP-Nogo-66). PC12 cells were chosen for the biochemical screen based on their expression of MAI receptors (Hsieh et al., 2006), and their responsiveness

to Nogo in neurite outgrowth assays (GrandPre et al., 2000) and biochemical assays evaluating RhoGTP levels (Fournier et al., 2003). Proteins interacting with GST-RhoA63L were separated by SDS-PAGE and visualized by silver staining. A 75 kDa protein with enhanced affinity for GST-RhoA63L following Nogo-66 treatment (Fig. 1a) was identified as CRMP4b by tandem mass spectrometry. The enhanced CRMP4–RhoA interaction was validated with a pan-CRMP antibody (generously provided by Dr. Peter McPherson, McGill University), which recognizes the 75 kDa CRMPb isoforms and the 65 kDa CRMPa isoforms (Fig. 1b). Both 75 kDa and 65 kDa CRMP isoform/s specifically precipitate with GST-RhoA63L while the interaction between the 75 kDa CRMPb isoform/s and RhoA is enhanced by Nogo stimulation (Fig. 1b).

To assess the specificity of the CRMP4b-RhoA interaction, we analyzed the ability of other CRMP members to interact with RhoA. Wild type myc-RhoA and CRMP-V5 constructs were co-transfected in HEK293T cells. Myc-RhoA was immunoprecipitated from cell lysates and immune complexes were analyzed for CRMP-V5. RhoA interacts preferentially with CRMP4 family members with stronger binding to CRMP4b when compared to CRMP4a (Fig. 2a). RhoA fails to bind to other CRMPa isoforms and weakly associates with CRMP1b (Fig. 2a). Amino-terminally-tagged GFP-CRMP4b fails to associate with RhoA suggesting that amino-terminal tags interfere with this binding interaction (data not shown).

We then asked whether CRMP4 specifically interacts with RhoA, by assessing its binding to Rac and Cdc42, two other members of the small Rho GTPases that regulate the actin cytoskeleton and positively regulate neurite outgrowth (Bishop and Hall, 2000). CRMP4a-V5 and CRMP4b-V5 were tested for binding to myc-tagged, GTP-bound active

forms of RhoA (myc-RhoA63L), Rac1 (myc-Rac1Q61L) and Cdc42 (myc-Cdc42Q61L). CRMP4 binding to RhoA is markedly stronger than to Rac1 or Cdc42 (Fig. 2b). Similarly, CRMP4 binding to wild type and GDP-bound forms Rac and Cdc42 is negligible (data not shown). Taken together, these results demonstrate that both CRMP4a and CRMP4b specifically bind to RhoA with CRMP4b binding more strongly than CRMP4a.

III.5.2. CRMP4b-RhoA binding is nucleotide-independent, phospho-dependent and direct

Rho GTPases cycle between an inactive GDP-bound state and an active GTP-bound state. Downstream effectors of Rho GTPases bind to Rho GTPases in the active GTP-bound state while guanine nucleotide exchange factors prefer Rho GTPases in the nucleotide free or GTP-bound state (Hall, 1994). To study the nucleotide-dependence of the CRMP4–RhoA interaction, CRMP4 binding to constitutively active myc-RhoA63L or dominant negative myc-RhoAN19 was assessed (Feig and Cooper, 1988). CRMP4a-V5 and CRMP4b-V5 interact with both myc-RhoA63L and myc-RhoAN19 (Fig. 2c) indicating that the interaction between CRMP4 and RhoA is nucleotide-independent.

To assess if the binding interaction between CRMP4 and RhoA is direct, we performed a RhoA overlay assay on CRMP-V5 immunoprecipitated from 293T cells (Fig. 2d). RhoA specifically binds to CRMP4b-V5 and CRMP4a-V5 and fails to bind to CRMP2a-V5 in the overlay assay indicating that the interaction between CRMP4b and RhoA is direct and specific.

The phospho-dependence of the interaction was evaluated by stimulating CRMP4b-V5-expressing 293T cells with Calyculin A, a serine/threonine phosphatase inhibitor, prior to immunoprecipitating CRMP4b-V5. Calyculin-dependent CRMP4b-V5 phosphorylation is indicated by an upward mobility shift of the CRMP4b-V5 protein (Fig 2d, V5 immunoblot). RhoA fails to bind to the phosphorylated species of CRMP4b-V5 in the overlay demonstrating that phosphorylated CRMP4b fails to bind to RhoA.

III.5.3. Nogo specifically modulates the CRMP4b-RhoA interaction

To further define the specificity and the time course of the Nogo effect on the CRMP4b–RhoA interaction, PC12 cells were co-transfected with wild type myc-RhoA and CRMP4-V5 and stimulated with Nogo-P4 peptide, the minimal sequence of Nogo-66 required for Nogo-66-dependent responses (GrandPre et al., 2000). Myc-RhoA was immunoprecipitated and immune complexes were analyzed for CRMP4-V5. We detect a rapid increase in CRMP4b-V5 immunoprecipitating with myc-RhoA by 1 minute following Nogo stimulation, which is maintained by 10 minutes (Fig. 3a). Nogo stimulation does not regulate the interaction between myc-RhoA and CRMP4a-V5 (Fig. 3b). Similar results were obtained with AP-Nogo-66 stimulation and with the active form of RhoA (data not shown) suggesting that CRMP4b is regulated independently of Nogo-dependent RhoA cycling to the GTP-bound state. The Nogo-regulated RhoA-CRMP4b interaction was further confirmed by co-immunoprecipation of endogenous proteins in Nogo-P4-treated P8 rat cerebellar cultures, where CRMP4b and RhoA form a complex 1 and 10 minutes following stimulation (Fig. 3c).

III.5.4. siRNA-mediated knockdown of CRMP4 attenuates neurite outgrowth inhibition

To determine if CRMP4 function is necessary for Nogo-dependent responses we assessed neurite outgrowth on myelin substrates in the context of CRMP4-specific siRNA. The efficacy of CRMP4-specific siRNAs was validated in transfected 293T cells. We identified one siRNA, which robustly inhibits both CRMP4a and CRMP4b expression (Fig. 4a). Lipofectamine-mediated transfection of siRNAs efficiently targets P4 rat dorsal root ganglion neurons (DRGs) and CRMP4 siRNA introduced in this manner diminishes the expression of CRMP4b-GFP introduced by HSV-mediated infection (Fig. 4b). Rat DRGs were grown for 24 hours following siRNA transfection, removed from the substrate with EDTA and re-seeded on myelin substrates for an additional 18 hours. Rat DRG neuronal outgrowth is inhibited by approximately 50% on a substrate coated with 1 μg/ml of myelin using this protocol (Fig. 4e), a more modest inhibitory response than when dissociated DRG neurons are plated immediately on myelin substrates (Fig. 8; (Hsieh et al., 2006)). Neuronal outgrowth on the control laminin substrate is not significantly affected by the introduction of CRMP4 siRNA (Fig. 4d). However, CRMP4 siRNA-transfected P4 rat DRGs grow significantly better on myelin substrates than those transfected with scrambled siRNA (Fig. 4c, e) indicating that CRMP4 is necessary for myelin-dependent inhibition. The efficacy of the CRMP4 siRNA (Fig. 4e) may be underestimated due to the failure to transfect 100% of the DRG neurons (Fig. 4b).

III.5.5. CRMP4b affects the growth cone actin cytoskeleton

CRMP proteins have been broadly implicated in the regulation of microtubule polymerization, actin bundling and endocytosis, three processes that influence growth cone dynamics and neurite outgrowth (Fukata et al., 2002c; Nishimura et al., 2003; Rosslenbroich et al., 2005). To gain insight into how CRMP4b may influence neurite outgrowth inhibition we assessed the distribution of CRMP4b in DRG growth cones. As previously described, endogenous CRMP4b has a punctate pattern within the growth cone extending throughout the central and peripheral domains (Fig. 5a) (Quinn et al., 2003). CRMP4b-V5 (Fig. 5b) and CRMP4b-GFP (Fig. 5c, d) fusion proteins also label the entire growth cone with a less distinct punctate profile, likely due to elevated cytosolic CRMP4b levels in the overexpression paradigm. Carboxy-terminally tagged CRMP4b-GFP labels the growth cone more broadly than tubulin (Fig. 5d) and extends into the actin-rich peripheral domain (Fig. 5c) co-localizing with actin at a subset of punctae within the growth cone (Fig. 5c, arrows). Intriguingly, CRMP4b-V5 overexpression promotes the extension of filopodia from the growth cone that are on average 70% longer than filopodia in GFP-infected growth cones (Fig. 5e, f, g). This phenotype is also manifested in the DRG neurite where ectopic actin-rich branches are formed (Fig. 5 e, f, g; arrowheads). A similar phenotype was promoted by CRMP4b-GFP overexpression (data not shown). On average, 8 branches per neurite can be detected on CRMP4b-infected neurites compared to 2 branches per neurite on GFP-infected neurites (Fig. 5g). The localization of CRMP4b within the growth cone is consistent with a role in microtubule or actin dynamics. However, the filopodial and branching phenotypes promoted by CRMP4b overexpression suggest that CRMP4b may affect neuronal

phenotype through an actin-based mechanism, an important observation since CRMPs have been implicated in modulating both microtubule and actin dynamics (Fukata et al., 2002b; Rosslenbroich et al., 2005). While we cannot rule out the possibility that the fusion proteins may behave differently from native CRMP4b, both CRMP4b-V5 and CRMP4b-GFP have similar distributions to endogenous CRMP and promote similar growth cone phenotypes suggesting that CRMP4b functionally interacts mainly with the actin cytoskeleton.

To further address the potential role for CRMP4b-RhoA complexes in neurite outgrowth inhibition, the distribution of CRMP4b and RhoA during Nogo-dependent growth cone collapse was examined. Uninfected and CRMP4b-V5-infected DRG growth cones were fixed and stained for endogenous RhoA and CRMP4b or the V5 epitope tag. Prior to stimulation, endogenous RhoA and CRMP4b have distinct distributions within the growth cone with negligible colocalization (Fig. 6a). In unstimulated (control) growth cones, overexpressed CRMP4b-V5 also has a distinct distribution compared to endogenous RhoA (Fig. 6b). Following myelin stimulation, RhoA and CRMP4b-V5 co-localize at a subset of distinct punctae within the growth cone central and peripheral domain (Fig. 6b, arrows) suggesting that a RhoA-CRMP4b complex forms in the growth cone where it may regulate actin cytoskeletal dynamics in response to inhibitory challenges.

III.5.6. C4RIP-V5 attenuates CRMP4b-RhoA binding

Nogo regulation of the CRMP4b-RhoA interaction raises the possibility that CRMP4b-RhoA complex formation is critical for inhibitory signaling. The specific enhancement of

CRMP4b-RhoA binding suggests that the increased affinity may be mediated by the CRMP4b amino terminal extension. We therefore assessed the ability of the CRMP4b amino terminus to mediate RhoA binding. We generated a chimeric CRMP molecule consisting of the CRMP4b amino terminus fused to CRMP2 (CRMP4bNCRMP2) and a CRMP4 construct consisting of the common region of CRMP4a and CRMP4b (CRMP4aΔN; Fig. 7a). Both CRMP4bNCRMP2 and CRMP4aΔN co-immunoprecipitate with RhoA, however both proteins bind RhoA more weakly than full length CRMP4b (Fig. 7b). This indicates that two independent RhoA binding sites within the CRMP4b molecule are sufficient for RhoA binding and that the two sites may co-operate to mediate maximal RhoA binding; however the amino-terminal region of CRMP4b appears to be the critical site mediating Nogo-dependent recruitment to RhoA (Fig. 3).

To specifically disrupt the Nogo-dependent interaction between full length CRMP4b and RhoA, we generated a construct to express the unique amino terminal domain of CRMP4b (C4RIP – CRMP4b-RhoA Inhibitory Peptide; Fig. 7a) fused to a V5-epitope tag. As predicted, co-transfection of C4RIP-V5 significantly diminishes RhoA binding to full length CRMP4b without affecting binding to CRMP4a (Fig. 7c), presumably by competing for the RhoA binding site/s.

The specificity of C4RIP-V5 was then evaluated by examining its effect on binding between FLAG-RhoA63L and myc-Rho kinase (myc-ROCK), a critical RhoA effector molecule for myelin inhibition. C4RIP-V5 does not diminish binding between FLAG-RhoA63L and myc-ROCK (Fig. 7d). Similarly, overexpression of CRMP4b-V5 does not affect the binding between FLAG-RhoA63L and myc-ROCK indicating that CRMP4b does not alter the ability of RhoA to interact with this downstream effector.

III.5.7. C4RIP-V5 attenuates neurite outgrowth inhibition

The ability of C4RIP-V5 to attenuate CRMP4b-RhoA binding provides a valuable tool to determine if CRMP4b-RhoA complex formation is necessary for its role in myelin inhibition. CRMP4b-V5 or C4RIP-V5 was introduced into dissociated E13 chick DRG neurons via recombinant HSV virus (Fig. 8a) and neurite outgrowth was assessed on myelin substrates (Fig. 8b, d). CRMP4b-V5 is not sufficient to mimic myelin inhibitory responses (Fig. b, c), presumably due to its failure to increase its interaction with RhoA (Fig. 6b). C4RIP significantly attenuates myelin-dependent outgrowth inhibition compared to GFP- or full length CRMP4b-infected neurons (Fig. 8b, d). Unlike previously characterized Rho and ROCK antagonists, C4RIP does not promote basal DRG outgrowth on permissive control substrates (Lehmann et al., 1999; Fournier et al., 2003). DRG neurons infected with HSVC4RIP or with HSV-dominant negative ROCK (Alabed et al., 2006), are both protected from myelin inhibition; however, HSV-DNROCK promotes basal outgrowth by approximately 50%.

An advantage to targeting intracellular mediators of neurite outgrowth inhibition is their potential as convergent targets, which may attenuate multiple inhibitory influences. To evaluate if C4RIP-V5 may block additional inhibitory signals associated with CNS injury, we examined the effect of C4RIP-V5 on neurite outgrowth inhibition mediated by aggrecan, an inhibitory chondroitin sulphate proteoglycan of the glial scar. Intriguingly, we find that C4RIP also promotes neurite outgrowth on aggrecan (Fig. 8e).

To assess the specificity of C4RIP we tested its ability to block Sema3A-dependent growth cone collapse in E8 chick DRG neurons. Sema3A-stimulation of neurons engages Rac1 GTPase and the CRMP2 isoform (Huber et al., 2003). We find

that C4RIP has no effect on Sema3A-dependent growth cone collapse (Fig. 8f). Taken together, these results indicate that disruption of the CRMP4b-RhoA interaction protects neurons from inhibitory influences that signal through RhoA GTPase.

III.6. DISCUSSION

Regeneration following CNS trauma is limited by the activation of intracellular pathways within the injured neuron that block axonal extension through targeted modifications to the cytoskeleton (Hsieh et al., 2006). Development of antagonists to intracellular targets of axon outgrowth inhibitors is an effective approach to circumvent the inhibitory influence of the astroglial scar and MAIs (Dergham et al., 2002; Niederost et al., 2002; Borisoff et al., 2003; Fournier et al., 2003). In this study we have demonstrated that CRMP4b is a necessary intracellular mediator of neurite outgrowth inhibition. Our findings suggest that complex formation between CRMP4b and RhoA is critical for outgrowth inhibition and that this inhibition may be mediated through an actin-dependent phenotype. By targeting the critical CRMP4b-RhoA binding interaction, we have developed a competitive antagonist of CRMP4b-RhoA binding, which specifically promotes neurite outgrowth on inhibitory substrates suggesting an exciting new therapeutic target for nerve repair following CNS injury.

Dynamics of the CRMP4b-RhoA interaction

We have described a specific protein interaction between RhoA and CRMP4, which does not extend to other closely related family members of the Rho GTPases, nor to other CRMP family members. In the absence of Nogo stimulation the baseline interaction

between endogenous CRMP4b and RhoA is negligible in cerebellar neurons and DRG growth cones. In transfected 293T and PC12 cells, CRMP4b and RhoA do interact in the absence of Nogo; however, this interaction could be a function of protein overexpression. Intriguingly, the RhoA-CRMP4b interaction is not dependent on the nucleotide binding state of RhoA, rather the interaction is dependent on the phosphorylation status of CRMP4. This raises the additional possibility that the strength of the baseline interaction may vary in different cells types as a function of the complement of kinases and phosphatases.

Since the RhoA used to screen for protein interactors was purified in bacteria, it is also conceivable that dephosphorylated RhoA is a favored binding partner for CRMP4b. This raises the possibility of a novel level of RhoA regulation in response to myelin inhibitors where the focus has largely been on the ability of MAIs to convert RhoA to the GTP-bound form (Dergham et al., 2002; Niederost et al., 2002; Fournier et al., 2003). This idea is consistent with finding that RhoA is phosphorylated at Ser188 by PKA and that RhoA phosphorylation modifies its binding to its endogenous inhibitor, Rho guanine nucleotide dissociation factor (RhoGDI) (Ellerbroek et al., 2003b; Nusser et al., 2006). CRMP4 is subject to phosphorylation by GSK-3β and dephosphorylation by PP2A phosphatase (Hill et al., 2006) and it is reasonable to hypothesize that phosphorylation alters its binding properties based on similarities to CRMP2 (Uchida et al., 2005). Together this suggests a model whereby Nogo-dependent engagement of a CRMP- and/or Rho–directed phosphatase may promote formation of a CRMP4b-RhoA complex. A prediction of this model would be that CRMP4b over-expression may fail to mimic myelin inhibition in DRG neurons because its association with RhoA is not adequately

enhanced in the absence of appropriate post translational modifications, and this is what we observe (Fig. 6, 8).

Role of N-terminal variants of the CRMP proteins

The recent discovery of novel amino-terminal variants of the original CRMP members in rat and in chick (Quinn et al., 2003; Yuasa-Kawada et al., 2003) has revealed additional potential functions for CRMPs. The unique amino-terminal extensions of the CRMPb isoforms could impart supplementary functions to each CRMP variant by mediating additional protein interactions. A reasonable possibility is that CRMP4a and CRMP4b are capable of mediating similar cytoskeletal rearrangements; however the amino terminal region of CRMP4b is necessary for recruitment to the appropriate cytoskeletal elements within the growth cone. This is consistent with the finding that overexpression of CRMP4b but not CRMP4a leads to an increase in neurite branching (Quinn et al., 2003).

C4RIP as a therapeutic agent

Targeting CRMP4-RhoA with C4RIP is a potential avenue for therapeutic intervention. The ability of C4RIP to attenuate inhibition in response to both MAIs and CSPGs is an obvious advantage in the complex inhibitory environment following CNS injury. The enrichment of CRMP expression in the nervous system raises the possibility that additional side effects on other cell types may be limited when compared to targeting ubiquitous molecules such as RhoA or ROCK. A unique characteristic of C4RIP is its failure to affect basal neurite outgrowth. To our knowledge, this is the first example of an

intracellular molecule, which can be specifically ascribed to an inhibitor-dependent neurite outgrowth pathway.

The function of CRMP4b in myelin-dependent inhibition

Reasonable hypotheses for CRMP4 function in myelin inhibition include effects on microtubule dynamics, actin dynamics and/or endocytosis. CRMP2 can bind to tubulin heterodimers and is an important organizer of microtubule assembly for establishing axon-dendrite fate during development (Fukata et al., 2002c; Arimura et al., 2005b). This is partly mediated by binding to tubulin heterodimers and promoting microtubule assembly (Fukata et al., 2002c). In fact, CRMP2 undergoes ROCK-dependent phosphorylation in response to Nogo-66 and MAG and has been hypothesized to subsequently regulate microtubule dynamics (Mimura et al., 2006). Although all CRMP members can bind tubulin, CRMP4 is not a substrate for ROCK and we were unable to detect a change in affinity between CRMP4 and tubulin in response to Nogo-66 stimulation by co-immunoprecipitation analysis in PC12 cells (data not shown). This suggests that Nogo-66 may not affect CRMP4-dependent microtubule dynamics; however, if the interaction is locally regulated within the neurite or growth cone, we may have failed to detect this by co-immunoprecipitation.

Our data suggests that a CRMP4b-RhoA complex may participate in neurite outgrowth inhibition through actin-dependent processes. An association between CRMP4b and intersectin, an endocytic-exocytic adaptor protein (Quinn et al., 2003) raises the possibility that CRMP4b could play a role in regulating endocytosis. Endocytosis during neurite inhibition may be important for regulating membrane

dynamics (Fournier et al., 2000b), or may target the internalization of cell surface receptors or cell adhesion molecules enabling their temporal and spatial regulation in response to MAIs. A plausible model is one in which CRMP4b-RhoA complexes regulate localized actin rearrangements from the growth cone periphery to the central domain and subsequent endocytic events necessary for growth cone collapse and neurite withdrawal.

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III.7. REFERENCES

Alabed YZ, Grados-Munro E, Ferraro GB, Hsieh S, Fournier AE (2006) Neuronal responses to myelin are mediated by ROCK. J Neurochem 96:1616-1625.

Amano M, Kruse HJ, Kaibuchi K, Weber PC, Aepfelbacher M (1998) Myosin II activation promotes neurite retraction during the action of Rho and Rho-kinase. Journal of Biological Chemistry 273:21867-21874.

Amano M, Chihara K, Kimura K, Fukata Y, Nakamura N, Matsuura Y, Kaibuchi K (1997) Formation of actin stress fibers and focal adhesions enhanced by Rho-kinase. Science 275:1308-1311.

Amano M, Ito M, Kimura K, Fukata Y, Chihara K, Nakano T, Matsuura Y, Kaibuchi K (1996) Phosphorylation and activation of myosin by Rho-associated kinase (Rho-kinase). J Biol Chem 271:20246-20249.

Arimura N, Menager C, Kawano Y, Yoshimura T, Kawabata S, Hattori A, Fukata Y, Amano M, Goshima Y, Inagaki M, Morone N, Usukura J, Kaibuchi K (2005) Phosphorylation by Rho kinase regulates CRMP-2 activity in growth cones. Mol Cell Biol 25:9973-9984.

Arthur WT, Ellerbroek SM, Der CJ, Burridge K, Wennerberg K (2002) XPLN, a guanine nucleotide exchange factor for RhoA and RhoB, but not RhoC. Journal of Biological Chemistry 277:42964-42972.

Bartsch U, Bandtlow CE, Schnell L, Bartsch S, Spillmann AA, Rubin BP, Hillenbrand R, Montag D, Schwab ME, Schachner M (1995) Lack of evidence that myelin-associated glycoprotein is a major inhibitor of axonal regeneration in the CNS. Neuron 15:1375-1381.

Bishop AL, Hall A (2000) Rho GTPases and their effector proteins. Biochem J 348 Pt 2:241-255.

Borisoff JF, Chan CC, Hiebert GW, Oschipok L, Robertson GS, Zamboni R, Steeves JD, Tetzlaff W (2003) Suppression of Rho-kinase activity promotes axonal growth on inhibitory CNS substrates. Mol Cell Neurosci 22:405-416.

Brown ME, Bridgman PC (2003) Retrograde flow rate is increased in growth cones from myosin IIB knockout mice. J Cell Sci 116:1087-1094.

Byk T, Dobransky T, Cifuentes-Diaz C, Sobel A (1996) Identification and molecular characterization of Unc-33-like phosphoprotein (Ulip), a putative mammalian homolog of the axonal guidance-associated unc-33 gene product. Journal of Neuroscience 16:688-701.

Calautti E, Grossi M, Mammucari C, Aoyama Y, Pirro M, Ono Y, Li J, Dotto GP (2002) Fyn tyrosine kinase is a downstream mediator of Rho/PRK2 function in keratinocyte cell-cell adhesion. J Cell Biol 156:137-148.

Chen MS, Huber AB, van der Haar ME, Frank M, Schnell L, Spillmann AA, Christ F, Schwab ME (2000) Nogo-A is a myelin-associated neurite outgrowth inhibitor and an antigen for monoclonal antibody IN-1. Nature 403:434-439.

Cheung A, Dantzig JA, Hollingworth S, Baylor SM, Goldman YE, Mitchison TJ, Straight AF (2002) A small-molecule inhibitor of skeletal muscle myosin II. Nat Cell Biol 4:83-88.

Davies SP, Reddy H, Caivano M, Cohen P (2000) Specificity and mechanism of action of some commonly used protein kinase inhibitors. Biochem J 351:95-105.

Dent EW, Gertler FB (2003) Cytoskeletal dynamics and transport in growth cone motility and axon guidance. Neuron 40:209-227.

Dergham P, Ellezam B, Essagian C, Avedissian H, Lubell WD, McKerracher L (2002) Rho signaling pathway targeted to promote spinal cord repair. J Neurosci 22:6570-6577.

Diefenbach TJ, Latham VM, Yimlamai D, Liu CA, Herman IM, Jay DG (2002) Myosin 1c and myosin IIB serve opposing roles in lamellipodial dynamics of the neuronal growth cone. J Cell Biol 158:1207-1217.

Ellerbroek SM, Wennerberg K, Burridge K (2003) Serine phosphorylation negatively regulates RhoA in vivo. J Biol Chem 278:19023-19031.

Feig LA, Cooper GM (1988) Inhibition of NIH 3T3 cell proliferation by a mutant ras protein with preferential affinity for GDP. Mol Cell Biol 8:3235-3243.

Fouad K, Dietz V, Schwab ME (2001) Improving axonal growth and functional recovery after experimental spinal cord injury by neutralizing myelin associated inhibitors. Brain Res Brain Res Rev 36:204-212.

Fournier AE, GrandPre T, Strittmatter SM (2001) Identification of a receptor mediating Nogo-66 inhibition of axonal regeneration. Nature 409:341-346.

Fournier AE, Takizawa BT, Strittmatter SM (2003) Rho Kinase inhibition enhances axonal regeneration in the injured CNS. J Neurosci 23:1416-1423.

Fournier AE, Nakamura F, Kawamoto S, Goshima Y, Kalb RG, Strittmatter SM (2000) Semaphorin3A enhances endocytosis at sites of receptor-F-actin colocalization during growth cone collapse. J Cell Biol 149:411-422.

Fujita A, Hattori Y, Takeuchi T, Kamata Y, Hata F (2001) NGF induces neurite outgrowth via a decrease in phosphorylation of myosin light chain in PC12 cells. Neuroreport 12:3599-3602.

Fukata Y, Itoh TJ, Kimura T, Menager C, Nishimura T, Shiromizu T, Watanabe H, Inagaki N, Iwamatsu A, Hotani H, Kaibuchi K (2002a) CRMP-2 binds to tubulin heterodimers to promote microtubule assembly. Nat Cell Biol 4:583-591.

Fukata Y, Itoh TJ, Kimura T, Menager C, Nishimura T, Shiromizu T, Watanabe H, Inagaki N, Iwamatsu A, Hotani H, Kaibuchi K (2002b) CRMP-2 binds to tubulin heterodimers to promote microtubule assembly. Nature Cell Biology 4:583-591.

Gaetano C, Matsuo T, Thiele CJ (1997) Identification and characterization of a retinoic acid-regulated human homologue of the unc-33-like phosphoprotein gene (hUlip) from neuroblastoma cells. J Biol Chem 272:12195-12201.

Gallo G (2004) Myosin II activity is required for severing-induced axon retraction in vitro. Exp Neurol 189:112-121.

Goshima Y, Nakamura F, Strittmatter P, Strittmatter SM (1995) Collapsin-induced growth cone collapse mediated by an intracellular protein related to UNC-33. Nature 376:509-514.

GrandPre T, Li S, Strittmatter SM (2002) Nogo-66 receptor antagonist peptide promotes axonal regeneration. Nature 417:547-551.

GrandPre T, Nakamura F, Vartanian T, Strittmatter SM (2000) Identification of the Nogo inhibitor of axon regeneration as a Reticulon protein. Nature 403:439-444.

Hall A (1994) Small GTP-binding proteins and the regulation of the actin cytoskeleton. Annu Rev Cell Biol 10:31-54.

Hedgecock EM, Culotti JG, Thomson JN, Perkins LA (1985) Axonal guidance mutants of Caenorhabditis elegans identified by filling sensory neurons with fluorescein dyes. Developmental Biology 111:158-170.

Hill JJ, Callaghan DA, Ding W, Kelly JF, Chakravarthy BR (2006) Identification of okadaic acid-induced phosphorylation events by a mass spectrometry approach. Biochem Biophys Res Commun 342:791-799.

Hsieh S, Ferraro GB, Fournier AE (2006) Myelin-associated inhibitors regulate cofilin phosphorylation and neuronal inhibition through Lim kinase and Slingshot phosphatase. J Neurosci 26:1006-1015.

Huber A, Kolodkin A, Ginty D, Cloutier J-F (2003) Signaling at the growth cone: Ligand-receptor complexes and the control of axon growth and guidance. Ann Rev Neurosci 26:509-563.

Igarashi M, Strittmatter SM, Vartanian T, Fishman MC (1993) Mediation by G proteins of signals that cause collapse of growth cones. Science 259:77-79.

Inatome R, Tsujimura T, Hitomi T, Mitsui N, Hermann P, Kuroda S, Yamamura H, Yanagi S (2000) Identification of CRAM, a novel unc-33 gene family protein that associates with CRMP3 and protein-tyrosine kinase(s) in the developing rat brain. J Biol Chem 275:27291-27302.

Ishizaki T, Maekawa M, Fujisawa K, Okawa K, Iwamatsu A, Fujita A, Watanabe N, Saito Y, Kakizuka A, Morii N, Narumiya S (1996) The small GTP-binding protein Rho binds to and activates a 160 kDa Ser/Thr protein kinase homologous to myotonic dystrophy kinase. Embo J 15:1885-1893. abs.html.

Jin Z, Strittmatter SM (1997) Rac1 mediates collapsin-1-induced growth cone collapse. J Neurosci 17:6256-6263.

Khosravi-Far R, Chrzanowska-Wodnicka M, Solski PA, Eva A, Burridge K, Der CJ (1994) Dbl and Vav mediate transformation via mitogen-activated protein kinase pathways that are distinct from those activated by oncogenic Ras. Mol Cell Biol 14:6848-6857.

Kim JE, Liu BP, Park JH, Strittmatter SM (2004) Nogo-66 receptor prevents raphespinal and rubrospinal axon regeneration and limits functional recovery from spinal cord injury. Neuron 44:439-451.

Kim JE, Li S, GrandPre T, Qiu D, Strittmatter SM (2003) Axon regeneration in young adult mice lacking nogo-a/b. Neuron 38:187-199.

Kimura K, Ito M, Amano M, Chihara K, Fukata Y, Nakafuku M, Yamamori B, Feng J, Nakano T, Okawa K, Iwamatsu A, Kaibuchi K (1996) Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho- kinase). Science 273:245-248.

Kobayashi K, Takahashi M, Matsushita N, Miyazaki J, Koike M, Yaginuma H, Osumi N, Kaibuchi K, Kobayashi K (2004) Survival of developing motor neurons mediated by Rho GTPase signaling pathway through Rho-kinase. J Neurosci 24:3480-3488.

Kottis V, Thibault P, Mikol D, Xiao ZC, Zhang R, Dergham P, Braun PE (2002) Oligodendrocyte-myelin glycoprotein (OMgp) is an inhibitor of neurite outgrowth. J Neurochem 82:1566-1569.

Kranenburg O, Poland M, van Horck FP, Drechsel D, Hall A, Moolenaar WH (1999) Activation of RhoA by lysophosphatidic acid and Galpha12/13 subunits in neuronal cells: induction of neurite retraction. Mol Biol Cell 10:1851-1857.

Lee JK, Kim JE, Sivula M, Strittmatter SM (2004) Nogo receptor antagonism promotes stroke recovery by enhancing axonal plasticity. J Neurosci 24:6209-6217.

Lehmann M, Fournier A, Selles-Navarro I, Dergham P, Sebok A, Leclerc N, Tigyi G, McKerracher L (1999) Inactivation of Rho signaling pathway promotes CNS axon regeneration. J Neurosci 19:7537-7547.

Leung T, Manser E, Tan L, Lim L (1995) A novel serine/threonine kinase binding the Ras-related RhoA GTPase which translocates the kinase to peripheral membranes. J Biol Chem 270:29051-29054.

Li S, Liu BP, Budel S, Li M, Ji B, Walus L, Li W, Jirik A, Rabacchi S, Choi E, Worley D, Sah DW, Pepinsky B, Lee D, Relton J, Strittmatter SM (2004a) Blockade of Nogo-66, myelin-associated glycoprotein, and oligodendrocyte myelin glycoprotein by soluble Nogo-66 receptor promotes axonal sprouting and recovery after spinal injury. J Neurosci 24:10511-10520.

Li W, Walus L, Rabacchi SA, Jirik A, Chang E, Schauer J, Zheng BH, Benedetti NJ, Liu BP, Choi E, Worley D, Silvian L, Mo W, Mullen C, Yang W, Strittmatter SM, Sah DW, Pepinsky B, Lee DH (2004b) A neutralizing anti-Nogo66 receptor monoclonal antibody reverses inhibition of neurite outgrowth by central nervous system myelin. J Biol Chem 279:43780-43788.

Luo Y, Raible D, Raper JA (1993) Collapsin: a protein in brain that induces the collapse and paralysis of neuronal growth cones. Cell 75:217-227.

Mandemakers WJ, Barres BA (2005) Axon regeneration: it's getting crowded at the gates of TROY. Curr Biol 15:R302-305.

Matsui T, Amano M, Yamamoto T, Chihara K, Nakafuku M, Ito M, Nakano T, Okawa K, Iwamatsu A, Kaibuchi K, Ishizaki T (1996) Rho-associated kinase, a novel serine/threonine kinase, as a putative target for small GTP binding protein Rho
The small GTP-binding protein Rho binds to and activates a 160 kDa Ser/Thr protein kinase homologous to myotonic dystrophy kinase. EMBO Journal 15:2208-2216.

McKerracher L, David S, Jackson DL, Kottis V, Dunn RJ, Braun PE (1994) Identification of myelin-associated glycoprotein as a major myelin-derived inhibitor of neurite growth. Neuron 13:805-811.

McPherson PS, Czernik AJ, Chilcote TJ, Onofri F, Benfenati F, Greengard P, Schlessinger J, De Camilli P (1994) Interaction of Grb2 via its Src homology 3

domains with synaptic proteins including synapsin I. Proc Natl Acad Sci U S A 91:6486-6490.

Mi S, Lee X, Shao Z, Thill G, Ji B, Relton J, Levesque M, Allaire N, Perrin S, Sands B, Crowell T, Cate RL, McCoy JM, Pepinsky RB (2004) LINGO-1 is a component of the Nogo-66 receptor/p75 signaling complex. Nat Neurosci 7:221-228.

Mimura F, Yamagishi S, Arimura N, Fujitani M, Kubo T, Kaibuchi K, Yamashita T (2006) Myelin-associated Glycoprotein Inhibits Microtubule Assembly by a Rho-kinase-dependent Mechanism. J Biol Chem 281:15970-15979.

Minturn JE, Fryer HJ, Geschwind DH, Hockfield S (1995) TOAD-64, a gene expressed early in neuronal differentiation in the rat, is related to unc-33, a C. elegans gene involved in axon outgrowth. Journal of Neuroscience 15:6757-6766.

Mukhopadhyay G, Doherty P, Walsh FS, Crocker PR, Filbin MT (1994) A novel role for myelin-associated glycoprotein as an inhibitor of axonal regeneration. Neuron 13:757-767.

Nakamura F, Tanaka M, Takahashi T, Kalb RG, Strittmatter SM (1998) Neuropilin-1 extracellular domains mediate semaphorin D/III-induced growth cone collapse. Neuron 21:1093-1100.

Neve RL, Howe JR, Hong S, Kalb RG (1997) Introduction of the glutamate receptor subunit 1 into motor neurons in vitro and in vivo using a recombinant herpes simplex virus. Neuroscience 79:435-447.

Niederost B, Oertle T, Fritsche J, McKinney RA, Bandtlow CE (2002) Nogo-A and myelin-associated glycoprotein mediate neurite growth inhibition by antagonistic regulation of RhoA and Rac1. J Neurosci 22:10368-10376.

Nishimura T, Fukata Y, Kato K, Yamaguchi T, Matsuura Y, Kamiguchi H, Kaibuchi K (2003) CRMP-2 regulates polarized Numb-mediated endocytosis for axon growth. Nature Cell Biology 5:819-826.

Nusser N, Gosmanova E, Makarova N, Fujiwara Y, Yang L, Guo F, Luo Y, Zheng Y, Tigyi G (2006) Serine phosphorylation differentially affects RhoA binding to effectors: implications to NGF-induced neurite outgrowth. Cell Signal 18:704-714.

Park JB, Yiu G, Kaneko S, Wang J, Chang J, He Z (2005) A TNF Receptor Family Member, TROY, Is a Coreceptor with Nogo Receptor in Mediating the Inhibitory Activity of Myelin Inhibitors. Neuron 45:345-351.

Pignot V, Hein AE, Barske C, Wiessner C, Walmsley AR, Kaupmann K, Mayeur H, Sommer B, Mir AK, Frentzel S (2003) Characterization of two novel proteins,

NgRH1 and NgRH2, structurally and biochemically homologous to the Nogo-66 receptor. J Neurochem 85:717-728.

Prinjha R, Moore SE, Vinson M, Blake S, Morrow R, Christie G, Michalovich D, Simmons DL, Walsh FS (2000) Inhibitor of neurite outgrowth in humans. Nature 403:383-384.

Quinn CC, Gray GE, Hockfield S (1999) A family of proteins implicated in axon guidance and outgrowth. Journal of Neurobiology 41:158-164.

Quinn CC, Chen E, Kinjo TG, Kelly G, Bell AW, Elliott RC, McPherson PS, Hockfield S (2003) TUC-4b, a novel TUC family variant, regulates neurite outgrowth and associates with vesicles in the growth cone. Journal of Neuroscience 23:2815-2823.

Riento K, Ridley AJ (2003) Rocks: multifunctional kinases in cell behaviour. Nat Rev Mol Cell Biol 4:446-456.

Rosslenbroich V, Dai L, Baader SL, Noegel AA, Gieselmann V, Kappler J (2005) Collapsin response mediator protein-4 regulates F-actin bundling. Exp Cell Res 310:434-444.

Shao Z, Browning JL, Lee X, Scott ML, Shulga-Morskaya S, Allaire N, Thill G, Levesque M, Sah D, McCoy JM, Murray B, Jung V, Pepinsky RB, Mi S (2005) TAJ/TROY, an Orphan TNF Receptor Family Member, Binds Nogo-66 Receptor 1 and Regulates Axonal Regeneration. Neuron 45:353-359.

Siddiqui SS, Culotti JG (1991) Examination of neurons in wild type and mutants of Caenorhabditis elegans using antibodies to horseradish peroxidase. Journal of Neurogenetics 7:193-211.

Simonen M, Pedersen V, Weinmann O, Schnell L, Buss A, Ledermann B, Christ F, Sansig G, van der Putten H, Schwab ME (2003) Systemic deletion of the myelin-associated outgrowth inhibitor nogo-a improves regenerative and plastic responses after spinal cord injury. Neuron 38:201-211.

Song XY, Zhong JH, Wang X, Zhou XF (2004) Suppression of p75NTR does not promote regeneration of injured spinal cord in mice. J Neurosci 24:542-546.

Suzuki Y, Nakagomi S, Namikawa K, Kiryu-Seo S, Inagaki N, Kaibuchi K, Aizawa H, Kikuchi K, Kiyama H (2003) Collapsin response mediator protein-2 accelerates axon regeneration of nerve-injured motor neurons of rat. Journal of Neurochemistry 86:1042-1050.

Takahashi T, Nakamura F, Jin Z, Kalb RG, Strittmatter SM (1998) Semaphorins A and E act as antagonists of neuropilin-1 and agonists of neuropilin-2 receptors. Nat Neurosci 1:487-493.

Uchida Y, Ohshima T, Sasaki Y, Suzuki H, Yanai S, Yamashita N, Nakamura F, Takei K, Ihara Y, Mikoshiba K, Kolattukudy P, Honnorat J, Goshima Y (2005) Semaphorin3A signalling is mediated via sequential Cdk5 and GSK3beta phosphorylation of CRMP2: implication of common phosphorylating mechanism underlying axon guidance and Alzheimer's disease. Genes Cells 10:165-179.

Venkatesh K, Chivatakarn O, Lee H, Joshi PS, Kantor DB, Newman BA, Mage R, Rader C, Giger RJ (2005) The Nogo-66 receptor homolog NgR2 is a sialic acid-dependent receptor selective for myelin-associated glycoprotein. J Neurosci 25:808-822.

Vincent S, Settleman J (1997) The PRK2 kinase is a potential effector target of both Rho and Rac GTPases and regulates actin cytoskeletal organization. Molecular & Cellular Biology 17:2247-2256.

Wang KC, Kim JA, Sivasankaran R, Segal R, He Z (2002a) P75 interacts with the Nogo receptor as a co-receptor for Nogo, MAG and OMgp. Nature 420:74-78.

Wang KC, Koprivica V, Kim JA, Sivasankaran R, Guo Y, Neve RL, He Z (2002b) Oligodendrocyte-myelin glycoprotein is a Nogo receptor ligand that inhibits neurite outgrowth. Nature 417:941-944.

Wang LH, Strittmatter SM (1997) Brain CRMP forms heterotetramers similar to liver dihydropyrimidinase. J Neurochem 69:2261-2269.

Wennerberg K, Ellerbroek SM, Liu RY, Karnoub AE, Burridge K, Der CJ (2002) RhoG signals in parallel with Rac1 and Cdc42. J Biol Chem 277:47810-47817.

Wong ST, Henley JR, Kanning KC, Huang KH, Bothwell M, Poo MM (2002) A p75(NTR) and Nogo receptor complex mediates repulsive signaling by myelin-associated glycoprotein. Nat Neurosci 5:1302-1308.

Yoshimura T, Kawano Y, Arimura N, Kawabata S, Kikuchi A, Kaibuchi K (2005) GSK-3beta regulates phosphorylation of CRMP-2 and neuronal polarity. Cell 120:137-149.

Yuasa-Kawada J, Suzuki R, Kano F, Ohkawara T, Murata M, Noda M (2003) Axonal morphogenesis controlled by antagonistic roles of two CRMP subtypes in microtubule organization. European Journal of Neuroscience 17:2329-2343.

Zheng B, Ho C, Li S, Keirstead H, Steward O, Tessier-Lavigne M (2003) Lack of enhanced spinal regeneration in nogo-deficient mice. Neuron 38:213-224.

Zheng B, Atwal J, Ho C, Case L, He XL, Garcia KC, Steward O, Tessier-Lavigne M (2005) Genetic deletion of the Nogo receptor does not reduce neurite inhibition in vitro or promote corticospinal tract regeneration in vivo. Proc Natl Acad Sci U S A 102:1205-1210.

Zhu Y, Stolz DB, Guo F, Ross MA, Watkins SC, Tan BJ, Qi RZ, Manser E, Li QT, Bay BH, Teo TS, Duan W (2004) Signaling via a novel integral plasma membrane pool of a serine/threonine protein kinase PRK1 in mammalian cells. Faseb J 18:1722-1724.

Figure 1 A novel RhoA-CRMP4 interaction is enhanced by Nogo-66. (a) GSTRhoA63L pull down from PC12 cells stimulated with AP (+ lysate) or AP-Nogo-66 (+ Nogo lysate). Precipitation of a 75 kDa protein identified as CRMP4b by tandem mass spectrometry is enhanced in the AP-Nogo-66-stimulated lysate (arrow). The protein runs slightly below a non-specific protein from the GSTRhoA63L purification, which is visible in all lanes. The GSTRhoA63L lane represents beads that were not incubated with lysates. **(b)** GST and GSTRhoA63L pull downs from PC12 cell lysates immunoblotted with a pan-CRMP antibody. CRMPa (65 kDa) and CRMPb (75kDa) bands are indicated in the PC12 input and pull down lanes (arrows).

FIGURE 1

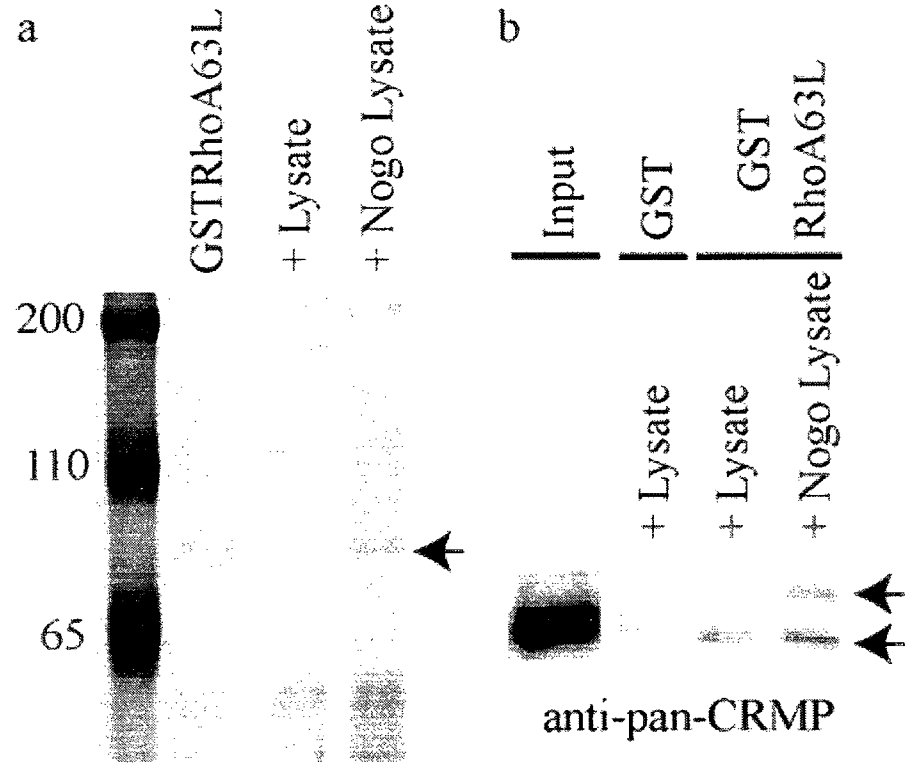

a
GSTRhoA63L
+ Lysate
+ Nogo Lysate
200
110
65
b
Input
GST
GST
RhoA63L
+ Lysate
+ Lysate
+ Nogo Lysate
anti-pan-CRMP

Figure 2 The RhoA-CRMP4 interaction is highly specific, nucleotide-independent, phospho-dependent and direct. (a-c) HEK293T cells co-transfected with CRMP-V5 constructs and myc-tagged versions of wild type and mutant Rho GTPases and subjected to myc-immunoprecipitation. **(a)** CRMP4 preferentially binds to RhoA. Data from two separate blots are separated by a vertical line. **(b)** RhoA is the preferred binding partner for CRMP4. **(c)** CRMP4 binding to RhoA is nucleotide-independent. **(d)** CRMP-V5 was immunoprecipitated from control or Calyculin-treated HEK293T cells, separated by SDS-PAGE and overlayed with recombinant RhoA. RhoA binds directly to CRMP4a and CRMP4b but fails to bind to phosphorylated CRMP4b.

FIGURE 2

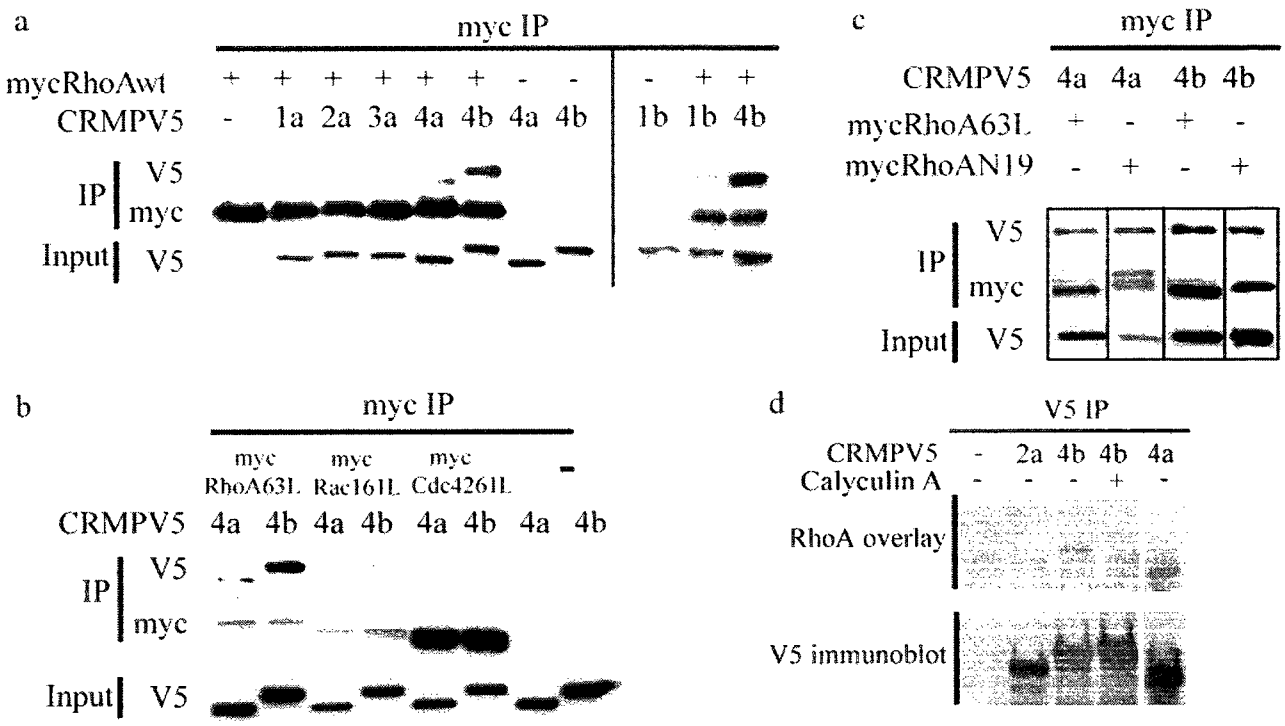

a
myc IP
mycRhoAwt + + + + + + - - - + +
CRMPV5 - 1a 2a 3a 4a 4b 4a 4b 1b 1b 4b
IP V5
myc
Input V5
b
myc IP
myc RhoA63L
myc Rac161L
myc Cdc4261L
CRMPV5 4a 4b 4a 4b 4a 4b 4a 4b
IP V5
myc
Input V5
c
myc IP
CRMPV5 4a 4a 4b 4b
mycRhoA63L + - + -
mycRhoAN19 - + - +
IP V5
myc
Input V5
d
V5 IP
CRMPV5 - 2a 4b 4b 4a
Calyculin A - - - + -
RhoA overlay
V5 immunoblot

Figure 3 Nogo-P4 peptide enhances the interaction between CRMP4b and RhoA. **(a)** PC12 cells transfected with myc-RhoA and CRMP4b-V5 or CRMP4a-V5 and, stimulated for 0, 1 or 10 minutes with Nogo-P4 peptide and subjected to myc-immunoprecipitation. **(b)** P8 rat cerebellar cultures stimulated with Nogo-P4 peptide, subjected to RhoA immunoprecipitation and analyzed for RhoA and CRMP4b.

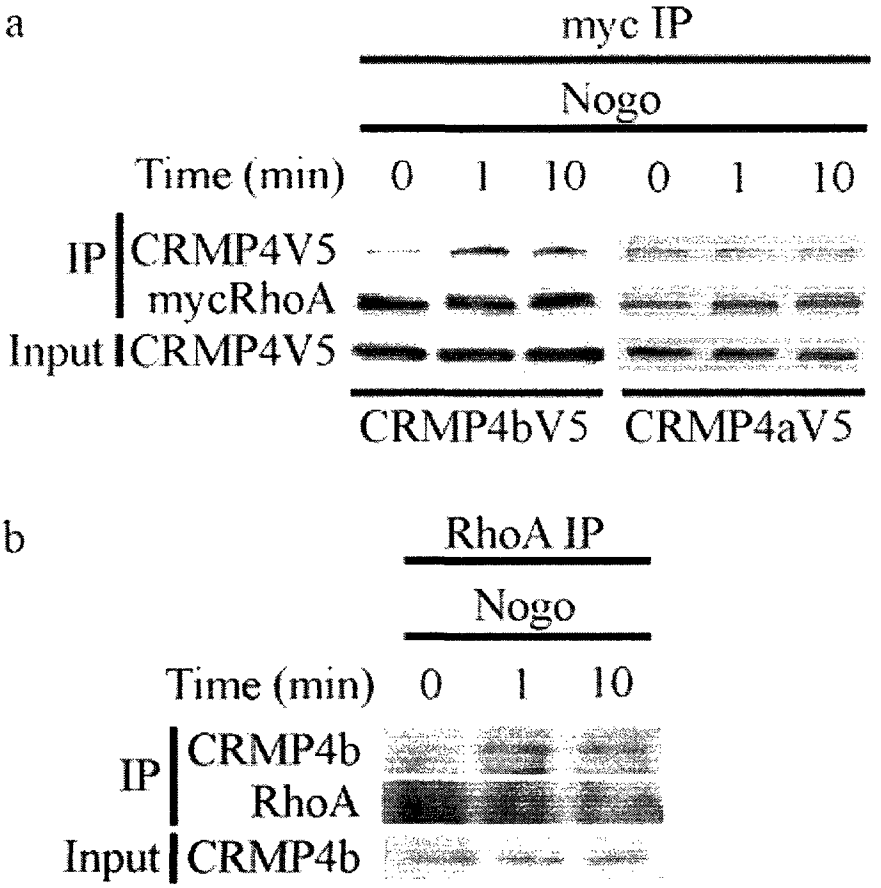

a
myc IP
Nogo
Time (min) 0 1 10 0 1 10
IP CRMP4V5
mycRhoA
Input CRMP4V5
CRMP4bV5
CRMP4aV5
b
RhoA IP
Nogo
Time (min) 0 1 10
IP CRMP4b
RhoA
Input CRMP4b

Figure 4 siRNA-mediated knockdown of CRMP4 expression promotes neurite outgrowth on myelin. **(a)** HEK293T cells co-transfected with CRMP4-V5 and scrambled- or CRMP4-targetted siRNA and analyzed by immunoblotting with anti-V5 antibody. **(b)** Dissociated P5 rat DRG neurons infected with HSVCRMP4b-GFP and transfected with scrambled or CRMP4 siRNA. Scale bar, 100 μm. **(c)** β-III tubulin-stained dissociated rat DRG neurons transfected with scrambled or CRMP4 siRNA and seeded on laminin (control) or myelin substrates for an 18 hour neurite outgrowth assay. Scale bar, 100 μm. **(d)** Quantitation of DRG outgrowth on control substrates with scrambled siRNA (Scram) or CRMP4 siRNA. **(e)** Quantitation of DRG neurite outgrowth on myelin from neurons transfected with scrambled or CRMP4 siRNA. Values are normalized to baseline outgrowth on the control laminin substrate for each experiment. Determinations are from 3 experiments performed in duplicate. *$p<0.01$ by student t-test compared to scrambled siRNA.

FIGURE 4

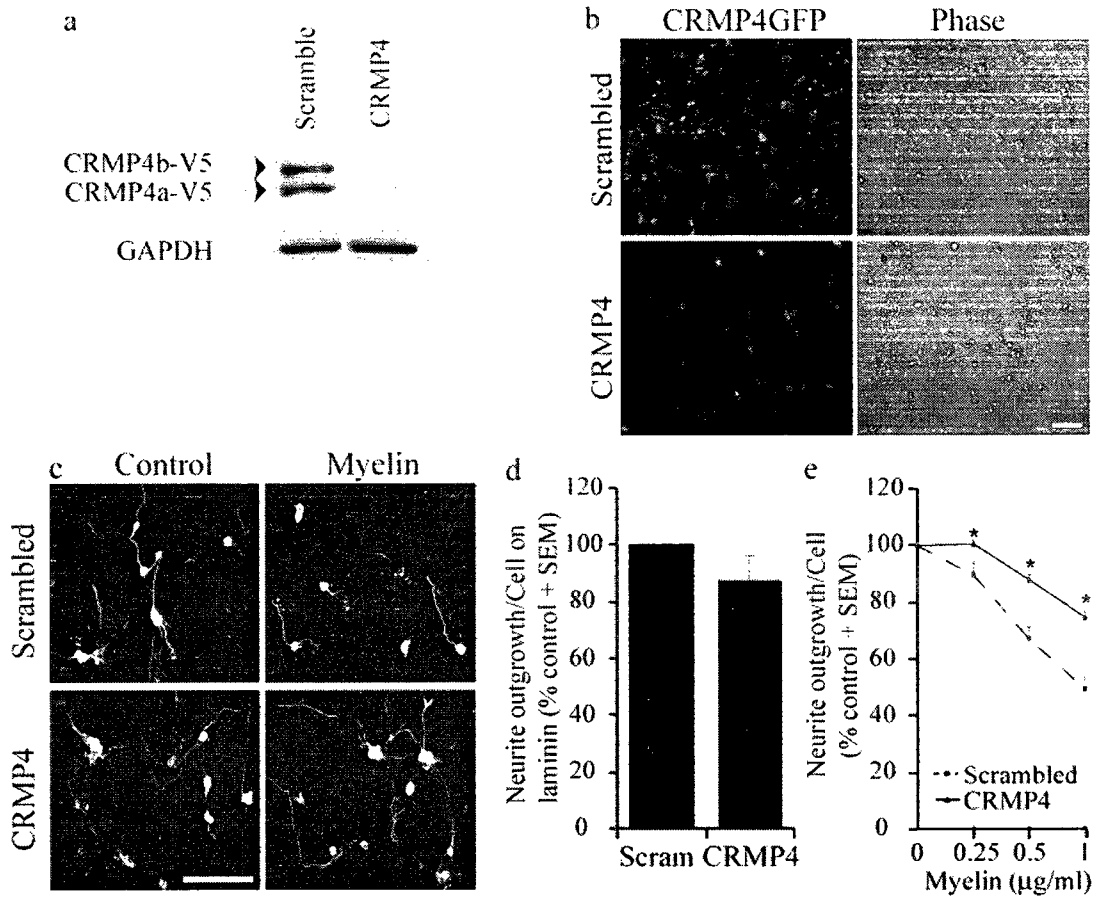

a
Scramble
CRMP4
CRMP4b-V5
CRMP4a-V5
GAPDH
b
CRMP4GFP
Phase
Scrambled
CRMP4
c
Control
Myelin
Scrambled
CRMP4
d
Neurite outgrowth/Cell on laminin (% control + SEM)
120
100
80
60
40
20
0
Scram
CRMP4
e
Neurite outgrowth/Cell (% control + SEM)
120
100
80
60
40
20
0
Scrambled
CRMP4
0
0.25
0.5
1
Myelin (µg/ml)

Figure 5 CRMP4b overexpression promotes an actin-based filopodial phenotype in the neuronal growth cone and neurite. **(a)** E13 chick DRG stained with an anti-CRMP4b antibody. **(b)** E7 chick DRG neurons infected with HSV-CRMP4b-V5 and stained with anti-V5 antibody. **(c-d)** E7 chick DRG neurons infected with HSV-CRMP4b-GFP and double stained with rhodamine phalloidin (c) to label F-actin or anti-βIII tubulin antibody (d). **(e)** E13 chick DRG neurons infected with HSV–GFP or HSV-CRMP4b-V5. GFP and CRMP4b-infected growth cones were stained with rhodamine phalloidin (red). CRMP4b-V5 was stained with anti-V5 antibody (green). Scale bar, 10 μm. **(f)** Magnification of boxed regions in panel (d) demonstrating enhanced neurite branching and filopodial extension in CRMP4b-V5-infected DRG neurons. **(g)** Quantitation of number of branches per neurite and filopodial length in GFP- or CRMP4b-V5-infected DRG neurons.

FIGURE 5

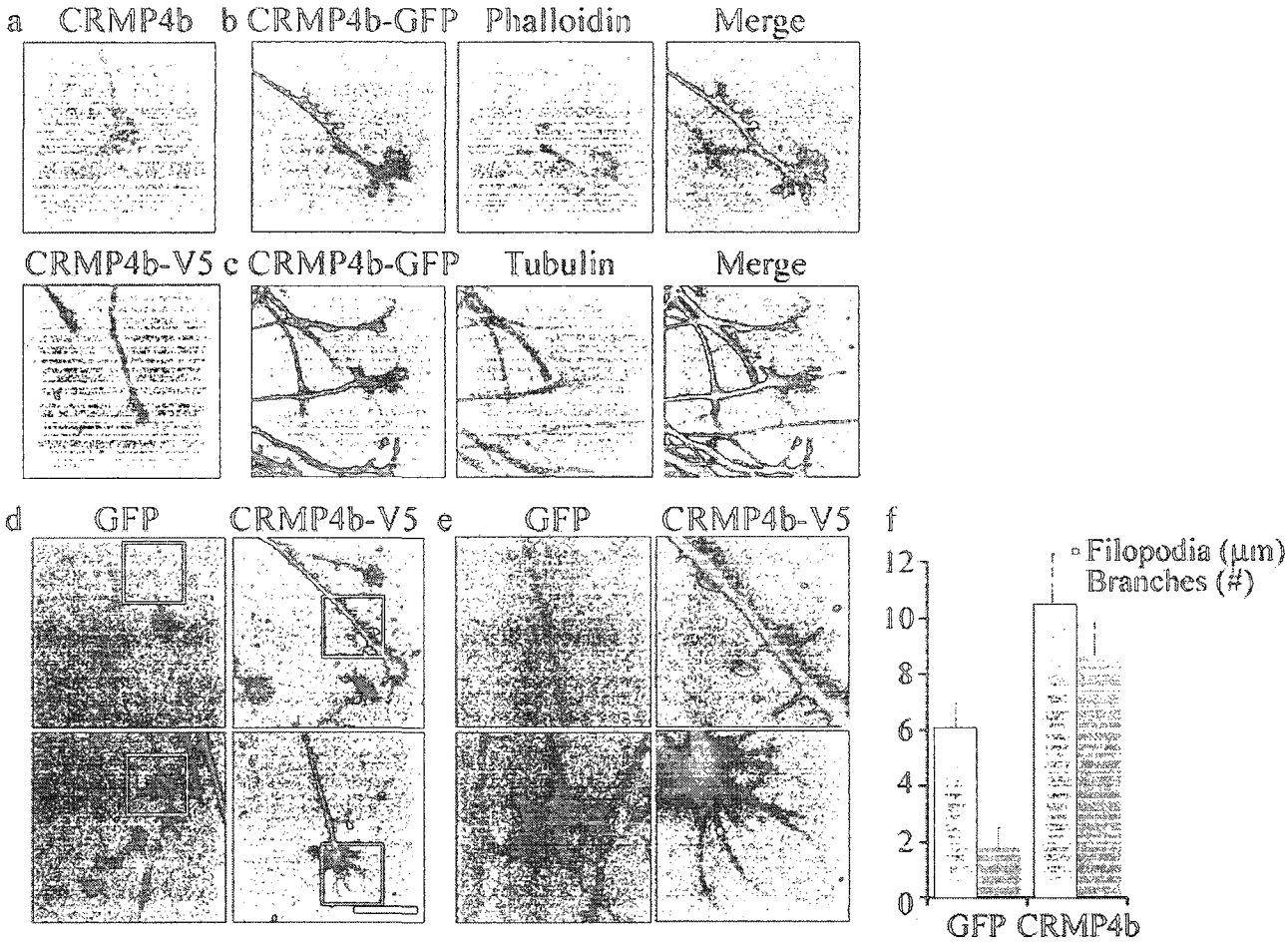

a CRMP4b
b CRMP4b-GFP
Phalloidin
Merge
CRMP4b-V5
c CRMP4b-GFP
Tubulin
Merge
d GFP
CRMP4b-V5
e GFP
CRMP4b-V5
f
Filopodia (μm)
Branches (#)
12
10
8
6
4
2
0
GFP
CRMP4b

Figure 6 CRMP4b and RhoA co-localize at discrete punctae during myelin-dependent growth cone collapse. Immunofluorescent detection of endogenous CRMP4b and RhoA **(a)** or CRMP4b-V5 and RhoA **(b)** in control or myelin-stimulated E13 chick DRG growth cones. Arrows in the merged panels indicate areas of CRMP4b-V5-RhoA co-localization.

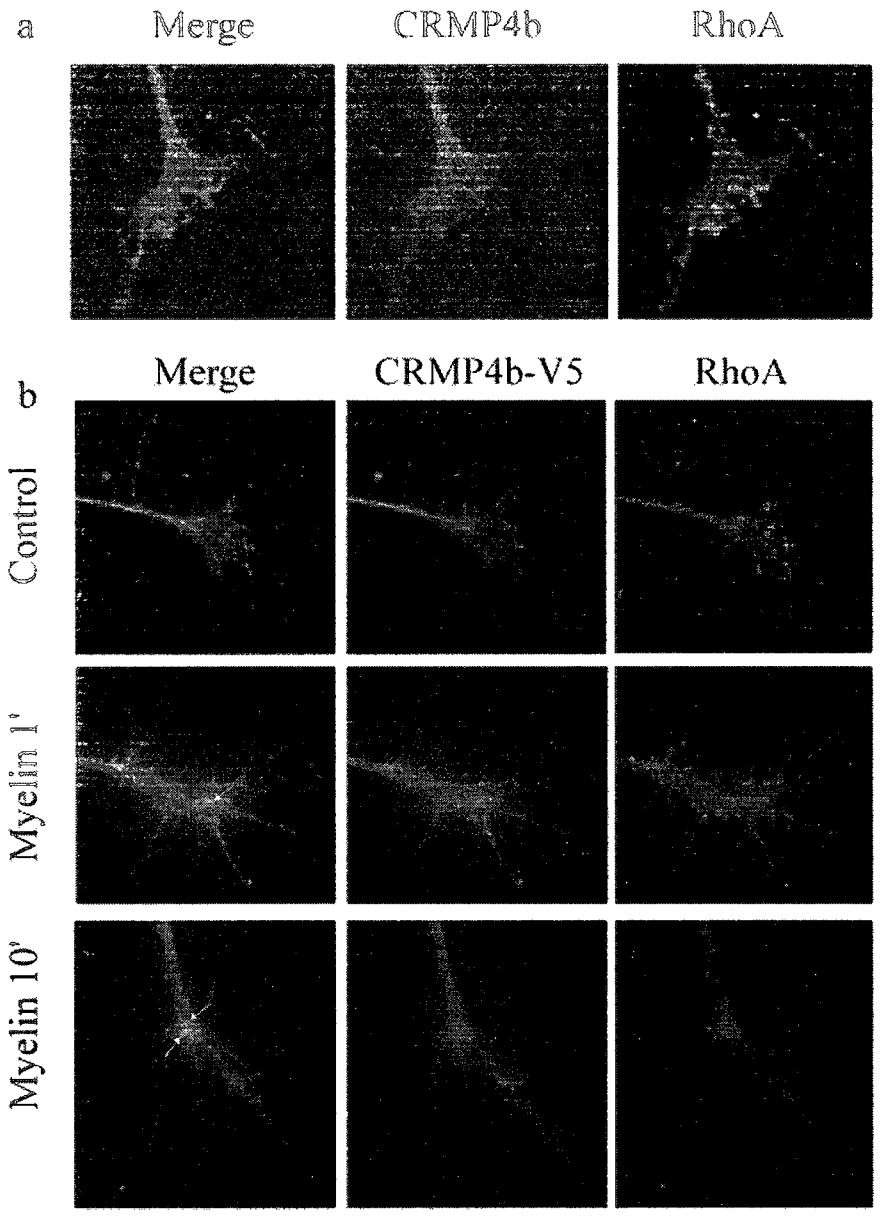

a
Merge
CRMP4b
RhoA
b
Merge
CRMP4b-V5
RhoA
Control
Myelin 1'
Myelin 10'

Figure 7 The amino terminal domain of CRMP4b is sufficient for RhoA binding and disrupts full length CRMP4b-RhoA binding when expressed as a recombinant fusion protein. **(a)** Schematic of CRMP constructs generated to assess CRMP4 domains involved in RhoA binding. **(b)** HEK293T cells co-transfected with myc-RhoA63L and CRMP-V5 constructs described in (a) and subjected to myc-immunoprecipitation. **(c)** HEK293T cells co-transfected with myc-RhoAWT, CRMP4-V5 and C4RIP-V5 to assess the effect of C4RIP on full length CRMP4 binding to RhoA. **(d)** HEK293T cells co-transfected with FLAG-RhoA63L, myc-ROCK and C4RIP-V5 to assess the effect of C4RIP on RhoA binding to ROCK.

FIGURE 7

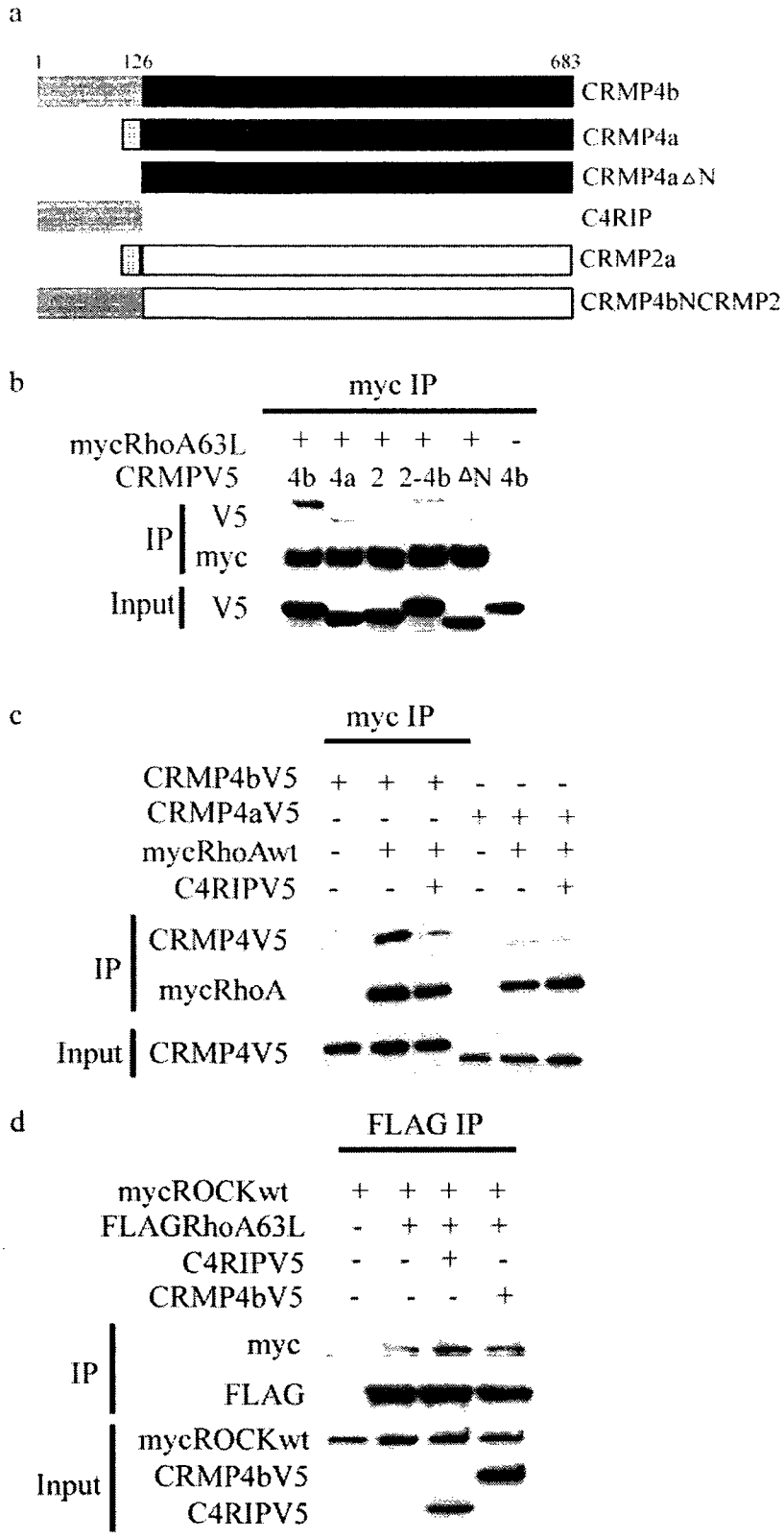
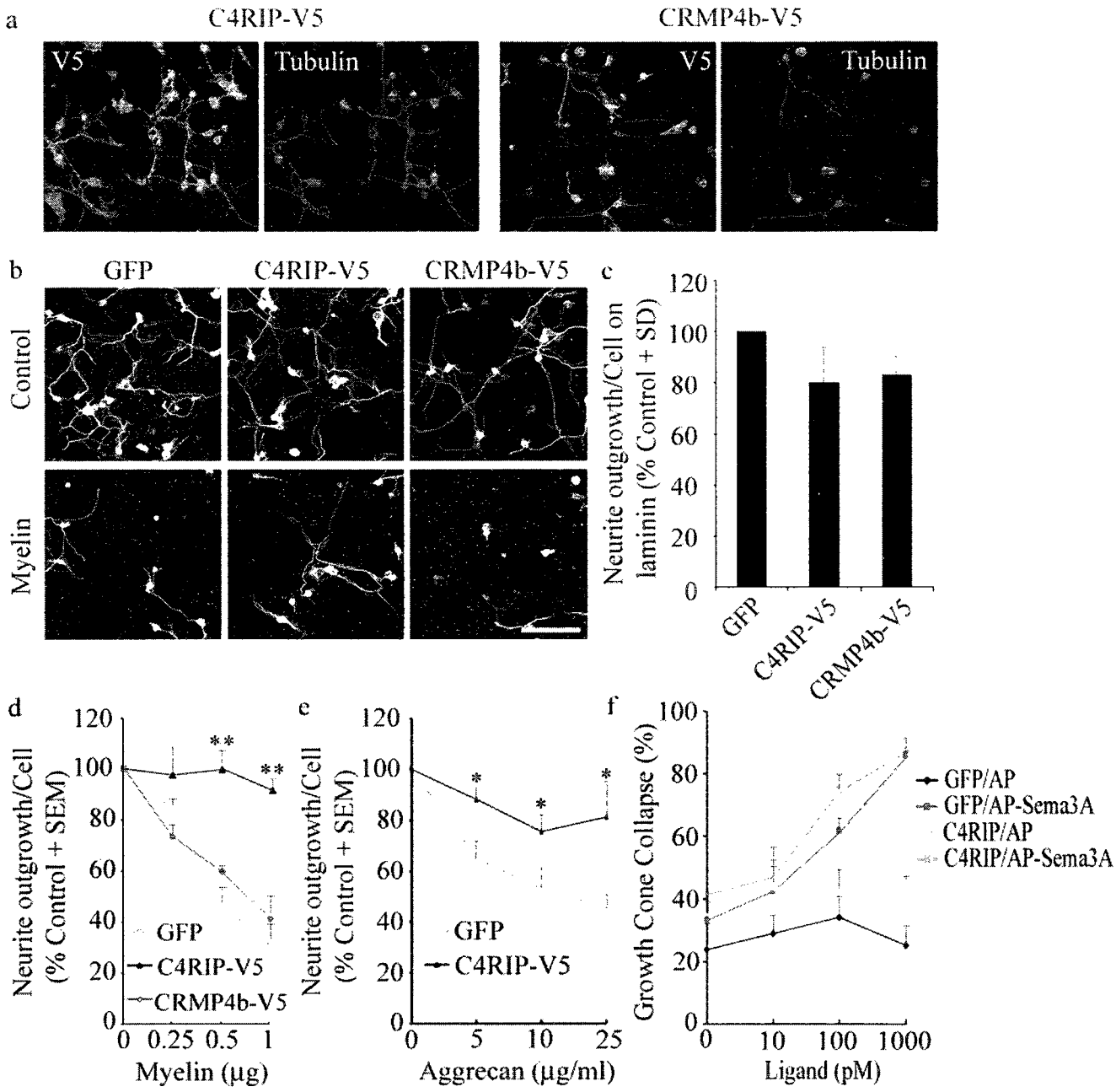

a
1
126
683
CRMP4b
CRMP4a
CRMP4aΔN
C4RIP
CRMP2a
CRMP4bNCRMP2
b
myc IP
mycRhoA63L + + + + + -
CRMPV5 4b 4a 2 2-4b ΔN 4b
IP V5
myc
Input V5
c
myc IP
CRMP4bV5 + + + - - -
CRMP4aV5 - - - + + +
mycRhoAwt - + + - + +
C4RIPV5 - - + - - +
IP CRMP4V5
mycRhoA
Input CRMP4V5
d
FLAG IP
mycROCKwt + + + +
FLAGRhoA63L - + + +
C4RIPV5 - - + -
CRMP4bV5 - - - +
IP myc
FLAG
Input mycROCKwt
CRMP4bV5
C4RIPV5

Figure 8 C4RIP-V5 attenuates myelin inhibition. **(a)** E13 dissociated chick DRG neurons infected with HSV-C4RIP-V5 or HSV-CRMP4b-V5 and double-stained with anti-V5 and anti-βIII tubulin antibodies to validate neuronal infection. **(b)** E13 dissociated chick DRG neurons infected with C4RIP-V5 or CRMP4b-V5 plated on laminin (control) or myelin substrates and stained with anti-βIII tubulin to visualize neurite outgrowth. **(c-e)** Quantitation of neurite outgrowth from GFP-, C4RIP-, or CRMP4b-infected neurons on laminin (c), myelin (d) or aggrecan (e) substrates. In (c) neurite outgrowth per cell is normalized to outgrowth in GFP-infected neurons for each experiment (100%). In (d) and (e), neurite outgrowth is normalized to growth on the laminin substrate for each dose curve (100%). Determinations are from 4 separate experiments. Scale bar, 100 μm. $*p<0.05$ and $**p<0.01$ by student t-test compared to GFP. **(f)** Quantitation of growth cone collapse in E8 chick DRG neurons infected with HSV-GFP or HSV-C4RIP and stimulated for 20' with control AP ligand or AP-Sema3A ligand. Determinations are from 3 separate experiments performed in duplicate.

FIGURE 8


a
C4RIP-V5
CRMP4b-V5
V5
Tubulin
V5
Tubulin
b
GFP
C4RIP-V5
CRMP4b-V5
Control
Myelin
c
Neurite outgrowth/Cell on laminin (% Control + SD)
120
100
80
60
40
20
0
GFP
C4RIP-V5
CRMP4b-V5
d
Neurite outgrowth/Cell (% Control + SEM)
120
100
80
60
40
20
0
**
**
GFP
C4RIP-V5
CRMP4b-V5
0 0.25 0.5 1
Myelin (µg)
e
Neurite outgrowth/Cell (% Control + SEM)
120
100
80
60
40
20
0
*
*
*
GFP
C4RIP-V5
0 5 10 25
Aggrecan (µg/ml)
f
Growth Cone Collapse (%)
100
80
60
40
20
0
GFP/AP
GFP/AP-Sema3A
C4RIP/AP
C4RIP/AP-Sema3A
0 10 100 1000
Ligand (pM)

CHAPTER 4

IV. GSK3β REGULATES CRMP4-RHOA COMPLEX FORMATION AND AXON OUTGROWTH INHIBITION.

Submitted to the Journal of Neuroscience

GSK3β regulates myelin-dependent axon outgrowth inhibition through CRMP4

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IV.1. PREFACE

We have identified a novel interaction between CRMP4b and RhoA that is specific to the Nogo-66 signalling cascade. Disruption of this interaction promotes axon growth on both myelin-based and CSPG substrates. This interaction offers a more specifc target for developing therapeutic avenues. A better understanding of the role of this interaction in mediating axon outgrowth inhibiton is necessary to further characterize the signalling cascade. The aim of next project was to dissect the regulatory mechanisms that govern this interaction.

IV.2. ABSTRACT

Myelin associated inhibitors (MAIs) contribute to failed regeneration in the central nervous system. The intracellular signaling pathways through which MAIs block axonal repair remain largely unknown. Here, we report that the kinase GSK3β is directly phosphorylated and inactivated by MAIs, consequently regulating protein-protein interactions that are critical for myelin-dependent inhibition. MAI-dependent phosphorylation and inactivation of GSK3β regulates CRMP4b phosphorylation and subsequent binding to RhoA. Inhibition of GSK3β mimics the neurite outgrowth inhibitory effect of myelin in a CRMP4b-dependent manner. Our findings also suggest that CRMP4b binding to RhoA is regulated by a phospho-dependent change in CRMP4b conformation. We present the first example of GSK3β inactivation in response to an inhibitory ligand and link the neurite outgrowth inhibitory effects of GSK3β inhibition directly to CRMP4b. These findings suggest that CRMP4b may be a more specific therapeutic target than GSK3β in the treatment of spinal cord injury.

Keywords: CRMP/myelin inhibition/regeneration/GSK3β/Nogo.

IV.3. INTRODUCTION

Inhibitory molecules at central nervous system (CNS) lesion sites including myelin-associated inhibitors (MAIs) and chondroitin sulfate proteoglycans (CSPGs) activate RhoA in injured neurons to mediate neurite outgrowth inhibition (Liu et al., 2006). Antagonism of RhoA or its downstream effector Rho kinase (ROCK) enhances neurite outgrowth and functional recovery following CNS trauma (Lehmann et al., 1999; Dergham et al., 2002; Fournier et al., 2003); however, the ubiquitous expression of RhoA and ROCK may limit the specificity of therapeutic agents targeting these molecules. In a screen to identify proteins that functionally interact with RhoA in the context of neurite outgrowth inhibition, we previously identified the cytosolic phosphoprotein CRMP4 (Collapsin Response Mediator Protein 4) as a protein that functionally interacts with RhoA to mediate neurite outgrowth inhibition (Alabed et al., 2007). The CRMP family consists of five family members (CRMP1-5) in vertebrates (Goshima et al., 1995b; Minturn et al., 1995; Byk et al., 1996; Gaetano et al., 1997; Inatome et al., 2000) that regulate aspects of axon pathfinding and neurite outgrowth (Hedgecock et al., 1985; Siddiqui and Culotti, 1991; Goshima et al., 1995b; Minturn et al., 1995; Quinn et al., 1999; Quinn et al., 2003; Yoshimura et al., 2005). CRMPs promote F-actin bundling (Rosslenbroich et al., 2005), affect endocytosis (Nishimura et al., 2003) and organize microtubule assembly to establish axon-dendrite fate during development (Fukata et al., 2002b; Arimura et al., 2005b).

Each CRMP allele produces two transcripts which differ in their amino terminus yielding a long (CRMP "b") and a short (CRMP "a") isoform (Yuasa-Kawada et al., 2003). CRMP4 consists of a 126 residue (CRMP4b) or 13 residue (CRMP4a) unique

amino terminal domain followed by 557 common residues. Treatment of neurons with the MAI Nogo, specifically enhances the association between RhoA and CRMP4b (Alabed et al., 2007). Overexpression of the unique 126 residue amino terminal domain of CRMP4b diminishes the interaction between CRMP4b and RhoA and enhances neurite outgrowth on myelin and CSPG substrates, indicating that this protein-protein interaction is critical for neurite outgrowth inhibition (Alabed et al., 2007). We found that CRMP4b-RhoA binding is not dependent on the nucleotide binding state of RhoA (Alabed et al., 2007). Therefore, we addressed the mechanism controlling CRMP4b-RhoA complex formation to gain insight into the signaling mechanisms mediating neurite outgrowth inhibition.

RhoA and CRMP4b rapidly associate following stimulation with Nogo leading us to investigate the possibility that this protein-protein interaction is regulated by phosphorylation. Both RhoA and CRMP4 are phosphoproteins. When RhoA is phosphorylated by Protein Kinase A (PKA) it binds tightly to Rho guanine dissociation inhibitor maintaining RhoA in its GDP-bound inactive state (Ellerbroek et al., 2003b). CRMPs are phosphorylated on multiple residues by Glycogen Synthase Kinase 3β (GSK3β) (Cole et al., 2004b). GSK3α and β are serine/threonine kinases originally identified as regulatory kinases for glycogen synthase and subsequently implicated in signaling cascades downstream of Wnts, NGF, EGF, Semaphorins and Hedgehog (Eickholt et al., 2002; Kockeritz et al., 2006). GSK3 has been widely studied as a potential therapeutic target for a variety of diseases including, cancer, Alzheimer's disease and nerve regeneration (Kockeritz et al., 2006). CRMP4 phosphorylation by GSK3β is dependent on a priming phosphorylation event (Cole et al., 2004b; Cole et al.,

2006). By analogy to other CRMPs, CRMP4 phosphorylation likely regulates its complement of binding partners. For example, CRMP2 phosphorylation by Rho kinase inhibits its binding to tubulin dimers and modifies its subcellular distribution (Arimura et al., 2005b).

Here, we report that CRMP4b is dephosphorylated in response to MAI stimulation in a GSK3-dependent manner and provide the first demonstration of direct phosphorylation and inactivation of GSK3β in response to a neurite outgrowth inhibitory cue. We demonstrate that CRMP4b dephosphorylation enhances CRMP4b binding to RhoA and provide evidence that a phospho-dependent change in CRMP4b conformation likely regulates this change in affinity. We confirm previous reports that inhibition of GSK3β activity inhibits outgrowth of cerebellar and Dorsal Root Ganglion (DRG) neurons and demonstrate that this inhibition can be markedly attenuated by antagonizing CRMP4b.

IV.4. METHODS AND MATERIALS

IV.4.1. Plasmids and antibodies

CRMP4, C4RIP and RhoA constructs were described previously (Alabed et al., 2007). CRMP4 mutants (CRMP4bS101A, CRMP4bAAA, CRMP4bAAAA, CRMP4bS101E, CRMP4bEEE, C4RIPS01A) and GSK3βR96A were generated using a site-directed mutagenesis kit (Stratagene, La Jolla, CA). pcDNA 3.1 GSK3βS9A-HA was generously provided by Dr. Dennis Stacey (The Cleveland Clinic Foundation).

CRMP4b antibody was generated by injecting rabbits with the following antigen RPGTTDQVPRQKYG as per (Quinn et al., 2003). Antiserum was affinity purified on an

antigen-Sepharose column. Phosphospecific antibody that recognizes CRMP4b phosphorylated at Ser 101 was generated in rabbit with the phosphopeptide ESREPVPEpSPKPAGV (where pS is phosphoserine). Antiserum was affinity purifed on a non-phosphorylated peptide column to remove antibodies that recognize unphosphorylated CRMP4b followed by affinity purification on a phosphopeptide antigen-Sepharose column.

IV.4.2. Preparation of recombinant proteins

Stimulations to examine inhibitory responses were performed with Nogo-P4 peptide or myelin. Nogo-P4 (Alpha Diagnostics, San Antonio, TX) is a 25 aa inhibitory peptide sequence (residues 31–55 of Nogo-66) sufficient to mediate the inhibitory properties of Nogo-66, a potent inhibitory component of Nogo-A (GrandPre et al., 2000). Myelin extracts were prepared from a bovine brain as described previously (Igarashi et al., 1993; Hsieh et al., 2006). CRMP4b-V5His was purified from transfected HEK293T cell lysates by Ni^{2+} affinity chromatography using Probond Nickel-Chelating resin (Invitrogen, Burlington, Ontario, Canada) according to the manufacturer's protocol.

IV.4.3. Preparation of herpes simplex viruses

pHSVPrPUC plasmids were transfected into 2-2 Vero cells that were superinfected with 5*dl* 1.2 herpes simplex virus (HSV) helper virus 1 d later. Recombinant virus was amplified through three passages and stored at –80°C as described previously (Neve et al., 1997).

IV.4.4. CRMP–RhoA coimmunoprecipitation assay

HEK293T cells were grown to subconfluence and transfected with Lipofectamine 2000 according to manufacturer's instructions (Invitrogen), washed twice with ice-cold PBS, and lysed in lysis buffer A [in mM: 50 Tris, pH 7.4, 150 NaCl, 1 EDTA, 1% (v/v) Triton X-100, 1 Na3VO4, 50 NaF, 1 PMSF, 100 nM Calyculin A and Complete protease inhibitors (Roche Diagnostics, Laval, Quebec, Canada)]. Lysates were precleared with protein A/G-agarose (Santa Cruz Biotechnology, Santa Cruz, CA) and subjected to immunoprecipitation with myc-agarose or V5-agarose (Sigma, Oakville, ON). After washing three times with ice-cold lysis buffer, bound protein was eluted with SDS and immunoblotted with anti-Myc (9E10, 1:1000; Sigma) or anti-V5 (1:5000; Invitrogen). For time-course experiments, PC12 cells were transfected for 24 h using Lipofectamine 2000 (Invitrogen) and differentiated with 50 ng/ml NGF (Cedarlane laboratories, Burlington, Ontario) for 24 h. Cells were treated with Nogo-P4 peptide for the indicated period of time at 37°C. Cells were then lysed, and proteins were immunoprecipitated as described above.

IV.4.5. Assessment of protein phosphorylation

PC12 cells were differentiated in RPMI/1% BSA/50 ng/ml NGF for 24 h before treatment with recombinant proteins. P8 rat cerebellar neurons were prepared as previously described (Hsieh et al., 2006). Cells were cultured for 18 h then serum starved for 6 hours before treatment. For CRMP4b-V5 infected neurons, dissociated cerebellar neurons were cultured in the presence of virus for 18 hours followed by serum starving for 6 hours.

Cell lysates were prepared by washing cells twice with ice-cold PBS and lysed in RIPA buffer containing 20 mM HEPES (pH 7.4), 150 mM NaCl, 1mM EDTA, 0.5% sodium deoxycholate, 0.1% SDS, 1% Triton X-100 with a protease inhibitor cocktail (Roche, Mannheim, Germany) and phosphatase inhibitors (1mM Na_3VO_4, 50 mM NaF, 100 nM Calyculin A). Lysates were analyzed by SDS-PAGE and immunoblotting with anti-phospho-threonine (Invitrogen), anti-phospho-GSK3β (Cell Signaling Technology, Inc., Danvers, MA), anti-GSK3β (Cell Signaling Technology, Inc., Danvers, MA), anti-phospho-CRMP4bT622 (generously provided by Dr. Calum Sutherland, University of Dundee, Dundee, Scotland), anti-phospho-CRMP4bS01 and anti-CRMP4b.

IV.4.6. Neurite outgrowth assay

For outgrowth assays using pharamacologic inhibitors, SB216763 (Sigma) and 6-Bromoindirubin-3'-acetoxime (EMD Biosciences, San Diego, CA) were added to the cultures after seeding. Dissociated embryonic day 13 (E13) chick dorsal root ganglion (DRG) neurons were cultured in DRG medium (F-12 medium, 10% FBS, 1% penicillin/streptomycin, 1% L-glutamine, 50 ng/ml NGF) in the presence of virus on PLL and laminin coated substrates, fixed with 4% paraformaldehyde/20% sucrose in PBS, and double stained with anti-ßIII tubulin (Covance, Berkeley, CA) and anti-V5 antibody (Sigma). Dissociated cerebellar neurons were cultured in serum-free SATO's medium. DRG neurite outgrowth lengths per cell were assessed using Image J, a public domain JAVA image processing program (http://rsb.info.nih.gov/ij/), as described previously (Fournier et al., 2003). Cerebellar outgrowth was analyzed with the neurite outgrowth module of MetaXpress.

IV.4.7. In Vitro Kinase Assay

Purified CRMP4b-V5 from transfected HEK293T cells was phosphorylated using recombinant GST-GSK3β (Cell Signaling Technology, Inc., Danvers, MA) and recombinant DYRK2 (Millipore, Billerica, MA) in kinase buffer containing 25 mM Tris-HCl (pH 7.5), 5 mM beta-glycerophosphate, 2 mM dithiothreitol (DTT), 0.1 mM Na3VO4, 10 mM MgCl2, 100 nM Calyculin A and 200 μM ATP. After incubation for 2 hours at 30°C, the reaction was terminated by addition of SDS, subjected to SDS-PAGE and immunoblot analysis.

IV.5. RESULTS

IV.5.1. CRMP4b-RhoA binding is regulated by Nogo-dependent dephosphorylation

As reported previously, the association between RhoA and CRMP4b is enhanced by stimulation with Nogo-P4 peptide, an inhibitory fragment of Nogo-A (GrandPre et al., 2000), in transfected PC12 cells (Fig. 1A) and cerebellar neurons (Alabed et al., 2007). The rapid enhancement of this protein-protein interaction led us to investigate the potential regulatory role of protein phosphorylation on this process. In 293T cells transfected with myc-wtRhoA and CRMP4b-V5, myc immunoprecipitates contain CRMP4b-V5 (Fig. 1B). Treatment of transfected 293T cells with the serine/threonine phosphatase inhibitor Calyculin A causes an upward mobility shift of CRMP4b-V5 indicative of CRMP4b phosphorylation. While there is no apparent mobility shift in wtRhoA following Calyculin A treatment this does not exclude the possibility that RhoA is also phosphorylated. Calyculin A treatment diminishes the CRMP4b-wtRhoA co-immunoprecipitation demonstrating that phosphorylation of CRMP4b and/or RhoA

disrupts their binding. When 293T cell lysates are treated with shrimp alkaline phosphatase to stimulate protein dephosphorylation, the association between CRMP4b-V5 and myc-wtRhoA is enhanced, similar to the effect of Nogo treatment. We then asked if dephosphorylation of RhoA and/or CRMP4b is capable of enhancing RhoA-CRMP4b binding. The binding properties of a RhoA mutant with the phospho-residue serine 188 mutated to alanine (non-phosphorylatable S188ARhoA) and of a CRMP4b triple alanine substitution mutant for the three carboxy terminal phospho-residues targeted by GSK3β (Thr622, Thr627, Ser631; CRMP4bAAA) were assessed. RhoAS188A binds more weakly than wtRhoA to wtCRMP4b (Fig. 1C). However, CRMP4bAAA binds more strongly than wtCRMP4b to WTRhoA (Fig. 1C). Together this indicates that dephosphorylation of CRMP4b favors CRMP4b-RhoA binding.

To evaluate the effect of Nogo stimulation on CRMP4b phosphorylation, PC12 cells or CRMP4b-V5-infected cerebellar neurons were treated with Nogo-P4 peptide and CRMP4b phosphorylation was assessed by Western blotting with a phospho-specific antibody recognizing pThr622 of CRMP4b (correlating with Thr 509 of CRMP4a) (Cole et al., 2004b). Nogo-P4 stimulation diminishes CRMP4b phosphorylation in both PC12 cells (Fig. 1D) and cerebellar neurons (Fig. 1E).

IV.5.2. CRMP4b is dephosphorylated in a GSK3β-dependent manner in response to Nogo

Dephosphorylation of CRMP4b suggests engagement of a CRMP4-directed phosphatase and/or inactivation of a CRMP4b-directed kinase in response to Nogo. CRMP4b phosphorylation is sequentially regulated by GSK3β on residues Ser631,

Thr627 and Thr622 following a priming phosphorylation event which may be mediated by DYRK2 (Cole et al., 2004b). Inactivation of GSK3β by phosphorylation on Ser9 leads to a rapid decrease in phospho content of its substrates. GSK3β phosphorylation and inactivation is an important regulatory step in response to many factors including NGF and Wnt (Cohen & Frame, 2001; Zhou et al, 2004), therefore we assessed the role of GSK3β in Nogo signaling. We find that GSK3β is phosphorylated coincident with reduced CRMP4b phosphorylation in Nogo-P4-stimulated PC12 (Fig. 2A) and cerebellar lysates (Fig. 2B). This suggests that CRMP4b is dephosphorylated as a consequence of GSK3β inactivation in response to Nogo-P4 peptide stimulation. To test this, we overexpressed a constitutively active form of GSK3β (GSK3βS9A) and examined the effect of Nogo. Overexpression of GSK3βS9A blocks the Nogo-P4-dependent decrease in CRMP4b dephosphorylation indicating that the dephosphorylation is GSK3β-dependent (Fig. 2C).

GSK3β phosphorylation is regulated by the AGC family members that include protein kinase C (PKC) and PKC contributes to myelin and CSPG inhibitory responses in injured neurons (Goode et al., 1992; Cohen and Frame, 2001; Sivasankaran et al., 2004), therefore we tested if Nogo-dependent GSK3β phosphorylation is dependent on PKC activation by pre-treating cerebellar neurons with the PKC inhibitor Go6976. We find that Nogo-P4-dependent phosphorylation of GSK3β is delayed but not blocked in the presence of the PKC inhibitor Go6976 (Fig. 2B).

IV.5.3. Inactivation of GSK3β inhibits neurite outgrowth in a CRMP4b- dependent manner

Our data suggests that Nogo induces GSK3β inactivation, resulting in CRMP4b dephosphorylation, and enhances CRMP4b-RhoA complex formation. If this is the case, then GSK3β inactivation will increase CRMP4b association with RhoA and inhibit neurite outgrowth. To test this, PC12 cells were treated with the GSK3–specific inhibitors SB216763 or 6-bromoindirubin-3'-acetoxime. Figure 3A shows that an overnight stimulation of PC12 cells with SB216763 or 6-bromoindirubin-3'-acetoxime increases the association of RhoA with CRMP4b but not CRMP4a. The specific effect of the pharmacologic GSK3β inhibitors on the long isoform of CRMP4 mimics that of Nogo treatment (Alabed et al., 2007). Similarly, overexpression of GSK3β specifically diminishes binding between RhoA and CRMP4b but not CRMP4a (Fig. 3A). Previous reports have shown that strong GSK3 inhibition reduces neurite outgrowth (Kim et al., 2006). Consistent with this, Figure 3B-E show that treatment of cerebellar neurons (Fig. 3B, D) or DRG neurons (Fig. 3C, E) with 5 μM SB216763 or 300 nM 6-bromoindirubin-3'-acetoxime diminishes neurite outgrowth. To test if the reduced neurite outgrowth that accompanies GSK inhibition requires CRMP4b, we assessed the effects of C4RIP (**C**RMP4b-**R**hoA **I**nterfering **P**eptide), an antagonist of CRMP4b-RhoA binding which does not affect neurite outgrowth on permissive laminin substrates (Alabed et al., 2007). This experiment was performed in DRG neurons, which are robustly infected by HSV. Remarkably, the neurite outgrowth inhibitory effect induced by GSK3β inhibition is dramatically attenuated by infecting neurons with HSV-C4RIP (Fig. 3C, E). This

indicates that the neurite outgrowth inhibition induced by GSK3β inhibitors requires CRMP4b.

IV.5.4. The unique amino terminus of CRMP4b is phosphorylated by GSK3β

CRMP4 is phosphorylated by GSK3β on three target residues that are within the carboxy terminus of the molecule and are common to CRMP4a and CRMP4b (Fig. 4A) (Cole et al., 2004b; Cole et al., 2006). This presents an apparent paradox as overexpression or pharmacologic inhibition of GSK3β specifically regulates binding of RhoA to the long "b" isoform of CRMP4. We therefore asked if GSK3β phosphorylates CRMP4b within the unique amino terminus. By using mass spectrometry analysis we identified several potential phospho-residues within C4RIP and by using site directed mutagenesis we identified residue Ser101 as a phospho-residue within C4RIP that confers a molecular weight shift (Fig. 4B). We generated a phospho-specific antibody to Ser101, which only recognizes CRMP4b in a phospho-dependent fashion (Fig. 4C) and used this to ask whether GSK3β phosphorylates Serine 101 of CRMP4b *in vitro*. We find that recombinant GSK3β phosphorylates Ser101 of CRMP4b *in vitro* as well as residue Thr622, as previously reported (Fig. 4D; (Cole et al., 2004b; Cole et al., 2006)). GSK3β-dependent phosphorylation of CRMP4b in the carboxy terminus may be dependent on the priming kinase DYRK2 (Cole et al., 2004b). To test if phosphorylation of Ser101 is dependent on the priming kinase DYRK2, we tested the effect of DYRK2 in the *in vitro* kinase assay (Fig. 4D) and found that DYRK2 is capable of directly phosphorylating Ser101. We then examined the phosphorylation of Ser101 in cells, by co-transfecting HEK293T cells with CRMP4b-V5 and GSK3βR96A, a GSK3β mutant that exclusively

phosphorylates substrates on non-primed sites (Frame et al., 2001). We find that GSK3βR96A phosphorylates CRMP4b-V5 at Ser101 (Fig. 4E), confirming that this residue is a substrate for GSK3β and that this event does not require a priming kinase. CRMP4bAAA is also phosphorylated by GSK3β at Ser101 (Fig. 4E) indicating that Ser101 phosphorylation by GSK3β is independent of the other GSK3β sites on CRMP4b. This data suggests that different regions of CRMP4b are primed and unprimed substrates of GSK3β as has previously been described for Tau, another physiologic substrate of GSK3β (Cho and Johnson, 2003).

IV.5.5. Ser101 is dephosphorylated by Nogo-P4 and regulates CRMP4b-RhoA binding

We then tested if CRMP4 phosphorylation is regulated on Ser101 by Nogo-P4 and found that Ser101 is dephosphorylated in PC12 cells and cerebellar neurons stimulated with Nogo-P4 (Fig. 5A, B). Further, we tested the effects of GSK3β overexpression on the Nogo-mediated dephosphorylation of Ser101 of CRMP4b. Surprisingly, introduction of GSK3βR96A fails to attenuate the Nogo-P4-dependent dephosphorylation of Ser101 (Fig. 5A) suggesting that Nogo-dependent dephosphorylation of this residue may not be directly regulated by GSK3β but rather by inactivation of DYRK2 or by an unidentified phosphatase.

To better understand the contribution CRMP phosphorylation at Ser101 or the carboxy terminus to RhoA binding, we generated CRMP Alanine substitution mutants and assessed their binding to RhoA. We tested binding between RhoA and a Ser101-Alanine substitution mutant (CRMP4bS101A) or CRMP4bAAA. Both CRMP4bS101A

and CRMP4bAAA bind to RhoA better than WTCRMP4B (Fig. 5C) indicating that dephosphorylation of Ser101 or the carboxy terminus of CRMP4b is sufficient to enhance CRMP4b-RhoA binding. Mutation of all four residues (Ser101, Thr622, Thr627, Ser631; CRMP4bAAAA) does not further enhance binding to RhoA (Fig. 5C). Binding between RhoA and CRMP4b mutants with glutamic acid substitutions at the same sites (CRMP4bS101E or CRMP4bEEE) was indistinguishable from WTCRMP4b (Fig. 5C). A possible interpretation of this result is that the Glutamic Acid substitutions do not effectively mimic CRMP4b phosphorylation since Calyculin-dependent CRMP4b phosphorylation blocks its binding to RhoA (Fig. 1). A second possibility is that unidentified Calyculin-sensitive phospho-residues within CRMP4b affect binding to RhoA.

We then assessed the Nogo-dependent association of RhoA with the CRMP4b mutants. Unlike WTCRMP4b, CRMP4bS101E does not show enhanced RhoA association following Nogo treatment (Fig. 5D). Similarly, CRMP4bEEE also failed to recruit more RhoA following Nogo-P4 stimulation (Fig. 5D). These findings indicate that while CRMP4bS101A or CRMP4bAAA bind more strongly to RhoA, dephosphorylation of both regions is necessary for the Nogo-dependent recruitment of RhoA.

IV.5.6. The amino terminus of CRMP4b binds to CRMP4, not RhoA

Since overexpression GSK3β is insufficient to block Nogo-dependent dephosphorylation of CRMP4b on Ser101, the specificity of GSK3β overexpression and inhibition towards CRMP4b binding to RhoA remained unresolved. We therefore considered the possibility that RhoA binding to CRMP4b may be affected by a phospho-dependent change in

CRMP4b conformation. We first investigated where RhoA binds to CRMP4b. We have demonstrated previously that RhoA binds specifically to the CRMP4 isoform, failing to bind to CRMP1, 2 and 3 and that RhoA binds to CRMP4b with higher affinity than CRMP4a. Further, the unique amino terminal region of CRMP4b confers RhoA binding to CRMP2a when fused to the amino terminus (Alabed et al., 2007). This originally suggested the presence of two RhoA binding sites within CRMP4b; one in the unique amino terminus, and one in region common to CRMP4a and CRMP4b. To identify the RhoA binding sites within CRMP4b, we assessed the ability of individual CRMP4b domains to interact with RhoA (Fig. 6A). We detected a binding site for RhoA in the common dihydropyrimidinase (DHP) region of CRMP4 but failed to detect binding between RhoA and the unique amino terminal domain of CRMP4b (C4RIP) even with a S101A mutation (Fig. 6B). The ability of the unique amino terminal domain of CRMP4b to confer binding to RhoA when in the context of a CRMP4-CRMP2 fusion construct (Alabed et al., 2007) together with its failure to bind to RhoA in isolation further suggested that protein conformation may regulate the strength of CRMP4b-RhoA binding.

We therefore considered the possibility that the amino terminal domain of CRMP4b (C4RIP) may fold back on CRMP4b to generate a binding pocket for RhoA by assessing the ability of C4RIP to co-immunoprecipitate CRMP4a (Fig. 6C). Indeed, C4RIP co-immunoprecipitates CRMP4a and equally co-immunoprecipitates CRMP4b (Fig. 6C). Alanine substitutions for GSK3β target phospho residues in the CRMP4 carboxy terminus enhance the interaction between CRMP4b and C4RIP (Fig. 6D). This enhancement is attenuated by Calyculin stimulation, indicating that an additional phospho-residue within CRMP4b also affects protein folding (Fig. 6D). Together these

data suggest the existence of a binding site for RhoA in the DHP domain of CRMP4b and that CRMP4b folds back on itself into a phospho-dependent conformation that generates a higher affinity binding site for RhoA (See model; Supplementary Figure 1). Further the finding that CRMP4bS101E fails to recruit additional RhoA in response to Nogo stimulation (Fig. 5D) suggests that dephosphorylated Ser101 may expose a key site within the binding pocket.

IV.6. DISCUSSION

We find that Nogo induces phosphorylation and inactivation of GSK3β which in turn causes CRMP4b dephosphorylation. Binding between RhoA and the dephosphorylated form of CRMP4b is enhanced, and this can be attributed to a phospho-dependent conformational change in the CRMP4b molecule. Inhibition of GSK3β mimics the effect of myelin on neurite outgrowth from cerebellar neurons and DRG neurons and we show that this effect requires CRMP4b. Together, these findings provide novel insights into the neuronal mechanism of action of GSK3β and suggest additional molecular sites of intervention to promote neuronal repair following CNS injury.

Nogo-dependent regulation of GSK3β

MAIs signal through a trimeric receptor complex to activate RhoA and regulate the axonal cytoskeleton; however little is known about the molecular links between the receptor complex and RhoA. GSK3α/β activity is negatively regulated by phosphorylation at Ser21/9 and positively regulated by phosphorylation at Tyr279/216 by multiple protein kinases (Jope and Johnson, 2004). In addition, GSK3 activity can be

regulated by interactions with physiological inhibitory proteins such as Dsh and FRAT (Thomas et al., 1999; Cohen and Frame, 2001). Our data suggests that Nogo regulation of GSK3β may be enhanced but not exclusively regulated by PKC-dependent phosphorylation. This is intriguing in the context of previous data demonstrating that PKC is activated in response to stimulation with MAIs and that blockade of PKC with Go6976 attenuates myelin-dependent inhibition (Sivasankaran et al., 2004). GSK3β-mediated phosphorylation of the carboxy terminus of CRMP4b is dependent on priming phosphorylation by DYRK2 at Ser635 and DYRK2 directly regulates phosphorylation of Ser101 *in vitro*. Whether DYRK2 may be directly regulated in response to MAI stimulation or whether it may exist in limiting concentrations to regulate CRMP4b phosphorylation and conformation remains unknown.

Neurite outgrowth inhibitors and GSK3β inactivation

We provide the first example of a neurite outgrowth inhibitory ligand which stimulates phosphorylation and inactivation of GSK3β. Our findings are consistent with several reports demonstrating that pharmacologic inhibition of GSK3 inhibits neurite outgrowth, but differ from the reported effects of GSK3 inhibition on axon branching (Owen and Gordon-Weeks, 2003; Shi et al., 2004; Kim et al., 2006). In an elegant study to examine why GSK3 inhibition has been associated with either axon branching or outgrowth inhibition phenotypes in different studies, Snider and colleagues have described a compelling correlation between GSK3 activity towards primed or non-primed substrates and neuronal phenotypes (Kim et al., 2006). Specifically, introduction of a GSK3 mutant that selectively phosphorylates non-primed substrates (GSK3R96A) results in reduced

axon branching. Further, low concentrations of pharmacologic GSK3 inhibitors that influence axon branching mainly affect the phosphorylation of primed GSK3 substrates. GSK3β regulates CRMP4b phosphorylation on priming-dependent and -independent residues and it is not clear if the sites may be differentially affected by various concentrations of GSK3β inhibitors. It is also possible that Nogo-dependent inactivation of GSK3β may impact additional priming-independent substrates leading to neurite outgrowth inhibition; however, this is difficult to reconcile with the ability of C4RIP to reverse Nogo- and SB216763-dependent outgrowth inhibition.

Spatial targeting of active and inactive GSK3β

A commonly accepted view is that GSK3 may be regulated at discrete sites within the axon and growth cone to target specific subsets of substrates. The engagement of distinct spatially segregated pools of target substrates could potentially explain how inhibitory MAIs and growth promoting neurotrophins both phosphorylate and inactivate GSK3β (Zhou et al., 2004). Indeed, following neurotrophin stimulation the inactive pool of GSK3β is discretely localized at the distal portion of the axon. This is a particularly important issue when one considers the multiple ligands that regulate GSK3 activity.

The fact that GSK3 can increase or decrease axonal growth, depending on the circumstances, is also an important consideration when considering GSK3β as a potential therapeutic target to promote regeneration following CNS injury or repair in the context of Alzheimer's disease or spinal cord injury (Cole et al., 2004b; Zhou et al., 2004). Based on previous findings indicating growth promoting and neuroprotective effects of GSK3β inhibition, clinical trials in spinal cord injury using a combination of stem cells

and the GSK3β inhibitor lithium are being pursued (Cyranoski, 2007). The findings we present here suggest that high doses of lithium may in fact impede axon extension raising concerns regarding the efficacy of such a treatment. The substrate-dependent effects of GSK3 activation and inactivation indicate that targeting of GSK3 substrates, particularly CRMP4 may represent a more effective therapeutic strategy than directly targeting GSK3.

CRMP4b phosphorylation and neurite outgrowth inhibition

Our data suggests that overexpression of GSK3β inhibits formation of a CRMP4b-RhoA complex and may be protective in the context of myelin inhibition. However, the previously reported pro-apoptotic function of GSK3β (Zhou et al., 2004), makes its overexpression an unlikely route for therapeutics. Further, GSK regulates the phosphorylation and activation of many microtubule-associated proteins including APC, CRMP2, CRMP4, MAP1b, MAP2, NF, Tau and Kinesin light chain which would be affected in an overexpression paradigm (Cole et al., 2004a; Zhou and Snider, 2005). Intriguingly, CRMP2 is hyperphosphorylated in Alzheimer's disease coincident with elevated GSK3 activity, although the cellular consequences of this hyperphosphorylation *in vivo* remain to be determined (Cole et al., 2004a). Phosphorylation of CRMP2 negatively regulates its activity by inhibiting its binding to microtubule heterodimers and to subsequently enhance microtubule polymerization (Fukata et al., 2002b). In fact, CRMP2 is phosphorylated in a ROCK-dependent manner by Nogo or MAG and may contribute to neurite outgrowth inhibition via dysregulated microtubule dynamics (Mimura et al., 2006). While CRMP4 is capable of binding to microtubules (Fukata et al., 2002b) it is not a ROCK substrate and its *in vivo* function likely differs from CRMP2 for

several reasons. First, overexpression of CRMP4a in hippocampal neurons or SHSY5Y cells has a modest effect on axon outgrowth when compared to the robust elongation effect of CRMP2a (Cole et al., 2004a). Second, CRMP4b overexpression promotes an actin based phenotype in DRG neurons promoting the extension of filopodia and neurite branches (Alabed et al., 2007). This actin-based phenotype is consistent with the ability of CRMP4 to bundle F-actin (Rosslenbroich et al., 2005) and to bind to RhoA (Alabed et al., 2007). Third, CRMP4b colocalizes with SV2 positive vesicles and binds to the endocytic adaptor protein intersectin suggesting a potential role in endocytosis (Quinn et al., 2003). Finally, it is worth noting that the long isoforms of CRMPs can serve different functions from the short isoforms, perhaps even serving as CRMPa antagonists (Yuasa-Kawada et al., 2003). The ability of C4RIP to inhibit CRMP4b-RhoA binding and to attenuate Nogo- and SB216763-dependent outgrowth inhibition suggests that the role of dephosphorylated CRMP4b in mediating neurite outgrowth inhibition may be linked to its ability to bind to RhoA and is suggestive of an actin-dependent phenotype; however, our additional finding that CRMP4b folds upon itself raises the possibility that C4RIP may regulate binding between CRMP4b and additional protein interactors.

It is reasonable to propose that GSK3β-dependent dephosphorylation of CRMP4b may influence a Rho-dependent modification of the actin cytoskeleton which may precede and be necessary for subsequent destabilization of the microtubule cytoskeleton. Mechanistically, this differs from the effects of simultaneous inhibition of all GSK3β substrates. Pharmacologic inhibition of GSK3 at concentrations resulting in dephosphorylation of all GSK3 substrates, results in impaired axon growth which is associated with enlarged lamellipodia indicative of enhanced actin polymerization (Kim

et al, 2006). This differs from the neuronal response to Nogo which elicits both growth cone collapse and neurite outgrowth inhibition and is not associated with obvious actin polymerization. This indicates that only a subset of GSK3 substrates are affected by Nogo stimulation and while the neurite outgrowth inhibitory phenotype mimics pharmacological inhibition of GSK, these two events are mechanistically distinct. Substrate selection and mechanistic differences between growth cone collapse and neurite outgrowth inhibition are also likely to account for the observation that Sema3A-dependent GC collapse is associated with increased GSK-3 activity (Eickholt et al., 2002).

CRMP4 Structure

The crystal structures of murine CRMP1 (Deo et al., 2004) and human CRMP2 (Stenmark et al., 2007) have been solved, but in both cases the structure does not include the amino terminal extension of the long isoforms, nor the carboxy terminal region containing the GSK3β sensitive phospho-residues. The lack of structural data for the carboxy terminus of the CRMP proteins is a function of proteolytic susceptibility of this region (Deo et al., 2004). Our findings suggest that full length CRMPb isoforms may undergo a fold resulting in a phospho-dependent conformation that regulates additional protein-protein interactions. For simplicity, our model is presented in the context of a single CRMP molecule, however it is known that CRMPs form heterotetramers (Wang and Strittmatter, 1997b). It is possible that intermolecular binding of RhoA to the amino terminus of one CRMP4b molecule and the DHP region of a second molecule may occur. Further, it is possible that phosphorylation of CRMP4b in the carboxy terminus may affect the oligomerization properties of CRMP4b and that RhoA may favor binding to

CRMP4b monomers or oligomers. Additional protein interactions conferred by phospho-dependent conformational changes in CRMP4b could play a key role in CRMP function by regulating binding affinities to upstream regulators such as GSK3 and/or to potential effectors such as RhoA. We have also identified an additional novel phospho residue in the unique amino terminal domain of CRMP4b which is a substrate for GSK3β and an *in vitro* substrate for DYRK2. A better understanding of the impact of amino terminal phosphorylation on CRMP4b binding interactions will likely yield additional insights into CRMP4b function and into intracellular mechanisms regulating neurite outgrowth inhibition.

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IV.7. REFERENCES

Alabed YZ, Grados-Munro E, Ferraro GB, Hsieh S, Fournier AE (2006) Neuronal responses to myelin are mediated by ROCK. *J Neurochem* **96:** 1616-1625

Alabed YZ, Pool M, Ong Tone S, Fournier AE (2007) Identification of CRMP4 as a convergent regulator of axon outgrowth inhibition. *J Neurosci* **27:** 1702-1711

Arimura N, Menager C, Kawano Y, Yoshimura T, Kawabata S, Hattori A, Fukata Y, Amano M, Goshima Y, Inagaki M, Morone N, Usukura J, Kaibuchi K (2005) Phosphorylation by Rho kinase regulates CRMP-2 activity in growth cones. *Mol Cell Biol* **25**(22): 9973-9984

Borisoff JF, Chan CC, Hiebert GW, Oschipok L, Robertson GS, Zamboni R, Steeves JD, Tetzlaff W (2003) Suppression of Rho-kinase activity promotes axonal growth on inhibitory CNS substrates. *Mol Cell Neurosci* **22**(3): 405-416.

Byk T, Dobransky T, Cifuentes-Diaz C, Sobel A (1996) Identification and molecular characterization of Unc-33-like phosphoprotein (Ulip), a putative mammalian homolog of the axonal guidance-associated unc-33 gene product. *Journal of Neuroscience* **16**(2): 688-701

Cho JH, Johnson GV (2003) Glycogen synthase kinase 3beta phosphorylates tau at both primed and unprimed sites. Differential impact on microtubule binding. *J Biol Chem* **278**(1): 187-193

Cohen P, Frame S (2001) The renaissance of GSK3. *Nat Rev Mol Cell Biol* **2**(10): 769-776

Cole A, Frame S, Cohen P (2004a) Further evidence that the tyrosine phosphorylation of glycogen synthase kinase-3 (GSK3) in mammalian cells is an autophosphorylation event. *Biochem J* **377**(Pt 1): 249-255

Cole AR, Causeret F, Yadirgi G, Hastie CJ, McLauchlan H, McManus EJ, Hernandez F, Eickholt BJ, Nikolic M, Sutherland C (2006) Distinct priming kinases contribute to differential regulation of collapsin response mediator proteins by glycogen synthase kinase-3 in vivo. *J Biol Chem* **281**(24): 16591-16598

Cole AR, Knebel A, Morrice NA, Robertson LA, Irving AJ, Connolly CN, Sutherland C (2004b) GSK-3 phosphorylation of the Alzheimer epitope within collapsin response mediator proteins regulates axon elongation in primary neurons. *J Biol Chem* **279**(48): 50176-50180

Cyranoski D (2007) Chinese network to start trials of spinal surgery. *Nature* **446**(7135): 476-477

Deo RC, Schmidt EF, Elhabazi A, Togashi H, Burley SK, Strittmatter SM (2004) Structural bases for CRMP function in plexin-dependent semaphorin3A signaling. *Embo J* **23**(1): 9-22

Dergham P, Ellezam B, Essagian C, Avedissian H, Lubell WD, McKerracher L (2002) Rho signaling pathway targeted to promote spinal cord repair. *J Neurosci* **22**(15): 6570-6577.

Domeniconi M, Zampieri N, Spencer T, Hilaire M, Mellado W, Chao MV, Filbin MT (2005) MAG induces regulated intramembrane proteolysis of the p75 neurotrophin receptor to inhibit neurite outgrowth. *Neuron* **46**(6): 849-855

Eickholt BJ, Walsh FS, Doherty P (2002) An inactive pool of GSK-3 at the leading edge of growth cones is implicated in Semaphorin 3A signaling. *J Cell Biol* **157**(2): 211-217

Ellerbroek SM, Wennerberg K, Burridge K (2003) Serine phosphorylation negatively regulates RhoA in vivo. *J Biol Chem* **278**(21): 19023-19031

Fournier AE, Takizawa BT, Strittmatter SM (2003) Rho Kinase inhibition enhances axonal regeneration in the injured CNS. *J Neurosci* **23**: 1416-1423

Frame S, Cohen P, Biondi RM (2001) A common phosphate binding site explains the unique substrate specificity of GSK3 and its inactivation by phosphorylation. *Mol Cell* **7**(6): 1321-1327

Fukata Y, Itoh TJ, Kimura T, Menager C, Nishimura T, Shiromizu T, Watanabe H, Inagaki N, Iwamatsu A, Hotani H, Kaibuchi K (2002) CRMP-2 binds to tubulin heterodimers to promote microtubule assembly. *Nat Cell Biol* **4**(8): 583-591

Gaetano C, Matsuo T, Thiele CJ (1997) Identification and characterization of a retinoic acid-regulated human homologue of the unc-33-like phosphoprotein gene (hUlip) from neuroblastoma cells. *J Biol Chem* **272**(18): 12195-12201

Goode N, Hughes K, Woodgett JR, Parker PJ (1992) Differential regulation of glycogen synthase kinase-3 beta by protein kinase C isotypes. *J Biol Chem* **267**(24): 16878-16882

Goshima Y, Nakamura F, Strittmatter P, Strittmatter SM (1995) Collapsin-induced growth cone collapse mediated by an intracellular protein related to UNC-33. *Nature* **376**(6540): 509-514.

GrandPre T, Nakamura F, Vartanian T, Strittmatter SM (2000) Identification of the Nogo inhibitor of axon regeneration as a Reticulon protein. *Nature* **403**(6768): 439-444.

Hedgecock EM, Culotti JG, Thomson JN, Perkins LA (1985) Axonal guidance mutants of Caenorhabditis elegans identified by filling sensory neurons with fluorescein dyes. *Developmental Biology* **111**(1): 158-170

Hsieh S, Ferraro GB, Fournier AE (2006) Myelin-associated inhibitors regulate cofilin phosphorylation and neuronal inhibition through Lim kinase and Slingshot phosphatase. *J Neurosci* **26**(3): 1006-1015

Igarashi M, Strittmatter SM, Vartanian T, Fishman MC (1993) Mediation by G proteins of signals that cause collapse of growth cones. *Science* **259**(5091): 77-79.

Inatome R, Tsujimura T, Hitomi T, Mitsui N, Hermann P, Kuroda S, Yamamura H, Yanagi S (2000) Identification of CRAM, a novel unc-33 gene family protein that associates with CRMP3 and protein-tyrosine kinase(s) in the developing rat brain. *J Biol Chem* **275**(35): 27291-27302

Jope RS, Johnson GV (2004) The glamour and gloom of glycogen synthase kinase-3. *Trends Biochem Sci* **29**(2): 95-102

Kim WY, Zhou FQ, Zhou J, Yokota Y, Wang YM, Yoshimura T, Kaibuchi K, Woodgett JR, Anton ES, Snider WD (2006) Essential roles for GSK-3s and GSK-3-primed substrates in neurotrophin-induced and hippocampal axon growth. *Neuron* **52**(6): 981-996

Kockeritz L, Doble B, Patel S, Woodgett JR (2006) Glycogen synthase kinase-3--an overview of an over-achieving protein kinase. *Curr Drug Targets* **7**(11): 1377-1388

Lehmann M, Fournier A, Selles-Navarro I, Dergham P, Sebok A, Leclerc N, Tigyi G, McKerracher L (1999) Inactivation of Rho signaling pathway promotes CNS axon regeneration. *J Neurosci* **19**(17): 7537-7547.

Liu BP, Cafferty WB, Budel SO, Strittmatter SM (2006) Extracellular regulators of axonal growth in the adult central nervous system. *Philos Trans R Soc Lond B Biol Sci* **361**(1473): 1593-1610

Liu BP, Fournier A, GrandPre T, Strittmatter SM (2002) Myelin-associated glycoprotein as a functional ligand for the Nogo-66 receptor. *Science* **297**(5584): 1190-1193.

Mimura F, Yamagishi S, Arimura N, Fujitani M, Kubo T, Kaibuchi K, Yamashita T (2006) Myelin-associated Glycoprotein Inhibits Microtubule Assembly by a Rho-kinase-dependent Mechanism. *J Biol Chem* **281**(23): 15970-15979

Minturn JE, Fryer HJ, Geschwind DH, Hockfield S (1995) TOAD-64, a gene expressed early in neuronal differentiation in the rat, is related to unc-33, a C. elegans gene involved in axon outgrowth. *Journal of Neuroscience* **15**(10): 6757-6766

Neve RL, Howe JR, Hong S, Kalb RG (1997) Introduction of the glutamate receptor subunit 1 into motor neurons in vitro and in vivo using a recombinant herpes simplex virus. *Neuroscience* **79**(2): 435-447

Niederost B, Oertle T, Fritsche J, McKinney RA, Bandtlow CE (2002) Nogo-A and myelin-associated glycoprotein mediate neurite growth inhibition by antagonistic regulation of RhoA and Rac1. *J Neurosci* **22**(23): 10368-10376.

Nishimura T, Fukata Y, Kato K, Yamaguchi T, Matsuura Y, Kamiguchi H, Kaibuchi K (2003) CRMP-2 regulates polarized Numb-mediated endocytosis for axon growth. *Nature Cell Biology* **5**(9): 819-826

Owen R, Gordon-Weeks PR (2003) Inhibition of glycogen synthase kinase 3beta in sensory neurons in culture alters filopodia dynamics and microtubule distribution in growth cones. *Mol Cell Neurosci* **23**(4): 626-637

Quinn CC, Chen E, Kinjo TG, Kelly G, Bell AW, Elliott RC, McPherson PS, Hockfield S (2003) TUC-4b, a novel TUC family variant, regulates neurite outgrowth and associates with vesicles in the growth cone. *Journal of Neuroscience* **23**(7): 2815-2823

Quinn CC, Gray GE, Hockfield S (1999) A family of proteins implicated in axon guidance and outgrowth. *Journal of Neurobiology* **41**(1): 158-164

Rosslenbroich V, Dai L, Baader SL, Noegel AA, Gieselmann V, Kappler J (2005) Collapsin response mediator protein-4 regulates F-actin bundling. *Exp Cell Res* **310**(2): 434-444

Shi SH, Cheng T, Jan LY, Jan YN (2004) APC and GSK-3beta are involved in mPar3 targeting to the nascent axon and establishment of neuronal polarity. *Curr Biol* **14**(22): 2025-2032

Siddiqui SS, Culotti JG (1991) Examination of neurons in wild type and mutants of Caenorhabditis elegans using antibodies to horseradish peroxidase. *Journal of Neurogenetics* **7**(4): 193-211

Sivasankaran R, Pei J, Wang KC, Zhang YP, Shields CB, Xu XM, He Z (2004) PKC mediates inhibitory effects of myelin and chondroitin sulfate proteoglycans on axonal regeneration. *Nat Neurosci* **7**(3): 261-268

Stenmark P, Ogg D, Flodin S, Flores A, Kotenyova T, Nyman T, Nordlund P, Kursula P (2007) The structure of human collapsin response mediator protein 2, a regulator of axonal growth. *J Neurochem*

Thomas GM, Frame S, Goedert M, Nathke I, Polakis P, Cohen P (1999) A GSK3-binding peptide from FRAT1 selectively inhibits the GSK3-catalysed phosphorylation of axin and beta-catenin. *FEBS Lett* **458**(2): 247-251

Wang LH, Strittmatter SM (1997) Brain CRMP forms heterotetramers similar to liver dihydropyrimidinase. *J Neurochem* **69**(6): 2261-2269.

Yamashita T, Tohyama M (2003) The p75 receptor acts as a displacement factor that releases Rho from Rho-GDI. *Nat Neurosci* **6**(5): 461-467.

Yoshimura T, Kawano Y, Arimura N, Kawabata S, Kikuchi A, Kaibuchi K (2005) GSK-3beta regulates phosphorylation of CRMP-2 and neuronal polarity. *Cell* **120**(1): 137-149

Yuasa-Kawada J, Suzuki R, Kano F, Ohkawara T, Murata M, Noda M (2003) Axonal morphogenesis controlled by antagonistic roles of two CRMP subtypes in microtubule organization. *European Journal of Neuroscience* **17**(11): 2329-2343

Zhou FQ, Snider WD (2005) Cell biology. GSK-3beta and microtubule assembly in axons. *Science* **308**(5719): 211-214

Zhou FQ, Zhou J, Dedhar S, Wu YH, Snider WD (2004) NGF-induced axon growth is mediated by localized inactivation of GSK-3beta and functions of the microtubule plus end binding protein APC. *Neuron* **42**(6): 897-912

FIGURE LEGENDS

Figure 1 CRMP4b dephosphorylation regulates CRMP4b-RhoA binding and is stimulated by Nogo-P4. (A-C) PC12 cells **(A)** or HEK293T cells **(B,C)** were co-transfected with myc-RhoA constructs and CRMP4b-V5 and subjected to immunoprecipitation with anti-myc antibody. **(A)** PC12 cells were stimulated for 8 minutes with Nogo-P4 peptide. **(B)** Cells were treated for 30 minutes with 200 nM Calyculin A prior to lysis or cell lysates were treated for 30 minutes with 10 U shrimp alkaline phosphatase (SAP) at 34° C prior to immunoprecipitation . **(D, E)** PC12 cells **(D)** or cerebellar neurons infected with HSV-CRMP4b-V5 **(E)** were stimulated for 1 to 8 minutes with Nogo-P4 peptide. Cell lysates were separated by SDS-PAGE and total- and phosphorylated- CRMP4b (pThr622) was detected by immunoblot.

FIGURE 1

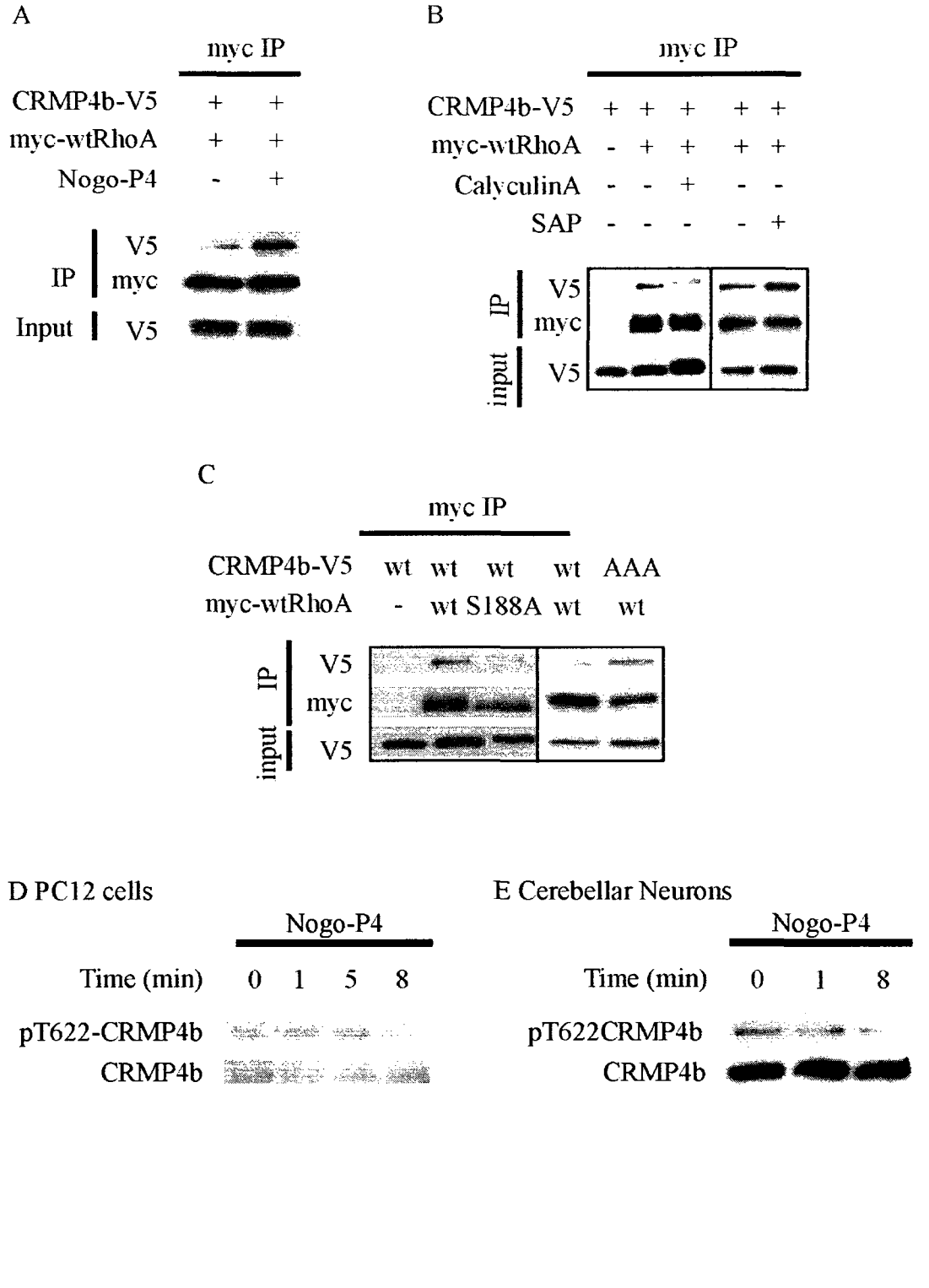

A
myc IP
CRMP4b-V5 + +
myc-wtRhoA + +
Nogo-P4 - +
IP V5
myc
Input V5
B
myc IP
CRMP4b-V5 + + + + +
myc-wtRhoA - + + + +
CalyculinA - - + - -
SAP - - - - +
IP V5
myc
input V5
C
myc IP
CRMP4b-V5 wt wt wt wt AAA
myc-wtRhoA - wt S188A wt wt
IP V5
myc
input V5
D PC12 cells
Nogo-P4
Time (min) 0 1 5 8
pT622-CRMP4b
CRMP4b
E Cerebellar Neurons
Nogo-P4
Time (min) 0 1 8
pT622CRMP4b
CRMP4b

Figure 2 Nogo-P4 stimulates CRMP4b dephosphorylation by phosphorylating GSK3β. (A,B) PC12 cells **(A)** or cerebellar neurons **(B)** were stimulated for 1 to 8 minutes with Nogo-P4 peptide. Cell lysates were separated by SDS-PAGE and total- and phosphorylated- GSK3β was detected by immunoblot. PKC activity was inhibited prior to Nogo-P4 stimulation of cerebellar neurons by incubating for 1 hour with 100 nM Go6976. **(C)** PC12 cells were transiently transfected with CRMP4b-V5 and GSK3β-S9A for 24 hours then treated with Nogo-P4 peptide for 8 minutes. CRMP4b-V5 was immunoprecipitated, separated by SDS-PAGE and total- and phosphorylated- CRMP4b was detected by immunoblot.

FIGURE 2

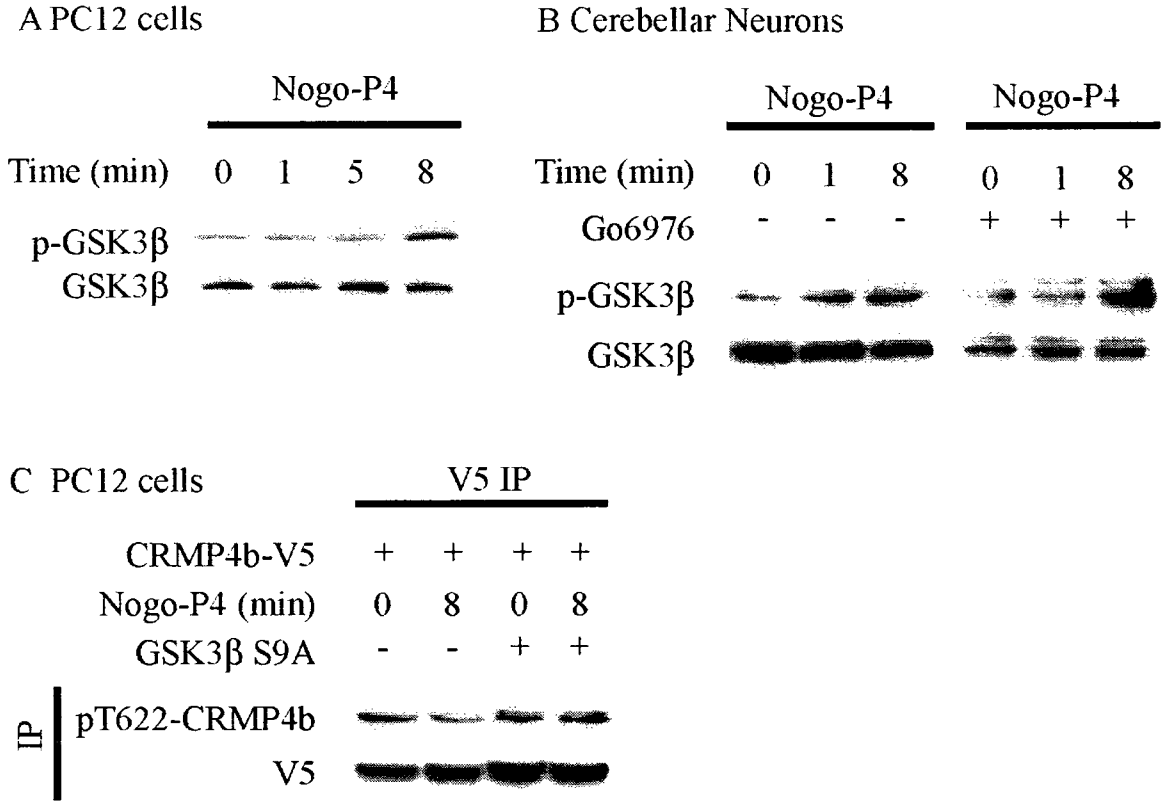
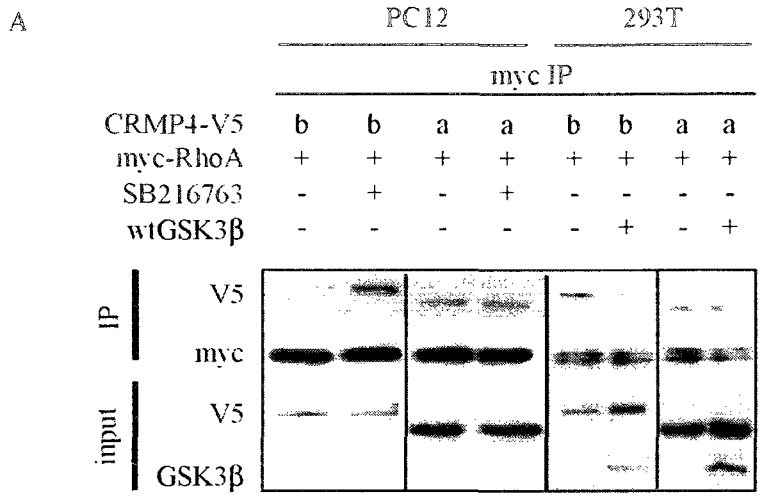

A PC12 cells
Nogo-P4
Time (min) 0 1 5 8
p-GSK3β
GSK3β
B Cerebellar Neurons
Nogo-P4
Nogo-P4
Time (min) 0 1 8 0 1 8
Go6976 - - - + + +
p-GSK3β
GSK3β
C PC12 cells
V5 IP
CRMP4b-V5 + + + +
Nogo-P4 (min) 0 8 0 8
GSK3β S9A - - + +
IP
pT622-CRMP4b
V5

Figure 3 Pharmacologic inhibition of GSK3β inhibits neurite outgrowth in a CRMP4b dependent manner. (A) Myc-RhoA (WT or 63L), CRMP4-V5 and GSK3β constructs were transiently co-expressed in PC12 cells or 293T cells. To inhibit GSK3β, cells were treated overnight with 5 μM SB216763 or 300 nM 6-bromoindirubin-3'-acetoxime. Cell lysates were subjected to immunoprecipitation with anti-myc antibody and analyzed by immunoblot with anti-V5 or anti-myc antibodies. **(B)** Dissociated cerebellar neurons were treated overnight in serum-free SATO with 5 μM SB216763 or 300 nM 6-bromoindirubin-3'-acetoxime, fixed and stained with anti-βIII tubulin antibody to visualize neurite outgrowth. Scale bar, 50 μm. **(C)** HSV-GFP- or HSV-C4RIP-infected dissociated DRG neurons were grown overnight in the presence of 5 μM SB216763 or 300 nM 6-bromoindirubin-3'-acetoxime, fixed and stained with anti-β-III tubulin antibody. Scale bar, 100 μm. **(D,E)** Quantification of cerebellar **(D)** or DRG **(E)** outgrowth/cell. Values are normalized to baseline outgrowth with no GSK3β inhibitor for each experiment. Determinations are from 3 experiments performed in duplicate. $^{**}p<0.01$ by student t-test.

FIGURE 3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293T
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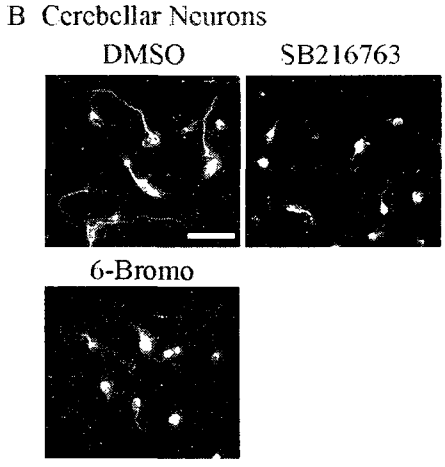
IP
V5
myc
input
V5
GSK3β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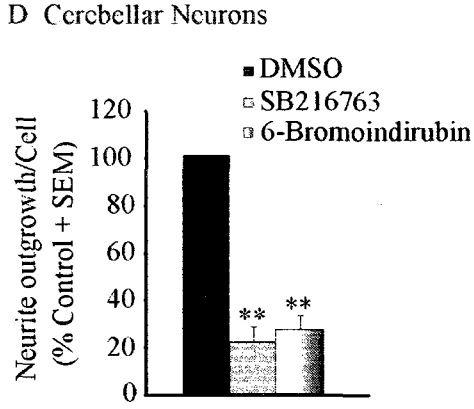
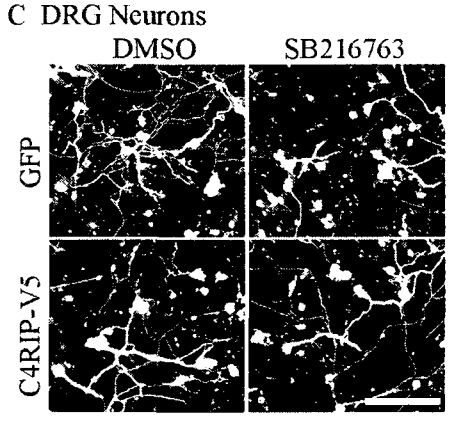
SB216763
6-Bromo


D Cerebellar Neurons
DMSO
SB216763
6-Bromoindirubin
Neurite outgrowth/Cell
(% Control + SEM)
120
100
80
60
40
20
0
**
**


C DRG Neurons
DMSO
SB216763
GFP
C4RIP-V5

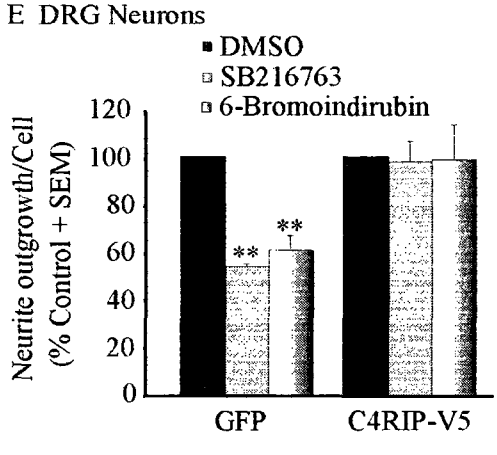

E DRG Neurons
DMSO
SB216763
6-Bromoindirubin
Neurite outgrowth/Cell
(% Control + SEM)
120
100
80
60
40
20
0
**
**
GFP
C4RIP-V5

Figure 4 The amino terminus of CRMP4b is phosphorylated. (A) Schematic representation of CRMP4b, CRMP4a and C4RIP. Phospho-residues are indicated in red with adjacent sequence in white. Phospho-residues are vertically aligned between CRMP4b and CRMP4a to directly compare the phospho-residue designations. **(B)** HEK293T cells were transiently transfected with wild type C4RIP-V5 or C4RIP-V5 alanine substitution mutants and treated for 30 minutes with 200 nM Calyculin A. Cell lysates were subjected to immunoprecipitation with anti-V5 antibody and immunoblotted with anti-V5 antibody. **(C)** HEK293T cells were transfected with pcDNA or CRMP4b-V5 constructs. Lysates were subjected to immunoprecipitation with anti-V5 antibody and immunoblotted with a phospho-specific antibody to Ser101 of CRMP4b. Prior to immunoprecipitation, cells or cell lysates were stimulated with Calyculin A or SAP, respectively. **(D)** CRMP4b-V5 was purified from transfected HEK293T cells and combined with recombinant GSK3β and DYRK2. The protein extract was separated by SDS-PAGE and immunoblotted with phospho-specific antibodies for Ser101 and Thr622 of CRMP4b. **(E)** HEK293T cells were transiently cotransfected with GSK3βR96A and CRMP4b-V5 constructs. Lysates were immunoblotted with the phospho-specific antibody for Ser101 of CRMP4b.

FIGURE 4

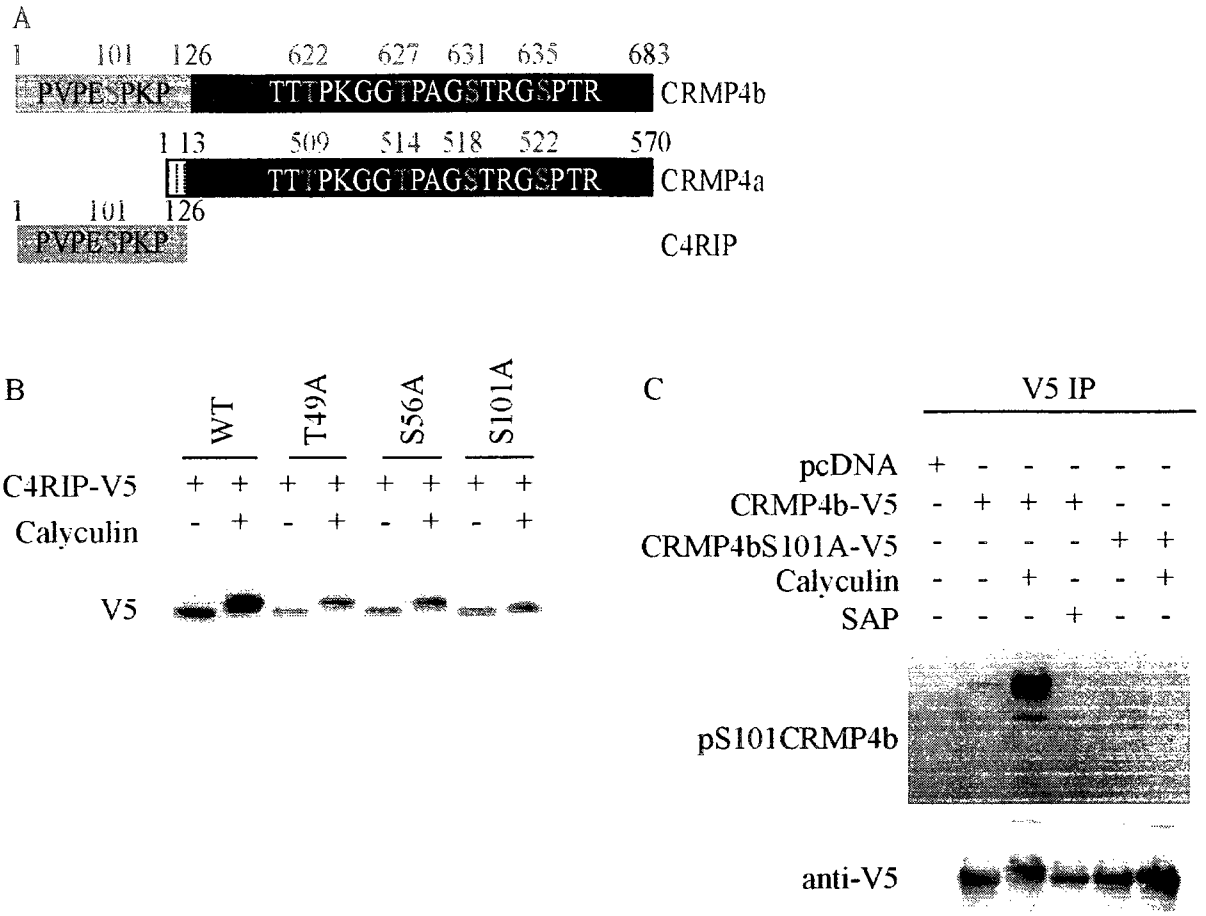
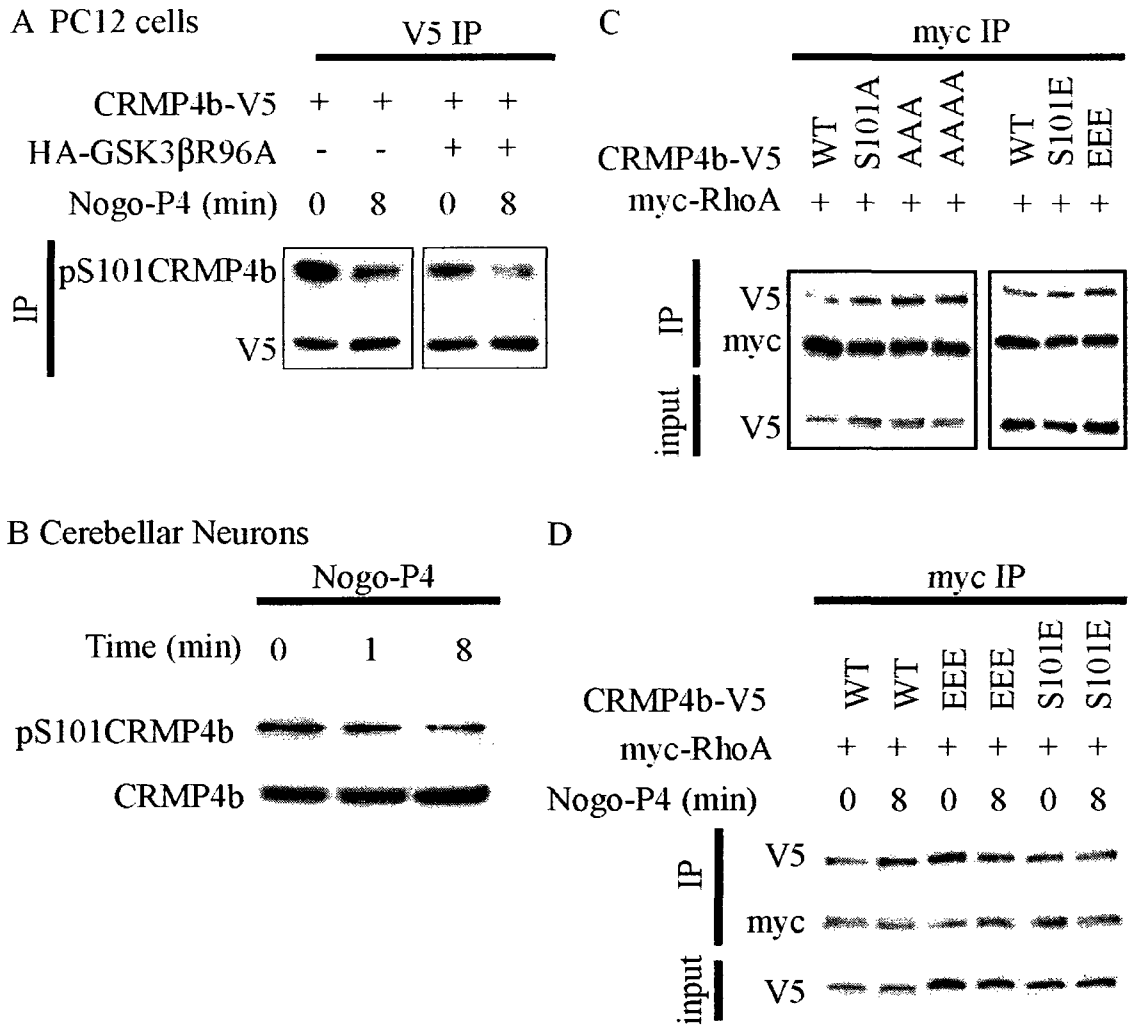

A
1 101 126 622 627 631 635 683
PVPESPKP
TTTPKGGTPAGSTRGSPTR
CRMP4b
1 13 509 514 518 522 570
TTTPKGGTPAGSTRGSPTR
CRMP4a
1 101 126
PVPESPKP
C4RIP
B
WT
T49A
S56A
S101A
C4RIP-V5 + + + + + + + +
Calyculin - + - + - + - +
V5
C
V5 IP
pcDNA + - - - - -
CRMP4b-V5 - + + + - -
CRMP4bS101A-V5 - - - - + +
Calyculin - - + - - +
SAP - - - + - -
pS101CRMP4b
anti-V5
D
CRMP4b-V5 + + + +
GSK3β - + + -
DYRK2 - - + +
pS101CRMP4b
pT622CRMP4b
E
CRMP4b-V5 + + - -
CRMP4bAAA-V5 - - + +
HA-GSK3βR96A - + - +
pS101CRMP4b
V5
HA

Figure 5 CRMP4bSer101 is dephosphorylated in response to Nogo-P4 and regulates binding to RhoA. (A,B) PC12 cells transfected with CRMP4b-V5 **(A)** or cerebellar neurons infected with HSV-CRMP4b-V5 **(B)** were stimulated for 8 minutes with Nogo-P4 peptide. For PC12 cells CRMP4b-V5 was immunoprecipitated from cell lysates, separated by SDS-PAGE and total- and phosphorylated- CRMP4b (pSer101) were detected by immunoblot. For cerebellar neurons, CRMP4b was analyzed directly from cell lysates. **(C)** HEK293T cells were transiently co-transfected with CRMP4b-V5 phospho-mutants and myc-RhoA. Cell lysates were subjected to immunoprecipitation with anti-myc antibody and were immunoblotted with anti-V5 and anti-myc antibodies. **(D)** PC12 cells were transiently cotransfected with CRMP4b-V5 phospho-mutants and mycRhoA and stimulated for 8 minutes with Nogo-P4 peptide. Lysates were subjected to immunoprecipitation with anti-myc antibody and immunoblotted with anti-myc or anti-V5 antibodies.

FIGURE 5


A PC12 cells
V5 IP
CRMP4b-V5 + + + +
HA-GSK3βR96A - - + +
Nogo-P4 (min) 0 8 0 8
IP
pS101CRMP4b
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C
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CRMP4b-V5 WT S101A AAA AAAA WT S101E EEE
myc-RhoA + + + + + + +
IP
V5
myc
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B Cerebellar Neurons
Nogo-P4
Time (min) 0 1 8
pS101CRMP4b
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CRMP4b-V5 WT WT EEE EEE S101E S101E
myc-RhoA + + + + + +
Nogo-P4 (min) 0 8 0 8 0 8
IP
V5
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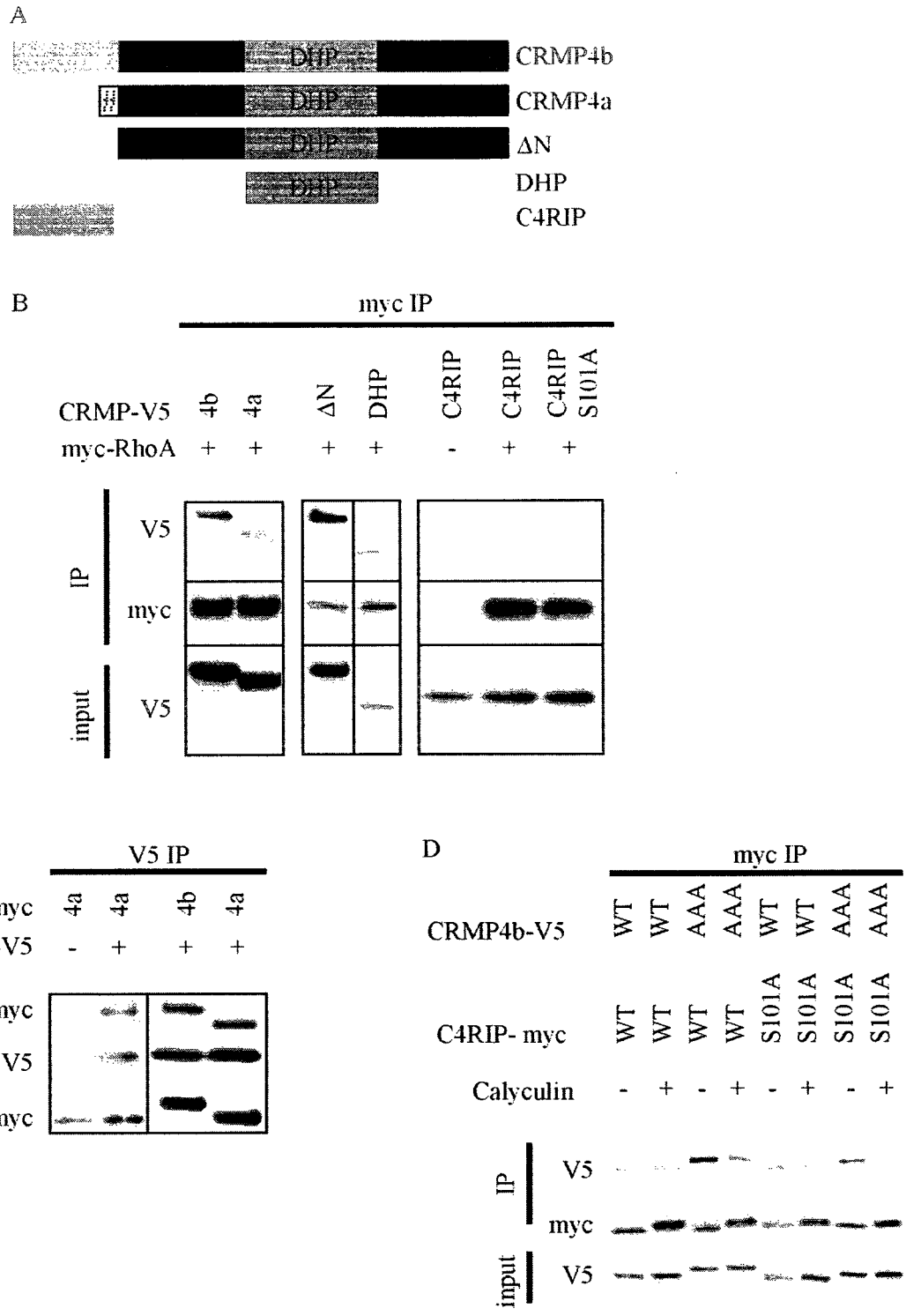
V5

Figure 6 C4RIP binds to CRMP4, not RhoA. **(A)** Schematic representation of full length and mutant CRMP4 constructs. **(B,C)** HEK293T cells were transiently cotransfected with myc-RhoA **(B)** or C4RIP-myc **(C)** and CRMP-V5 constructs. Lysates were subjected to immunoprecipitation with anti-myc antibody and immunoblotted with anti-myc or anti-V5 antibodies. **(D)** HEK293T cells were transiently transfected with CRMP4b-V5 and C4RIP-myc constructs and subjected to immunoprecipitation with anti-myc antibody. Prior to immunoprecipitation cells were stimulated for 30' with Calyculin A.

FIGURE 6


A
DHP
CRMP4b
DHP
CRMP4a
DHP
ΔN
DHP
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C4RIP
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myc
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C4RIP-V5
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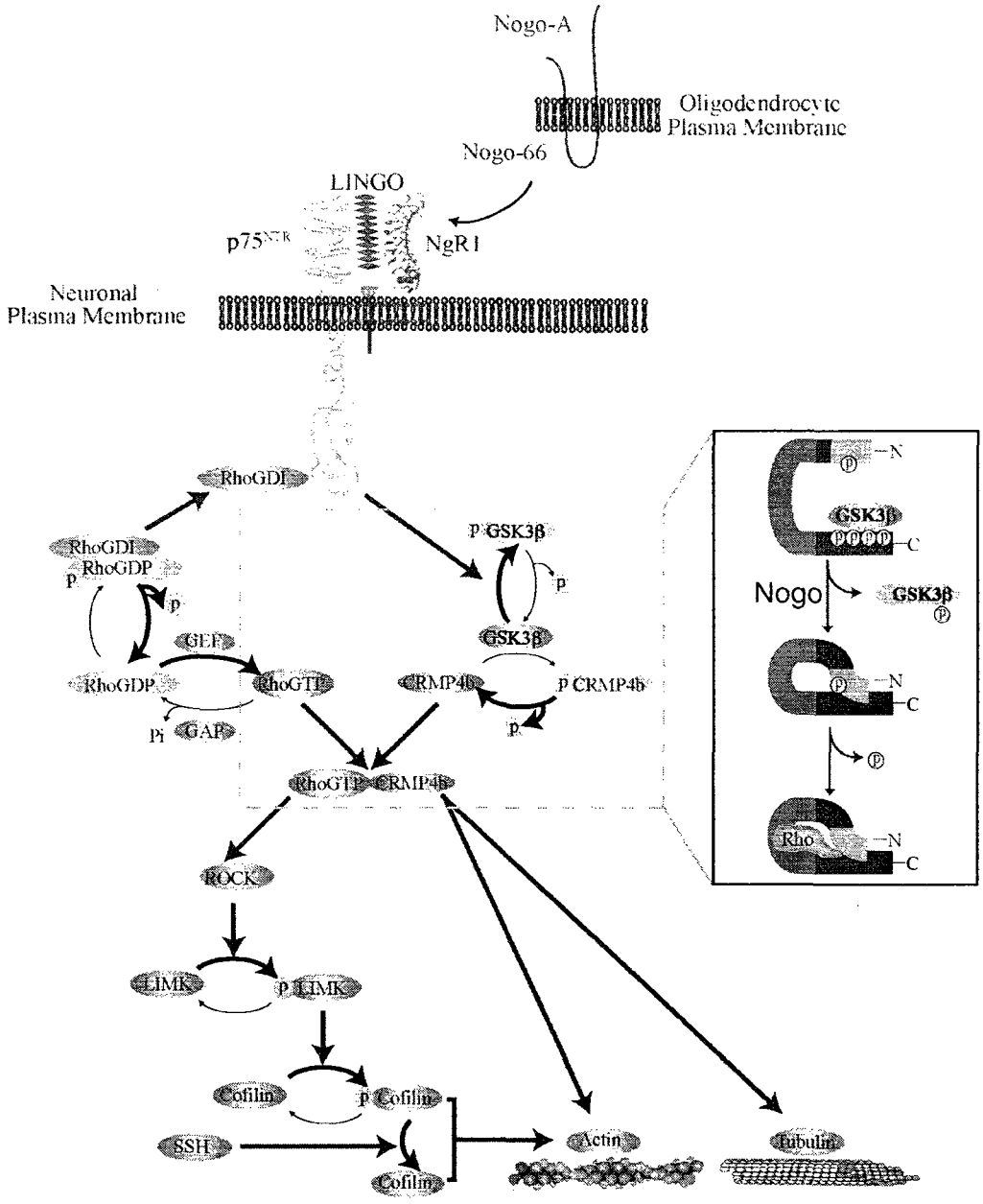
Supplementary Figure 1 Schematic representation of neurite outgrowth inhibitory signaling. Schematic representation of neurite outgrowth inhibition. Previous studies have demonstrated that inhibitory ligands including Nogo-A bind and signal through an NgR-containing neuronal cell surface receptor complex (Liu et al., 2002). Both CSPGs and MAIs signal via activation of the small GTPase RhoA (Dergham et al., 2002; Niederost et al., 2002; Borisoff et al., 2003; Fournier et al., 2003; Alabed et al., 2006). MAI-dependent activation of RhoA is mediated by enhanced intracellular proteolysis of $p75^{NTR}$ (Domeniconi et al., 2005), and sequestration of RhoGDI (Yamashita and Tohyama, 2003). LIM kinase- and Slingshot phosphatase-dependent regulation of cofilin phosphorylation contribute to cytoskeletal rearrangements underlying outgrowth inhibition (Hsieh et al., 2006). In this manuscript we demonstrate that phosphorylation and inactivation of GSK3β leads to CRMP4b dephosphorylation and CRMP4b-RhoA complex formation. CRMP4b-RhoA complex formation may impinge on both actin (Alabed et al., 2007) and microtubule (Fukata et al., 2002b; Cole et al., 2004b) dynamics to regulate cytoskeletal rearrangements required for outgrowth inhibition. Active and inactive versions of molecules are colored in green and red, respectively. Bolded arrows indicate the direction of signaling upon Nogo stimulation. We also find that CRMP4b binding to RhoA is regulated by a phosphorylation-dependent conformational change in CRMP4b (inset). Our data suggests a model whereby dephosphorylation of the carboxy terminal region of CRMP4b may enhance the folding interaction between the amino and carboxy termini of the protein and modify the protein conformation to favor dephosphorylation of CRMP4b at Ser101. Dephosphorylation of Ser101 may then expose a binding pocket for RhoA.

SUPPLEMENTAL FIGURE 1



CHAPTER 5

V. GENERAL DISCUSSION

V. 1. Summary

The major aim of this thesis was to study the intracellular events that link MAI signals to the cytoskeleton and to use this information to develop strategies to promote regeneration. We were interested in molecules that functionally interact with RhoA to mediate myelin-dependent inhibition, as potentially more specific targets for therapeutic intervention. The results presented herein address the importance of two RhoA interacting proteins in translating the effect of MAIs on the cytoskeleton. The results also reveal new insights on alternative molecules induced by Nogo-66 that also contribute to failed regeneration in CNS neurons.

Chapter 2 of this thesis describes the contribution of the rho effector, ROCK to MAI responses in neurons. We provide direct evidence for the involvement of the RhoA-ROCK pathway in response to Nogo-66 signalling. Our results were consistent with previous studies indirectly implicating ROCK using pharmacologic inhibitors (Dergham et al., 2002; Borisoff et al., 2003; Monnier et al., 2003).

In Chapter 3 of this thesis, we identify the cytosolic phosphoprotein CRMP4b as a novel RhoA interacting partner that mediates neuronal responses to CNS inhibitors. This protein is known for roles in actin and microtubule cytoskeletal regulation and our results are in keeping with other studies also implicating the CRMP proteins with a role in the Nogo-66 signalling cascade (Mimura et al., 2006). By analyzing the specifics of the CRMP4b-RhoA association we have generated a molecular antagonist (C4RIP) of CRMP4b-RhoA binding that circumvents myelin- and CSPG-induced neurite outgrowth inhibition *in vitro*. Contrary to previous antagonists that target cytoskeletal proteins such C3 or Y-27632, the C4RIP antagonist does not affect basal neurite outgrowth. Our results

are in keeping with the notion that targeting pathway specific molecules reduces the potential for side effects. This C4RIP inhibitor has the potential to be a potent and more specific molecular therapeutic for spinal cord injury.

In Chapter 4 of this thesis, we identify glycogen synthase kinase 3β (GSK3β) as an important kinase in the MAI pathway that regulates the interactions between CRMP4b and RhoA. Our results uncover another role for the multitasking GSK3β protein and propose novel molecular players and regulatory mechanisms in the MAI pathway.

This thesis provides insights into the molecular machinery that is engaged in response to CNS inhibitors and reveals several novel therapeutic targets to promote axon regeneration following CNS injury.

V. 2. Pros and Cons of targeting intracellular proteins as a therapeutic approach

The initial discovery that multiple axon outgrowth inhibitors exist may limit the efficacy of therapeutic strategies aimed at neutralizing individual inhibitory proteins. This heterogeneity is further compounded by the finding that inhibitory molecules bind to several receptors. For instance, MAG can bind NgR1, NgR2 (Venkatesh et al., 2005) and the ganglioside GT1b (Vinson et al., 2001). In addition, NgR1 ligands: MAG, Nogo-66 and OMgp can bind the PirB receptor to mediate growth inhibition (Atwal et al., 2008). PirB and NgR1 are both coexpressed by similar neuronal cell types.

On the other hand, both MAIs and CSPGs mediate their growth-inhibitory effect through activation of RhoA identifying a convergent pathway of axon outgrowth inhibition (Winton et al., 2002). Circumvention of axon outgrowth blockade in the context of injury will undoubtedly require a more aggressive focus on the identification

of convergent and pathway-specific downstream intracellular signalling cues rather than events occurring at the cell surface. A careful balance will need to be achieved to identify convergent intracellular targets that can be targeted without affecting key endogenous functions. For example targeting of RhoA or direct actin cytoskeletal regulators will undoubtedly lead to side effects from affecting critical functions in healthy cells. For example, suppression of ROCK activity using Y-27632 has been used effectively to enhance axonal regeneration. Y-27632 treatment enhances sprouting by injured CST and dorsal column tract axons and accelerates locomotor recovery in adult rodents (Dergham et al., 2002; Fournier et al., 2003). However it was also noted that Y-27632 also increased the expression of inhibitory CSPGs within the extracellular matrix and activates astrocytes, which could counteract the growth promoting effects of ROCK inhibition on axonal growth (Chan et al., 2007). As we progress and explore downstream signals the general efficacy of the therapeutic approach increases but with a corresponding decrease in its specificity as undoubtedly several pathways crosstalk. One way to promote therapeutic specificity is to target neuronal specific molecules to limit side effects on non-neuronal cells. One advantage of targeting CRMP4b (Chapter 3) is its enrichment in neuronal cells and the finding that the CRMP4b antagonist C4RIP specifically affects neurite outgrowth on inhibitory myelin substrates without impacting outgrowth on control substrates.

We identified this novel interaction between CRMP4b and RhoA that appears downstream of the Nogo-66 signalling cascade. Atwal et al (2008) found that inhibition of PirB in NgR1-deficient cerebellar granule neurons only partially rescues the Nogo-induced axon outgrowth inhibition while completely rescuing axon growth on myelin

extracts. This suggests that Nogo's inhibitory effect on outgrowth might be mediated by additional unknown receptors. This reinforces the notion that identifying and targeting interactions such as the CRMP4b-RhoA association might be more beneficial and efficient at reversing axon outgrowth inhibition.

V. 3. C4RIP: CRMP4b antagonist and more

Our data demonstrates a near complete recovery of axonal growth on myelin substrates. We have also demonstrated biochemical evidence that overexpression of C4RIP (amino terminus of CRMP4b) selectively disrupts CRMP4b-RhoA association and not CRMP4a-RhoA. We speculate that C4RIP blocks the association between CRMP4b and RhoA perhaps by direct steric hindrance. However, our data could indicate that C4RIP may in fact function as a dominant negative form of CRMP4b, possibly by binding and sequestering endogenous CRMP4b from its interacting partners. In fact, the CRMP proteins are known to form homo- and hetero-oligomers, which may be critical to their function (Wang and Strittmatter, 1997b; Inatome et al., 2000; Ricard et al., 2001; Leung et al., 2002; Deo et al., 2004). This reinforces the notion that C4RIP could be binding endogenous wild type CRMP4b. Further, Yuasa-Kawada et al. (2003) found that overexpression of the amino terminal domain of CRMP2b into CRMP2b-expressing fibroblasts rescued the microtubule patterns and cell shapes induced by CRMP2b overexpression alone. Their findings also point towards a potential role for amino CRMP2b as a dominant-negative form of CRMP2b (Yuasa-Kawada et al., 2003).

We found that overexpression of C4RIP or knockdown of CRMP4 using siRNA in DRG neurons circumvents myelin-induced axon outgrowth inhibition. It is intriguing

that C4RIP overexpression appears to be more efficient than knockdown of endogenous CRMP4 at rescuing from myelin inhibition. Several reasons might account for this discrepancy: 1) Limitations in the transfection efficiency of the oligonucleotides versus the high level of infection using the HSV carrier. 2) Limitations in the degree of CRMP4b suppression versus the high level of C4RIP expression induced by the infection. 3) It is also conceivable that the overexpression paradigm of C4RIP overwhelms the total endogenous pool of CRMP4b and also sequesters additional target substrates that are critical for myelin-dependent inhibition.

V. 4. The contributions of CRMP4b to cytoskeletal dynamics

We found that disruption of the CRMP4b-RhoA interaction using C4RIP reverses myelin- and CSPG-based inhibition. Based on our current understanding of this interaction and of the C4RIP antagonist, the mediated effect could be through 1) the disruption of the CRMP4b-RhoA complex and the subsequent blockade of any signalling event downstream of this interaction 2) the disruption of the function of endogenous CRMP4b. In addition to acting as a dominant negative CRMP4b, C4RIP could also be mediating an effect because of the overexpression paradigm since the sequence contains several motifs such as an SH3 binding domain and therefore this could also impact other CRMP4b mediators.

By analogy to CRMP2, it is reasonable to hypothesize that CRMP4b's functions in regulating the cytoskeleton might be diverse. CRMP4b has a punctuate distribution and partially colocolizes with sv2 positive vesicles suggestive of a role in exocytosis or trafficking (Quinn et al., 2003). Endocytosis during neurite inhibition may be important

for regulating membrane dynamics, or may target the internalization of cell surface receptors or cell adhesion molecules enabling their temporal and spatial regulation in response to MAIs. Therefore a reasonable hypothesis for CRMP4b's function could be through the localization of RhoA to discreet areas within the growth cone to enable RhoA downstream signalling events. This would be in keeping with a role in trafficking. In fact under Sema3A signalling Rac1 and F-actin were shown to be recruited to membrane ridges and vacuoles (Fournier et al., 2000).

Independently from RhoA, CRMP4b could also be regulating the cytoskeleton. CRMP4a and CRMP4b might be capable of mediating similar cytoskeletal rearrangements; however the amino terminal region of CRMP4b is necessary for recruitment to the appropriate cytoskeletal elements within the growth cone. This is consistent with the finding that overexpression of CRMP4b but not CRMP4a leads to an increase in neurite branching (Quinn et al., 2003). CRMP4a was shown to promote F-actin bundling (Rosslenbroich et al., 2005), this would support the notion that CRMP4a might be an important regulator of collapse as general loss of actin bundling is a critical event for growth cone collapse (Zhou and Cohan, 2001). We have found that overexpression of CRMP4b increases neurite branching and filopodial length. This is suggestive of an actin based phenotype which could be in keeping with the fact that CRMP4b binds Intersectin via an SH3 binding motif within the unique amino terminal region (Quinn et al., 2003). Intersectin is known to activate Cdc42 and mediate actin polymerization and branching through N-WASP and Arp2/3 (Hussain et al., 2001).

Furthermore, all CRMP proteins including CRMP4 were shown to bind tubulin (Fukata et al., 2002). However the only member with a role in microtubule assembly is CRMP2.

This does not rule out a role for CRMP4 in regulating tubulin localization within the growth cone as these events could be localized within select sites.

V. 5. The role of GSK3β in axon outgrowth inhibition

Our results show that Nogo-66 stimulation induces the dephosphorylation of CRMP4b at S101 in the amino terminus and at T622 in the carboxy region. We also showed that Nogo-66 induced the phosphorylation of GSK3β at serine 9 and its subsequent inactivation towards primed sites. We show that pharmacologic inhibition of GSK3β mimics myelin-induced neurite outgrowth inhibition.

Surprisingly in a recent study by Dill et al. (2008), pharmacologic inhibition of GSK3β with lithium in a spinal cord transection paradigm promotes corticospinal and serotonergic axon sprouting accompanied with improved locomotor function. Their *in vitro* data shows pharmacologic inhibition of GSK3 enhances neurite outgrowth of adult mice dorsal root ganglion neurons and postnatal cerebellar granule neurons on CNS inhibitors (Dill et al., 2008). Several explanations could account for the differences: 1) it is conceivable that the regulation of GSK3β by Nogo-66 according to our data accounts for the acute phase of growth inhibition as is currently accepted for the NgR1 signalling pathway. 2) The level of lithium (3mEq/kg/day) used in the *in vivo* Dill study may not achieve complete inhibition of GSK3β activity *in vivo*. These conditions would promote a different spectrum of growth phenotype according to the results by Kim et al. (2006), whereby in adult DRG neurons, low levels of GSK3β inhibition leads to axon growth, whereas strong inhibition of GSK3β activity results in reduced axon growth. This would mean that the growth promoting effect of GSK3β inhibition *in vivo* in the Dill study

could be attributed to indirect local regulation of additional GSK3β-dependent factors that may contribute to axon growth. Further, in a study by Yick et al. (2004) examining the effect of lithium and ChABC on the regeneration of axotomized rubrospinal tract (RST) neurons, treatment of lithium alone failed to promote the regeneration of severed RST axons. However, application of ChABC along with lithium promoted axonal regeneration of injured RST neurons suggesting that lithium may potentiate the neuroregenerative function of ChABC. It was suggested that lithium may protect the injured RST neurons from apoptosis therefore facilitating axon regeneration across the growth-permissive lesion scar following ChABC treatment (Yick et al., 2004). Interestingly, combined treatment of lithium together with ChABC relieved the asymmetry of forelimb use compared with ChABC treatment alone. However treatment with lithium alone did not improve forelimb function (Yick et al., 2004). This suggests that although there may be a potential use for lithium in neuroprotection, it is still not clear what is the contribution to regeneration?

Cole et al. (2006) found that the level of CRMP4a phosphorylation at Thr-514 (equivalent of Thr-622 in CRMP4b) in whole brain lysates of conditional GSK3βS9A knockin mice is similar to wild type mice. Similar results were found for brain lysates from transgenic mice that specifically overexpress GSK3β in the brain (Cole et al., 2006). One explanation for their findings was that CRMP4 might be maximally phosphorylated in rodent neurons or that the availability of the necessary priming kinase is the limiting step. In fact, Ser-522 of CRMP4a was also found to be fully phosphorylated in rat brain lysates as evidenced by the inability to further phosphorylate this site by exogenous Cdk5 (Cole et al., 2008). This same study found that Cdk5 phosphorylation site on CRMP2

might be resistant to dephosphorylation and therefore propose a mechanism to explain the high level of phosphorylated CRMP2 in rodent brain. Taken together, these data point towards the idea that *in vivo* the regulation of the phosphorylation of the CRMP molecules might not necessarily correlate with the observed changes *in vitro*. This suggests another degree of complexity that may help explain why exogenous manipulation of GSK3β activity with respect to the CRMP molecules does not represent the *in vitro* evidence. In fact, the observed growth recovery in the Dill study after lithium therapy could be attributed to the impact of inhibiting other GSK3β substrates *in vivo* and the subsequent growth promoting and neuroprotective effect induced. In fact lithium has been shown to be neuroprotective through multiple intersecting mechanisms in a large number of studies both *in vitro* and *in vivo*. The neuroprotective effect extends to multiple cell lines including cerebellar granule neurons, cerebral cortical cells and hippocampal neurons (Nonaka et al., 1998a). Protection is also promoted from various cellular insults as well including glutamate excitotoxicity and the subsequent NMDA-receptor-mediated calcium influx (Nonaka and Chuang, 1998; Nonaka et al., 1998b). The neuroprotective mechanisms of lithium are through multiple pathways which include an induction of the cell survival signalling pathways. Lithium increases cell survival by inducing brain-derived neurotrophic factor (BDNF) and the subsequent stimulation of anti-apoptotic pathways including the PI3K/AKT (Franke et al., 2003) and the mitogen-activated protein kinase pathways (Chang et al., 2003). Lithium also directly inhibits both GSK3α/β isoforms and indirectly activates a large number of downstream effectors and anti-apoptotic factors including CREB, NF-κB, Heat shock factor 1 (HSF-1), Activator protein 1 (AP-1) and β-catenin among others (Bijur and Jope, 2000; Xavier et al., 2000;

Chen et al., 2003; Gould et al., 2004; Hansen et al., 2004) and the Bcl-2 family of proteins (Chen and Chuang, 1999; Manji et al., 2000). In conclusion, it would be difficult in the clinical setting to separate neuroprotection from neurotrophic effect of lithium and it would be not be possible to ascribe one single functional effect to a single molecule following lithium treatment.

V. 6. A role for RhoA/CRMP4b in neuronal plasticity?

In the context of learning and memory, LTP/LTD and dendritic spine morphology are key underlying aspects. Cytoskeletal rearrangememt is a core mechanism for mediating several cellular key functions. Actin and microtubule dynamics mediate neuronal-repair inhibition. In addition, spine actin dynamics are crucial for translating and reflecting alterations in synaptic strength and dendritic spine morphology (Yuste and Bonhoeffer, 2001; Lamprecht and LeDoux, 2004). Therefore it is conceivable that many cytoskeletal mechanisms recruited in neuronal-injury paradigms could also be key mediators of other cellular processes. The modulation of the RhoA-CRMP4b interaction in the Nogo-66 pathway is rapid and transient, therefore suggesting a possible involvement in the regulation of acute insults to the nervous system. This would be in keeping with the evidence that NgR1 is required for the acute inhibitory activity of the myelin-derived inhibitors (Kim et al., 2004). However recent data suggest PirB, a receptor originally identified as an MHCI receptor involved in regulating immune cell activation (Takai and Ono, 2001), as a novel functional receptor for the MAIs (Atwal et al., 2008). PirB is the only mouse ortholog of the human leukocyte immunoglobulin (Ig)-like receptor B2 (LILRB2) that was identified as binding partner of Nogo-66 (Atwal et al., 2008). Thus an alternative possibility is that the RhoA/CRMP4b is engaged in response to stimulation of

PirB. More data is needed to elucidate the functional relationship between NgR1 and PirB in contributing to failed regeneration and to the contribution of RhoA/CRMP4b to plasticity. Several situations are possible: 1) It is conceivable that RhoA-CRMP4b recruitment might be modulated only downstream of one of the Nogo66 receptors or 2) it could represent a convergent step necessary for both receptors. Similarly, this same interaction may be more pronounced in other cellular mechanisms such as synaptic plasticity. In keeping with a role for NgR1 in ocular dominance plasticity (McGee et al., 2005), PirB also defines a critical period for the development of the visual cortex (Syken et al., 2006). These findings point toward the notion that myelin-inhibitory proteins are intricately linked to neuronal plasticity in addition to their growth inhibitory effect. The association between these myelin-based molecules and their receptors may constitute a limiting mechanism for short-term neuronal remodeling in the adult brain. Similarly, removal of CSPG GAGs using ChABC in the visual cortex partially reactivates ocular dominance plasticity (Pizzorusso et al., 2002). This connection between limiting neuronal growth and neuronal plasticity is also evident in other neural specific mediators of axon growth. For example, CRMP2 and CRMP4 expression correlate with adult cortical reorganization in response to deafferentiation of the visual cortex (Cnops et al., 2007). The CRMP proteins are also known to persist in areas with high degrees of synaptic remodeling, such as the cerebellum, the hippocampus and the olfactory system (Ricard et al., 2000; Cameron and McKay, 2001; Ricard et al., 2001). Further, CRMP1, CRMP2 and CRMP5 appear differentially regulated in newly generated neurons of the olfactory system in keeping with participating with systems requiring permanent neurogenesis in the adult brain (Veyrac et al., 2005). More recently, CRMP1 was implicated as well in

synaptic remodeling. CRMP1 may be important in LTP maintenance in the CA1 region for hippocampal-dependent spatial learning and memory (Su et al., 2007). Taken together these findings point towards the idea that molecules and interactions involved in axon outgrowth inhibition most likely contribute to the same extent or even more so to neuronal remodeling. Therefore, the CRMP4b-RhoA interaction could potentially contribute to neuronal plasticity and remodeling as well.

V. 7. A role for neuroprotection in reversing axonal growth inhibition

We found that C4RIP, an antagonist of the RhoA-CRMP4b complex, reverses myelin- and SB216763-induced neurite outgrowth inhibition. We find that pharmacological inhibition of GSK3β mimics the effect of myelin inhibition on outgrowth. It is intriguing that C4RIP is able to reverse the effect of drug-induced inactivation of GSK3β since its inactivation undoubtedly affects the phosphorylation status of numerous effectors and microtubule associated proteins (MAPs). It is conceivable that several mechanisms may be mediating this effect. It is possible that CRMP4b may play a more convergent position in integrating multiple signalling cues affecting the cytoskeleton or that several RhoA-dependent events require an initial CRMP4b-induced localization of RhoA to distinct locations within the growth cone. Therefore the observed reversal of outgrowth inhibition may be in fact attributed to a neuroprotective effect induced by the combination of several events caused by the blockade of the RhoA-CRMP4b complex as well as the antagonism of CRMP4b and its subsequent interactors. In fact, antagonism of CRMP4b might disrupt the function of the other CRMP isoforms since they exist almost exclusively as heterotetramers (Wang and

Strittmatter, 1997b; Fukata et al., 2002c). Recent evidence demonstrates that the CRMP proteins may, in addition of holding individualized functions, also display additional properties based on their intrinsic ability to form heteromers with other CRMP members. For example, CRMP1b was found to complex with CRMP2a and participate in antagonizing the effect of ROCK in mediating RhoA-dependent signalling (Leung et al., 2002). In fact CRMP1 was also found to function as an invasion-suppressor gene (Steeg, 2001) as levels of CRMP1 are downregulated in lung cancer tissue relative to normal tissue (Shih et al., 2001). These data suggest that blockade of CRMP proteins might not only protect from outgrowth inhibition by their ability to affect the cytokeleton but may also have a growth-promoting effect by stimulating the intrinsic growth capacity of neurons. In fact, in a recent study by He and colleagues (2008), conditional deletion of the tumor suppressor PTEN in an optic nerve injury paradigm enhanced the survival of retinal ganglion cells but also promoted robust axon extension (Park et al., 2008). This mechanism was thought to be mediated by stimulating and enhancing protein synthesis necessary for long-distance axon growth. This study suggests a novel potential mechanism of inducing protein synthesis for promoting axon growth after injury.

V. 8. Inhibitory molecules with multiple functions

More recent data have focused on demonstrating the involvement of the Nogo-66 receptor in multiple systems other than limiting injury-induced axonal growth. In the context of Alzheimer's disease, NgR1 physically interacts with both Aβ and the amyloid precursor protein (APP) to regulate the production and clearance of Aβ within the brain (Park et al., 2006b; Park et al., 2006a). Selected NgR1 genetic variants are also involved

with a possible association to Schizophrenia (Budel et al., 2008). Further, NgR1 is implicated as a regulator of synaptic plasticity. The Nogo66/NgR1 complex regulates experience-dependent plasticity of the visual cortex. Deletion of NgR1 prolongs ocular dominance plasticity into adulthood (McGee et al., 2005). Consistent with the idea of limiting neuronal plasticity, NgR1 interacts with FGF1 and FGF2 to regulate activity-dependent synaptic strength by impinging on the pathways that regulate long-term potentiation (LTP) (Lee et al., 2008). The notion that molecules, previously implicated in neuronal-injury, might also impinge on other systems, is therefore suggestive that even previously characterized protein-protein interactions might also be involved in mediating several other functions. In fact numerous studies have demonstrated the benefit of interfering with the Nogo-66/NgR1 complex for stroke recovery (Papadopoulos et al., 2002; Li et al., 2004a; Seymour et al., 2005). This is in keeping with the finding that Nogo-A expression is significantly increased after focal ischemic stroke lesion in the adult rat (Cheatwood et al., 2008). Similarly, Nogo-A appears crucial for oligodendrocyte differentiation and myelin formation *in vivo* (Pernet et al., 2008). Genetic deletion or antibody-mediated neutralization of Nogo-A reduces the appearance of experimental autoimmune encephalomyelitis (EAE) symptoms potentially by locally decreasing inflammatory processes (Karnezis et al., 2004). Therefore antagonizing the interaction between CRMP4b and RhoA using C4RIP would have the potential application towards other paradigms including stroke, multiple sclerosis and traumatic brain injury.

V. 9. General conclusion

The identification and characterization of novel proteins in the Nogo-66 signaling pathway is critical to understanding the process of axon regeneration. In this thesis we focused on the role of RhoA interacting proteins and we have identified a novel interaction between CRMP4b and RhoA that is regulated by MAIs. We found that disruption of this complex using C4RIP reverses myelin- and CSPG-based axon outgrowth inhibition. We further characterized the specifics of this interaction and determined that GSK3β, under Nogo-66 stimulation, regulates the affinity between CRMP4b and RhoA by affecting the phosphorylation state of CRMP4b. Characterization of this interaction has revealed insights into novel regulatory mechanisms between proteins that will ultimately increase our understanding of protein-protein interactions. Substantial progress has been made in elucidating the molecular identity of negative cues at the CNS injury site following injury. Based on the current knowledge of inhibitory influences, a number of interventions have been tested to promote recovery in models of CNS trauma. These advances represent the first steps in developing viable therapies to promote axon regeneration following CNS trauma.

V. 10. REFERENCES

Atwal JK, Pinkston-Gosse J, Syken J, Stawicki S, Wu Y, Shatz C, Tessier-Lavigne M (2008) PirB is a functional receptor for myelin inhibitors of axonal regeneration.[see comment]. Science 322:967-970.

Bijur GN, Jope RS (2000) Opposing actions of phosphatidylinositol 3-kinase and glycogen synthase kinase-3beta in the regulation of HSF-1 activity. Journal of Neurochemistry 75:2401-2408.

Budel SP, T. Liu, B.P. Feng, Z. Hu, F. Johnson, S. Lauren, J. Park, J.H. McGee, A.W. Liao, J. Stillman, A. Kim, J.E. Yang, B.Z. Sodi, S. Gelernter, J. Zhao, H. Hisama, F. Arnsten, A.F. Strittmatter, S.M. (2008) Genetic variants of Nogo-66 receptor with possible association to schizophrenia block myelin inhibition of axon growth. J Neurosci 28:13161-13172.

Borisoff JF, Chan CC, Hiebert GW, Oschipok L, Robertson GS, Zamboni R, Steeves JD, Tetzlaff W (2003) Suppression of Rho-kinase activity promotes axonal growth on inhibitory CNS substrates. Mol Cell Neurosci 22:405-416.

Cameron HA, McKay RD (2001) Adult neurogenesis produces a large pool of new granule cells in the dentate gyrus. Journal of Comparative Neurology 435:406-417.

Chan CC, Wong AK, Liu J, Steeves JD, Tetzlaff W (2007) ROCK inhibition with Y27632 activates astrocytes and increases their expression of neurite growth-inhibitory chondroitin sulfate proteoglycans. GLIA 55:369-384.

Chang F, Steelman LS, Shelton JG, Lee JT, Navolanic PM, Blalock WL, Franklin R, McCubrey JA (2003) Regulation of cell cycle progression and apoptosis by the Ras/Raf/MEK/ERK pathway (Review). International Journal of Oncology 22:469-480.

Cheatwood JL, Emerick AJ, Schwab ME, Kartje GL (2008) Nogo-A expression after focal ischemic stroke in the adult rat. Stroke 39:2091-2098.

Chen RW, Chuang DM (1999) Long term lithium treatment suppresses p53 and Bax expression but increases Bcl-2 expression. A prominent role in neuroprotection against excitotoxicity. Journal of Biological Chemistry 274:6039-6042.

Chen RW, Qin ZH, Ren M, Kanai H, Chalecka-Franaszek E, Leeds P, Chuang DM (2003) Regulation of c-Jun N-terminal kinase, p38 kinase and AP-1 DNA binding in cultured brain neurons: roles in glutamate excitotoxicity and lithium neuroprotection. Journal of Neurochemistry 84:566-575.

Cnops L, Hu TT, Eysel UT, Arckens L (2007) Effect of binocular retinal lesions on CRMP2 and CRMP4 but not Dyn I and Syt I expression in adult cat area 17. European Journal of Neuroscience 25:1395-1401.

Cole AR, Causeret F, Yadirgi G, Hastie CJ, McLauchlan H, McManus EJ, Hernandez F, Eickholt BJ, Nikolic M, Sutherland C (2006) Distinct priming kinases contribute to differential regulation of collapsin response mediator proteins by glycogen synthase kinase-3 in vivo. J Biol Chem 281:16591-16598.

Cole AR, Soutar MP, Rembutsu M, van Aalten L, Hastie CJ, McLauchlan H, Peggie M, Balastik M, Lu KP, Sutherland C (2008) Relative resistance of Cdk5-phosphorylated CRMP2 to dephosphorylation. Journal of Biological Chemistry 283:18227-18237.

Deo RC, Schmidt EF, Elhabazi A, Togashi H, Burley SK, Strittmatter SM (2004) Structural bases for CRMP function in plexin-dependent semaphorin3A signaling. Embo J 23:9-22.

Dergham P, Ellezam B, Essagian C, Avedissian H, Lubell WD, McKerracher L (2002) Rho signaling pathway targeted to promote spinal cord repair. J Neurosci 22:6570-6577.

Dill J, Wang H, Zhou F, Li S (2008) Inactivation of glycogen synthase kinase 3 promotes axonal growth and recovery in the CNS. Journal of Neuroscience 28:8914-8928.

Fournier AE, Nakamura F, Kawamoto S, Goshima Y, Kalb RG, Strittmatter SM (2000) Semaphorin3A enhances endocytosis at sites of receptor-F-actin colocalization during growth cone collapse. J Cell Biol 149:411-422.

Fournier AE, Takizawa BT, Strittmatter SM (2003) Rho Kinase inhibition enhances axonal regeneration in the injured CNS. J Neurosci 23:1416-1423.

Franke TF, Hornik CP, Segev L, Shostak GA, Sugimoto C (2003) PI3K/Akt and apoptosis: size matters. Oncogene 22:8983-8998.

Fukata Y, Itoh TJ, Kimura T, Menager C, Nishimura T, Shiromizu T, Watanabe H, Inagaki N, Iwamatsu A, Hotani H, Kaibuchi K (2002) CRMP-2 binds to tubulin heterodimers to promote microtubule assembly. Nature Cell Biology 4:583-591.

Gould TD, Chen G, Manji HK (2004) In vivo evidence in the brain for lithium inhibition of glycogen synthase kinase-3. Neuropsychopharmacology 29:32-38.

Hansen TO, Rehfeld JF, Nielsen FC (2004) GSK-3beta reduces cAMP-induced cholecystokinin gene expression by inhibiting CREB binding. Neuroreport 15:841-845.

Hussain NK, Jenna S, Glogauer M, Quinn CC, Wasiak S, Guipponi M, Antonarakis SE, Kay BK, Stossel TP, Lamarche-Vane N, McPherson PS (2001) Endocytic protein intersectin-l regulates actin assembly via Cdc42 and N-WASP. Nature Cell Biology 3:927-932.

Inatome R, Tsujimura T, Hitomi T, Mitsui N, Hermann P, Kuroda S, Yamamura H, Yanagi S (2000) Identification of CRAM, a novel unc-33 gene family protein that associates with CRMP3 and protein-tyrosine kinase(s) in the developing rat brain. J Biol Chem 275:27291-27302.

Karnezis T, Mandemakers W, McQualter JL, Zheng B, Ho PP, Jordan KA, Murray BM, Barres B, Tessier-Lavigne M, Bernard CC (2004) The neurite outgrowth inhibitor Nogo A is involved in autoimmune-mediated demyelination. Nat Neurosci 7:736-744.

Kim JE, Liu BP, Park JH, Strittmatter SM (2004) Nogo-66 receptor prevents raphespinal and rubrospinal axon regeneration and limits functional recovery from spinal cord injury. Neuron 44:439-451.

Lamprecht R, LeDoux J (2004) Structural plasticity and memory. Nature Reviews Neuroscience 5:45-54.

Lee H, Raiker SJ, Venkatesh K, Geary R, Robak LA, Zhang Y, Yeh HH, Shrager P, Giger RJ (2008) Synaptic function for the Nogo-66 receptor NgR1: regulation of dendritic spine morphology and activity-dependent synaptic strength. Journal of Neuroscience 28:2753-2765.

Leung T, Ng Y, Cheong A, Ng CH, Tan I, Hall C, Lim L (2002) p80 ROKalpha binding protein is a novel splice variant of CRMP-1 which associates with CRMP-2 and modulates RhoA-induced neuronal morphology. FEBS Lett 532:445-449.

Li S, Liu BP, Budel S, Li M, Ji B, Walus L, Li W, Jirik A, Rabacchi S, Choi E, Worley D, Sah DW, Pepinsky B, Lee D, Relton J, Strittmatter SM (2004) Blockade of Nogo-66, myelin-associated glycoprotein, and oligodendrocyte myelin glycoprotein by soluble Nogo-66 receptor promotes axonal sprouting and recovery after spinal injury. J Neurosci 24:10511-10520.

Manji HK, Moore GJ, Chen G (2000) Lithium up-regulates the cytoprotective protein Bcl-2 in the CNS in vivo: a role for neurotrophic and neuroprotective effects in manic depressive illness. Journal of Clinical Psychiatry 61 Suppl 9:82-96.

McGee AW, Yang Y, Fischer QS, Daw NW, Strittmatter SM (2005) Experience-driven plasticity of visual cortex limited by myelin and Nogo receptor. Science 309:2222-2226.

Mimura F, Yamagishi S, Arimura N, Fujitani M, Kubo T, Kaibuchi K, Yamashita T (2006) Myelin-associated Glycoprotein Inhibits Microtubule Assembly by a Rho-kinase-dependent Mechanism. J Biol Chem 281:15970-15979.

Monnier PP, Sierra A, Schwab JM, Henke-Fahle S, Mueller BK (2003) The Rho/ROCK pathway mediates neurite growth-inhibitory activity associated with the chondroitin sulfate proteoglycans of the CNS glial scar. Mol Cell Neurosci 22:319-330.

Nonaka S, Chuang DM (1998) Neuroprotective effects of chronic lithium on focal cerebral ischemia in rats. Neuroreport 9:2081-2084.

Nonaka S, Hough CJ, Chuang DM (1998a) Chronic lithium treatment robustly protects neurons in the central nervous system against excitotoxicity by inhibiting N-methyl-D-aspartate receptor-mediated calcium influx. Proceedings of the National Academy of Sciences of the United States of America 95:2642-2647.

Nonaka S, Katsube N, Chuang DM (1998b) Lithium protects rat cerebellar granule cells against apoptosis induced by anticonvulsants, phenytoin and carbamazepine. Journal of Pharmacology & Experimental Therapeutics 286:539-547.

Papadopoulos CM, Tsai SY, Alsbiei T, O'Brien TE, Schwab ME, Kartje GL (2002) Functional recovery and neuroanatomical plasticity following middle cerebral artery occlusion and IN-1 antibody treatment in the adult rat. Ann Neurol 51:433-441.

Park JH, Widi GA, Gimbel DA, Harel NY, Lee DH, Strittmatter SM (2006a) Subcutaneous Nogo receptor removes brain amyloid-beta and improves spatial memory in Alzheimer's transgenic mice. Journal of Neuroscience 26:13279-13286.

Park JH, Gimbel DA, GrandPre T, Lee JK, Kim JE, Li W, Lee DH, Strittmatter SM (2006b) Alzheimer precursor protein interaction with the Nogo-66 receptor reduces amyloid-beta plaque deposition. J Neurosci 26:1386-1395.

Park KK, Liu K, Hu Y, Smith PD, Wang C, Cai B, Xu B, Connolly L, Kramvis I, Sahin M, He Z (2008) Promoting axon regeneration in the adult CNS by modulation of the PTEN/mTOR pathway.[see comment]. Science 322:963-966.

Pernet V, Joly S, Christ F, Dimou L, Schwab ME (2008) Nogo-A and myelin-associated glycoprotein differently regulate oligodendrocyte maturation and myelin formation. Journal of Neuroscience 28:7435-7444.

Pizzorusso T, Medini P, Berardi N, Chierzi S, Fawcett JW, Maffei L (2002) Reactivation of ocular dominance plasticity in the adult visual cortex.[see comment]. Science 298:1248-1251.

Quinn CC, Chen E, Kinjo TG, Kelly G, Bell AW, Elliott RC, McPherson PS, Hockfield S (2003) TUC-4b, a novel TUC family variant, regulates neurite outgrowth and associates with vesicles in the growth cone. Journal of Neuroscience 23:2815-2823.

Ricard D, Rogemond V, Charrier E, Aguera M, Bagnard D, Belin MF, Thomasset N, Honnorat J (2001) Isolation and expression pattern of human Unc-33-like phosphoprotein 6/collapsin response mediator protein 5 (Ulip6/CRMP5): coexistence with Ulip2/CRMP2 in Sema3a- sensitive oligodendrocytes. Journal of Neuroscience 21:7203-7214.

Ricard D, Stankoff B, Bagnard D, Aguera M, Rogemond V, Antoine JC, Spassky N, Zalc B, Lubetzki C, Belin MF, Honnorat J (2000) Differential expression of collapsin response mediator proteins (CRMP/ULIP) in subsets of oligodendrocytes in the postnatal rodent brain. Molecular & Cellular Neurosciences 16:324-337.

Rosslenbroich V, Dai L, Baader SL, Noegel AA, Gieselmann V, Kappler J (2005) Collapsin response mediator protein-4 regulates F-actin bundling. Experimental Cell Research 310:434-444.

Seymour AB, Andrews EM, Tsai SY, Markus TM, Bollnow MR, Brenneman MM, O'Brien TE, Castro AJ, Schwab ME, Kartje GL (2005) Delayed treatment with monoclonal antibody IN-1 1 week after stroke results in recovery of function and corticorubral plasticity in adult rats. Journal of Cerebral Blood Flow & Metabolism 25:1366-1375.

Shih JY, Yang SC, Hong TM, Yuan A, Chen JJ, Yu CJ, Chang YL, Lee YC, Peck K, Wu CW, Yang PC (2001) Collapsin response mediator protein-1 and the invasion and metastasis of cancer cells.[see comment]. Journal of the National Cancer Institute 93:1392-1400.

Steeg PS (2001) Collapsin response mediator protein-1: a lung cancer invasion suppressor gene with nerve.[comment]. Journal of the National Cancer Institute 93:1364-1365.

Su KY, Chien WL, Fu WM, Yu IS, Huang HP, Huang PH, Lin SR, Shih JY, Lin YL, Hsueh YP, Yang PC, Lin SW (2007) Mice deficient in collapsin response mediator protein-1 exhibit impaired long-term potentiation and impaired spatial learning and memory. Journal of Neuroscience 27:2513-2524.

Syken J, Grandpre T, Kanold PO, Shatz CJ (2006) PirB restricts ocular-dominance plasticity in visual cortex. Science 313:1795-1800.

Takai T, Ono M (2001) Activating and inhibitory nature of the murine paired immunoglobulin-like receptor family. Immunological Reviews 181:215-222.

Venkatesh K, Chivatakarn O, Lee H, Joshi PS, Kantor DB, Newman BA, Mage R, Rader C, Giger RJ (2005) The Nogo-66 receptor homolog NgR2 is a sialic acid-dependent receptor selective for myelin-associated glycoprotein. J Neurosci 25:808-822.

Veyrac A, Giannetti N, Charrier E, Reymond-Marron I, Aguera M, Rogemond V, Honnorat J, Jourdan F (2005) Expression of collapsin response mediator proteins 1, 2 and 5 is differentially regulated in newly generated and mature neurons of the adult olfactory system. European Journal of Neuroscience 21:2635-2648.

Vinson M, Strijbos PJ, Rowles A, Facci L, Moore SE, Simmons DL, Walsh FS (2001) Myelin-associated glycoprotein interacts with ganglioside GT1b. A mechanism for neurite outgrowth inhibition. J Biol Chem 276:20280-20285.

Wang LH, Strittmatter SM (1997) Brain CRMP forms heterotetramers similar to liver dihydropyrimidinase. J Neurochem 69:2261-2269.

Winton MJ, Dubreuil CI, Lasko D, Leclerc N, McKerracher L (2002) Characterization of new cell permeable C3-like proteins that inactivate Rho and stimulate neurite outgrowth on inhibitory substrates. J Biol Chem 277:32820-32829.

Xavier IJ, Mercier PA, McLoughlin CM, Ali A, Woodgett JR, Ovsenek N (2000) Glycogen synthase kinase 3beta negatively regulates both DNA-binding and transcriptional activities of heat shock factor 1. Journal of Biological Chemistry 275:29147-29152.

Yick LW, So KF, Cheung PT, Wu WT (2004) Lithium chloride reinforces the regeneration-promoting effect of chondroitinase ABC on rubrospinal neurons after spinal cord injury.[erratum appears in J Neurotrauma. 2007 Aug;24(8):1415 Note: dosage error in text]. Journal of Neurotrauma 21:932-943.

Yuasa-Kawada J, Suzuki R, Kano F, Ohkawara T, Murata M, Noda M (2003) Axonal morphogenesis controlled by antagonistic roles of two CRMP subtypes in microtubule organization. European Journal of Neuroscience 17:2329-2343.

Yuste R, Bonhoeffer T (2001) Morphological changes in dendritic spines associated with long-term synaptic plasticity. Annual Review of Neuroscience 24:1071-1089.

Zhou FQ, Cohan CS (2001) Growth cone collapse through coincident loss of actin bundles and leading edge actin without actin depolymerization. Journal of Cell Biology 153:1071-1084.

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J. Neurosci., Feb 2007; 27: 1702 - 1711 ;
doi:10.1523/JNEUROSCI.5055-06.2007
I would like to request a permission to include the text and the figures of this article as part of my thesis for my PhD.

Sincerely,

Yazan Z. Alabed
McGill University
Department of Neurology and Neurosurgery
Montreal Neurological Institute, BT-105
Montreal, Quebec
H3A 2B4
tel: 514-398-8436

Re: Paper Authorship in Thesis

Madeline Pool [madeline.pool@mail.mcgill.ca]

Sent: January 30, 2009 3:13 PM

To: Zi Yazan Alabed, Mr

Hello Yazan,

This is to confirm that I give my permission to have my name printed in your thesis as a co-author on the unpublished manuscript entitled: "GSK3b regulates CRMP4-RhoA complex formation and axon outgrowth inhibition".

Sincerely,
Madeline Pool

On 29-Jan-09, at 11:07 PM, Zi Yazan Alabed, Mr wrote:

> Hello Madeline,
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>
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>
> Yazan Alabed

APPENDIX: Compliance Certificates

5. RESEARCH PERSONNEL: (attach additional sheets if preferred)

Name	Department	Job Title/Classification	Trained in the safe use of biological safety cabinets within the last 3 years? If yes, indicate training date.
Ferraro	Neurology and Neurosurgery	Student/PhD	yes/oct 2005
Chris Kent	Neurology and Neurosurgery	Student/MSc	yes/dec 2005
Yazan Alabed	Neurology and Neurosurgery	Student/MD/PhD	yes/feb 2005
Madeline Pool	Neurology and Neurosurgery	Post Doc	yes/oct 2005
Isabel Rambaldi	Neurology and Neurosurgery	Research Assistant	yes/oct 2005
Steph Ongtone	Neurology and Neurosurgery	Student/MD/PhD	yes/mar 2007
Horia Pribiag	Neurology and Neurosurgery	Student/MSc	yes/sept 2006

6. Briefly describe:

i) the biohazardous material involved (e.g. bacteria, viruses, human tissues, toxins of biological origin) & designated biosafety risk group

~~tissue culture waste~~
~~non-pathogenic bacterial waster~~
~~broken glass/sharps~~
~~organic solvents~~

E. Coli DH5α
HEK 293T
Cos7
PC-12
HSA24 (neuronal cells)
Primary cell line (Rat) Div 190307

Mammalian cell lines are Level 2

ii) the procedures involving biohazards

cell lines will be used to express recombinant DNA fragments produced in vitro by standard molecular biology techniques. The constructs to be used represent previously identified proteins that show no ability to transform cells.
- tissue culture will be performed in an approved laminar flow hood located in a dedicated room for this purpose
- personnel working in this area will be suitably trained in sterile technique
- biohazardous waster will be disposed of separately from regular garbage. Cell and bacterial culture waste is placed in biohazard autoclave bags and autoclaved prior to disposal. Liquid waster is neutralized with 0.1% Roccal or sodium hypochlorite solution (5.25% bleach diluted 1:10)
- containers/equipment leaving the lab will be decontaminated with 1% bleach and 70% ethanol
- working areas will be regularly wiped with 70% ethanol
- sharps are disposed in impermeable sealed plastic containers; glass is sealed in cardboard boxes
- organic/caustic chemicals are stored in a reinforced cabinet and used in a fume hood

iii) the protocol for decontaminating spills

spills will be decontaminated by:
- allowing aerosols to settle
- covering spill with paper towel and then applying 1% bleach from the periphery inwards
- after a 30 minute incubation period in the applied bleach, the paper towel will be disposed of in a biohazard bin and subsequently autoclaved.
- spills on clothing will be decontaminated by autoclaving.

7. Does the protocol present conditions (e.g. handling of large volumes or high concentrations of pathogens) that could increase the hazards?

NO

8. Do the specific procedures to be employed involving genetically engineered organisms have a history of safe use?

not applicable

9. What precautions will be taken to reduce production of infectious droplets and aerosols?

biosafety cabinets are being used

10. Will the biohazardous materials in this project expose members of the research team to any risks that might require special training, vaccination or other protective measures? If yes, please explain.

NO

11. Will this project produce combined hazardous waste – i.e. radioactive biohazardous waste, biohazardous animal carcasses contaminated with toxic chemicals, etc.? If yes, please explain how disposal will be handled.

NO

12. List the biological safety cabinets to be used.

Building	Room No.	Manufacturer	Model No.	Serial No.	Date Certified
		FormaScientific	1286	19727-301	sept 2006
Montreal Neurological		FormaScientific	1286	17719-312	sept 2006
Institute, BTRC	BT-106	FormaScientific	1286	17719-311	sept 2006

5 e) KEYWORDS: Using keywords only, list the procedures used on animals (e.g. anaesthesia, breeding colony, injection IP, gavage, drug administration, major survival surgery, euthanasia by exsanguination, behavioural studies). For a more complete list of suggested keywords refer to Appendix 1 of the Guidelines (www.mcgill.ca/research/compliance/animal/forms).

uthanasia. primary neuronal culture.

6. Animals Use data for CCAC

6 a) Purpose of Animal Use (Check most appropriate one):

1. ☒ **Studies of a fundamental nature/basic research**
2. ☐ **Studies for medical purposes relating to human/animal diseases/disorders**
3. ☐ **Regulatory testing**
4. ☐ **Development of products/appliances for human/veterinary medicine**
5. **If for Teaching, use the Animal Use Protocol form for Teaching (www.mcgill.ca/research/compliance/animal/forms)**

6 b) Will field studies be conducted? NO ☒ YES ☐ If yes. complete "Field Study Form"
Will the project involve genetically altering animals? NO ☒ YES ☐ If yes. complete SOP #5 or #6
Will the project involve breeding animals? NO☒ YES ☐ If breeding transgenics or knockouts. complete SOP#4

7. Animal Data

7 a) Please justify the need for live animals versus alternate methods (e.g. tissue culture, computer simulation)

Tissue culture of PC12 cells will be used for biochemical experiments. however these cells only provide a model for neurons. It is unclear if primary neurons respond to myelin inhibitors in the same way as model cell lines. To develop antagonists to myelin-associated inhibitors. it is critical that we understand how they signal in their native cellular context. All results will therefore be validated using primary neuronal cell culture.

7 b) Describe the characteristics of the animal species selected that justifies its use in the proposed study (consider characteristics such as body size, species, strain, data from previous studies or unique anatomic/physiological features)

Post natal day 5 (P5)to day8 (P8) mice and rats will be used to culture neurons derived from their dorsal root ganglia , cerebellum and retina. These ages will be used because at this stage neurons express receptors to myelin-associated inhibitors and become sensitive to these molecules. The majority of commercially available antibodies that will be used in the study react with rat and mouse antigens. Rat or mouse will be used depending on the efficacy of the antibody in each species.

7 c) Description of animals

Quality Control Assurance: To prevent introduction of infectious diseases into animal facilities. a health status report or veterinary inspection certificate may be required prior to receiving animals from all non-commercial sources or from commercial sources whose animal health status is unknown or questionable. Quarantine and further testing may be required for these animals.

If more than 6 columns are needed, please attach another page

	Sp/strain 1	Sp/strain 2	Sp/strain 3	Sp/strain 4	Sp/strain 5	Sp/strain 6
Species	Mouse	Rat				
Supplier/Source	Charles River	Charles River				
Strain	CD-1	Sprague Dawley				
Sex	1 female with litter	1 female with litter				
Age/Wt	P5 toP8	P5 toP8				
# To be purchased	75. females with litter	75. females with litter				
# Produced by in-house breeding	none	none				
# Other (e.g. field studies)						

#needed at one time	2 litters	2 litters				
# per cage	1 litter	1 litter				
TOTAL# /YEAR	**75, females with litter**	**75, females with litter**				

7 d) Explanation of Animal Usage: BASED ON THE EXPERIMENTAL OBJECTIVES OF THE PROJECT, describe the number of animals required for one year. Include information on experimental and control groups, # per group, and failure rates.

For breeding, specify how many adults are used, number of offspring produced, and how many offspring are used in experimental procedures.

The arithmetic explaining how the total of animals for each column in the table above is calculated should be made clear.
(Space will expand as needed)

We will produce 1or2 early postnatal mouse or rat cultures per week for neurite outgrowth assays or for biochemical assays. Each culture requires 1 litter of pups(for a total of 75 females and litter per year).

8. Animal Husbandry and Care

8 a) If projects involves non-standard cages, diet and/or handling, please specify

N/A

8 b) Is there any component to the proposed procedures which will result in immunosuppression or decreased immune function (e.g. stress, radiation, steroids, chemotherapeutics, genetic modification of the immune system)?

NO ☒ YES ☐ **if yes, specify:**

8 c) Indicate area(s) where animal use procedures will be conducted:

Building: MNI **Room:** BT 105

Indicate area(s) all facilities where animals will be housed:

Building: MNI **Room:** Animal Facility

If animal housing and animal use are in different locations, briefly describe procedures for transporting animals:

9. Standard Operating Procedures (SOPs)

Complete this section if you plan to use any of the UACC SOPs listed below. IT IS UACC POLICY THAT THESE SOPS BE USED WHEN APPLICABLE. Any proposed variation of the SOPs must be described and justified. The Standard Operating Procedures can be found at the UACC website at www.mcgill.ca/research/compliance/animal/procedures . The completed and signed SOP form must be attached to the protocol.

Check all SOPs that will be used:

Blood Collection UACC#1	☐	**Collection of Amphibian Oocytes UACC#9**	☐
Anaesthesia in rodents UACC#2	☐	**Rodent Survival Surgery UACC#10**	☐
Analgesia in rodents UACC#3	☐	**Anaesthesia & Analgesia Neonatal Rodents UACC#11**	☐
Breeding transgenics/knockouts UACC#4	☐	**Stereotaxic Survival Surgery in Rodents UACC#12**	☐
Transgenic Generation UACC#5	☐	**Field Studies Form**	☐
Knockout/in Generation UACC#6	☐	**Phenotype Disclosure Form**	☐
Production of Monoclonal Antibodies UACC#7	☐	**Other, specify:**	☐
Production of Polyclonal Antibodies UACC#8	☐		☐

10. Description of Procedures

10 a) . IF A PROCEDURE IS COVERED BY AN SOP, WRITE "AS PER SOP", NO FURTHER DETAIL IS REQUIRED.

FOR EACH EXPERIMENTAL GROUP, DESCRIBE ALL PROCEDURES AND TECHNIQUES, WHICH ARE NOT PART OF THE SOPs, IN THE ORDER IN WHICH THEY WILL BE PERFORMED – surgical procedures, immunizations, behavioural tests, immobilization and restraint, food/water deprivation, requirements for post-operative care, sample collection, substance administration, special monitoring, etc Appendix 2 of the Guidelines (www.mcgill.ca/research/compliance/animal/forms) provides a sample list of points that should be addressed in this section.

The rodents will be euthanised (as described in section 10g below) and brains will be used for neuronal cultures.

10 b) Experimental endpoint – for each experimental group indicate survival time

N/A

10 c) Clinical endpoint – describe the conditions, complications, and criteria (e.g. >20% weight loss, maximum tumour size, vocalizing, lack of grooming) that would lead to euthanasia of an animal before the expected completion of the experiment (specify per species and project if multiple projects involved)

Frequency of monitoring: N/A

10 d) Specify person(s) who will be responsible for animal monitoring and post-procedural care ***(must also be listed in section 4)***

Name:	**Phone #:**

10 e) Pre-Anesthetic/Anaesthetic/Analgesic Agents: List all drugs that will be used to minimize pain, distress or discomfort. *(Table will expand as needed)*

Species	Agent	Dosage (mg/kg)	Total volume(ml) per administration	Route	Frequency/Duration
N/A					

10 f) Administration of ALL other substances: List all non-anaesthetic agents under study in the experimental component of the protocol, including but not limited to drugs, infectious agents, viruses. *(Table will expand as needed)*

Species	Agent	Dosage (mg/kg)	Total volume(ml) per administration	Route	Frequency/Duration
none					

10 g) Method of Euthanasia

Specify Species	
	☐ **Anaesthetic overdose, list agent/dose/route:**
	☐ **Exsanguination with anaesthesia, list agent/dose/route:**
mouse and rat P5-P8	☒ **Decapitation without anaesthesia *** ☐ **Decapitation with anesthesia, list agent/dose/route (including CO_2):**
adult mouse/rats	☐ **Cervical dislocation without anaesthesia *** ☒ **Cervical dislocation with anaesthesia, list agent/dose/route (including CO_2):** CO2 chamber followed by cervical dislocation
	☐ **CO_2 chamber only**
	☐ **Other, specify:**
	☐ **Not applicable, explain:**

***For physical method of euthanasia without anaesthesia, please justify:** animals are under 10days old

11. Category of Invasiveness:	**B** ☒	**C** ☐	**D** ☐	**E** ☐

Categories of Invasiveness (from the CCAC *Categories of Invasiveness in Animal Experiments*). Please refer to this document for a more detailed description of categories.

Category A: Studies or experiments on most invertebrates or no entire living material.

Category B: Studies or experiments causing little or no discomfort or stress. *These might include holding animals captive, injection, percutaneous blood sampling, accepted euthanasia for tissue harvest, acute non-survival experiments in which the animals are completely anaesthetized.*

Category C: Studies or experiments involving minor stress or pain of short duration. *These might include cannulation or catheterizations of blood vessels or body cavities under anaesthesia, minor surgery under anaesthesia, such as biopsy; short periods of restraint, overnight food and/or water deprivation which exceed periods of abstinence in nature; behavioural experiments on conscious animals that involve short-term stressful restraint.*

Category D: Studies or experiments that involve moderate to severe distress or discomfort. *These might include major surgery under anaesthesia with subsequent recovery, prolonged (several hours or more) periods of physical restraint; induction of behavioural stresses, immunization with complete Freund's adjuvant, application of noxious stimuli, procedures that produce pain, production of transgenics (in accordance with University policy).*

Category E: Procedures that involve inflicting severe pain, near, at or above the pain threshold of unanaesthetized, conscious animals. *Not confined to but may include exposure to noxious stimuli or agents whose effects are unknown; exposure to drugs or chemicals at levels that (may) markedly impair physiological systems and which cause death, severe pain or extreme distress or physical trauma on unanaesthetized animals.* According to University policy, E level studies are not permitted.

12. Potential Hazards to Personnel and Animals It is the responsibility of the investigator to obtain the necessary Biohazard and/or Radiation Safety permits before this protocol is submitted for review.

A copy of these certificates must be attached, if applicable.

No hazardous materials will be used in this study: ☒

12 a) Indicate which of the following will be used in animals:

☐ Toxic chemicals ☐ Radioisotopes ☐ Carcinogens
☐ Infectious agents (includes vectors) ☐ Transplantable tumours and/or tissues

12 b) Complete the following table for each agent to be used (use additional page as required):

Agent name			
Dosage			
Route of administration			
Frequency of administration			
Duration of administration			
Number of animals involved			
Survival time after administration			

12 c) After administration the animals will be housed in:

☐ **the animal care facility** ☐ **laboratory under supervision of laboratory personnel**

Please note that cages must be appropriately labeled at all times.

12 d) Describe potential health risk (s) to humans or animals:

none

12 e) Describe measures that will be used to reduce risk to the environment and all project and animal facility personnel:

N/A

12 f) If using cell lines, have they been tested?

☐ **Yes If yes, What human and/or animal pathogens have been tested?**

☒ **No If no, justify:** We are using well characterized cell lines that are being handled using containment level 2 facilities and work practices.

Reviewer's Comments and Modifications (to be completed by ACC only): The Animal Care Committee has made the following modification(s) to this animal use procedure protocol during the review process. Please make these changes to your copy and comply with the recommended changes as a condition of approval.