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The construction and characterization of new permeable Rho antagonists and their roles after spinal cord injury

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Cette thèse de doctorat intitulée:

The construction and characterization of new permeable Rho antagonists and their roles after spinal cord injury

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RÉSUMÉ

Un des facteurs important qui contribue au manque de régénération par suite d'une lésion du système nerveux central (SNC) est la présence de protéines inhibitrices de croissance dans l'environnement cellulaire endommagé. Récemment, plusieurs études ont identifié la petite GTPase Rho, qui régule principalement le cytosquelette d'actine, comme une molécule clé de la réponse à ces protéines inhibitrices. Cette GTPase peut être spécifiquement inactivée par la C3-transférase (C3), une exoenzyme produite par la bactérie *C.botulinum*. L'inactivation de Rho par C3 promeut la croissance neuritique en culture malgré la présence de protéines inhibitrices de croissance et stimule la régénération axonale par suite de lésions de la moelle épinière chez la souris. Cependant, C3 traverse difficilement les membranes cellulaires et des méthodes d'application drastiques, comme la trituration cellulaire et la microinjection sont necéssaires pour le traitemnet des cellules en culture. Afin de faciliter l'utilisation de la toxine, nous avons donc construit et caractérisé cinq versions modifiées de C3 qui pénètrent facilement les membranes cellulaires et qui sont biologiquement efficaces à des concentrations 1000 fois plus faibles que celle à laquelle C3 inactive Rho.

au site de la lésion. Ces résultats suggèrent que l'activation de Rho contribue à la mort des cellules par suite de lésions de la moelle épinière. Même s'il est connu que les protéines de myéline qui inhinbent la neuronale activent la petite GTPase Rho, on ignore si ces inhibiteurs contribuent à l'apoptose médieé par Rho. Pour déterminer si l'activation de Rho par la myéline peut entraîner la mort neuronale, nous avons examiné la survie de neurones mis en culture sur des substrats de myéline en présence ou non d'une variété de facteurs apoptotiques. Nous avons auisi constalé que la myéline augmente le reponse apoptotique à la présence du facteur de necrore tumorale-alpha (TNF-α) de manière Rho-dépendante, ce qui, suggère que l'excitotoxicité cellulaire peut être aussi accrue par la myéline. L'ensemble de ces résultants indique que les protéins inhibitrices de la croissance dérivées de la myéline augmentent l'apoptose neuronale par activation de Rho.

Mots clés: Rho GTPase, C3-transférase, peptides permeable à la membrane

SUMMARY

It is now well established that one major reason for the lack of regeneration after central nervous system (CNS) trauma is the presence of potent growth inhibitory proteins in the damaged CNS environment. Recent studies have implicated Rho, a small GTPase best characterized for its ability to regulate the actin cytoskeleton, as a key-signaling molecule involved in the cellular response to growth inhibitory molecules. The bacteria *C. botulinum* produces an exoenzyme, C3-transferase (C3), that ADP-ribosylates and specifically inactivates Rho. The inactivation of Rho by C3 promotes neurite outgrowth in the presence of growth inhibitory substrates *in vitro* and stimulates axonal regeneration *in vivo*. C3 is not cell permeable and as a result, is difficult to deliver efficiently into cells. Presently, invasive methods such as scrape loading or microinjection are commonly used to treat cultured cells. Therefore we constructed and characterized five new cell-permeable C3-like chimeric proteins that are effective at doses of at least a 1,000-fold lower than those required for the inactivation of Rho by unmodified C3.

Upon characterization of these C3-like chimeric proteins the most effective construct tested, C3-05, was further examined in both *in vitro and in vivo* studies. Making

dramatic reduction in the number of apoptotic cells at the lesion site. These results suggest that the activation of Rho after CNS trauma contributes to cell death.

Although myelin is known to activate Rho, it is not understood if myelin-derived growth inhibitory proteins contribute to Rho-mediated apoptosis. To investigate if Rho activation by growth inhibitory proteins affects neuronal cell death, we studied cell survival of neurons plated on myelin substrates in the presence or absence of various apoptotic factors. We provide evidence that myelin augments the apoptotic response of neurons to tumor necrosis factor-alpha (TNF-α) in a Rho-dependent manner, and suggest that excitotoxic cell death may also be augmented by myelin. These results indicate that myelin-derived growth inhibitory proteins increase neuronal apoptosis by activation of Rho.

Key words: Rho GTPase, C3-transferase, cell-permeable peptides, myelin, MAG, Nogo receptor, Campenot chambers, apoptosis, excitotoxicity, TNF- α,

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LIST OF ABBREVIATIONS

AD.....Alzheimer's disease

ADF.....actin depolymerizing factor

ALS.....amyotrophic lateral sclerosis

AMPA.....α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

Antp.....Antennapedia homeodomain

Apaf-1.....apoptotic protease activating factor-1

ATP.....adenosine triphosphate

C3.....C3-transferase

cAMP.....cyclic adenosine monophosphate

CHO.....Chinese hamster ovary

CNF.....cytotoxic necrotizing factor

CNS.....central nervous system

CSPG.....chondrotin sulphate proteoglycans

DH.....Dbl homology

DNA.....deoxyribonucleic acid

DNT.....dermonecrotic toxin

DRG.....dorsal root ganglion

GEF.....guanine nucleotide exchange factors

GFP.....green fluorescent proteins

GPI.....glycosylphosphatidylinositol

GTPases.....small GTP-binding proteins

HD.....Huntington's disease

KDa.....kilo Daltons

LCC.....large clostridal cytotoxins

LINGO-1.....LRR and Ig domain, containing Nogo receptor-interacting protein

LPA.....lysophosphatidic acid

LRR.....leucine rich repeats

mAb.....monoclonal antibody

MAG.....myelin associated glycoprotein

MBS.....myosin binding subunit

MLC.....myosin light chain

MS.....multiple sclerosis

MTS.....membrane transport sequence

NGF.....nerve growth factor

NgR.....Nogo receptor

NMDA.....N-methyl D-Aspartate

PKN.....protein kinase N

RGC.....retinal ganglion cells

RAG.....regeneration associated gene

Robo.....roundabout

SCG.....superior cervical ganglion

SCI.....spinal cord injury

Sema 3A.....semaphorin 3A

Tat.....transcription activator

TNF-α.....tumor necrosis factor-alpha

TNFR.....tumor necrosis factor receptor

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"The main ingredient of stardom is the rest of the team."
- John Wooden

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PREFACE

The overall scope of my doctoral studies have focused on the examination of the small GTPase Rho as a potential therapeutic target to promote the regeneration and survival of injured axons after spinal cord injury. This thesis provides a detailed introduction of the structure and functions of Rho in both neuronal and non-neuronal cells, a general review of the current literature in the field of spinal cord regeneration, as well as evidence demonstrating the benefits of targeting the Rho signaling pathway after spinal cord injury. This thesis is written in a manuscript-based structure and therefore contains copies of original research and review articles that I published in peer-reviewed journals over the course of my PhD.

Chapter 1

1 General Introduction

1.1 Small GTP-binding proteins

Small GTP-binding proteins (GTPases) are a series of monomeric G proteins that are ubiquitously expressed across species, from yeast to worms, plants to humans. To date, more than 100 small GTPase proteins have been identified (for review see Burridge and Wennerberg, 2004). These low molecular mass proteins (20-40 kDa) participate in a wide spectrum of cellular functions and are structurally classified into 5 main families: the Ras family, the Rho family, the Rab family, the Sar1/Arf families and the Rnd family. These main families of GTPases are best characterized for their ability to regulate gene expression, the actin cytoskeleton, vesicle trafficking, vesicle budding and nucleocytoplasmic transport, respectively.

1.2 The Ras family of GTPases

The family of Ras genes was first identified in the mid-1960s as key transforming elements in rat sarcoma virus. Detailed investigation into the biological functions of Ras began in 1980s, when mutations in 3 mammalian Ras genes (H-ras, K-ras and N-ras)

interest in Ras and Ras-related GTP-binding proteins and eventually led to the discovery of the Rho family of GTPases (Hall, 1994; Madaule and Axel, 1985).

1.3 The Rho family of GTPases

Members of the Rho (Ras homologue) family of GTPases were first identified in the marine snail *Aplyshia* (Madaule and Axel, 1985) and shown to be 35 % homologous to mammalian H-ras. The sequence homology between these genes is clustered in specific regions, resulting in areas of strong conservation and areas of complete divergence (Madaule and Axel, 1985). Amongst the conserved areas, the regions possessing the greatest levels of homology are responsible for GTP binding and hydrolysis, suggesting that Rho proteins are members of a larger superfamily of Ras GTPases that share common biochemical properties (Takai et al., 2001). In addition to the high degree of homology found at the nucleotide-biding site, highly conserved regions between Ras and Rho GTPases are also present at the C-terminal (Hancock et al., 1989). In Ras proteins, the C-terminal region is responsible for lipid attachment and membrane binding. To promote its biologically effects, Ras must localize to the inner

of critical cellular functions. Currently available information from the mammalian genome-sequencing project suggests a total of 22 Rho family members. Of these proteins, the RhoA, -B, -C (Rho), Rac1, -2 (Rac) and Cdc42 are the most widely expressed and best-characterized members (Wherlock and Mellor, 2002). Rho A, -B, -C are isoforms of Rho that share 85 % sequence homology. All 3 isoforms are thought to have similar actions in cells, but have different tissue distributions and expression profiles (Takai et al., 2001). In the central nervous system (CNS), RhoA is the most widely expressed isoform (Lehmann et al., 1999).

1.3.1 Structure

Rho GTPases have 2 distinct conformations: a GTP-bound active form and a GDP-bound inactive form (Bourne et al., 1990; Hall, 1990; Takai et al., 1992). In response to extracellular stimuli, Rho proteins translocate from the cytosol to the plasma membrane. At the membrane, Rho GTPases bind and hydrolyse GTP, cycling between active and inactive states (see Figure 1). Once activated, Rho GTPases can bind and activate various downstream targets and effector proteins (Etienne-Manneville and Hall, 2002; Hall

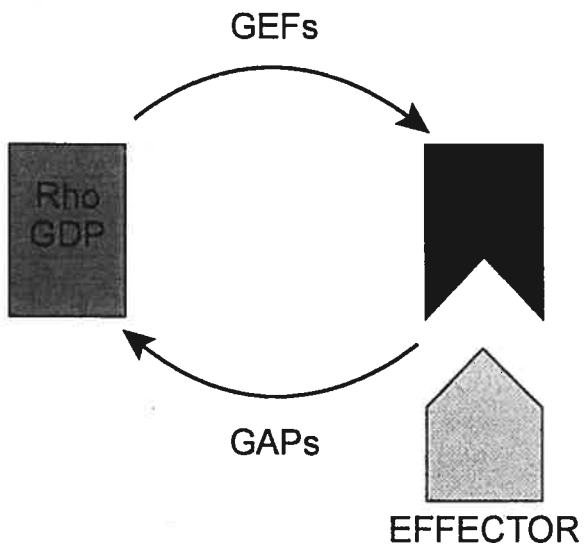


Figure 1. Regulation of Rho GTPase activity.

Cycling of Rho GTPase between inactive GDP-bound and active GTP-bound states is regulated by Guanine exchange factors (GEFs) and GTPase-activating proteins (GAPs). Active GTP-bound Rho interacts with downstream effector molecules to mediate various cellular responses.

Several studies have identified two short regions, which border the nucleotide-binding site of Rho proteins, termed Switch I (amino acids 28-44) and Switch II (amino acids 62-69) (Ihara et al., 1998). As GTPases only interact with downstream effectors

the γ-phosphate group of GTP and amine groups located in the switch region of Rho proteins (Wei et al., 1997).

1.3.2 Post-translational modifications

Similar to members of the Ras superfamily, Rho GTPases undergo post-translational modifications, resulting in the addition of unique C-terminal sequences (Adamson et al., 1992). Geranyl-geranyl transferase catalyzes the addition of a geranyl-geranyl moiety to the cysteine-rich C-terminal region of RhoA, -B, -C, Rac1, -2 and Cdc42. Rho B is unique in that it can be modified by both the addition of a 20-carbon chain geranyl-geranyl group, or a 15-carbon chain farnesyl group (Adamson et al., 1992). Post-translational modifications of Rho GTPases are important for various biological functions, such as membrane interaction (Glomset and Farnsworth, 1994; Takai et al., 1992; Zhang and Casey, 1996). Studies have shown that unmodified forms of Rho fail to bind to the membrane and prevent interactions with RhoGDI, a cytosolic Rho-GDP stabilizing protein (Hori et al., 1991).

Aelst and D'Souza-Schorey, 1997). In contrast, Rho proteins are inactivated by GTPase-activating proteins (GAPs), which hydroloyse GTP by stimulating the low intrinsic GTPase activity (Fidyk and Cerione, 2002; Peck et al., 2002). While Rho GTPases are expressed in all cell types, GEFs and GAPs are developmentally regulated and exhibit cell-type specificity. Guanine nucleotide exchange dissociation inhibitors (GDIs) are a third group of proteins that affect the activation states of Rho GTPases (Scheffzek et al., 2000; Van Aelst and D'Souza-Schorey, 1997). GDIs interact with GDP bound Rho, inhibiting GTP exchange and regulate the cellular location of Rho (i.e. cytosol vs. membrane) by sequestering the GTPase to the cytosol.

1.4.1 Guanine exchange factors

Guanine exchange factors (GEFs) belong to a rapidly growing family of proteins that share both a common function and a common motif, the Dbl homology (DH) domain (Cerione and Zheng, 1996). The Dbl oncogene was first identified during studies attempting to oncogenically transform NIH 3T3 cells (Eva and Aaronson, 1985) and was than shown to possess significant sequence homology with CDC 24, an upstream

The majority of Rho GEFs also possess another important functional domain, the pleckstrin homology (PH) domain, which lies adjacent to the DH domain (Michiels et al., 1997; Zheng et al., 1996). The suggested function of PH domains is to target GEFs to their appropriate intracellular location. However, recent structural and biochemical studies provide evidence that PH domains can also affect the catalytic activity of DH domains (Cerione and Zheng, 1996). Trio, a GEF for Rho, Rac and Cdc42 that plays an important role in axon formation and guidance, contains 2 separate DH-PH domains (Debant et al., 1996). Studies examining the GEF activity of Trio provide evidence that, together, the DH and PH domains increase the activity of Trio by 100-fold, compared to the DH domains alone. Further, deletion or mutation of the PH domain in other Rho GEFs, such as Dbs, Dbl, Lsc, Lfc and Lbc abolishes their GEF activity *in vivo* (Olson et al., 1997; Whitehead et al., 1995a; Whitehead et al., 1995b; Zheng et al., 1996). As a result of such studies, it is currently believed that the DH-PH motif is the minimal structure unit needed to promote nucleotide exchange *in vivo* (Schmidt and Hall, 2002; Zheng, 2001).

With the continuous discovery of new GEFs, it is becoming clear that as a group,

exhibits exchange activity toward Rho, Rac and Cdc42 *in vitro*, but its activity is specific to Rac *in vivo* (Michiels et al., 1995). Additional studies will be required to better understand the mechanisms responsible for determining the specificity of GEFs towards specific different Rho GTPases.

In addition to DH and PH domains many GEFs also possess domains commonly found in other signaling molecules, such as SH2, SH3, PDZ, Ser/Thr kinase, Ras-GEF and Rho-GAP domains (Schmidt and Hall, 2002). These domains are known to participate in protein-protein interactions suggesting that in addition to promoting nucleotide exchange, GEFs may have additional cellular functions (Cerione and Zheng, 1996; Schmidt and Hall, 2002). Vav2 a Rho family GEF with broad range specificity, possesses a SH2 domain (Bustelo et al., 1992; Schuebel et al., 1996). The binding of Vav2 with SHP-2, a tyrosine phosphatase that also contains SH2 domains, reduces its ability to activate GTPases (Kodama et al., 2000).

The number of GEFs currently identified in higher eukaryotic cells is 3 times greater then the number of identified GTPase substrates. At first glance, the mechanism of nucleotide exchange appears to be functionally redundant, as more than one GEF can

cell types, and/or to be present as isoforms in various cell types (e.g., Vav 2 andVav3 are found in most cell types) (Movilla and Bustelo, 1999; Schuebel et al., 1996). A second possibility is that, through different receptors, extracellular signals may use different GEFs to activate the same GTPase (Liu and Burridge, 2000). A large number of GEFs would therefore be needed to interact with many different receptors and signaling complexes. However, other studies have shown that individual GEFs can promote nucleotide exchange in response to the activation of various receptor-signaling complexes (Bustelo, 2000). A final possibility is that GEFs, through their cellular location and protein-protein interactions, mediate the activation of specific downstream pathways. Rho has many different downstream effectors and targets, which carry out a wide range of cellular functions. How activated Rho distinguishes between its many downstream effectors is still a major outstanding issue in the field and, although not directly proven, GEFs, could be partly be responsible for this selectivity.

1.4.2 GTPase activating proteins

The activity of Rho proteins (i.e. GTP-bound state) is terminated by GTP

cells (Bernards, 2003). Biochemical analysis of Rho GAPs indicates that the RhoGAP domain is the minimum structural unit that is necessary for GTP hydrolysis activity. To date, over 30 Rho GAPs have been identified. However, the mechanisms by which these proteins catalyze GTP hydrolysis are still poorly understood. By analogy with GEFs, changes in subcellular localization are likely to be a key determinant in the function of Rho GAPs (Bernards, 2003; Moon and Zheng, 2003; Tanabe et al., 2000).

The substrate specificity of GAPs towards different members of the Rho family of GTPases varies amongst individual GAP proteins. Similar to GEFs, several studies have shown that individual GAPs regulate multiple Rho family proteins *in vitro*, but results show tighter substrate specificity *in vivo* (Ridley et al., 1993). This observed difference between the two experimental methods raises questions as to whether other factors regulate GAP activity and, as a result, may provide additional specificity *in vivo*. Recent evidence suggests that phospholipids strongly regulate the function of p190RhoGAP, a GAP for both Rho and Rac proteins (Ligeti et al., 2004). In this novel mechanism of GAP regulation, acidic phospholipids and fatty acids inhibit the Rho GAP activity of p190RhoGAP, but increase its Rac GAP activity (Ligeti et al., 2004), demonstrating that

Nishiyama et al., 1994). Recently, 2 additional GDIs were discovered: the hematopoietic cell specific D4-GDI (Lelias et al., 1993; Scherle et al., 1993), which shows activity for Rho, Rac and Cdc42 and RhoGDIγ (Adra et al., 1993), which is expressed in the brain and pancreas and displays activity towards RhoA, RhoB, Cdc42 and RhoG.

In resting cells, Rho proteins are localized to the cytosol in a GDP-bound state through their interaction with RhoGDIs (Olofsson, 1999; Takai et al., 1995). RhoGDIs sequester Rho proteins and maintain their inactive state by two direct interactions. First, an immunoglobulin domain located on the RhoGDI molecule binds to the C-terminal geranyl-geranyl moiety, anchoring the GTPase to the cytosol (Hoffman et al., 2000). Second, the N-terminal region of RhoGDI binds to the switch region of Rho proteins, preventing GDP dissociation (Gosser et al., 1997; Keep et al., 1997). Deletion and mutant analysis studies suggest that the last 6 amino acids near the C-terminal region of GDI molecules are critical in determining the binding specificity of GTPases (Lin et al., 2003). Rho GTPases are maintained in an inactive state in the cytosol until an appropriate stimulus induces the dissociation of the Rho/RhoGDI complex. Once the GDP-bound form is released, it travels to the membrane, where it is converted to the GTP-bound form

The exact mechanism by which GDP-bound Rho is released from the RhoGDI complex is not completely understood. However, one proposed model involves the ezrin, radaxin, moesin (ERM) family of proteins (Takahashi et al., 1997). Several studies have shown that RhoGDI co-immunopercipitates with moesin (Hirao et al., 1996; Takahashi et al., 1997). The binding of RhoGDI with the N-terminal regions of ERM proteins reduces GDI activity and results in the displacement of GDP-bound Rho from GDI. Therefore, members of the ERM family of proteins may play an important role in regulating the activation states of Rho GTPases (Hirao et al., 1996; Tsukita and Yonemura, 1997). As ERM proteins are located near, or at the cell membrane, one possible mechanism for this regulation may involve the translocation of RhoGDI to the membrane, where it can be dissociated and activated by GEFs.

1.5 Bacterial toxins that modify Rho activation

Rho GTPases are the target of several classes of bacterial protein toxins (Aktories, 1997; Aktories et al., 2000) (see Table 1). The majority of toxins catalyze covalent modifications, such as deamidation, transglutamination, glucosylation and ADP-

Table 1. Toxins targeting Rho GTPases

	Specificity
Toxins inactivating Rho GTPases by covalent modification	
ADP-RIBOSYLTRANSFERASES:	
C. botulinum C3 exoenzyme	Rho
- *DC3B*	Rho
- *C2IN-C3*	Rho
- *C3-05*	Rho
C. limosum transferase	Rho
B. cereus transferase	Rho
S. aureus transferase	Rho
- C3stau2	Rho, RhoE, Rnd1
LARGE CLOSTRIDIAL CYTOTOXINS:	
C. difficile toxin A	Rho, Rac, Cdc42
C. difficile toxin B	Rac, Cdc42
C. sordellii haemorrhagic toxin	Rho, Rac, Cdc42
C. sordellii lethal toxin	Rac, Cdc42
C. novyi α-toxin	Rho, Rac, Cdc42
Toxins activating Rho GTPases by covalent modification	
DEAMIDATING TOXINS:	
E.coli CNF1	Rho, Rac, Cdc42
E.coli CNF2	Rho, Rac, Cdc42
TRANSGLUTAMINATING/DEAMIDATING TOXINS:	
B. bronchiseptica, B. parapertussis, B. pertussis DNT	Rho, Rac, Cdc42
Toxins non-covalently acting on Rho GTPases	
GAP-LIKE TOXINS:	
P. aeruginosa exoenzyme S (ExoS)	Rho, Rac, Cdc42

1.5.1 Rho-activating toxins that covalently modify Rho

Cytotoxic-necrotizing factors (CNF) are Rho activating toxins produced from pathological strains of *E.coli*. Members of the CNF family of toxins include CNF1 (Caprioli et al., 1983), CNF2 (De Rycke et al., 1990), and dermonercrotic toxin (DNT) (Pullinger et al., 1996), respectively produced by *B. pertussis*, *B. parapertussis* and *B. bronchiseptica*. CNF1 and CNF2 are 115 kDa proteins that share approximately 85 % homology and contain a C-terminal catalytic domain and a N-terminal cell-binding domain. CNF1 and CNF2 catalyze the deamidation of Rho proteins, removing a carboxamide nitrogen group from residue Gln 63 in Rho and from residue Gln 61 in Rac and Cdc42, respectively (Flatau et al., 1997; Schmidt et al., 1997; Schmidt et al., 1998a). These amino acids are essential for intrinsic and GAP-stimulated GTPase activities.

DNT is a 160 kDa protein that possesses a C-terminal catalytic domain highly homologous to the catalytic domains of CNF1 and CNF2 (Pullinger et al., 1996). Like CNF1 and CNF2, DNT catalyzes the deamidation of Rho GTPase; however, in addition, it also displays transglutaminase activity (Schmidt et al., 1999). Therefore, DNT not only removes carboxamide nitrogen from specific residues in Rho, Rac and Cdc42, it also

exchange (Hardt et al., 1998). SopE has been shown to possess such 'GEF-like' properties for Rac1 and Cdc42. Recently a second isoform of SopE, SopE2, was identified, which possesses 'GEF-like' activity for only Cdc42. Interestingly, SopE proteins share no apparent homology with GEFs, but have evolved to mimic their mode of action and have even developed rates of activity similar to endogenous GEFs (Hardt et al., 1998).

1.5.3 Rho-inactivating toxins that covalently modify Rho

The family of large clostridial cytotoxins (LCC) is among the largest family of protein toxins identified to date (250-308 kDa). Members of this family of glucosylating toxins include: toxins A and B from *C. difficle* (Lyerly et al., 1982), the lethal and hemorrhagic toxins from *C. sordellii* (Genth et al., 1996), and alpha toxin from *C. novyi* (Ball et al., 1993). All of the toxins in this family, which inactivate Rho GTPases by glucosylation, possess a N-terminal glucosyltransferase and a C-terminal receptor-binding domain (Ball et al., 1993; Genth et al., 1996; Lyerly et al., 1982). Although these toxins share a high degree of homology, LCCs have different substrate specificities

1.5.4 C3 transferase

C3 transferase (C3) was the first bacterial protein discovered that specifically blocks Rho activity (Aktories et al., 1989; Aktories and Hall, 1989). This 24-26 kDa exoenzyme was originally purified from strains C and D of the bacteria *Clostridium botulinum*. However, since its initial discovery, other bacteria, such as *Clostridium limosum* (Jung et al., 1993), *Bacillius cereus* (Just et al., 1992) and *Staphylococcus aureus* (Wilde et al., 2001a), have been also shown to produce related forms of C3. The effects of C3 are specific to RhoA, -B and -C (Chardin et al., 1989; Mohr et al., 1992), with the exception of C3stau2, which, in addition, modifies RhoE and Rnd1 (Wilde et al., 2001b). C3 catalyzes the transfer of an ADP-ribose moiety from NAD to an aspargine residue (Asn 41) located adjacent to the switch region of Rho, impairing the ability of GEFs to activate Rho (Aktories and Hall, 1989).

$$\mathbf{NAD^+ + Rho \rightarrow ADP\text{-}ribose\text{-}Asn41\text{-}Rho + nicotinamide + H^+}$$

In C3 treated cells, ADP-ribosylated forms of Rho accumulate in the cytoplasm, suggesting that ADP-ribosylation also affects the ability of Rho to translocate from the cytosol to the plasma membrane where it interacts with downstream effectors. Further, a

contains a catalytic domain, and therefore is considered to be an exoenzyme rather than a toxin. For C3 to be effective it must cross the plasma membrane and interact with intracellular Rho. However, C3 is not very cell permeable. To date, various methods have been used to help facilitate the entry of C3 into cells. In experiments using fibroblast cells, C3 was microinjected into individual cells (Ridley and Hall, 1992), whereas in studies using neuronal cells, triturating or scrape loading techniques are commonly used to aid cellular entry (Lehmann et al., 1999). The need for such disruptive methods and the inability to treat all cell types with equivalent techniques has limited the use of C3 as a tool for biological studies on Rho signaling. One solution has been to create fusion proteins that increase the efficiency of C3 delivery across the plasma membrane. One such protein, a fusion between C3 and the B subunit of diphtheria toxin (DT), binds to cell surface DT receptors and is internalized by an endocytosis-mediated mechanism (Boquet et al., 1995). This fusion protein is only effective in cells that contain DT receptors, therefore excluding most rodent cells. Another fusion protein, C2IN-C3, consisting of the bacterial toxin C2 and C3-transferase, also increases the ability of C3 to cross the cell membrane by receptor-mediated endocytosis (Barth et al., 1998). However,

peptides are part of larger proteins that are able to cross biological membranes. A series of different classes of transport peptides exist: 1) An HIV transcription activator (Tat) of 86 to 102 amino acids, required for viral replication, has been shown to posses cell penetrating properties (Fawell et al., 1994; Schwarze et al., 1999; Vives et al., 1997; Wender et al., 2000). Two specific sequences of the Tat, the larger of the two, spanning amino acids 37 to 72 (Fawell et al., 1994; Schwarze et al., 1999), and a shorter sequence, spanning amino acids 48 to 60, have been shown to translocate cargo to both the cytosol and nucleus (Vives et al., 1997). 2) The third helix of the Antennapedia homeodomain (Antp), a *Drosophila* homeoprotein, possesses the ability to cross biological membranes. Homeoproteins are a class of trans-activating DNA binding proteins encoded by homeotic genes. These genes are predominately expressed during development, but have also been observed after birth and in some cases throughout adulthood (Carrasco and Malacinski, 1987; Gehring, 1987; Price et al., 1992). The DNA binding properties of these proteins are due to a highly conserved 60 amino acid sequence present in many species including mammals. This 60 amino acid sequence consists of 3 α-helixes with a β-turn between helixes 2 and 3 (Bloch-Gallego et al., 1993). Experiments using

al., 2000). 5) The hydrophobic regions of several membrane transport sequences (MTS) can translocate across the cellular membrane and accumulate in the nucleus (Rojas et al., 1998). 6) Recently, a short amphipathic peptide carrier, Pep-1, was also shown to be able to translocate across cellular membranes (Morris et al., 2001).

1.5.6 Toxins acting as GAPs

To date, three homologous bacterial protein toxins, ExoS (Pederson et al., 1999), YopE and SptP (Kaniga et al., 1996), have been shown to inactive Rho GTPases by 'GAP-like' activity. Similar to the GEF-like toxins, these proteins share very little homology with endogenous GAPs, but hydrolyze GTP by a similar mechanism (Lerm et al., 2000). ExoS, produced by the bacteria *P.aeruginosa,* is a bifunctional protein that promotes both non-cytotoxic and cytotoxic activities. The N-terminal region of ExoS contains 'GAP-like' activity, specific for Rho, Rac and Cdc42 (Pederson et al., 1999), whereas its C-terminal region ADP-ribosylates Ras proteins (Ganesan et al., 1998). Both SptP, from *Salmonella typhimurium*, and the *Yersinia* protein YopE, contain similar N-

number of actin-binding and associated proteins, constitute the actin cytoskeleton. In response to specific extracellular signals, the actin cytoskeleton undergoes re-organization and plays a critical role in mediating many cellular functions, such as cell morphology, cell motility and cell adhesion (dos Remedios et al., 2003; Schmidt and Hall, 1998).

1.6.1 Role of Rho in regulating the cytoskeleton

Changes to the actin cytoskeleton induced by extracellular stimuli were the first effects of Rho GTPases characterized to be in mammalian cells (Ridley and Hall, 1992; Ridley et al., 1992). These landmark studies implicated Rho in the formation of actin stress fibres and focal adhesions (Ridley and Hall, 1992). Stress fibres are contractile bundles of actin filaments that transverse the cell and link to the extracellular matrix through cytoplasmic structures called focal adhesions (Kaibuchi et al., 1999). Focal adhesions are multi-protein complexes composed of a series of cytoplasmic proteins, including vinculin, α-actinin and talin, which assemble on the cell surface with integrins and other signaling molecules. Focal adhesions are the sites of strongest attach-

(Ridley and Hall, 1992). Confluent serum-starved fibroblast cells have very few visible stress fibres or focal adhesions; however, the stimulation of such cells with lysophosphatidic acid (LPA), a component of serum and a strong activator of Rho, results in the formation of an intense network of actin stress fibres and focal adhesions, as detected by phalloidin staining and an anti-vinculin antibody, respectively (Ridley and Hall, 1992). The formation of actin stress fibres and focal adhesion are prevented by treatment with C3 (Paterson et al., 1990; Ridley and Hall, 1992). The results from these studies implicate Rho as a key signaling molecule linking extracellular stimuli to the actin cytoskeleton through the regulation of actin stress fibres and focal adhesion complexes (Kaibuchi et al., 1999).

1.6.2 Downstream targets of Rho

The initial evidence documenting the ability of Rho to regulate the actin cytoskeleton was solely based on physical observations (Ridley and Hall, 1992) and, until recently, little was known about the molecular mechanisms by which Rho affected the actin cytoskeleton. Recently, however, numerous downstream effectors of Rho have b

Table 2. Downstream effectors of Rho

Effectors	Functions	Key References
Citron	Cytokinesis	(Madaule et al., 1998)
Kinectin	Kinesin binding	(Hotta et al., 1996)
MBS	MLC inactivation	(Kimura et al., 1996)
mDia	Actin organization	(Watanabe et al., 1997)
Phospholipase D	Phosphatidic acid levels	(Malcolm et al., 1994)
PI 3-kinase	PIP3 levels	(Zhang et al., 1993)
PI 4,5-kinase	PIP2 levels/actin	(Chong et al., 1994; Ren et al., 1996)
PKN	Unknown	(Amano et al., 1996b; Watanabe et al., 1996)
Rho kinase	Actin/myosin	(Ishizaki et al., 1996; Leung et al., 1995; Matsui et al., 1996)
Rhophilin	Unknown	(Watanabe et al., 1996)
Rhotekin	Unknown	(Reid et al., 1996)

Of particular interest has been the identification of Rho kinase, a serine/threonine protein kinase activated by GTP-bound Rho (Ishizaki et al., 1996; Leung et al., 1995; Matsui et al., 1996; Nakagawa et al., 1996). Rho kinase contains a coiled-coil domain located at its C-terminal end that negatively regulates its kinase activity. The transfection of active mutant forms of Rho kinase into cells induces stress fibre and focal adhesion formation (Amano et al., 1997; Ishizaki et al., 1997; Leung et al., 1996). In contrast, the expression of inactive forms of Rho kinase, or Rho binding-defective mutant forms of Rho kinase,

phosphorylates MLC (Amano et al., 1996a). These two downstream activities of Rho kinase result in high cellular levels of phosphorylated MLC, increased actin filament binding and ultimately the formation of stress fibre and focal adhesion formation (Burridge and Chrzanowska-Wodnicka, 1996). Rho kinase also regulates the actin cytoskeleton through other signaling pathways. It directly phosphorylates and activates LIM kinase, which regulates the activity of cofilin. When phosphorylated, the actin depolymerizing activity of cofilin is inhibited and as a result induces the formation of actin stress fibres (Maekawa et al., 1999; Sumi et al., 1999) (Figure 2).

Interestingly, recent studies have shown that the formation of actin stress fibres and focal adhesions are in fact separate events (Burridge and Chrzanowska-Wodnicka, 1996). Therefore, it appears that Rho kinase activation alone may not be sufficient for the assembly of both actin stress fibres and focal adhesions and that other downstream effectors may be involved (Sahai et al., 1998; Watanabe et al., 1999). The activation of mDia, another downstream effector of Rho, by GTP-bound Rho induces the formation of thin and disorganized actin stress fibres. Watanabe et al. (1999) have provided evidence that both mDia and Rho kinase are necessary for the formation of Rho-induced stress

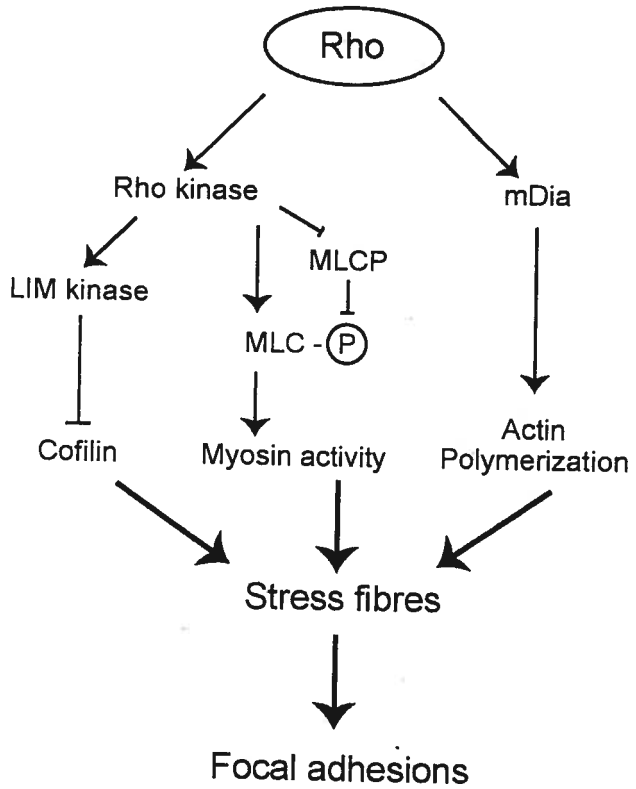


Figure 2. Signal-transduction pathways involved in Rho-induced stress fibre formation

(see text for details; modified from Takai *et al.* 2001)

1.6.3.1 Rac

Microinjection and transfection studies confirm that Rac regulates lamellipodia formation and membrane ruffling (Ridley et al., 1992). Lamellipodia are thin plasma membrane protrusions generated at the leading edge of migrating cells. Membrane ruffles are also present at the leading edge of cells and are caused by lamellipodia that lift up off the substrate and fold backwards. These dynamic, protrusive, actin sheets are rapidly induced in response to certain extracellular signals (Kaibuchi et al., 1999). Interestingly, microinjection studies using constitutively active forms of Ras induce membrane ruffling, which is blocked by the expression of dominant negative Rac. These results suggest that, at least in fibroblasts, Ras acts upstream of Rac (Zondag et al., 2000).

1.6.3.2 Cdc42

Cdc42 was originally isolated in yeast and shown to be essential for bud site assembly and cell polarity (Johnson and Pringle, 1990). In mammalian cells, several studies have shown that the activation of Cdc42 induces the formation of filopodia and actin spikes, which extend from the leading

of both Rac and Cdc42 results in the assembly of small focal complexes called point contacts, respectively localized to membrane ruffles and actin spikes (Nobes and Hall, 1995). Although composed of the same cytoskeleton proteins, these point contacts are smaller and morphologically different from the focal adhesions induced by Rho activation. The exact biological functions of these complexes are not clear, but they are likely relevant to neurons, where point contacts are the major adhesive structure of growth cones (Renaudin et al., 1999). In growth cones, Cdc42 has been shown to be important for the formation of filopodia (Kozma et al., 1995; Nobes and Hall, 1995). Cdc42 therefore appears to hve has a similar function in all cell types.

1.6.4 Crosstalk between Rho GTPases

Cellular effects are mediated through the interactions of various cell-signaling pathways (Matozaki et al., 2000). Consistent with this view, current evidence suggests that GTPases can directly regulate the activity of other GTPases, although the exact mechanism by which such crosstalk occurs is not completely understood. Initial studies

and Cdc42 modulate Rho activity in an unidirectional signaling pathway. In contrast, Rottner *et al.* (1999) have provided evidence of a bi-directional signaling pathway, in which an increase in Rho activation leads to a decrease in Rac activity and vice versa. In neurons, a more complex signaling cascade has been demonstrated (Li et al., 2002). Using a novel in situ GTP-Rho pulldown assay in Xenopus optic tectal cells, the Cline laboratory has shown that Rho activity is increased by the activation of Rac and inhibited by the activation of Cdc42. Rho activity has also been shown to inhibit Rac activation, suggesting that the activation of Rac in neurons may be part of a regulatory feedback loop (Li et al., 2002). Consistent with this finding, Rho and Rac pathways appear to control neurite outgrowth in PC-12 cells, with Rho inhibiting NGF-induced Rac activation (Yamaguchi et al., 2001). Subsequent studies will be necessary to further understand the exact Rho-like GTPase crosstalk pathways in various cell types.

1.7 Rho GTPases in neurons

Actin based motility is critical for the development of the nervous system, as both

et al., 1994; Kozma et al., 1997; Tigyi et al., 1996b). Growth cone collapse is prevented by pre-treatment with C3 (Jin and Strittmatter, 1997; Lehmann et al., 1999). Similar to their role in fibroblast cells, the activation of Rac and Cdc42 in neurons promotes the formation of lamellipodia and filopodia, respectively (Kozma et al., 1997). Therefore, it is believed that the activation of Rac and Cdc42 in neurons stimulates growth cone motility, which in turn, promotes growth. In contrast, the activation of Rho induces growth cone collapse, which prevents neuronal growth (Hall, 1998; Mueller, 1999).

1.7.1 Neuronal growth cone

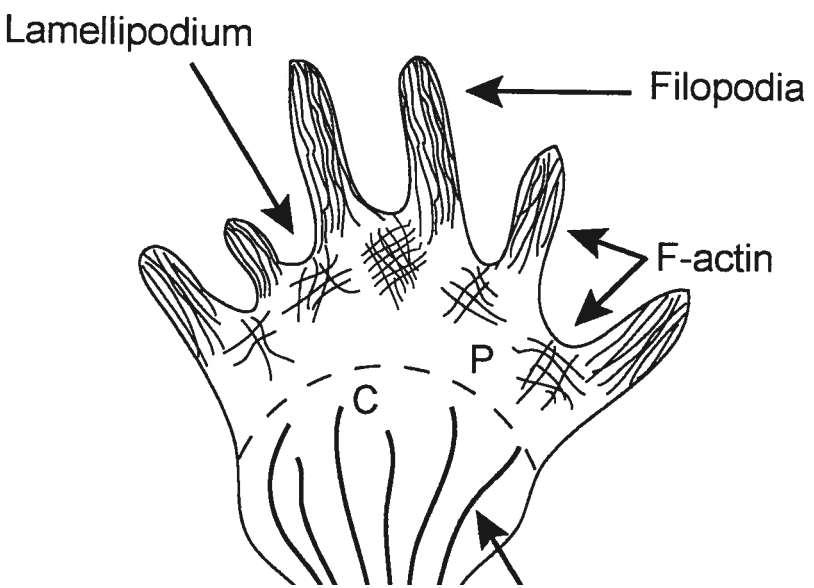
During nervous system development, neurons extend axons over long distances to reach their final targets. As neurons transport information to and from the brain, it is crucial for the proper functioning of the nervous system that they form appropriate connections with their target cells. The task of navigating through the developing nervous system is delegated to neuronal growth cones (Dontchev and Letourneau, 2003; Goodman, 1996; Mueller, 1999; Tessier-Lavigne and Goodman, 1996). Growth cones are specialized, highly dynamic, sensory structures located at the distal tips of axons (see

other environmental cues and the concurrent activation of additional intracellular signaling pathways (Hopker et al., 1999; Ming et al., 1997; Song et al., 1997; Stein et al., 2001).

1.7.1.1 Growth cone motility

Two major cytoskeletal components present in growth cones are filamentous actin (F-actin) and microtubules (Dent and Gertler, 2003). F-actin is predominantly located in the peripheral area (P-domain) of growth cones. These actin filaments are oriented with their faster growing ends towards the distal end of the growth cone and their slower growing ends towards the central domain (Bridgman and Dailey, 1989; Forscher and Smith, 1988; Lewis and Bridgman, 1992). Microtubules run straight and parallel inside axons, but splay out and extend into the central area (C-domain) at the base of the growth cone (Figure 3) (Gordon-Weeks, 1987; Li and Black, 1996). Several studies have demonstrated that both these cytoskeletal elements are necessary for the regulation of growth cone motility, axonal elongation and guidance (Bentley and Toroian-Raymond, 1986; Bradke and Dotti, 1999; Challacombe et al., 1997; Rochlin et al., 1996; Tanaka et

growth cone to the central domain, where it is depolymerized. This process of 'actin treadmilling' is controlled by the myosin family of motor proteins and has an important role in filopodia and lamellipodia retraction (Forscher and Smith, 1988; Lin et al., 1996). Third, in a process that is not yet completely understood, F-actin is recycled to the transitional zone of growth cones (Suter and Forscher, 1998). Therefore, it is suggested that the rate of actin polymerization and the rate of retrograde flow mediate filopodia and lamellipodia extension.



In order to interpret the extracellular environment, filopodia continuously protrude and retract from the leading edge of growth cones. The contact of filopodia with chemorepulsive guidance cues results in filopodia retraction. In most cases, additional filopodia will continue to extend in directions away from negative stimuli; however, if chemorepulsive guidance cue are extremely strong, the growth cone can undergo complete collapse, preventing any further growth (Mueller, 1999). It is suggested that contact of neurons with repulsive factors may result in the destabilization of F-actin located at the leading edge of growth cones (Fan et al., 1993). When filopodia contact positive stimuli, the retrograde transport of F-actin is slowed in the direction of growth cone extension and accumulates at the site of interaction. This process is followed by the formation of web-like lamellipodia between the extended filopodia. Lamellipodia act to stabilize filopodia while the body of the growth cone advances and axonal extension takes place (Gallo and Letourneau, 2000; Lin and Forscher, 1993; O'Connor and Bentley, 1993). Filopodia are further stabilized by the re-orientation and invasion of microtubule towards this future site of outgrowth (Sabry et al., 1991; Tanaka et al., 1995; Tanaka and Sabry, 1995).

Surprisingly, the disruption of microtubules also decreases the rate of lamellipodia formation (Gallo, 1998), suggesting that microtubule stability may also be involved in the regulation F-actin dynamics. Consistent with these results, actin-based structures located in the transition zone of growth cones, termed actin arcs, were recently shown to strongly affect microtubule dynamics and organization in a Rho dependent manner (Zhang et al., 2003). Further, in addition to their interaction with microtubules, certain microtubule-associated proteins (MAPs) also interact with actin filaments, proving a mechanical link between these two cytoskeletal components (Correas et al., 1990; Sattilaro, 1986). Therefore, growth cone motility and axonal outgrowth and guidance are mediated by F-actin and microtubules dynamics, which are in part regulated by upstream proteins such as the Rho family of Rho GTPases (Luo, 2002).

1.7.1 Biological mechanisms of growth cone collapse

Several studies have suggested two important Rho-dependent signaling pathways responsible for neurite retraction and growth cone collapse (Dent and Gertler, 2003; Gungabissoon and Bamburg, 2003; Meyer and Feldman, 2002). By interaction with

neurite outgrowth. In contrast, treatment with LPA phosporylates and inactivates ADF/cofilin, resulting in a dramatic decrease in neurite outgrowth (Meberg et al., 1998).

As discussed above, during neurite extension actin polymerization occurs at the leading edge of the growth cone and results in the formation of actin-rich filopodia and lamellipodia. Simultaneously, there is a constant retrograde flow of actin towards the cell interior, which is thought to be mediated by actomyosin-generated forces. Myosin II, the best-characterized family member of the non-skeletal muscle myosins, is highly concentrated in growth cones, located between the central domain and the actin rich peripheral domain (Miller et al., 1992). In neurons, the inhibition of myosin function slows the forward progress of the growth cone; treatment with antisense oligonucleotides against myosin reduces neurite extension in neuroblastoma cells (Wylie et al., 1998) and cultured neurons from myosin knockout mice show a reduction in growth cone spread (Bridgman et al., 2001).

1.7.2 Regulation of ADF/cofilin

The ability of the actin cytoskeleton to carry out diverse biological roles in

over-expressed in cultured rat cortical neurons, ADF/cofilin induces a dramatic increase in neurite outgrowth (Meberg and Bamburg, 2000). Further LIM kinase, a downstream substrate of Rho kinase, phosphorylates and inhibits ADF/cofilin. This phosphorylation results in a decrease in actin polymerization leading to growth cone collapse (Arber et al., 1998). Therefore, Rho GTPase can regulate growth cone motility through it effects on ADF/cofilin activity (Kuhn et al., 2000).

1.7.3 Regulation of myosin II

Although never experimentally proven, the rates of retrograde actin flow in filopodia and lamellipodia have been extensively implicated in Rho-dependent growth cone collapse (Goldberg, 2003; Huber et al., 2003). In a recent study, the Forscher laboratory (Zhang et al., 2003) suggests that this is not the case. These authors report data indicating evidence that the rate of retrograde actin flow is, in fact, unchanged during growth cone collapse and functions independently of the Rho signaling pathway. The same study also provides evidence that actin arcs and central actin bundles are responsible for Rho/Rho kinase-induced growth cone collapse (Zhang et al., 2003)

phosphorylation state of MLC (Kaibuchi et al., 1999). Further, the expression of constitutively active mutants of MLC activates myosin ATPase and promotes neurite retraction (Amano et al., 1998). Therefore, Rho-induced neurite retraction and growth cone collapse are driven,at least in part, by the contraction of the actin-myosin system which functions downstream of Rho kinase. The inhibition of Rho kinase by treatment with its specific inhibitor Y-27326, or the microinjection of dominant negative forms, blocks Rho-induced growth cone collapse (Bito et al., 2000; Hirose et al., 1998), indicating that Rho kinase has an important function in growth cone collapse. Interestingly, actomyosin contraction is also accompanied by the depolymerization of microtubules. Although the exact involvement of Rho in the regulation of microtubule dynamics is not completely understood, the Rho effector mDia has been implicated in the formation and orientation of stable microtubules (Palazzo et al., 2001).

1.8 Spinal cord injury

Spinal cord injury (SCI) disrupts or severs the axons of projection neurons, which act as fibres of communication between the brain and the rest of the body. While there is

1.8.1 PNS vs. CNS

Lesion of adult CNS axons results in permanent and irreparable injury. In contrast injured axons of the PNS are endowed with the ability to regenerate, which in certain cases, can result in functional recovery. The difference in regenerative ability between the two main branches of the nervous system is attributed to a series of both molecular and cellular factors. Adult CNS neurons possess a reduced intrinsic capacity for growth as compared to embryonic CNS neurons and all PNS neurons, independent of their developmental age. Recently it was shown that highly purified retinal ganglion cells (RGC) loose their ability to rapidly extend axons as they increase in age (Goldberg et al., 2002). This reduction in the intrinsic growth programme of CNS neurons can also be seen in the limited expression of regeneration-associated genes (RAGs), such as GAP-43, after CNS injury (Fernandes et al., 1999; Tetzlaff et al., 1991). In contrast, a massive up-regulation of such genes has been shown to occur after PNS injury, and suggested to be at least partly responsible for the regeneration of injured PNS neurons (Plunet et al., 2002).

The injured CNS environment is also clearly more hostile and less conducive to regeneration than that of the PNS. Several studies have shown that severed CNS axons

growth inhibitory protein, is also present in PNS myelin (Shen et al., 1998). However, there is substantially less MAG in PNS as compared to CNS myelin. Further, the increased immune response that follows PNS injury immediately clears any damaged white matter, which could potentially be growth inhibitory, from the lesion site. This is evident in the lack of regeneration observed in experiments using C57BL/Wld mice, which have a substantially reduced rate of myelin removal after injury (Brown et al., 1992; Brown et al., 1991). In the CNS, debris from injured and dying cells remain at the lesion site for an extended period of time, further contributing to the growth inhibitory environment (Fawcett and Keynes, 1990). Additional growth inhibitory molecules, such as chondrotin sulphate proteoglycans (CSPGs) and negative guidance cues, are also up regulated after CNS injury, and known to be present within the glial scar (Fawcett and Asher, 1999). The astroglial scar is specific to the CNS and as discussed earlier acts as both a physical and a chemical barrier to regeneration (Fawcett and Asher, 1999).

1.8.2 Cell death

Acute spinal cord trauma induces a rapid and massive necrotic response, resulting

that apoptotic cell death occurs after SCI injury. Apoptosis is responsible for the delayed cell death following SCI and extends well beyond the original site of injury (for reviews see (Beattie et al., 2000; Beattie et al., 2002b; Schwartz and Fehlings, 2002). The pathophysiology of this 'secondary injury' is very complex and involves many cellular factors and processes, such as the release of excitatory amino acids (EAA), increased levels of intracellular calcium, the generation of free radicals, lipid peroxidation, and edema (Anderson and Hall, 1993; Fehlings and Tator, 1995; Tator and Fehlings, 1991) (Figure 4). Since apoptosis is a cellular controlled, active process, further understanding of the molecular components and biochemical mechanisms involved in its execution may lead to new treatments aimed at reducing cell death, tissue damage and motor dysfunction after SCI. Fehlings and Tator have demonstrated that the rescue of as few as 10 % of neurons after SCI can result in significant functional recovery (Fehlings and Tator, 1995). As the apoptotic cascade provides a therapeutic window for treatment after SCI it will be discussed in some detail.

1.8.3 Apoptosis

proteins homologous to those of *Caenorhabditis elegans* (Hengartner and Horvitz, 1994). The concept of apoptosis was first introduced 30 years ago and was initially defined morphologically (Kerr et al., 1972). Distinct characteristic changes in cellular morphology occur during apoptosis, including cell shrinkage, chromatin condensation, DNA fragmentation and plasma membrane blebbing (Kerr et al., 1972; Wyllie, 1980a; Wyllie et al., 1980b). In contrast to necrotic cell death, which occurs from acute traumatic injury and results in tissue damage, rapid lysis of cellular membranes and leakage of the cellular content, apoptotic cell death occurs in the absence of any inflammatory response. Due to rapid progress over the last 10 to 15 years, key molecular components of this cell death pathway have now been identified, although ultrastructural morphology still remains the most reliable criteria for the identification of apoptosis.

1.8.3.1 Biochemical pathways of apoptosis

The majority of observed changes in the morphology of cells undergoing apoptosis is due to the activation of a highly regulated family of cysteine proteases called [illegible] 1998). Caspases are highly

and -14) (Earnshaw et al., 1999; Thornberry et al., 1997). All caspases are synthesized and exist in normal cells as relatively inactive enzymes termed procaspases. During apoptosis, procaspases undergo proteolytic cleavage, resulting in the production of the necessary subunits that constitute the heterotetramer form of active caspases (Earnshaw et al., 1999; Thornberry and Lazebnik, 1998). Once activated, caspases, namely the effector caspases, inactivate or activate various cellular proteins by caspase-mediated proteolysis (Budihardjo et al., 1999; Strasser et al., 2000). Mammalian cells have two distinct apoptotic pathways that converge on the activation of caspases: one is mediated by ligand binding to cell surface death receptors (Ashkenazi and Dixit, 1998; Nagata, 1997), and the other is initiated by stress-induced signals inside the cell (Desagher and Martinou, 2000; Wang, 2001).

Caspase-3 is widely distributed in many different cell types, with high a expression in neurons, suggesting that it may be an important mediator of apoptosis in the CNS. A series of biochemical fractionation and purification experiments using cell lysates have shown that 4 molecules are necessary for the activation of caspases *in vitro*; (i) dATP; (ii) Apaf-1 (apoptotic protease activating factor-1); (iii) caspase-9; and (iv) cytochrome c,

proteins (Faleiro et al., 1997). It is suggested that the complex formed between cytochrome c and Apaf-1 increases the cellular concentration of procaspase-9 available for cleavage, and reduces the possibility that occasional cytochrome c leakage from the mitochondria will cause the initiation of the apoptotic pathway and cell death (Li et al., 1997; Yoshida et al., 1998; Zou et al., 1997; Zou et al., 1999).

The Bcl-2 proteins are an evolutionary conserved family that is a critical upstream regulator of apoptosis (Gross et al., 1999; Merry and Korsmeyer, 1997). Currently, 15 mammalian family members have been identified, all of which contain at least one of the four conserved Bcl-2 homology domains (BH1-BH4). Members of the Bcl-2 family either promote (e.g. BAX and BAK) or inhibit apoptosis (e.g. Bcl-2, and Bcl-x_L). It has been suggested that, to promote survival, Bcl-2 proteins may regulate the integrity of mitochondrial membrane, thus preventing the release of cytochrome c and consequently blocking caspase activation (Desagher and Martinou, 2000; Gross et al., 1999). One proposed mechanism by which Bcl-2 proteins prevent the release of cytochrome c involves the ability of the anti-apoptotic proteins of the Bcl-2 family to associate with their pro-apoptotic counterparts. It is believed that the formation of this complex prevents

activator Apaf-1. These results suggest that Bcl-2 has a broader role in preventing apoptosis.

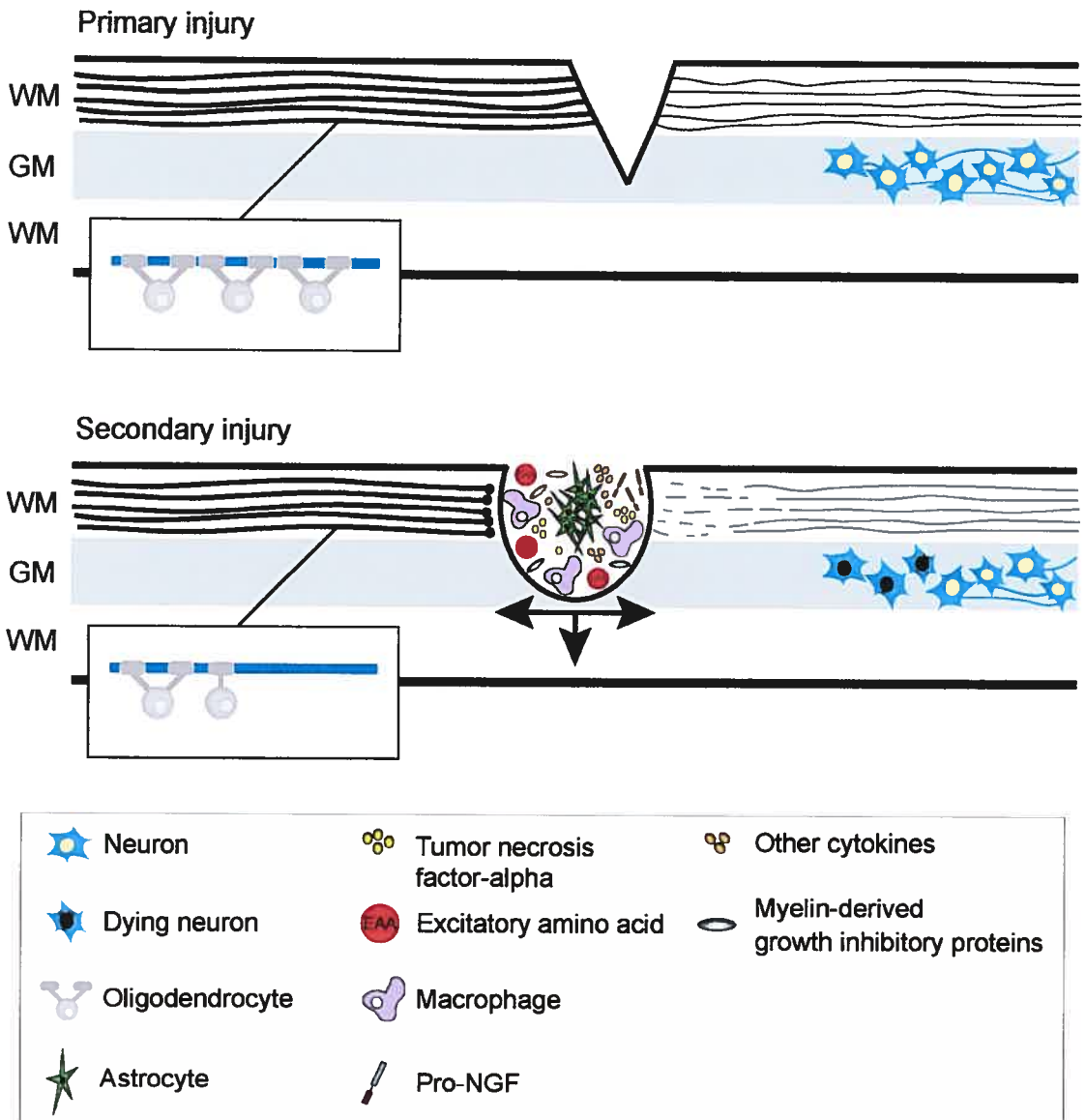
1.8.3.2 Apoptosis following spinal cord injury

Following the initial mechanical insult of SCI, two major events are well documented: an intense inflammatory response, which is mediated by a large scale invasion of inflammatory cells into the lesion site and surrounding tissue and a secondary wave of apoptotic cell death, increasing the extent of damage beyond the lesion area (Schwab and Bartholdi, 1996). The exact mechanisms underlying this secondary stage of degeneration are not completely understood. It is suggested that this prolonged cell death away from the lesion site is triggered by the presence of calcium, glutamate and other excitatory amino acids, free radicals, ionic fluxes, and pro-inflammatory cytokines, such as TNF-α, which all are consequences of the primary injury (Tator and Fehlings, 1991; Schwab and Bartholdi, 1996; Young, 1993) (Figure 4). By 8 days post injury, the lesion site is enlarged both rostrally and caudally by several millimetres, demonstrating that this secondary wave of cell death plays an important role in the extensive loss of both white

1.8.4 p75 neurotrophin receptor

As early as 4-8 hours after SCI, condensed chromatin and DNA fragmentation is observed in neurons located at the lesion site (Katoh et al., 1996). This finding is consistent with abundant data showing, which report that neuronal apoptosis occurs within the first 24 hours post injury (Crowe et al., 1997; Grossman et al., 2001b; Lee et al., 2000; Liu et al., 1997a; Yong et al., 1998). Neuronal apoptotic cell death after SCI is believed to be mediated by caspase-3. Springer *et al.* (1999) have documented a 600 % increase in the level of activated caspases as early as 1 hour after injury, which is maintained elevated for at least 24 hours. Further, Emery *et al.* (1998) provides evidence of the presence of caspase-3 in the spinal cord of human patients who died 3 hours to 15 months following a SCI. Increased levels of the pro-apoptotic protein Bax, cytochrome c, and caspase-9, all presmumably to be upstream of caspase-3, are also observed as early as 30 minutes post injury and stay elevated for a period of at least 24 hours (Isenmann et al., 1997; Springer et al., 1999). In contrast, expression of the anti-apoptotic protein Bcl-2 has been shown to reduce neuronal loss after SCI (Saavedra et al., 2000; Takahashi et al., 1999). These results indicate a rapid transduction of death signals following SCI.

axotomy. Consistent with this finding, Dubreuil *et al.* (2003) have found that this increase in the expression of $p75^{NTR}$ after SCI is associated with neuronal apoptosis. Further, the precursor protein of NGF, proNGF, is produced and secreted into the cerebral spinal fluid after spinal cord injury, and has been recently shown to induce the death of both oligodendrocytes and neurons in a $p75^{NTR}$ dependent manner (Beattie et al., 2002a) Taken together, these studies provide strong evidence for $p75^{NTR}$ dependent apoptosis after SCI. The delayed increase in the apoptotic cell death of oligodendrytes is thought to be associated with degenerating long white matter tracts and Wallerian degeneration. Wallerian degeneration is a classically defined change in axons after traumatic injury. This degeneration of the distal nerve occurs in both the PNS and CNS (Buss and Schwab, 2003). Warden *et al.* (2002) have provided evidence of increased levels of apoptosis in areas of Wallerian degeneration after corticalspinal or dorsal ascending tract lesions. During development, oligodendrocytes survival is dependent on trophic support from axons and, as a result, axonal loss after injury may decrease oligodendrocyte survival (Barres and Raff, 1999). In turn, the loss of oligodendrocytes may lead to further demyelination and dysfunction of injured and uninjured axons, and contribute to the


Primary injury
WM
GM
WM
Secondary injury
WM
GM
WM
Neuron
Dying neuron
Oligodendrocyte
Astrocyte
Tumor necrosis factor-alpha
Excitatory amino acid
Macrophage
Pro-NGF
Other cytokines
Myelin-derived growth inhibitory proteins

traumatic insult (Perry et al., 1995). TNF-α has been implicated in the death of CNS cells in various inflammatory and neurodegenerative disorders (Gonzalez-Scarano and Baltuch, 1999), such as multiple sclerosis (MS), Alzheimer's disease (AD) and Parkinson's disease (PD). After traumatic injury, reactive astrocytes, microglia and infiltrating macrophages all secrete TNF-α and promote cell death as well as failed regeneration in the CNS (Neumann et al., 2002a; Perry et al., 1995). Recently, TNF-α has also been shown to inhibit the elongation and branching of neurites in a Rho dependent manner (Neumann et al., 2002a).

Following SCI, several studies have documented an increase in both the expression of TNF-α, at the mRNA and protein levels, and its cell-surface receptor tumor necrosis factor receptor 1 (TNFR1). Increased levels of TNF-α are observed directly at the lesion site, as early as 30 minutes post injury and peak at 72 hours (Wang et al., 2002a). This increase in TNF-α has been implicated in the apoptotic cell death of neurons and glia (Lee et al., 2000). The treatment of cultured neurons and oligodendrocytes with TNF-α induces apoptosis, and the injection of TNF-α into the optic nerve (Jenkins and Ikeda, 1992) or spinal cord (Simmons and Willenborg, 1990) causes demyelination.

caspase-8 and caspase-3 (Varfolomeev and Ashkenazi, 2004). In contrast, neuroprotective effects have been observed by the deletion of TNF receptors after CNS trauma (Fontaine et al., 2002; Gary et al., 1998; Raivich et al., 2002).

1.8.6 Glutamate

Traumatic SCI causes an immediate and rapid increase in the concentration of excitatory amino acids (EAA), such as glutamate. This increase results in the excitotoxic cell death of neurons and glial cells (Figure 4) (Panter et al., 1990; Zipfel et al., 2000). Cellular events such as exocytosis, inhibited or reverse glutamate uptake, the breakdown of the blood brain barrier, and cell lysis are suggested to be responsible for this increase. Several studies have also confirmed that a high concentration of extracellular glutamate after SCI induces apoptotic cell death of both neurons and oligodendrocytes (Liu et al., 1999; McDonald et al., 1998; Wrathall et al., 1994). Interestingly, both *in vitro* (Chao and Hu, 1994; Gelbard et al., 1993) and *in vivo* (Hermann et al., 2001) studies have shown that glutamate can act in synergy with TNF-α to promote increased cell death

There are two classes of cell membrane glutamate receptors: the metabotropic

2002; Grossman et al., 2001a; Mills et al., 2001). Pharmacological blockage of glutamate receptors after SCI reduces damage to both white and gray matter (Wrathall et al., 1994; Wrathall et al., 1996; Wrathall et al., 1997). Such studies provide strong evidence for the involvement of glutamate-induced excitotoxicity in the secondary wave of apoptotic cell death that follows SCI.

1.8.7 Rho and apoptosis

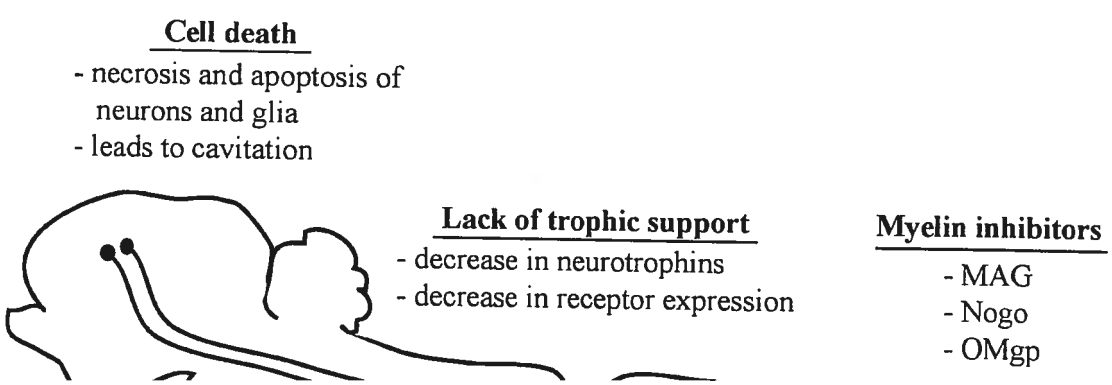
Multiple studies have implicated the activation of Rho in both pro-apoptotic (Donovan et al., 1997; Dubreuil et al., 2003; Mills et al., 1998; Petrache et al., 2003) and anti-apoptotic (Bobak et al., 1997; Coleman and Olson, 2002) signaling pathways, providing evidence that the role of Rho in apoptosis is cell-type dependent. In endothelial cells undergoing apoptosis, the inactivation of either Rho or Rho Kinase reduced the levels of cleaved caspase 3, 7 and 8 and the presence of nucleosomal fragmentation (Petrache et al., 2003). The inactivation of Rho kinase in TF-1 cells prevents Phorbal ester-induced caspase-3 activation and apoptosis (Lai et al., 2003). In PC-12 cells, Rho activation is involved in mediating the morphological changes that occur during the

of SCI, inactivation of Rho with C3-07 treatment reduced the number of apoptotic cells by approximately 50% (Dubreuil et al., 2003). These data support a possible role for Rho in mediating apoptosis after SCI.

1.9 Inhibitors of axon regeneration

It is now well established that one major reason for the lack of regeneration in the CNS is the presence of myelin-derived growth inhibitory proteins (for reviews, see McKerracher and Winton, 2002; David and Lacroix, 2003; He, 2004). Using peripheral nerve grafts to replace the damaged CNS environment, the Aguayo group elegantly demonstrated that, when provided with an appropriate growth environment, CNS axons possess an intrinsic capacity for regeneration (David and Aguayo, 1981). The inability of the CNS environment to support growth was initially thought to be solely due to the lack of growth-promoting signals. However, Schwab and colleagues have demonstrated that, in addition to a lacking positive growth cues, the injured CNS also contains growth inhibitory proteins that block regeneration (Schwab and Thoenen, 1985; Caroni and Schwab, 1988b).

myelin prior to SCI (Huang et al., 1999). Taken together, these findings suggest that the inhibitory environment of the CNS is somehow, associated with myelin. To date, three separate myelin-derived growth inhibitory proteins have been identified: myelin associated glycoprotein (MAG) (McKerracher et al., 1994; Mukhopadhyay et al., 1994), Nogo-A (Chen, 2000; GrandPré, 2000; Prinjha et al., 2000), and oligodendrocyte-myelin glycoprotein (OMgp) (Kottis et al., 2002; Wang et al., 2002c). Other inhibitory proteins, such as the CSPGs (Fawcett and Asher, 1999; McKeon et al., 1991) that are expressed by cells forming the scar at the lesion site, and chemorepulsive guidance factors, which are known to inhibit axon growth during development and are re-expressed after injury, contribute to growth inhibition after adult CNS trauma (Tang, 2003).



1.9.1 Myelin associated glycoprotein

Myelin associated glycoprotein (MAG), a previously identified myelin protein, was the first myelin-derived growth inhibitory protein identified. MAG is a transmembrane sialic acid dependent adhesion molecule that is part of the immunoglobulin family (Lai et al., 1987; Salzer et al., 1990). Utilizing distinct experimental procedures two groups, McKerracher and colleagues (McKerracher et al., 1994) and the Filbin laboratory (Mukhopadhyay et al., 1994), independently reported the inhibitory activity of MAG. Employing a non-denaturing extraction method, McKerracher *et al.* (1994) purified MAG from bovine CNS myelin, and documented its inhibitory activity by neurite outgrowth assays and immunodepletion studies. The Filbin group demonstrated the inhibitory activity of MAG with CHO cells that express MAG on their surface (Mukhopadhyay et al., 1994). Recently, a soluble proteolytic fragment corresponding to the extracellular domain of MAG (dMAG) was shown to be present after SCI. dMAG is a potent growth inhibitory molecule, and likely to account for the majority of MAG-induced growth inhibition after injury (Tang et al., 2001).

The initial discovery that MAG possessed growth inhibitory activity was not

deficient myelin (Li et al., 1996; Shen et al., 1998), where as a third group reported no change (Bartsch et al., 1995). Regardless of the differences between these studies, all three groups reported poor levels of axonal regeneration in MAG $^{-/-}$ mice after SCI, suggesting the presence of additional growth inhibitory protein in CNS myelin.

Over the years, several MAG binding partners, such as sialic acid and gangliosides, have been suggested to mediate MAG-induced growth inhibitory signaling (Vinson et al., 2001; Vyas et al., 2002). GD1a and GT1b are widely expressed gangliosides present on the cell surface of neuronal cells. Vyas *et al.* (2002) have provided strong evidence that GD1a and GT1b play an important role in MAG-induced growth inhibition. The clustering of GD1a or GT1b in the absence of ligand mimicked neurite outgrowth inhibition, suggesting a possible role for GD1a and GT1b as functional MAG receptors. A presumtive role for gangliosides in mediating the inhibition of growth by other myelin-derived growth inhibitory proteins remains to be determined.

1.9.2 Mechanisms of MAG inhibition

MAG-induced inhibition of neurite outgrowth appears to be dependent on both

levels of cAMP (Cai et al., 2001a). During development, neurons contain high levels of cAMP, which may override the growth inhibitory activity of MAG. In adult neurons, cAMP levels are dramatically decreased, making them more sensitive to MAG inhibition (Cai et al., 2001a). Treatment with cAMP (Cai et al., 2001a) or specific drugs known to enhance cAMP signaling (Neumann et al., 2002b; Qiu et al., 2002) block MAG-induced growth inhibition in adult neurons, and increases the regeneration of lesioned DRG *in vivo*. Pretreatment of neurons with high levels of neurotrophins can also blocks myelin and MAG-induced growth inhibition (see Figure 6) (Cai et al., 1999). It is suggested that such priming of cells increases the intracellular levels of cAMP and the activity of protein kinase A (PKA), a downstream effector of cAMP. Blocking PKA activation re-establishes MAG-induced growth inhibition (Cai et al., 1999). In addition, studies examining the response of Xenopus growth cones to various guidance cues demonstrate that the growth cone turning responses are dependent on cyclic nucleotide levels, with high levels favouring attraction and low levels favouring repulsion (Imperato-Kalmar et al., 1997; Song et al., 1998; Song et al., 1997).

In a possible mechanism explaining how increased levels of cAMP overcome

1.9.3 Nogo-A

Using SDS-PAGE fractionation techniques, Schwab and colleagues isolated two minor membrane proteins with molecular weights of 35 and 250 kDa from rat brain myelin (Caroni and Schwab, 1988b). These proteins were later named NI-35 and NI-250, respectively. NI-35 and NI-250 are expressed by differentiated oligodendrocytes and are particularly enriched in adult CNS white matter. Biochemical analysis of NI-35 and NI-250 showed that both proteins were, in part, responsible for the non-permissive properties of myelin (Schwab and Thoenen, 1985; Caroni and Schwab, 1988b; Schwab et al., 1993). To block the inhibitory properties of these myelin-derived proteins, a monoclonal antibody, named IN-1, was raised. IN-1, recognizes both NI-35 and NI-250 and blocks myelin and NI 35/250-induced growth cone collapse (Caroni and Schwab, 1988a; Schnell and Schwab, 1990). When applied to various CNS lesions in adult rat, IN-1 enhances sprouting of injured and uninjured axons (Buffo et al., 2000; Raineteau and Schwab, 2001) and promotes axon regeneration and functional recovery (Bregman et al., 1995; Schnell and Schwab, 1990; Schnell and Schwab, 1993).

For over a decade the exact identity of IN-1 antigens remained a mystery, until

Reticulons are highly concentrated in the endoplasmic reticulum of neurons and endocrine cells; however, the exact functions of this family of proteins are largely unknown (Oertle et al., 2003a; Oertle and Schwab, 2003).

Nogo-A, the longest Nogo isoform, is mainly located on the cell surface of oligodendrocytes and highly expressed in the CNS. In contrast, Nogo-B is widely expressed in various tissues outside the CNS, whereas, Nogo-C is mainly expressed in skeletal muscle (Huber et al., 2002). Unexpectedly, *in situ* studies have also documented the presence of Nogo-A on several groups of neurons located in the brain, spinal cord and peripheral ganglia. Neuronal expression of Nogo-A is highest during development and decreases in the adult nervous system; however, the exact role, if any, that neuronal Nogo-A has in growth inhibition remains unclear (Josephson et al., 2001; Huber et al., 2002). Consistent with the role of Nogo-A as a potent myelin-derived inhibitory protein mice overexpressing Nogo-A in the PNS show delayed regeneration after sciatic nerve lesion (Pot et al., 2002; Kim et al., 2003).

Nogo-A contains two functional inhibitory domains: a short 66 amino acid region, termed Nogo- 66 that is common to all 3 isoforms, and a Nogo-A specific N-terminal

et al., 2000), whereas the entire N-terminal region of Nogo- A is surprisingly proposed to be cytoplasmic (Chen, 2000). Two scenarios have been suggested by which intracellular Amino-Nogo induces growth inhibition. One possibility is that injury to the CNS results in oligodendrocyte damage, such that both inhibitory regions are exposed. As a result, Amino-Nogo would only contribute to growth inhibition in pathological states. A second possibility is that Nogo-A may under go conformational changes, which would allow for the extracellular exposure of the N-terminal domain (Huber and Schwab, 2000).

Recently, using different techniques, three independent groups developed Nogo-A knockout mice (Nogo $^{-/-}$) (Kim et al., 2003; Simonen et al., 2003; Zheng et al., 2003). None of mutant mice were shown to have any detectable neurological deficits or abnormalities in their myelin, suggesting that Nogo-A is not involved in the development of the CNS, or in the maintenance of axonal pathways. One group reported substantial improvements in regeneration, but this improvement was restricted to mice less then 9 weeks old (Kim et al., 2003). Of the two other groups, one reported significant, but modest improvements in axonal growth, with a very limited number of regenerating axons observed and no improvements in functional recovery (Oertle et al., 2003b). The

1.9.4 Oligodendrocyte myelin glycoprotein

Oligodendrocyte myelin glycoprotein (OMgp), the most recently identified inhibitory component of myelin, is a glycosylphosphatidylinositol (GPI)-liked protein expressed on the surface of mature oligodendrocytes (Wang et al., 2002c). Similar to most myelin-derived growth inhibitory proteins OMgp is located in the myelin membrane directly adjacent to the axon (Kottis et al., 2002). During the purification of MAG from CNS myelin, the McKerracher group noted an additional protein fraction, termed *Arretin*, which possessed growth inhibitory activity (McKerracher et al., 1994). *Arretin* was subsequently identified by Kottis *et al.* as the previously identified myelin protein OMgp (Kottis et al., 2002). Using a different approach, the He laboratory independently identified OMgp as a potent inhibitor of neurite outgrowth, during a search for GPI-linked inhibitors in CNS myelin (Wang et al., 2002c). The inhibitory activity of OMgp has only been examined *in vitro* and the effects of OMgp on axonal regeneration *in vivo* have yet to be determined. However, the inhibitory activity of OMgp *in vitro* appears to be as potent as that of MAG and Nogo, and all three proteins have a similar distribution in the myelin sheath, suggesting that they all contribute to growth inhibition in the adult

cDNA expression library was transfected into non-neuronal cells and screened for interaction with Nogo, Fournier *et al.* (2002) have identified a high affinity receptor for Nogo-66, termed Nogo-66 receptor (NgR). Currently, no receptor has been identified for Amino-Nogo. Northern blot analysis has revealed a strong presence of NgR in the adult brain, with the strongest expression observed in neurons. Low levels of expression were also detected in the heart and kidneys (Fournier et al., 2001). The N-terminal region of this 473 amino acid protein contains a membrane translocation signal sequence, followed by 8 leucine-rich-repeat (LRR) domains and a cystine rich LRR carboxy-terminal flanking domain. The C-terminal contains a unique region located prior to a GPI anchorage site (Fournier et al., 2001). Deletion studies have suggested that all of the NgR LRR domains are required for Nogo binding and that the unique C-terminal portion of NgR is necessary but not sufficient for NgR signaling. The transfection of NgR in RGC from E7 chick embryos, which normally are insensitive to Nogo-66, results in growth cone collapse. Further, treatment with phosphatidylinositol-specific phospholipase C (PI-PLC) cleaves this protein from the cell surface and abolishes Nogo-66 binding and Nogo-66 induced growth cone collapse.

and NgR. Subsequent binding studies have suggested that OMgp and Nogo-66 bind to overlapping regions of NgR (Fournier et al., 2001; Domeniconi et al., 2002; Fournier et al., 2002; Wang et al., 2002 c). Immediately following, two groups identified MAG as a functional ligand of NgR (Domeniconi et al., 2002; Liu et al., 2002). Both groups independently showed that NgR is necessary and sufficient for MAG inhibition. These results were confirmed by loss of function studies demonstrating that the removal or blockage of NgR abolishes MAG-induced growth cone collapse and promotes neurite outgrowth (Domeniconi et al., 2002; Liu et al., 2002).

Currently, it is not know whether other functional receptors exist for Nogo, MAG and OMgp and it is a challenge to understand how one receptor can bind 3 unrelated ligands and activate similar signaling pathways. Two GPI-linked, neuronal expressed proteins, NgR2 and NgR3, which share extensive sequence homology with NgR, have been recently discovered (He, 2003; Barton, 2003). These NgR related proteins do not bind Nogo, MAG, or OMgp. The biological function of these two proteins remains to be identified, but considering fact that they do not posses binding affinity for any of the 3 myelin-derived growth inhibitory proteins, it is unlikely that NgR2 and NgR3 are

receptor for NgR (Wang et al., 2002b; Wong et al., 2002). Co-immunoprecipitation studies showed that NgR and p75 ^{NTR} form a receptor complex, mediated through the extracellular domain of p75 ^{NTR}. The formation of this NgR/ p75 ^{NTR} receptor complex is enhanced in the presence of myelin-derived growth inhibitory proteins, and is responsible for mediating for neurite outgrowth inhibition (Wang et al., 2002b; Wong et al., 2002).

p75 ^{NTR} was originally identified as a low-affinity receptor for neurotrophins that modulates the activity of TrkA receptors (for review see Kaplan and Miller, 2000; Miller and Kaplan, 2001). Since its initial discovery, p75 ^{NTR} has been implicated in several biological functions (Barker, 2004). Yamashita *et al.* (2002) have shown that neurons isolated from p75 ^{NTR}-deficient mice are insensitive to MAG, and that p75 ^{NTR} co-localizes to sites of MAG binding, although there is no direct interaction between p75 ^{NTR} and MAG. Subsequent signaling studies have demonstrated that the intracellular domain of p75 ^{NTR} mediates the inhibitory effects of myelin-derived growth inhibitory proteins. The deletion of the intracellular domain of p75 ^{NTR} enables neurite outgrowth on MAG, OMgp and myelin substrates, further supporting the role of p75 ^{NTR} as a co-receptor for NgR (Wang et al., 2002b). These results suggest that blocking p75 ^{NTR} signaling would

Recently, a second co-receptor for NgR, termed LINGO-1 (LRR and Ig domain containing, Nogo Receptor -interacting protein) has been identified and shown to act as part of the NgR/ p75 ^{NTR} signaling complex (Mi et al., 2004). LINGO-1 is a nervous system specific transmembrane protein that is highly expressed in brain. LINGO-1 binds to both NgR and p75 ^{NTR}, as indicated by direct binding assays and immunopercipitation experiments. LINGO-1 is required for the inhibitory activities of myelin-derived growth inhibitory proteins, since transfection of dominant negative LINGO-1 prevents the inhibition of neurite outgrowth by OMgp (Mi et al., 2004).

1.9.7 Lipid rafts

The individual components which make-up the NgR signaling complex associate with and are localized to specialized cholesterol- and sphingolipid-rich lipid signaling microdoamins termed lipid rafts (Brown and London, 1998; Simons and Toomre, 2000). It is suggested that binding of myelin-derived growth inhibitory proteins to NgR results in the recruitment of co-receptors to lipid rafts, where most GPI-linked surface molecules, including NgR, are located (Simons and Toomre, 2000; McKerracher and

2002;Vinson et al., 2003). Function blocking with GT1b antibodies prevent MAG-induced growth inhibition, and neurons that lack complex gangliosides, an important component of lipid rafts, are insensitive to MAG (Vyas et al., 2002).

1.9.8 Inhibition of Nogo receptor

The identification of specific ligand binding domains of NgR has led to the development of several NgR antagonists (see Figure 6). Using regions required for high affinity binding, the Strittmatter lab has developed two inhibitors of NgR: NEP1-40, a synthetic peptide containing residues 1-40 from Nogo-66 (GrandPre et al., 2002), and NgREcto, a soluble truncated from of NgR (Fournier et al., 2002). NEP1-40 prevents the binding of Nogo-66 to NgR, therefore blocking its inhibitory effects. After SCI, treatment with NEP1-40 promotes regeneration and functional recovery. Similar to NEP1-40, NgREcto also blocks Nogo-66/NgR interactions. Treatment with NgREcto reverses the inhibitory activity of Nogo-66 and promotes neurite outgrowth of E13 chick dorsal root ganglia in the presence of Nogo-66 (Fournier et al., 2002). The effects of NgREcto *in vivo* NgR have yet to be established

1.9.10 The glial scar

Damage to the CNS initiates a series of cellular and molecular events, which results in the recruitment of glial cells to the lesion site, and ultimately leads to the formation of a glial scar (Fawcett and Asher, 1999). The glial scar is an evolving structure, composed of cellular debris, microglia, meningeal cells, oligodendrocyte precursor cells and reactive astrocytes. It is not fully formed untill approximately 2 weeks after injury in an adult rat, and acts as both a physical and chemical barrier to regeneration (Fawcett and Asher, 1999; McKeon et al., 1991). Reactive astrocytes, the main component of the mature scar, express many different CSPG, such as versican, phosphican, neurocan, agrican, brevican and NG2, which have all been shown to posses growth inhibitory activity (Fawcett and Asher, 1999; McKeon et al., 1999). Meningeal and oligodendrocyte precursor cells also express various growth inhibitory proteins, such as NG2 (Levine, 1994) and semaphorin 3A (Tang et al., 2003). In addition, the invasion of the lesion site by meningeal cells also affects the growth properties of other cells. To date a specific extracellular receptor for CSPG has remained elusive, and the intracellular signaling pathways and molecular mechanisms by which CSPG induces growth

1.9.11 Chondrotin sulfate proteoglycans

CSPG are a family of widely expressed extracellular matrix molecules that are composed of a core protein with covalently attached sulfate glycosaminoglycans (GAG) side chains (Yamaguchi, 2000). In tissue culture studies. reactive astrocytes (Snow et al., 1990;McKeon et al., 1991), astroglial scar (Rudge and Silver, 1990; McKeon et al., 1995) and CSPG substrates (Snow et al., 1996; Winton et al., 2002; Sango et al., 2003) have been shown to inhibit the neurite outgrowth of various classes of cultured neurons. After SCI there is a rapid increase in the expression of CSPG, limiting the capacity for axonal regeneration (Fitch and Silver, 1997; Lemons et al., 1999; Moon et al., 2002). Subsequent immunolabelling studies have indicated that different CSPG are expressed at different times following injury (Jones et al., 2003; Tang et al., 2003). Increases in the levels of versican, neurocan and brevican are detected within days of injury, and peak after 2 weeks. Versican and neurocan levels remain elevated for 4 weeks post injury, whereas brevican levels remain elevated for up to 1 month (Jones et al., 2003). In contrast, phosphocan expression initially decreases after SCI, but increases at later time points, peaking at 2 months post injury (Jones et al., 2003).

transplantation technique in which neurons are experimentally grafted in a way that prevents scaring, Davies et al. (1997) showed that transplanted neurons could extend their axons for long distances on undamaged CNS white matter. Axonal growth was then only blocked when the neurons contacted regions rich in CSPG, suggesting that, in addition to myelin derived growth inhibitory proteins the inhibitory environment of the glial scar also represents a major barrier to CNS regeneration (Davies et al., 1997).

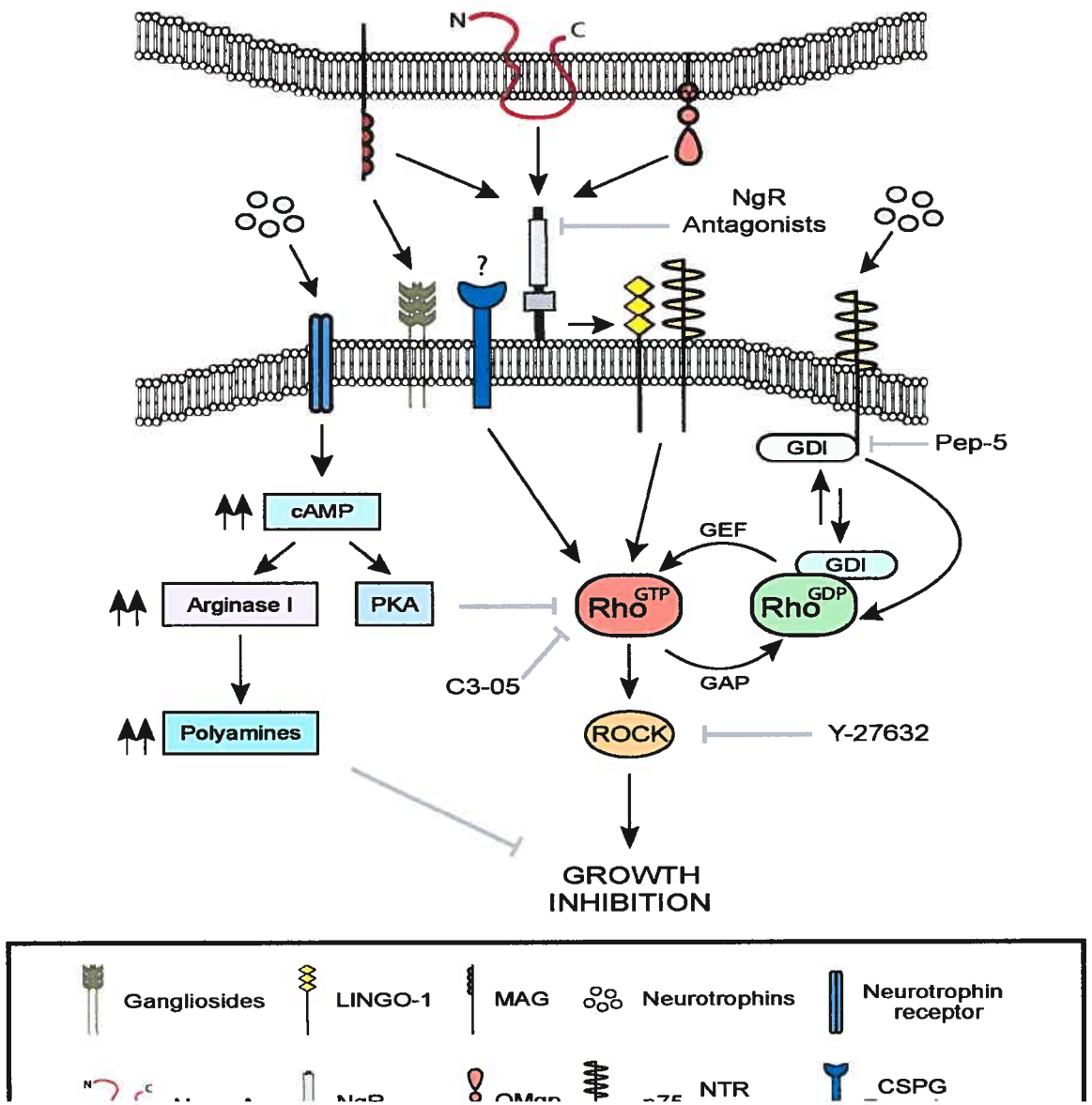
1.10 Rho and growth inhibition

The first indications that Rho might play a key role in growth inhibition originated from studies, showing that the transfection of dominant negative Rho, or the inactivation of Rho by treatment with C3, promotes neurite outgrowth on MAG and myelin substrates (Lehmann et al., 1999). Subsequent biochemical studies have now confirmed that neuronal contact with myelin (Winton et al., 2002), or with any of its inhibitory components (i.e. MAG, Niederost et al., 2002; Nogo-66, Fournier et al. 2003; Amino-Nogo, Niederost et al., 2002; and Omgp, Sivasankaran et al., 2004, results in a rapid increase in Rho activation, occurring as early as 30 minutes after contact (Wint

Further, recent studies provide evidence that CSPG mediate their growth inhibitory activity through the Rho signaling pathway (Sivasankaran et al., 2004). These results demonstrate that the activation of Rho and its downstream effectors are key elements in the cellular response to growth inhibitory proteins (see Figure 6).

1.10.1 Mechanisms of Rho activation by growth inhibitory proteins

The identification of NgR and its co-receptors has led to a greater understanding of the molecular mechanisms and of signaling pathways involved in the cellular response to growth inhibitory proteins and the inhibition of axon regeneration in the adult CNS (for review, see David and Lacroix, 2003). As NgR does not span the plasma membrane it is suggested that it uses its co-receptors, p75 ^{NTR} and LINGO, to mediate its inhibitory activity (Fournier et al., 2001; Wang et al., 2002b; Wong et al., 2002; Mi et al., 2004). Although very little is known about possible intracellular signaling pathways involving the newly discovered LINGO, the over expression of p75 ^{NTR} has been shown to induce Rho activation in neuronal cells. Recently, studies using p75 ^{NTR} knockout mice have suggested an involvement of p75 ^{NTR} in the early activation of Rho after SCI (Dubreuil et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GROWTH
INHIBITION
Gangliosides
LINGO-1
MAG
Neurotrophins
Neurotrophin
receptor
NTR
CSPG

In a follow-up study, Yamashita and Tohyama (2003) have provided evidence that, in neurons p75 ^{NTR} forms a complex with RhoGDI, and that this interaction is enhanced by and is essential for the growth inhibitory activity of MAG and Nogo-A. They have suggested that p75 ^{NTR} binds to and sequesters RhoGDI, allowing for the activation of Rho by a currently unidentified GEF(s). In this study Yamashita and and Tohyama (2003) also identified the 5th alpha-helix region on the intracellular domain of p75 ^{NTR} as the site of interaction between RhoGDI and p75 ^{NTR}, and developed a competitive peptide antagonist, Pep5, to this region. Treatment with Pep5 completely blocked MAG and Nogo-A induced Rho activation and growth inhibition (see Figure 6) (Yamashita and Tohyama, 2003). These results suggest a model in which ligand binding to NgR is responsible for the activation of Rho by p75 ^{NTR}.

1.10.2 Rho signalling and repulsive guidance molecules

Rho GTPases also play an important role in growth cone guidance. Recently, three well-characterized repulsive guidance molecules, ephrins, semaphorins and slits, have been shown to exert their inhibitory effects through the activation of the Rh

1.10.2.1 Ephrins

Ephrins are a large family of guidance molecules for which a role has been demonstrated in determining the intricate pattern of retino-tectal projections. These repulsive guidance cues interact with and signal through the Eph family of tyrosine kinase receptors (Flanagan and Vanderhaeghen, 1998). Using a GTP-bound Rho pulldown assay, Whal *et al.* (2000) have recently shown that the binding of EphrinA results in a robust activation of Rho. Subsequent studies have revealed that Eph receptors interact with ephexin (Eph- interacting exchange factor), a novel RhoGEF, establishing a direct link between Eph receptors and Rho GTPases (Shamah et al., 2001). *In vitro*, ephexin has been found to possess GEF activity for Rho, Rac and Cdc42. However, the binding of EphrinA blocked its activity towards Rac and Cdc42 and stimulated ephexin's activity towards Rho, inducing growth cone collapse (Shamah et al., 2001).

1.10.2.2 Semaphorins

The axon repulsive effects of semaphorins have been well documented in both *in vitro* and *in vivo* studies, and to date more than 20 different members of this family

results in the phosphorylation and inactivation of Rho (Dong et al., 1998; Lang et al., 1996) and, in turn, of Rho kinase. Further, Aizawa et al. (2001) shown that cofilin is phosphorylated in response to Sema 3A treatment and the inhibition of Rho kinase, and that LIM kinase reduces Sema 3A-induced growth cone collapse. In both *Drosophila* and mammalian cells the cytoplasmic domain of the Sema 3A receptor complex binds directly to and sequesters active Rac, preventing it from binding to its downstream effectors. In *Drosophila* plexin, part of the Sema 3A receptor complex associates with DRho and the loss of DRho, blocks the effects of plexin gain of function mutants (Hu et al., 2001). These results suggest that similar to the mechanism used by Ephrins to induce growth cone collapse, Sema 3A blocks Rac signaling and, by a still unknown mechanism, enhances Rho activation. In addition, semaphorins have also been shown to induce neuronal cell death after axotomy (Gagliardini and Fankhauser, 1999; Shirvan et al., 1999; Shirvan et al., 2002).

1.10.2.3 Robo/Slit

Recent evidence has implicated a newly discovered GAP, srGAP, in regulating

1.11 Research objectives

1.11.1 Objective 1

One of the major reasons for the lack of regeneration in the CNS of adult mammals is the presence of growth inhibitory proteins. It is known that the small GTPase Rho is an important intracellular signaling molecule involved in negative signaling pathway triggered by growth inhibitory proteins. The McKerracher lab has previously demonstrated that the inactivation of Rho by C3 promotes neurite outgrowth from PC-12 cells and RGC plated on inhibitory MAG or myelin substrates. In vivo, treatment with C3 stimulates regeneration of RGC after optic nerve microlesion (Lehmann et al., 1999). However, this regenerative response is short-lived and, we suggest, may be due to the lack of permeability of C3. Therefore, we proposed to make a series of recombinant, chimeric C3-like proteins that can freely enter the cell and inactivate Rho. The results of this study are now published under the title: *Characterization of new cell permeable C3-like proteins that inactivate Rho and stimulated neurite outgrowth on inhibitory substrates*, in The Journal of Biological Chemistry, Vol.277, No. 36, pp. 32820-32829, 2002 (see Chapter 2.)

Campenot chambers isolate cell bodies and distal axons of neurons into independent compartments, and allow for the investigation of responses when cell bodies and axons are exposed separately to test compounds (Campenot, 1992). Therefore, to examine the effects of Rho inactivation, on neurite growth, when isolated to specific cellular locations (i.e. cell body vs. distal axons), we cultured SCG in Campenot chambers, treated the various compartments with C3-07 independently, and measured the effect of isolated Rho inactivation on axonal growth after axotomy and in the presence of inhibitory substrates. The results of this study are described in the manuscript entitled *Inactivation of Rho signaling in neuronal cell bodies promoted neurite growth on inhibitory substrates and regeneration of retinal ganglion cell axons in the optic nerve of adult rats*, which is currently in preparation and to be submitted to the Journal of Neuroscience (see Appendix A).

1.11.3 Objective 3

We have shown in mice that the inactivation of Rho by C3 promotes anatomical regeneration and stimulates functional recovery after spinal cord injury, as determined on

treatment on apoptosis after spinal cord injury in both rat and mouse models. The results of this study are reported in the article entitled: *Rho activation patterns after spinal cord injury and the role of activated Rho in apoptosis*, published in The Journal of Cell Biology, Vol.162, No. 2, pp. 233-243, 2003 (see Appendix B).

1.11.4 Objective 4

Recently, Dubreuil et al. (Dubreuil et al., 2003) have demonstrated that Rho is abnormally activated in both neuronal and glial cells after SCI, and that this inactivation of Rho is cell protective. Such results implicate abnormal Rho activation in apoptotic cell death after neuronal trauma (Dubreuil et al., 2003). Myelin is known to activate Rho. (Winton et al., 2002) However, it is not known if myelin-derived growth inhibitory proteins contribute to Rho mediated apoptosis. To investigate if Rho activation by growth inhibitory proteins affects neuronal cell death, we studied the survival of neurons plated on myelin substrates, in the presence or absence of various apoptotic factors. The results of this study are reported in the manuscript entitled *Growth inhibitory proteins contribute to Rho-dependent apoptosis in the presence of TNF-α*, which was submitted for

Chapter 2

2 First Article

« Characterization of new cell permeable C3-like proteins that inactivate Rho and stimulated neurite outgrowth on inhibitory substrates. Matthew J. Winton, Catherine I. Dubreuil, Dana Lasko, Nicole Leclerc and Lisa McKerracher. J Biol Chem 277(36): 32820-9; 2002. »

Characterization of New Cell Permeable C3-Like Proteins That Inactivate Rho and Stimulate Neurite Outgrowth on Inhibitory Substrates

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Running Title: New C3-like Chimeric Proteins

2.1 Summary

The activation state of Rho is an important determinant of axon growth and regeneration in neurons. Axons can extend neurites on growth inhibitory substrates when Rho is inactivated by C3-ADP-ribosyltransferase (C3). We found by Rho-GTP pull-down assay that inhibitory substrates activate Rho. To inactivate Rho scrape loading of C3 was necessary, as it does not freely enter cells. To overcome the poor permeability of C3, we made and characterized five new recombinant C3-like chimeric proteins designed to cross the cell membrane by receptor-independent mechanisms. These proteins were constructed by the addition of short transport peptides to the carboxyl terminal of C3 and tested using a bioassay measuring neurite outgrowth of PC-12 cells plated on growth inhibitory substrates. All five constructs stimulated neurite outgrowth, but with different dose-response profiles. Biochemical properties of the chimeric proteins were examined using C3-05, the most effective construct tested. Gel shift assays showed that C3-05 retained the ability to ADP-ribosylate Rho. Western blots and immunocytochemistry were used to verify the presence of C3 inside treated cells. C3-05 was also effective at promoting neurite outgrowth in primary neuronal cultures, as well as causing the

2.2 Introduction

Rho GTPase regulates the actin cytoskeleton and cell motility in response to extracellular signals. Initial studies using Swiss 3T3 fibroblasts demonstrate the ability of Rho to regulate the formation of actin stress fibers and focal adhesion complexes in non-neuronal cells (1). In neurons, Rho plays a key role in determining the response of axons to growth inhibitory proteins. GTPases have two conformations: a GDP-bound inactive state and a GTP-bound active state (2). The activation of Rho in neurons causes growth cone collapse, neurite retraction and cell body rounding (3-5). Treatment with C3-ADP-ribosyltranferase (C3), a specific inhibitor of Rho, stimulates axon growth and regeneration (6-7). To be effective, this 24 kDa protein must cross the plasma membrane and interact with intracellular Rho, however, C3 does not easily enter cells. To date, various methods have been used to help facilitate the entry of C3 into cells. In experiments using fibroblasts, C3 is microinjected into individual cells (1), whereas in studies using neuronal cells, triturating (8), or scrape loading techniques (7) are used to aid cellular entry. The need for such disruptive methods to inactive Rho by C3, and the inability to treat all cell types with equivalent techniques has limited the use of C3 as a tool for biochemical studies on Rho

independently cross the cell membrane because it requires the presence of the C2 toxin-binding component, C2II, to enter cells (10, 11). Both of these fusion proteins enter cells by receptor-mediated endocytotic pathways, and therefore, may be trapped within vesicles, which may lessen efficient interaction with Rho.

Small peptides can act as carriers by transporting large protein cargo across cell membranes. Such peptides are part of larger proteins that are able to cross biological membranes. A series of different classes of transport peptides exist: 1) the human immunodeficiency virus transcription activator (Tat) contains a region spanning amino acids 37 to 72, which translocates its cargo to both the cytosol and nucleus (12, 13). A shorter Tat sequence, spanning amino acids 48 to 60, is also effective (14). 2) The third helix of the Antennapedia homeodomain (Antp), a Drosophila homeoprotein, possesses the ability to cross biological membranes. Experiments using biotinylated forms of this 16 amino acid peptide have confirmed its ability to penetrate cells and locate in both the cytosol and nucleus (15-17). 3) Prolines are functional participants in some transport peptides (18). Proline residues act as helix breakers and form turn structures within peptides. Peptides rich in proline can form conformations that help in membrane

and a highly basic, arginine-rich sequence corresponding to the reverse Tat sequence (C3-06). We provide evidence that these five new C3-like chimeric proteins all cross the plasma membrane, inactivate Rho, and stimulate neurite outgrowth on inhibitory substrates.

2.3 Experimental procedures

2.3.1 Materials

The pGEX-2T and pGEX-4T vector systems were obtained from Amersham Pharmacia (Baie d`Urfé, Québec, Canada). Oligonucleotides were purchased from Gibco Life Technologies (Burlington, Ontario, Canada), polymerase chain reaction (PCR) was carried out using a DNA Thermal Cycler (Perkin Elmer, Montréal, Québec, Canada), restriction enzymes and T4-DNA ligase were purchased from New England Biolabs (Mississauga, Ontario, Canada) and Gibco Life Technologies (Burlington, Ontario, Canada). DNA sequencing was provided by University of Ottawa Biotechnology Centre (Ottawa, Ontario, Canada) and Bio S&T (Montréal, Québec, Canada). RhoA and Cdc42 antibodies were purchased from Santa CruzBio Technology Inc. (Santa Cruz, California). Secondary

additional sequences to the 3' end, the stop codon was replaced with an EcoRI restriction site by polymerase chain reaction (PCR) using the primers:

5'GAATTCTTTAGGATTGATAGCTGTGCC 3' and

5'GGTGGCGACCATCCTCCAAAA 3'. The PCR, product was cloned into an EcoRV restriction site in the pSTBlue-1 vector (Novagen, Madison, WI). The C3 PCR product was then sub-cloned into pGEX-4T (Amersham Pharmacia, Québec, Canada) using BamHI and EcoRI restriction sites, creating pGEX-4T/C3.

The shorter of the two Tat constructs, corresponding to aa 48-60, was made by annealing the oligonucleotides sequences 5'AATTCTATGGTCGTAAAA

AACGTCGTCAACGTCGTCGTG 3'and 5'GATACCAGCATTTTTTGCAG

CAGTTGCAGCAGCACAGCT 3'. The annealed sequence was ligated into pGEX-4T/C3 at EcoRI and SalI restriction sites. A longer Tat construct (C3-03), corresponding to aa 27 to 72, was made by PCR. The template was plasmid SVCMV-Tat, a generous gift from Dr. Eric Cohen (Department of Microbiology and Immunology, Université de Montréal) and 5'GAATCCAAGCATCCAGGAAGTCAG 3' and 5'TCAGTTCTCCTT

CTTCCACTTCATGCG 3' were used as primers. The PCR product was cloned into an

Antennapedia sequence cloned into pET-3a (pAHI), was a generous gift from Alain Prochiantz (Ecole Normal Supérieure, Paris, France). This vector was used to isolate, by PCR, a 60 amino acid region encoding the full-length homeodomain, using primers: 5'GGAATCCCGCAAACGCGCAAGGCAG 3' and 5'TCAGTTCTCCTTCTT CCACTTCATGCG 3'. The PCR product was cloned into pSTBlue-1 and subcloned into pGEX-4T/C3 using *EcoRI*, creating C3-05. Sequencing of this construct revealed a deletion mutation that altered the primary amino acid sequence, giving a proline-rich sequence resembling fusogenic peptides (23), and thus it was kept and tested. C3-06 was constructed by oligonucleotide sequences that coded for a highly basic and arginine-rich peptide corresponding to the reverse Tat sequence, 5'AATTCAGAAGGAAAC AAAGAAGAAAAGAAGACTGCAGGC 3'and 5' GGCCGCCTGCAGTCTTCTTTTTCTTCTTTGTTTCCTTCTG 3'. These oligonucleotide sequences were annealed and ligated into pGEX-4T/C3, at *EcoRI* and *NotI* restriction sites. Plasmids were transformed into XL-1 blue competent cells except C3-06, in which DH5α competent cells were used. Plasmids were sequenced through the fusion region to the end of the peptide.

induce the production of recombinant protein, and then the cultures were incubated for another 6 hours at 37°C and 250 rpm. The bacteria were pelleted by centrifugation in a GSA rotor (Sorval, Superspeed Centrifuge) at 7000 rpm for 6 minutes at 4°C. Each pellet was re-suspended in 10 ml of buffer A (50 mM Tris, pH 7.5, 50 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT plus PMSF (1 mM). All re-suspended pellets were pooled and transferred to a 100 ml plastic beaker on ice. The bacterial suspension was sonicated for 6 x 20 seconds, on ice, using a Branson Sonifier 450 probe sonicator (VWR, Québec, Canada). The lysate was centrifuged twice in a Sorvall SS-34 rotor at 16,000 rpm for 12 minutes at 4°C to clarify the supernatant. Glutathione-agrose beads (Sigma, Oakville, Ontario, Canada) were added to the cleared lysate and the preparation was placed on a rotator for 2-3 hours. The beads were washed 4 times with Buffer B, (Buffer A + 100 mM NaCl) and 2 times with Buffer C (Buffer B + 2.5mM $CaCl_2$). The final wash was removed until a thick slurry was created, and 20 U of thrombin (Calbiochem, San Diego, California) was added and the beads were shaken overnight at 4°C. The beads were loaded into an empty 20 ml column and 1 ml aliquots were collected after elution with PBS. The fractions containing the protein peak were pooled. To remove the thrombin form the protein sample, 100 µl of p-

Mississauga, Ontario, Canada). Purity of the sample was determined by SDS-PAGE, and confirmed by western blot with anti-C3 antibody (eg. Fig.1).

2.3.4 Cell culture

PC-12 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) with 10% horse serum (HS), 5% fetal bovine serum (FBS), 1% penicillin-streptomycin (P/S), and differentiated by addition of 50 ng/ml of nerve growth factor (NGF). One day prior to use, 8 well chamber Lab-Tek slides (Nunc, Naperville, IL) were prepared by incubating 150 µl of poly-l-lysine (0.025 µg) (Sigma, Oakville, Ontario, Canada) for 4 hours and then drying 8g of myelin overnight in the laminar flow hood. To detach cells for plating, 2 ml of trypsin-EDTA (0.05%) was added and approximately 7500 cells were plated on the coated chamber slides. The cells were left at 37°C for 3-4 hours to allow them to settle. After the cells had adhered to the test substrate, the media was aspirated and fresh DMEM with 1% FBS, 1% P/S, 50 ng/ml of NGF, together with the appropriate amount of the test C3-like chimeric proteins were added. After 24 hours, the cells were fixed with 4% paraformaldehyde, 0.5% glutaraldehyde in 0.1 M phosphate buffer. For control

were collected with a 20X objective using a Zeiss Axiovert microscope (Oberkochen, Germany). The percent of cells with neurites of at least one cell body diameter in length were counted, and the longest neurite per cell was measured using Northern Eclipse Data analysis (Empix Imaging, Mississauga, Ontario, Canada) and statistical unpaired student-t tests were completed with Microsoft Excel (Microsoft) and Sigmaplot.

NIH 3T3 and CHO cells were grown in DMEM with 10% FBS and 1% P/S. To detach the cells for plating 2ml of trypsin-EDTA (0.05%) was added and approximately 5000 cells were plated on the 8 well chamber slides. The cells were left at 37°C for 3-4 hours to allow them to settle. After the cells had adhered to the slides, the media was aspirated and fresh DMEM with 10% FBS, 1% P/S, together with varying concentrations of C3-05 was added. In experiments where Rho was activated by the addition of LPA, the cells were plated on 8 well chamber slides at a concentration of approximately 5000 cells per well, and then serum starved for 24 hours prior to treatment. The cells were then stimulated with LPA (100nM) for 15 minutes (24). The media was then aspirated and fresh DMEM with 1% P/S, together with 0.0025 μg/ml of C3-05 was added. After 24 hours the cells were fixed in 4% paraformaldehyde, 0.1 M phosphate buffer, and stained with

To culture retinal ganglion neurons (RGCs), retinas were removed from postnatal day zero (P0) rat pups, and the cells were dissociated with 12.5 U/ml Papain in Hanks balanced serum solution (HBSS), 0.2 mg/ml DL-cysteine and 20 ug/ml bovine serum albumin (BSA). The dissociated cells were washed, added to DMEM with 10% FBS, 1% P/S, 50 ug/ml BDNF and plated on inhibitory substrates, myelin (4 μg) or CSPG, in the presence or absence of C3-like chimeric proteins for 24 hours. The CSPG substrate (Chemicon,Temecula, California) was plated at a concentration of 0.001 μg in PBS and incubated at 37°C overnight.

2.3.5 Immunocytochemistry

A polyclonal anti-C3 antibody was made by injecting a rabbit subcutaneously with 0.5 mg of C3 protein in Freund's complete adjuvant and the rabbit was boosted four times in incomplete Freund's adjuvant with 0.5 mg C3. Blood samples were tested until a high antibody titter was reached and then the serum was collected, purified and verified by ELISA (Sheldon Biotechnology Centre, McGill University). The specificity of the antibody was tested by western blot (Fig. 1). PC-12 cells were grown and plated on myelin coated

2.3.6 Western blots

PC-12 cells treated for 24 hours with 25 μg/ml of C3, by scrape-loading, or 10 μg/ml of C3-05, added directly to the media, were washed twice with cold TBS, and lysed in 20mM Tris pH 8, 125 mM NaCl, 10% glycerol, 1% NP40, 1 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 μg/ml pepstatin. CHO and NIH 3T3 cells were treated with 10 μg/ml of C3-05 and cell lysates were prepared as described above. Lysates were clarified by centrifugation and 10 μg of protein was separated on 12% acrylamide gels. After transfer to nitrocellulose, the membranes were either blocked with TBS containing 0.1% Tween 20 (TBS-T) and 3% BSA and incubated in blocking buffer with an anti-RhoA antibody (1:1000) (Santa Cruz, Santa Cruz, California), or blocked with 5% powered milk and incubated in blocking buffer with an anti-C3 antibody (1:4000). The signals were revealed by an HRP-based chemiluminescent reaction (Pierce, Rockford, IL). Membranes probed with anti-RhoA antibody were stripped and re-probed with an anti-Cdc42 antibody (1:1000) (Santa Cruz, Santa Cruz, California).

2.3.7 Pull down assays to detect Rho-GTP

and lysed in RIPA buffer (50 mM Tris pH 7.2, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 500 mM NaCl, 10 mM $MgCl_2$, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM PMSF. Cell lysates were clarified by centrifugation at 13,000 g for 10 minutes at 4°C and incubated for 50 minutes at 4°C with GST- Rho binding domain (RBD) (a generous gift from John Collard, Division of Cell Biology, Netherlands Cancer Institute) coupled to glutathione-agarose beads (Sigma, Oakville, Ontario, Canada). The beads were washed 4 times with cold Tris buffer containing 1% Triton X-100, 150 mM NaCl, 10 mM $MgCl_2$, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 0.1 mM PMSF and eluted in sample buffer containing 40 mM DTT. Bound Rho-GTP eluted from the beads and total Rho from the original cell lysate was detected by western blot analysis using a monoclonal anti RhoA antibody (1:1000 in TBS-T, Santa Cruz). The quantification of Rho inactivation by C3-05 was by densitometry using IQ MAC 1.2 software (Molecular Dynamics, Sunnyvale, Ca). Densitometry values for untreated cells plated on myelin were normalized to correspond to100% Rho activation. C3-05 treated cells were calculated as the percent inactivation compared to the normalized values.

2.4 Results

We chose to test a number of different strategies to design C3-like chimeric proteins that could cross the plasma membrane by receptor independent mechanisms. Five C3-like chimeric proteins were constructed by adding DNA sequences encoding known membrane translocating peptides derived from Tat (C3-02, C3-03), Antennapedia (C3-04), a proline-rich fusogenic peptide (C3-05) and a basic, arginine-rich peptide (C3-06) to the 3' end of the C3 cDNA. All five cDNAs encoding the C3-like chimeric proteins were expressed as GST fusion proteins in *E.coli*, purified, and their molecular weights verified by SDS-PAGE gel (eg. Fig.1A). To confirm the presence of C3 in all five constructs, western blots using a polyclonal antibody raised against C3 were completed (eg. Fig.1B).

PC-12 cells typically extend neurites in response to NGF, but when plated on inhibitory substrates, this outgrowth is inhibited and the cells remain round (7). The ability to inactivate Rho and promote neurite outgrowth on inhibitory substrates was used as a bioassay to test the effectiveness of the new C3-like chimeric proteins. First, we examined the dose-response profile of unmodified C3. In previous experiments to inactivate Rho, we determined that scrape-loading was necessary to treat PC-12 cells with C3 (7). We found

any mechanical disruption of the cells. To establish the effective concentration ranges, preliminary experiments included a test concentration of 0.00025 μg/ml were completed, but none of the C3-like chimeric proteins were effective at this dose. Surprisingly, concentrations of 0.0025 μg/ml of C3-03, C3-05, and C3-06 lead to significant increases in both the number of cells extending neurites and the length of neurites compared to cells plated on myelin without treatment (Fig. 3A-B). This effective dose is 10,000-fold lower than that required with unmodified C3 using scrape-loading techniques (Fig. 2). At the highest concentrations tested, C3-03 was not effective, perhaps because of toxicity associated with the Tat sequence (14). C3-02 and C3-04 promoted significant neurite outgrowth at concentrations of 0.25 μg/ml and 2.5 μg/ml respectively and an increase in neurite length was observed at concentrations between the range of 0.025 μg/ml and 2.5 μg/ml (Fig.3A-B). At all concentrations, C3-05 and C3-06 stimulated neurite outgrowth on myelin substrates, with C3-05 giving the best results. These results indicated that all of the new C3-like proteins had some capacity to penetrate cells, inactivate Rho, and promote neurite outgrowth on inhibitory substrates. As C3-05 gave the best results, it was used for further testing.

re-probed the same blots for another member of the Rho GTPase family, Cdc42 (Fig. 4, bottom). Cdc42 did not show any change in mobility after treatment with C3-05, demonstrating that C3-05 maintains the same ADP-ribosylation specificity as unmodified C3.

We compared by immunocytochemistry the ability of C3 and C3-05 to enter cells and ADP-ribosylate Rho. PC-12 cells were plated on myelin substrates and treated with C3, C3-05, or left untreated. The cells were washed twice in cold TBS before preparing cell lysates. The proteins were separated on 12 % polyacrylamide gels, transferred to nitrocellulose and probed with a polyclonal anti-C3 antibody. A 27-kDa band was observed in the lysates of PC-12 cells treated with C3-05 (Fig. 5A). No band was detected in PC-12 cells treated with unmodified C3, indicating that no detectable amount of unmodified C3 was able to penetrate PC-12 cells without scrape loading. CHO and NIH 3T3 cells were incubated with 10 μg/ml of C3-05 for 24 hours, or left untreated. C3-05 was clearly detected in the cell lysates of both fibroblast cell lines tested (Fig. 5B). By immunocytochemistry of PC-12 cells plated on myelin, we observed no intracellular staining with unmodified C3, but staining was visible when cells were treated with C3-05

formed actin stress fibers were present (Fig. 6). In both cell lines the addition of C3-05 at concentrations as low as 0.0025 μg/ml dramatically reduced actin stress fiber formation. At concentrations of 0.025 μg/ml, or 0.25 μg/ml disassembly of actin stress fibers were almost complete. At 25 μg/ml, the highest concentration tested, CHO cells treated with C3-05 showed an altered morphology (Fig. 6).

The serum component lysophosphatidic acid (LPA) is a strong activator of Rho (25). Serum starved fibroblasts have very few visible stress fibers, however, the stimulation of such cells by LPA produces an intense network of actin stress fibers (1). To test if C3-05 is effective in reversing Rho activation by extracellular stimuli in fibroblasts, we stimulated serum starved CHO and NIH 3T3 cells with LPA (100nM) and then treated them with 0.0025 μg/ml of C3-05. The cells were stained with phalloidin to visualize the formation of actin stress fibers (Fig. 7A). In both cell lines, serum starved cells in the absence of LPA displayed a limited number of stress fibers. In contrast, when Rho was activated by LPA stimulation a dramatic increase in the formation of stress fibers occurred. The treatment of LPA stimulated cells with 0.0025 μg/ml of C3-05 resulted in a substantial decrease in the formation of actin stress fibers visualized by phalloidin stain (Fig. 7A). Therefore C3-05

to the serum starved controls (Fig. 7B). Treatment of the LPA stimulated cells with 0.0025 μg/ml C3-05 prevented the LPA-induced augmentation of focal contacts (Fig. 7B).

Rho activity can be measured using GTP-bound Rho pull down assays. When cells are plated on myelin substrates, an increase in the amount of active Rho was observed compared to cells plated on poly-l-lysine (Fig. 8A). When PC-12 cells were plated on myelin and treated with 0.0025 μg/ml, 0.025 μg/ml, and 0.25 μg/ml of C3-05, there was a dramatic reduction in the levels of active Rho. Treatment with C3-05 decreased the amount of active Rho below levels observed from PC-12 cells plated on growth promoting poly-l-lysine substrates (Fig. 8A). To determine the duration of this C3-05 induced Rho inactivation, PC-12 cells plated on growth inhibitory substrates were incubated with C3-05 and lysed at different time points after the addition of C3-05. Rho activation was completely reversed at 0.025 μg/ml and 0.25 μg/ml after 24 hours. At all concentrations tested, the ability of C3-05 to inactivate Rho peaked at 24 hours. Rho inactivation began to diminish after 36 hours with the complete activation of Rho regained in cells treated with 0.0025 μg/ml at 48 hours (Fig. 8B). Cells treated with all three concentrations maintained their differentiation state for at least 48 hours after treatment (data not shown). These

0.0025 μg/ml, 0.025 μg/ml and 0.25 μg/ml for 24 hours decreased Rho activation (Fig. 8C-D). These results confirm that C3-05 inactivated Rho in fibroblasts.

To further support the ability of C3-like chimeric proteins to promote neurite outgrowth on inhibitory substrates, we examined the response of primary cultures plated on inhibitory substrates to C3-05 treatment. Purified retinal ganglion cells (RGCs) were plated on myelin, or CSPG substrates and treated with varying concentrations of C3-05 for 24 hours. During the RGC dissection great care was taken in order to try to limit the amount of mechanical manipulation of the cells, however, the isolation protocol requires that some triturating take place in order to dissociate and separate the cells. When RGCs are plated on inhibitory substrates, they maintained a similar round appearance to PC-12 cells plated on myelin. Treatment of RGCs with C3-05 promoted neurite outgrowth and increased neurite length on both myelin and CSPG substrates (Fig. 9A-F). In contrast to the wide range of concentrations shown to be effective in experiments with PC-12 cells, a narrower range of C3-05, 0.025 μg/ml to 50 μg/ml, promoted neurite outgrowth and increased neurite length on myelin (Fig. 9A-D). In the case of RGCs plated on CSPG effective concentration ranges of 0.0025 μg/ml to 50 μg/ml were observed (Fig. 9A,B, E,F).

which must be scrape loaded, we suggest that the ability of these new C3-like proteins to promote neurite outgrowth at such low concentrations is due to their increased cellular permeability. The differences observed in promoting neurite outgrowth between the five C3-like chimeric proteins might result from the different methods used by the various transmembrane carrier peptides to enter cells.

We tested two different transport sequences derived from the Tat protein of the human immunodeficiency virus (HIV). This protein has been reported to enter cells, carry protein cargo into cells, and even cross blood brain barrier (13). The mechanism whereby Tat transports cargo across the plasma membrane is still not completely understood. Tat internalization is not decreased at 4 °C, or in the presence of endocytosis inhibitors (14). There is uncertainty, however, whether its uptake is receptor mediated because Tat binds to specific cell membrane proteins (28). We found that C3-03, the longer Tat peptide sequence, was more efficient at promoting neurite outgrowth then C3-02, the shorter Tat peptide. However, the longer sequence may have some cellular toxicity (14), a finding consistent with the decreased ability to promote neurite growth at high concentrations (Fig. 3).

translocating ability when they bind to double stranded DNA (30). We found that C3-04 containing the Antp sequence was an effective carrier, but only within a narrow concentration range (Fig. 3).

Proline-rich peptides can also act as receptor independent delivery peptides. Fusogenic peptides contain both hydrophobic and hydrophilic amino acids, which form amphiphilic α-helical structures. A critical component of these proteins are proline residues (19, 23). Studies where site-directed mutation changing single proline residues of the PH-30α fusogenic protein, active in sperm-egg fusion, shows that prolines are critical for the fusogenic activity (23, 31). The membrane translocating sequence (MTS) of Kaposi fibroblast growth factor, a known transport peptide, contains 3 proline residues spaced 5 to 7 amino acids apart (21). The spacing of prolines in this MTS peptide are similar to that in C3-05, which also possesses 3 prolines spaced 6 to 8 amino acids apart. Furthermore, these proline residues may explain why C3-05 was the most effective C3-like chimeric protein tested. When proline residues were added to Antp translocating sequences, Antp and its cargo were only present in the cytoplasm and not in the nucleus (32). The possibility that proline residues may restrict the membrane translocating peptides and their cargo to the

transport sequence than either Tat, or Antennapedia. C3-06 contains three arginine residues at the amino end of the peptide end, compared to the Tat translocation peptide used in C3-02, which contains one arginine, and two lysine residues at the amino terminal. The increased ability of C3-06 to penetrate PC-12 cells and promote neurite outgrowth suggests that N-terminal residues are important for cellular uptake. Furthermore, arginine residues are more effective then lysine residues in inducing cellular uptake (20).

Neurite outgrowth and neurite length profiles of PC-12 cells treated with C3-02 and to a lesser degree C3-03 and C3-04 had a normal distribution, (Fig.3) showing increased neurite outgrowth when cells were treated with low to moderate concentrations, but not with high concentrations. High concentrations of C3-02, C3-03 and C3-04 may have toxic effects on PC-12 cells. Previously, two other permeable C3 fusion proteins have been produced, one with Diphtheria-toxin B subunit, called DC3B (9) and another that is a C2 toxin-C3 fusion protein, called C2IN-C3 (10). Our three most effective C3-like chimeric proteins, C3-03, C3-05, and C3-06 all worked at a much lower dose than DC3B (0.6 μg/ml) (9) and C2IN-C2 (0.2-0.3 μg/ml) (10), being effective at 0.0025 μg/ml. The lower effective dose of our C3-like proteins might be because they enter cells by receptor-

When neuronal cells are plated on myelin they become round and do not grow neurites (7). Previously, we have suggested that myelin-derived growth inhibitory proteins directly activate Rho (33). Here we demonstrate the first evidence that inhibitory substrates activate Rho. GTP-bound Rho assays showed that myelin alone activated Rho when compared to cells plated on poly-l-lysine substrates (Fig. 7). Cells plated on myelin showed a 4 to 5-fold increase in cellular active Rho compared to cells plated on poly-l-lysine (Fig. 7). Treatment with C3-like chimeric proteins not only reversed the myelin induced Rho activation, but sustained this decrease for 36 to 48 hours. For all 3 concentrations tested, peak Rho inactivation appeared 24 hours after treatment, and then began to decrease. Possibly, the decrease occurred because the C3-like chimeric proteins were no longer active, or had all been taken up. We did not test whether the addition of more C3-05 to the culture media could sustain Rho inactivation for longer periods.

In previous *in vivo* experiments using unmodified C3, a robust period of regeneration of retinal ganglion cells in the optic nerve was observed after treatment (7). Future studies will address the ability of these new C3-like proteins to help axon regeneration and repair after CNS injury. These C3-like chimeric proteins, therefore, may

and inflammatory breast cancer (34, 37, 38). In culture, fibroblasts transfected with active Rho develop alterations in morphology and grow at higher densities than untransfected cells (35, 39). The regulation of cell proliferation by active Rho in fibroblasts is inhibited by C3 treatment. This inhibition of cell proliferation is evident one day after treatment with C3, correlating with the ADP-ribosylation of Rho (41). Other studies suggest Rho plays a role in tumor metastasis. In contrast to neuronal cells, where Rho activation inhibits cell motility (Fig. 3C), Rho activation in tumor cells can increases invasiveness and motility of cells. Experiments with metastatic tumors induced in mice show that the RhoC gene is over expressed compared to the expression profiles of other genes as determined by DNA array analysis (40). The transfection of dominant negative Rho in highly metastatic human tumor cells suppresses the number of metastatic tumors observed in mice (Clark et al., 2000). The C3-like chimeric proteins described here may prove useful therapeutically as several experiments have demonstrated that the inactivation of Rho has a role in limiting abnormal cell growth and metastasis (40-42).

The characterization of five C3-like chimeric proteins demonstrates biochemical properties similar to those seen in previous experiments using C3 (7). Addition of the

used without disruptive methods, resulting in possible therapeutic roles in the regulation of tumor formation in cancers and the regeneration of neurons after traumatic injury.

2.6 References

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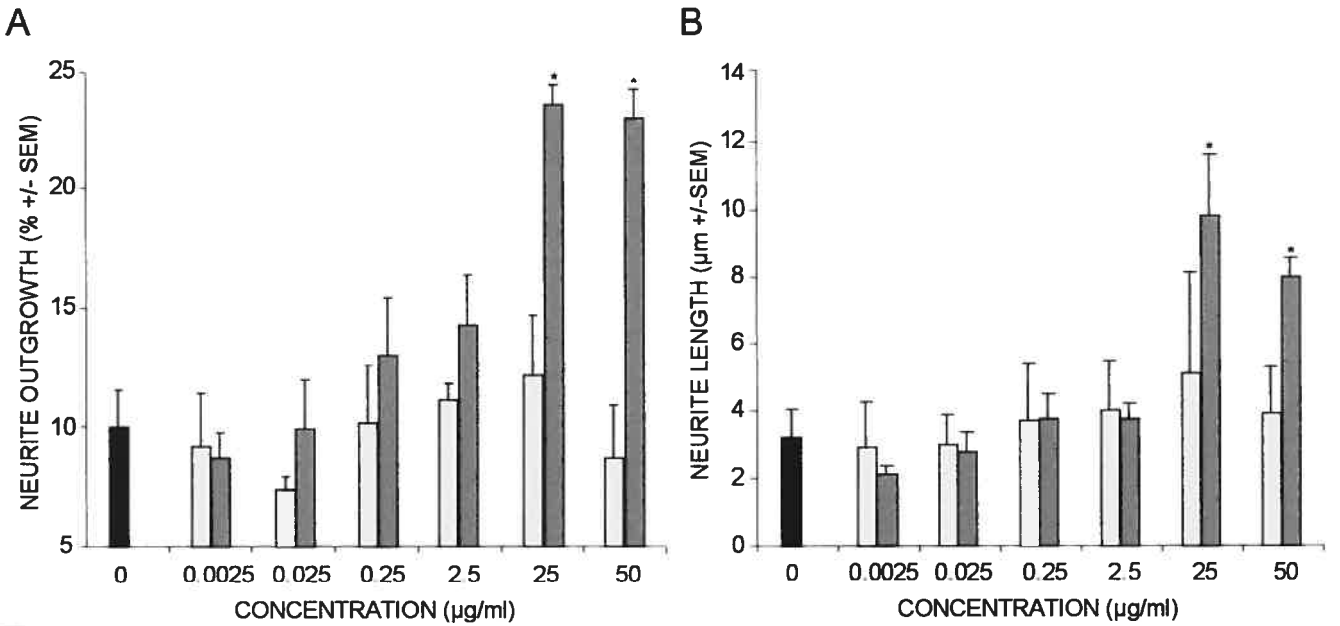
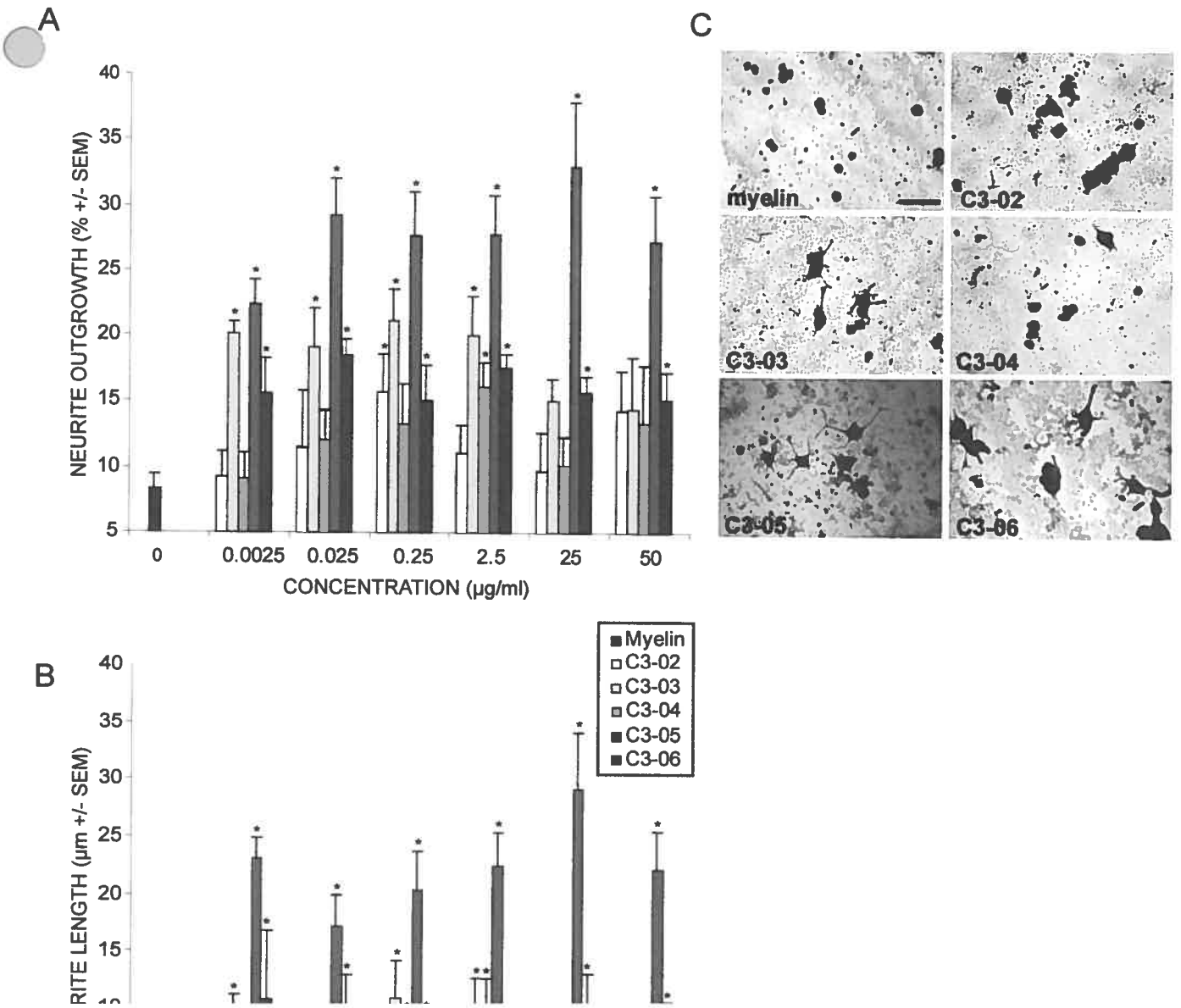
A
LYSATE 1
LYSATE 2
GST-TAG CLEAVAGE
PURIFIED PROTEIN
B
LYSATE 1
PURIFIED PROTEIN


A
NEURITE OUTGROWTH (% +/- SEM)
25
20
15
10
5
0
0.0025
0.025
0.25
2.5
25
50
CONCENTRATION (µg/ml)
B
NEURITE LENGTH (µm +/-SEM)
14
12
10
8
6
4
2
0
0
0.0025
0.025
0.25
2.5
25
50
CONCENTRATION (µg/ml)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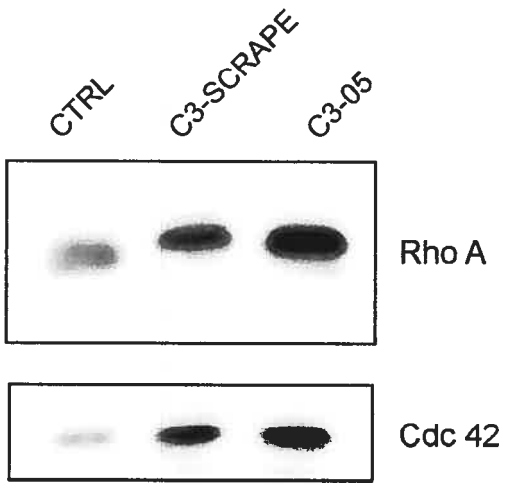
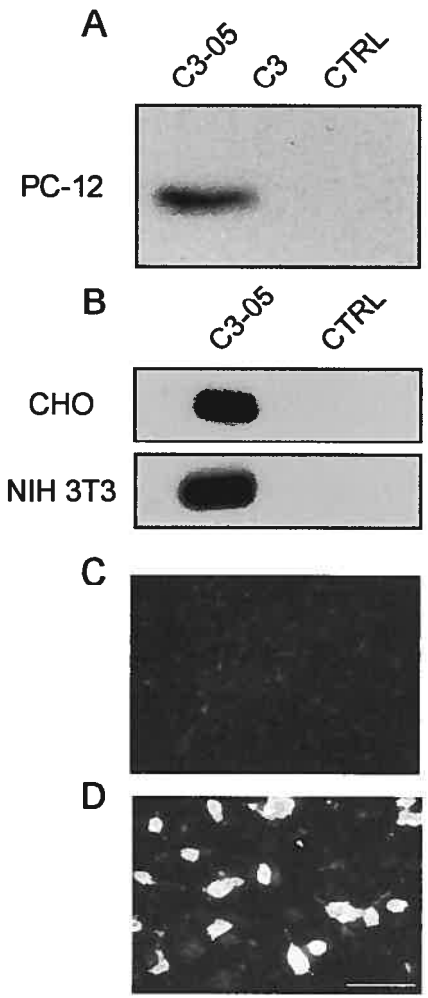
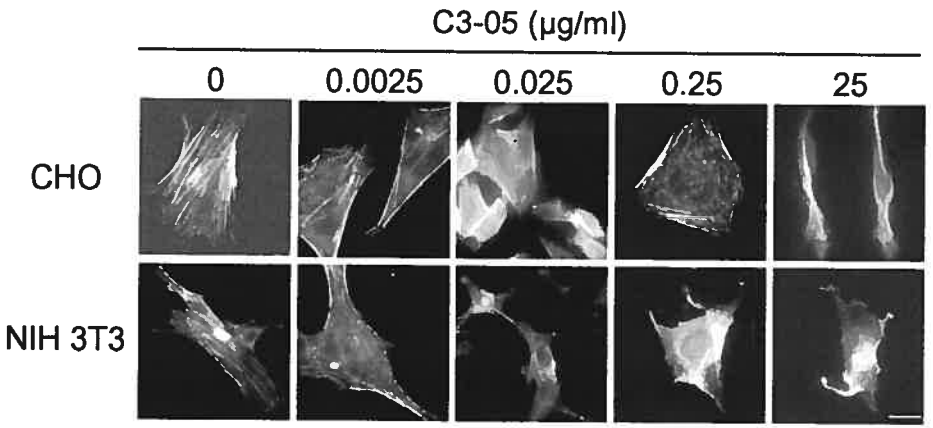
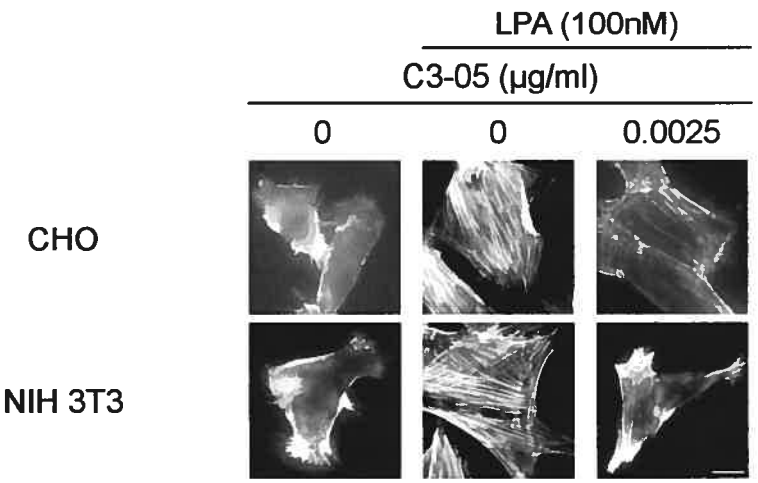
35
30
25
20
15
10
5
0
0.0025
0.025
0.25
2.5
25
50
CONCENTRATION (µg/ml)
B
RITE LENGTH (µm +/- SEM)
Myelin
C3-02
C3-03
C3-04
C3-05
C3-06
C
myelin
C3-02
C3-03
C3-04
C3-05
C3-06


CTRL
C3-SCRAPE
C3-05
Rho A
Cdc 42

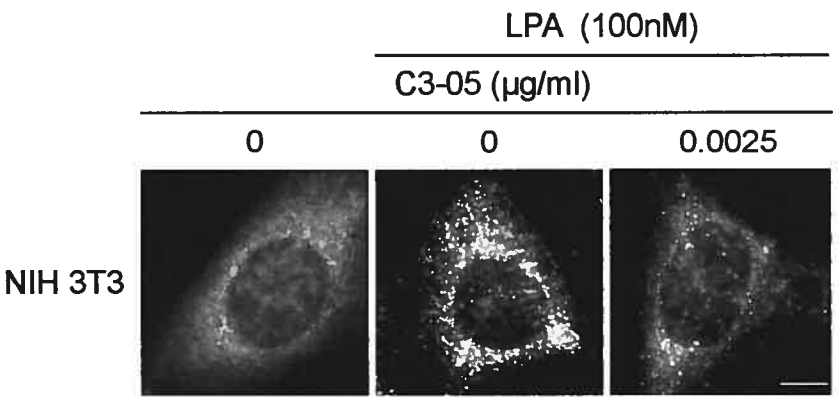

A
C3-05
C3
CTRL
PC-12
B
C3-05
CTRL
CHO
NIH 3T3
C
D


C3-05 (μg/ml)
0
0.0025
0.025
0.25
25
CHO
NIH 3T3

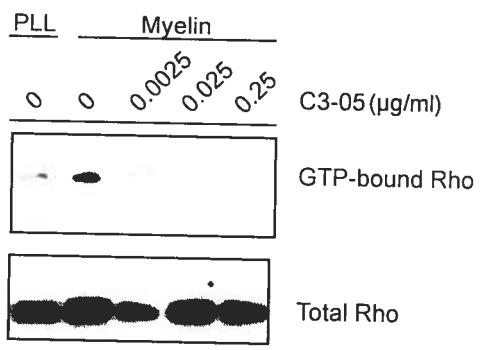
A


LPA (100nM)
C3-05 (μg/ml)
0
0
0.0025
CHO
NIH 3T3

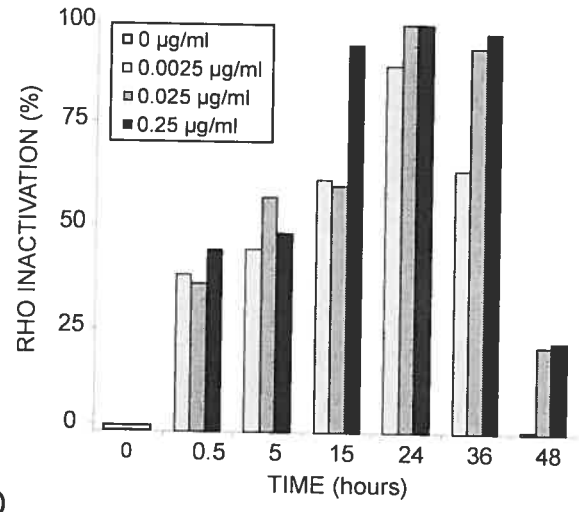
B


LPA (100nM)
C3-05 (μg/ml)
0
0
0.0025
NIH 3T3

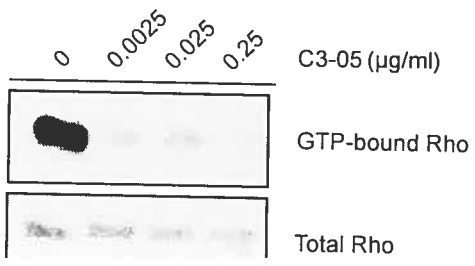
A


PLL
Myelin
0
0
0.0025
0.025
0.25
C3-05 (µg/ml)
GTP-bound Rho
Total Rho

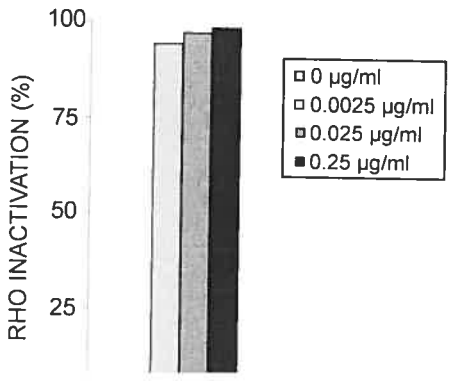
B

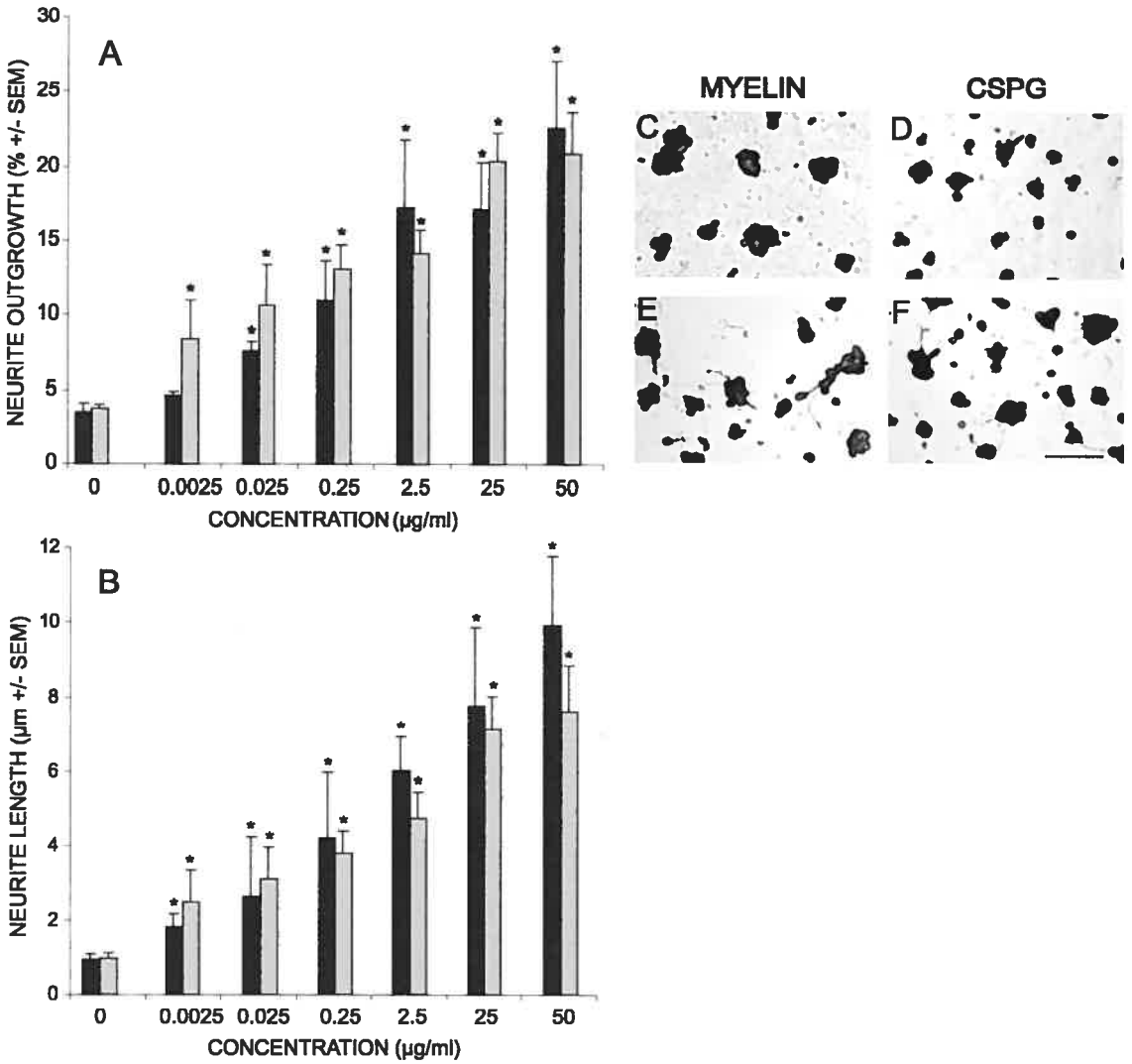

0 µg/ml
0.0025 µg/ml
0.025 µg/ml
0.25 µg/ml
RHO INACTIVATION (%)
100
75
50
25
0
0
0.5
5
15
24
36
48
TIME (hours)

C


0
0.0025
0.025
0.25
C3-05 (µg/ml)
GTP-bound Rho
Total Rho

D


0 µg/ml
0.0025 µg/ml
0.025 µg/ml
0.25 µg/ml
RHO INACTIVATION (%)
100
75
50
25


A
NEURITE OUTGROWTH (% +/- SEM)
30
25
20
15
10
5
0
0
0.0025
0.025
0.25
2.5
25
50
CONCENTRATION (µg/ml)
B
NEURITE LENGTH (µm +/- SEM)
12
10
8
6
4
2
0
0
0.0025
0.025
0.25
2.5
25
50
CONCENTRATION (µg/ml)
MYELIN
CSPG
C
D
E
F

2.7 Figure legends

Fig. 1. **Purification of recombinant C3.** C3 was expressed as a GST fusion protein and purified from bacterial lysates by affinity chromatography. *A*, protein samples from the bacterial lysate (*LYSATE*) and the protein after purification and filter sterilization (*PURIFIED PROTEIN*). *B*, Western blot of samples of cell lysates (*LYSATE*) and purified protein (*PURIFIED PROTEIN*) probed with a C3 antibody.

Fig. 2. **C3 promotes neurite growth on inhibitory substrates only after scrape loading.** PC-12 cells plated on myelin substrates, were treated with C3 by scrape loading (*dark gray bars*), or by its addition directly into the culture media (*light gray bars*). Concentrations of C3 between 0.0025 μg/ml and 50 μg/ml were tested (*x-axis*). After 24 hours cells were fixed, stained and counted. *A*, the percentage of cells that extended neurites longer then 1 cell body diameter (*neurite outgrowth*); *B*, the length of the longest neurite per cell (*neurite length*). The average counts are shown for four or more experiments each performed in duplicate. Error bars represent the SEM, * is statistically significant ($p \leq 0.05$).

more experiments performed in duplicate +/- the SEM. * indicates significance differences compared to untreated cells ($p \leq 0.05$). *C*, micrographs showing PC-12 cells treated at the lowest concentration of the test C3-like proteins (*0.0025 μg/ml*). *Scale bar*, 50 μm.

Fig. 4. **ADP-ribosylation of Rho in PC-12 cells.** PC-12 cells plated on myelin substrates, were untreated (CTRL), scrape loaded with 25 μg/ml unmodified C3 (C3-SCRAPE), or treated with 10 μg/ml C3-05 added directly to the media, and incubated for 24 hours. *Top*, western blot of cell lysates probed with an anti-RhoA antibody. ADP ribosylation causes a shift in the apparent molecular weight of RhoA (Lehmann et al., 1999). *Bottom*, the same membrane was stripped and re-probed with an anti-Cdc42 antibody.

Fig. 5. **Transmembrane delivery of C3-05 into PC-12, CHO and NIH 3T3 cells.** *A*, western blot of PC-12 cell lysates prepared from cells treated with 10 μg/ml of C3-05 (*C3-05*), 10 μg/ml of C3 (*C3*), and untreated cells (CTRL) and probed with a polyclonal anti-C3 antibody. *B*, western blots of CHO and NIH 3T3 cell lysates prepared from cells treated with 10 μg/ml of C3-05 (*C3-05*) and untreated cells (*CTRL*) and probed with a polyclonal

to the media of pre-plated cells. All experiments were performed in duplicate at least 3 separate times. Micrographs show individual cells representative of the total cell population observed. *Scale bar*, 50 μm.

Fig. 7. **C3-05 reverses LPA induced stress fibers and focal adhesions.** *A*. Serum starved CHO and NIH 3T3 cells were stimulated with 100 nM of LPA, treated with 0.0025 μg/ml C3-05 for 24 hours and stained with phalloidin to visualize stress fiber formation. *B*, NIH 3T3 cells were stimulated with 100 nM of LPA, treated with 0.0025 μg/ml of C3-05 and immunostained with an anti-vinculin antibody. Three experiments were performed in duplicate. Micrographs show individual cells representative of the total cell population observed. *Scale bar,* 50 μm.

Fig. 8. **Inactivation of Rho by C3-05.** *A*, assay for cellular GTP-bound Rho. PC-12 cells were plated on poly-l-lysine (*lane 1)*, or myelin, (*lanes 2-5)* substrates with increasing concentrations, between 0.0025 μg/ml and 0.25 μg/ml, of C3-05 for 24 hours. The cells were washed, lysed, and GTP-bound Rho was isolated by pull-down assay. GTP-bound

Fig. 9. **C3-05 promotes neurite outgrowth from retinal neurons plated on inhibitory myelin or CSPG substrates.** Retinal neurons plated on myelin substrates, (*black bars)*; or CSPG substrates, (*gray bars)*, were treated with increasing concentrations, between 0.0025 μg/ml and 50 μg/ml, of C3-05 for 24 hours. *A*, the percentage of cells with neurites longer than 1 cell body diameter (*neurite outgrowth*); *B*, the length of the longest neurite per cell (*neurite length*). The data represents the average of four or more experiments performed in duplicate +/- the SEM. * indicates significance differences compared to untreated cells ($p \leq 0.05$). *C-F*, micrographs of retinal neurons plated on (*C, E*) myelin, or (*E, F*) CSPG substrates. *C-D*, Controls without treatment do not grow neurites. *D-F*, 2.5 μg/ml of C3-05 added to the culture media. *Scale bar*, 50 μm.

Chapter 3

3 Second Article

« Growth inhibitory proteins contribute to Rho-dependent TNF-α induced apoptosis. Matthew J. Winton, Catherine I. Dubreuil, and Lisa McKerracher. Submitted to Nature Neuroscience . »

Growth inhibitory proteins contribute to Rho-dependent apoptosis in the presence of TNF-α

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Département de pathologie et biologie cellulaire, Université de Montréal, Montréal Québec, H3T 1J4,

3.1 Abstract

Rho is an intracellular signaling molecule that is activated when neurons come in contact with growth inhibitory proteins found in central nervous system (CNS) myelin. After CNS injury, apoptotic neurons have high Rho activation levels, but cell death resulting from neuronal contact with myelin has not been reported. Here we show that myelin enhances cell death in the presence of tumor necrosis factor-alpha (TNF-α), a pro-inflammatory cytokine known to induce neuronal apoptosis *in vitro* and *in vivo*. Myelin increased the level of apoptosis in PC-12 cells and primary neurons treated with TNF-α, and this increase in cell death was blocked by treatment with Rho antagonist (C3-07), Nogo receptor competing peptide (NEP1-40), or AMPA receptor antagonist (CNQX). Treatments that improved cell survival reduced Rho activation levels. Combined contact with myelin and TNF-α may contribute to neuronal death and excitotoxicity in trauma and neurodegenerative disease.

3.2 Introduction

It is now well established that one major reason for the lack of regeneration after trauma in the central nervous system (CNS) is the growth inhibitory environment of myelin (McKerracher and Winton, 2002). To date, three separate myelin-derived growth inhibitory proteins have been discovered: myelin associated glycoprotein (MAG) (McKerracher et al., 1994b; Mukhopadhyay et al., 1994), Nogo-A (Chen et al., 2000; GrandPre et al., 2000; Prinjha et al., 2000) and oligodendrocyte-myelin glycoprotein (OMgp) (Domeniconi et al., 2002; Kottis et al., 2002), all of which bind to the Nogo-66 receptor (NgR) (Domeniconi et al., 2002; Liu et al., 2002; Wang et al., 2002c). Other inhibitory proteins, such as chondroitin sulphate proteoglycans (CSPGs), that act by NgR independent mechanisms are also important in growth inhibition in the CNS. Chemorepulsive guidance factors such as ephrins, semaphorins, netrins and slits are potential inhibitors of axon growth after CNS injury, and some are re-expressed after traumatic injury (Tang, 2003). Evidence from our lab (Dubreuil et al., 2003; Lehmann et al., 1999; Winton et al., 2002) and others (Fournier et al., 2003; Niederost et al., 2002; Shearer et al., 2003; Wahl et al., 2000; Wang et al., 2002c) suggests that both NgR-dependent and independent growth inhibitory proteins share

(Haviv and Stein, 1999). The cellular effects of TNF-α are mediated by two transmembrane receptors, TNFR1 and TNFR2. TNFR1 posses a cytoplasmic death domain, which upon ligand binding, activates a caspase dependent apoptotic pathway, and therefore, is mainly responsible for TNF-α induced cytotoxic effects. TNFR2 does not posses a death domain, but can enhance TNFR1-induced cytotoxicity (Ashkenazi and Dixit, 1998).

Apoptosis is an important and highly regulated biological process, and several culture models of apoptosis are useful to investigate cell-signaling pathways. Three common methods of inducing apoptosis in culture involve treatment with TNF-α (Haviv and Stein, 1999), with staurosporine (STS) (Taylor et al., 1997), a broad-spectrum protein kinase inhibitor, or the withdrawal of growth factors, such as NGF, from growth factor-dependent cultures (Miller and Kaplan, 2001).

Studies with myelin, Nogo and MAG show that neurons in the presence of growth inhibitory protein have high levels of Rho-GTP (Fournier et al., 2003; Niederost et al., 2002; Winton et al., 2002). Recently, Dubreuil et al. (Dubreuil et al., 2003) demonstrated that Rho is abnormally activated in both neuronal and glial cells after spinal cord injury (SCI). Moreover, the inactivation of Rho after spinal cord injury (SCI) is cell protective,

apoptotic response of neurons to TNF-α in a Rho-dependent manner, and show that excitotoxic cell death may also be augmented by myelin.

3.3 RESULTS

3.3.1 Inhibitory substrates alone do not cause apoptosis

We have previously shown that cells plated on myelin have elevated levels of active Rho (Winton et al., 2002) and that *in vivo*, Rho activation contributes to apoptosis after SCI (Dubreuil et al., 2003). To examine if myelin substrates induced apoptosis, we plated PC-12 cells on myelin for 48 hours and then quantified cell survival by MTT assay, which measures mitochondrial function. There was no significant difference in the survival of PC-12 cells plated on myelin substrates compared to poly-L-lysine (PLL) controls (Fig. 1a). Therefore, although myelin activates Rho, contact with myelin does not directly induce apoptosis.

3.3.2 Myelin increases cell death of TNF-α treated PC-12 cells

We next investigated if myelin affected the susceptibility of neurons to undergo cell death. We examined the response of PC-12 cells plated on myelin or PLL substrates to conditions known to initiate apoptosis. Cells were plated on test substrates

3.3.3 Treatment with TNF-α activates Rho

TNF-α increased the cell death of PC-12 cells plated on myelin (Fig. 1b) and both myelin and TNF-α are reported to activate Rho (Neumann et al., 2002a; Winton et al., 2002). We determined, by GTP-Rho pull down assay, the effect of TNF-α, STS or NGF withdrawal on Rho activation levels in PC12 cells. PC-12 cells were plated on uncoated culture dishes and exposed to TNF-α, STS or NGF withdrawal for 0.5 hours or 18 hours. We observed an increase in Rho activation 0.5 hours after TNF-α treatment (Fig. 2a), as compared to controls. At 18 hours after TNF-α treatment, Rho activation returned to control levels. By contrast, treatment with STS, or NGF withdrawal failed to increase levels of GTP-Rho at all time points tested (Fig. 2a). Therefore, the change in Rho activation with TNF-α, but not STS or NGF withdrawal, is correlated with the synergistic effects of TNF-α and myelin on cell death.

After SCI injury, Rho is abnormally activated and Rho-GTP is correlated with apoptosis (Dubreuil et al.). We examined the possibility that the combination of two Rho activating factors, myelin and TNF-α, resulted in a heightened level of Rho activation, which may induce cell death. PC-12 cells were plated on myelin substrates overnight and

3.3.4 Active Rho and myelin mediate TNF-α induced apoptosis

To better understand the role that Rho plays in myelin-dependent increase in cell death after TNF-α treatment, we made use of PC-12 cells transfected with constitutively active (V14RhoA), or dominant negative (N19RhoA) Rho. V14RhoA PC-12 cells remain round and do not extend neurites on growth promoting substrates, whereas, N19RhoA PC-12 cells show enhanced neurite outgrowth and can extend neurites on growth inhibitory substrates (Lehmann et al., 1999; Sebok et al., 1999). TNF-α treated V14RhoA PC-12 cells plated on myelin showed a significant 18 % increase in cell death compared to TNF-α treated V14RhoA PC-12 cells on PLL (Fig. 2c). This suggests that the activation of Rho alone does not account for the increase in apoptosis. Dominant negative N19RhoA PC-12 cells showed no difference in survival with TNF-α treatment when plated on PLL (88 %) or myelin substrates (87 %) (Fig. 2d). Together these results suggest that both active Rho and myelin are needed to augment the apoptotic responses to TNF-α.

3.3.5 Myelin increases cell death of TNF-α treated granule neurons

To further investigate the [illegible]

compared to 89 % survival on PLL (Fig. 3b). There was no significant difference between the survival of CGN plated on PLL or myelin substrates after KCl withdrawal or STS treatment (Fig. 3b). The percent survival of CGN plated on PLL after KCl withdrawal was 68 % and 69 %, respectively, and for STS treatment, 68 % and 65%, respectively. To determine if CGN cells die by apoptosis when exposed to myelin and TNF-α, we plated cells on test substrates with or without TNF-α. The cells were then fixed and TUNEL labeled, which detects fragmented DNA. Positive TUNEL staining of TNF-α treated CGN plated on myelin substrates confirmed that cell death was apoptotic (Fig. 3c).

3.3.6 Antagonists of Rho GTPase and NgR prevent cell death

Signaling by myelin-derived growth inhibitory proteins can be blocked with antibodies (Caroni and Schwab, 1988a), Nogo-66 receptor (NgR) antagonists (GrandPre et al., 2002), or by the inactivation of the Rho signaling pathway (Winton et al., 2002). We made use of C3-07, a permeable Rho antagonist and NEP1-40 (GrandPre et al., 2002), a commercially available competitive peptide antagonist of NgR, to block signaling by

cells ($p \leq 0.05$, $n = 5$) (Fig. 4c). Figure 4b shows representative micrographs of cell plated on test substrates and treated with C3-07 or NEP1-40.

To further understand the role that Rho activation and myelin/NgR signaling have on apoptosis, we treated neurons with C3-07 or NEP 1-40, and plated the cells on test substrates overnight prior to TNF-α exposure. Inactivation of Rho by C3-07 increased the survival of TNF-α treated CGN plated on myelin by 21 % (Fig. 5). NEP1-40 treatment increased the survival of CGN plated on myelin substrates by 22 % (Fig. 5). There was no synergistic effect on survival when neurons were treated with both antagonists simultaneously (Fig. 5), suggesting that the increase in survival of the neurons by C3-07 and NEP1-40 is due to the blockage of the same signaling pathway.

3.3.7 Antagonists of the AMPA glutamate receptor prevent cell death

Previous studies have reported that TNF-α can enhance glutamate neurotoxicity, resulting in an increase in cell death (Chao and Hu, 1994; Gelbard et al., 1993; Hermann et al., 2001). We examined whether NMDA or AMPA glutamate receptors have a role in the increased cell death of TNF-α treated neurons plated on myelin substrates. CGN were

substrates and treated with CNQX or MK-801 were completed. Controls were plated on myelin substrates and left untreated. Treatment with CNQX decreased the levels of active Rho as compared to myelin controls (Fig. 6b). In contrast, treatment with MK-801 had no effect on Rho activation levels (Fig. 6b).

3.4 DISCUSSION

Here we report that myelin increases the number of dying cells when PC-12 cells and cerebellar granule neurons are exposed to TNF-α. Neurons *in vivo* are exposed to both growth inhibitory proteins and TNF-α after traumatic injury, as well as in degenerative disease, so these finding have wide implications to our understanding of environmental factors that cause neuronal death. Rho is a key intracellular signaling molecule that regulates cellular responses to growth inhibitory proteins (Fournier et al., 2003; Lehmann et al., 1999; Niederost et al., 2002; Shearer et al., 2003; Wahl et al., 2000; Wang et al., 2002c; Winton et al., 2002). By pull down assay we show that TNF-α synergistically increases Rho activation when cells are in contact with myelin. Further, data from constitutively active and dominant negative PC-12 cells suggests that Rho activation is necessary for the increase in apoptosis when cells are plated on myelin substrates and exposed to TNF-α. Inactivating Rho or blocking AMPA glutamate receptor activation prevented both Rho activation and the increase in cell death of TNF-α treated cells plated on myelin substrates.

Apoptosis plays a key role in cell death after traumatic CNS injury (Crowe et al., 1997)

Rho, reduced the levels of cleaved caspase 3, 7 and 8 and the presence of nucleosomal fragmentation (Petrache et al., 2003). In PC-12 cells, Rho is involved in mediating morphological changes that occur during the execution phase of apoptosis. The inactivation of Rho prevented such morphological changes (Mills et al., 1998). Treatment of cultured astrocytes and hippocampal neurons with thrombin induces apoptosis in a Rho dependent manner, and inactivation of Rho with C3-transferase prevented cell death (Donovan et al., 1997). Recently, we provided evidence that the inactivation of Rho of after SCI is cell protective (Dubreuil et al., 2003). In both rat and mouse models of SCI, inactivation of Rho with C3-07 treatment reduced the number of apoptotic cells by approximately 50% (Dubreuil et al., 2003).

TNF-α is a multifunctional cytokine that plays an important role in regulating immune response in the CNS. In the normal intact CNS, TNF-α expression is low, but is dramatically increased in response to disease and traumatic insult (Perry et al., 1995). TNF-α has been implicated in the death of CNS cells in various inflammatory and neurodegenerative disorders (Gonzalez-Scarano and Baltuch, 1999), such as multiple sclerosis, Alzheimer's disease and Parkinson's disease. In multiple sclerosis, TNF-α is

treatments to inactivate Rho may be interesting neuroprotective therapies for inflammatory disorders (Zhou et al., 2003).

Many studies have documented the presence of TNF-α after traumatic injury, however, the time course of TNF-α up-regulation is both injury and cell-type dependent. In animal models of traumatic brain injury, TNF- α was identified 1-6 hours after lateral fluid percussion injury (Fan et al., 1996), whereas, after focal brain ischemia, TNF-α was first expressed by microglia, between 8 and 24 hours (Buttini et al., 1996), and then by neurons, up to 5 days after initial injury (Liu et al., 1994). Following SCI, both TNF-α levels and TNF-α mRNA levels increase at the lesion site as early as 30 minutes post injury, peaking at 72 hours (Wang et al., 2002a). In SCI, TNF-α induces apoptotic cell death of neurons and glia (Lee et al., 2000). In addition, TNF-α from peripheral sources may also enter into the lesion site after SCI, due to tissue damage and the disruption of the blood brain barrier (Pan et al., 1999).

The contact of neurons with myelin-derived growth inhibitory proteins results in a NgR-dependent increase in free intracellular Ca^{2+} (Bandtlow et al., 1993; Wong et al., 2002). Recently, it was shown that protein kinase C (PKC) mediates the inhibitory effect of

pathway may prevent the sensitization of cells by Ca^{2+}, and as a result protect neurons from the myelin-dependent increase in cell death after TNF-α treatment as reported in this study.

In vitro (Chao and Hu, 1994; Gelbard et al., 1993) and *in vivo* (Hermann et al., 2001) studies provide evidence that TNF-α can act in synergy with glutamate to promote cell death. This is consistent with our results showing that TNF-α treatment alone does not result in substantial levels of apoptosis, and suggests that TNF-α alone is not significantly neurotoxic. However, in combination with other factors (i.e. glutamate or myelin) TNF-α induces increased levels of cell death. Further, TNF-α has also been shown to exert neurotoxic effects by indirect activation of AMPA receptors (Gelbard et al., 1993). In this study, we are the first to report that treatment of CGN with CNQX resulted in Rho inactivation (Fig. 6b). This novel finding suggests that AMPA glutamate receptors can signal to activate Rho. We propose that indirect AMPA receptor activation by TNF-α resulted in increased GTP-Rho levels. Therefore, blocking AMPA receptors with CNQX protected TNF-α treated CGN plated on myelin from Rho-dependent apoptosis. This may help to explain why treatment with CNQX prevented the increase of TNF-α-induced cell death on myelin substrates.

data presented here show that another important effect of treatment with C3-07 or NEP1-40 *in vivo* may be a reduction in cell death caused by the pro-inflammatory cytokine TNF-α acting on cells in contact with myelin inhibitors.

3.5 METHODS

3.5.1 Culture of PC-12 cells

PC-12 cells were grown in Dulbecco`s Modified Eagle Medium (DMEM) supplemented with 10 % horse serum (HS), 5 % fetal bovine serum (FBS), 1 % penicillin-streptomycin (P/S) (Gibco, Burlington, Ontario, Canada). Cells were differentiated overnight in DMEM supplemented with 1 % P/S, 1 % FBS and 20 ng/ml of nerve growth factor (NGF) (CedarLane Labs, Hornby, Ontario, Canada).

PC-12 cells stablely transfected with constitutively active (V14RhoA), or with dominant negative (N19RhoA) Rho (Sebok et al., 1999) were grown in DMEM with 1 % P/S, 5 % FBS, 10 % HS, and 400 mg/ml Geneticin (Gibco).

3.5.2 Culture of cerebellar granule neurons

Primary cultures of rat cerebellar granule neurons (CGN) were prepared from postnatal day 6 to 8 Sprague Dawley rats. Following digestion in 1 % trypsin / 0.1 % DNAse1 and

3.5.3 Preparation of myelin substrates

Myelin coated plates were prepared one-day prior to use. Bovine CNS myelin was plated on poly-L-lysine (PLL) (Sigma, Oakville, Ontario, Canada) coated plates and allowed to dry overnight in a laminar flow hood (8 μg total protein for PC-12 cells and 4 μg total protein for primary cells). PLL coated plates were prepared by incubating PLL (0.025 μg) at 37°C for 2 to 24 hours.

3.5.4 Measurement of neuronal apoptosis

PC-12 cells or CGN were plated on PLL or myelin coated 24-well plates and incubated overnight at 37°C to allow cells to adhere to test substrates. To induce apoptosis, PC-12 cells, were treated for 24 hours with 300 nm staurosporine (STS) (Sigma), 100 ng/ml tumor necrosis factor-alpha (TNF-α) (Chemicon, Temecula, CA) or underwent nerve growth factor (NGF) withdrawal. NGF was removed by washing cultures three times in DMEM over a 2-hour period. Cultures were then incubated in fresh media lacking NGF for the experimental period. In the case of granule cells, neurons were treated for 24 hours with

isoproponal: HCl (500:1). Specific and non-specific absorbance was measured by spectrophotometry (PowerwaveX, Bio-Tek Instruments, Winooski, VT) at 570 nm and 630 nm, respectively. Non-specific absorbance was subtracted as background. The data were normalized to there respective untreated controls. For each experiment the number of surviving neurons was expressed as a percentage (i.e. percent survival) and statistical unpaired student-t tests ($p \leq 0.05$) were completed with Graph Pad InStat (San Diego, Ca). For all conditions at least four separate experiments were performed in duplicate.

3.5.5 TUNEL assay

Purified CGN were plated on PLL or myelin substrates in 8-well chamber slides (Nunc, Naperville, IL). Neurons were incubated overnight on test substrates prior to 24-hour treatment with TNF-α (100 ng/ml). Control cells were left untreated. Cells were fixed with 4% paraformaldehyde (PFA) in 0.1M-phosphate buffer and TUNEL labeling was carried out using the Fluorescein-FragEL DNA Fragmentation kit (Oncogene, Boston, MA). Cells were co-stained with Hoechst 33342 (Sigma), visualized and photographed. For each well, 8 images were collected with a 20X objective using a Zeiss Axiovert

05 was sub cloned into the pET9a expression vector to obtain pET9a-C3-07, which was transformed into the *E. coli* strain BL21 (DE3). The recombinant protein was recovered by sonication of the bacterial pellets, precipitation steps with Polymin P and ammonium sulfate remove nucleic acids, and purification by FPLC AktaExplorer 100 with three columns: cation exchange, size exclusion and anion exchange. The purified C3-07 is approximately 99% pure, and the activity of each batch of protein was assessed by neurite outgrowth bioassay and measurement of the glycohydrolysis activity. C3-07 differs from C3-05 in that it contains silent mutations that do not affect the enzymatic activity.

3.5.7 Measurement of neurite outgrowth

CGN were plated on PLL or myelin substrates, treated with C3-07, or NEP 1-40 for 48 hours and then fixed with 4 % PFA. NEP1-40, a competitive antagonist of NgR was purchased from Alpha Diagnostic International (San Antonio, TX). C3-07 was added to the culture medium (2.5 μg/ml) at time of cell platting. Neurons treated with NEP1-40 were incubated in media containing 1μM NEP1-40 for 10 minutes prior to contact with growth inhibitory proteins, as reported (GrandPre et al., 2002). As myelin is phase

3.5.8 Measurement of neuronal survival

Prior to plating on test substrates, CGN were treated with C3-07 (2.5 μg/ml), NEP 1-40 (1 μm), the NDMA receptor antagonist diozocilpine (MK-801) (10 μm) (Sigma), or the AMPA receptor antagonist 6-cyano-7-nitroquinoxaline 2,3-(1H, 4H)-dione (CNQX) (10 μm) (Sigma) and incubated overnight. TNF-α was then added to the culture media for 24 hours and cell survival was quantified by MTT assay. The data were normalized to untreated controls. For each experiment the number of surviving neurons was expressed as a percentage. Statistical unpaired student-t tests ($p \leq 0.05$) were completed with Graph Pad InStat. For all conditions at least four separate experiments were performed in duplicate.

3.5.9 Pull down assays

Rho-GTP pull down assay was performed as previously described (Dubreuil et al., 2003). Briefly, cell lysates from PC-12 cells and CGN were prepared and incubated for 50 minutes at 4 °C with the GST-Rho binding domain (RBD) coupled to glutathion agarose beades (Sigma). The beads were then washed several times and eluted in sample buffer.

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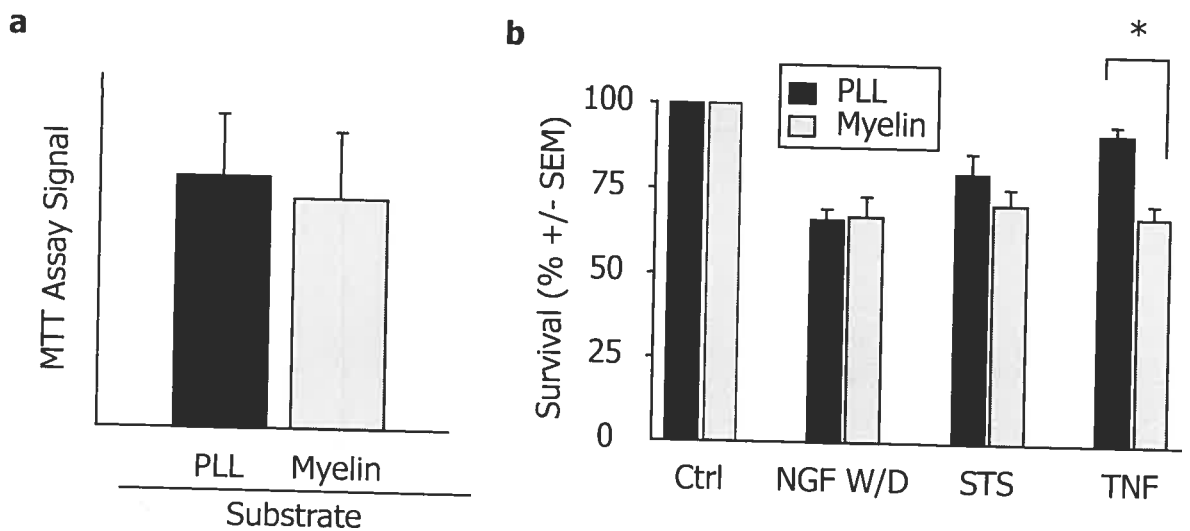
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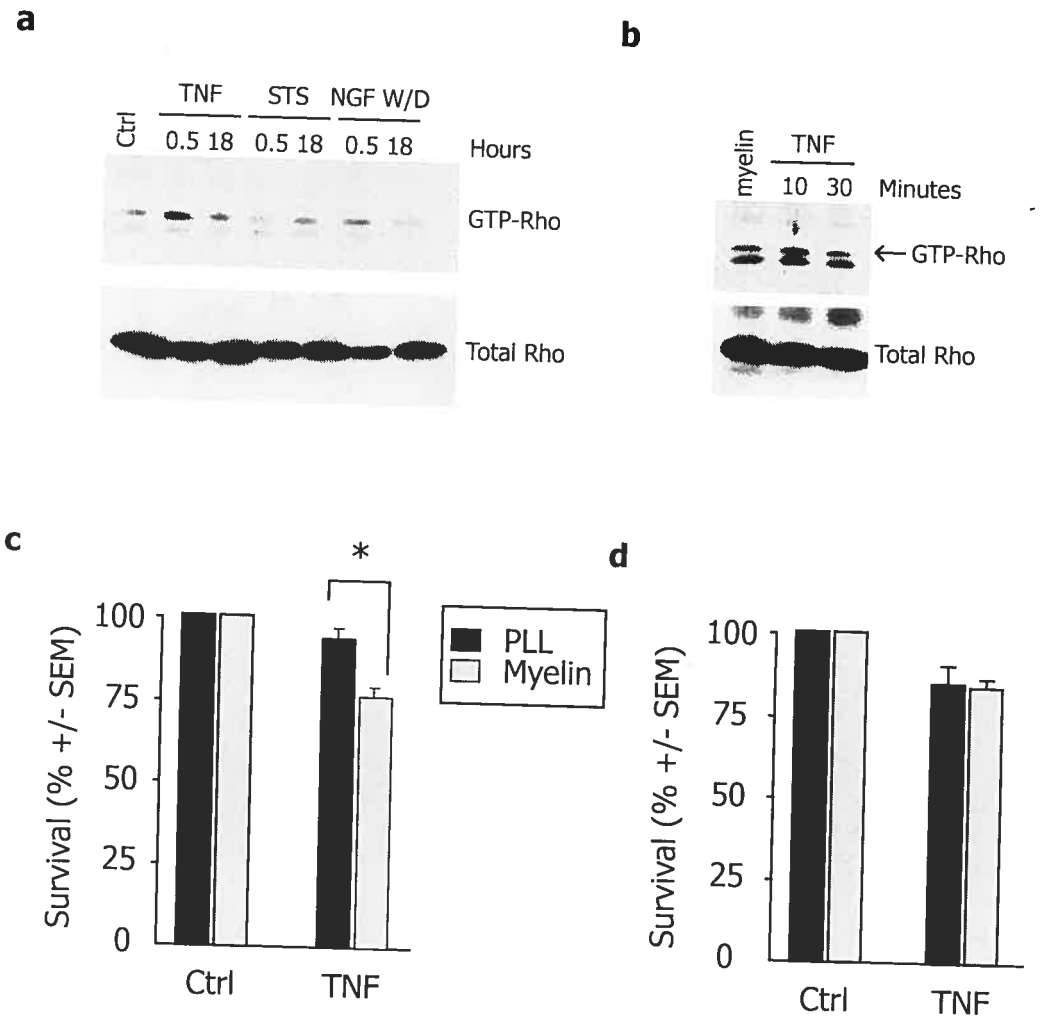
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Total Rho
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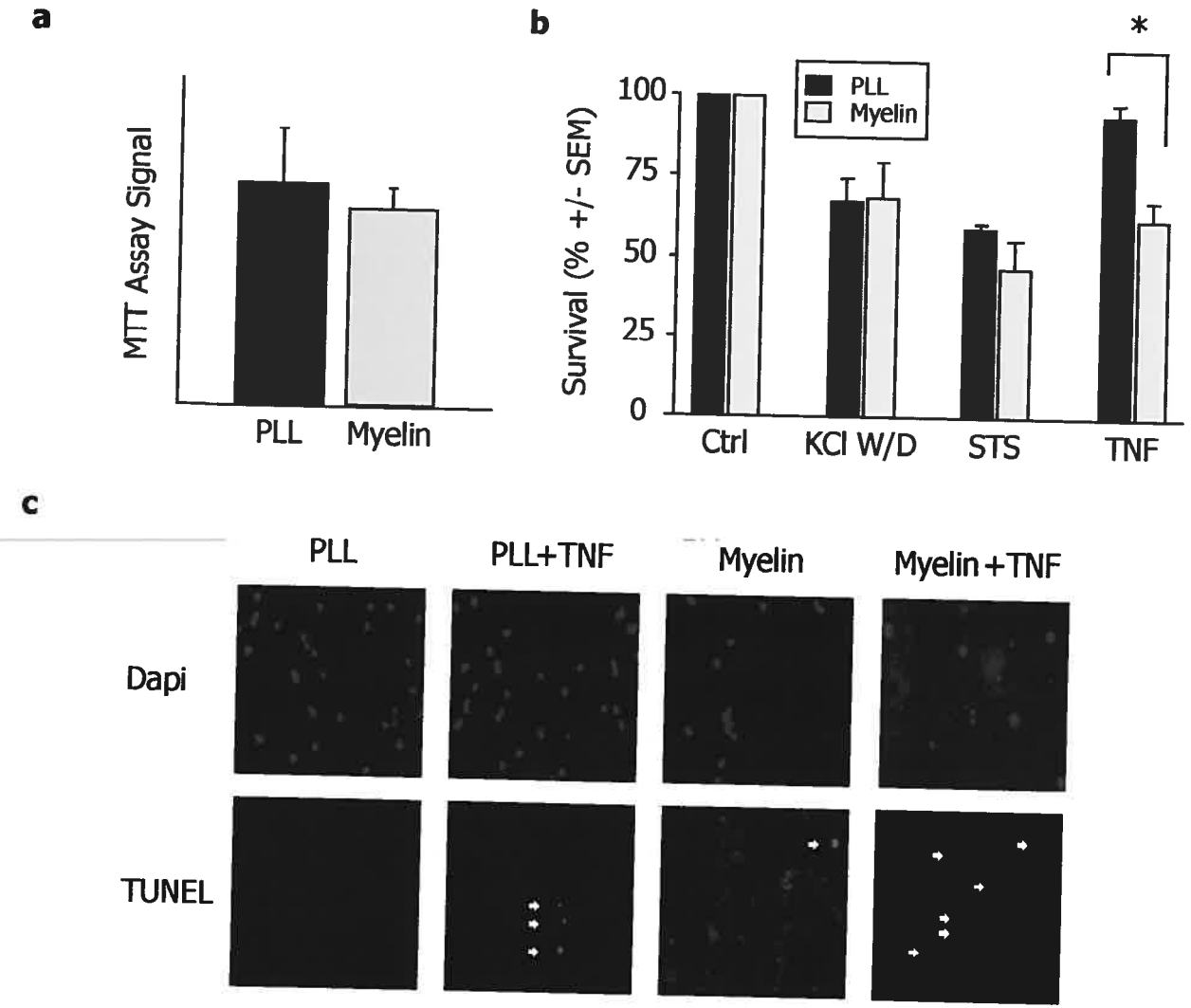
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MTT Assay Signal
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Myelin
b
Survival (% +/- SEM)
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75
50
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TNF
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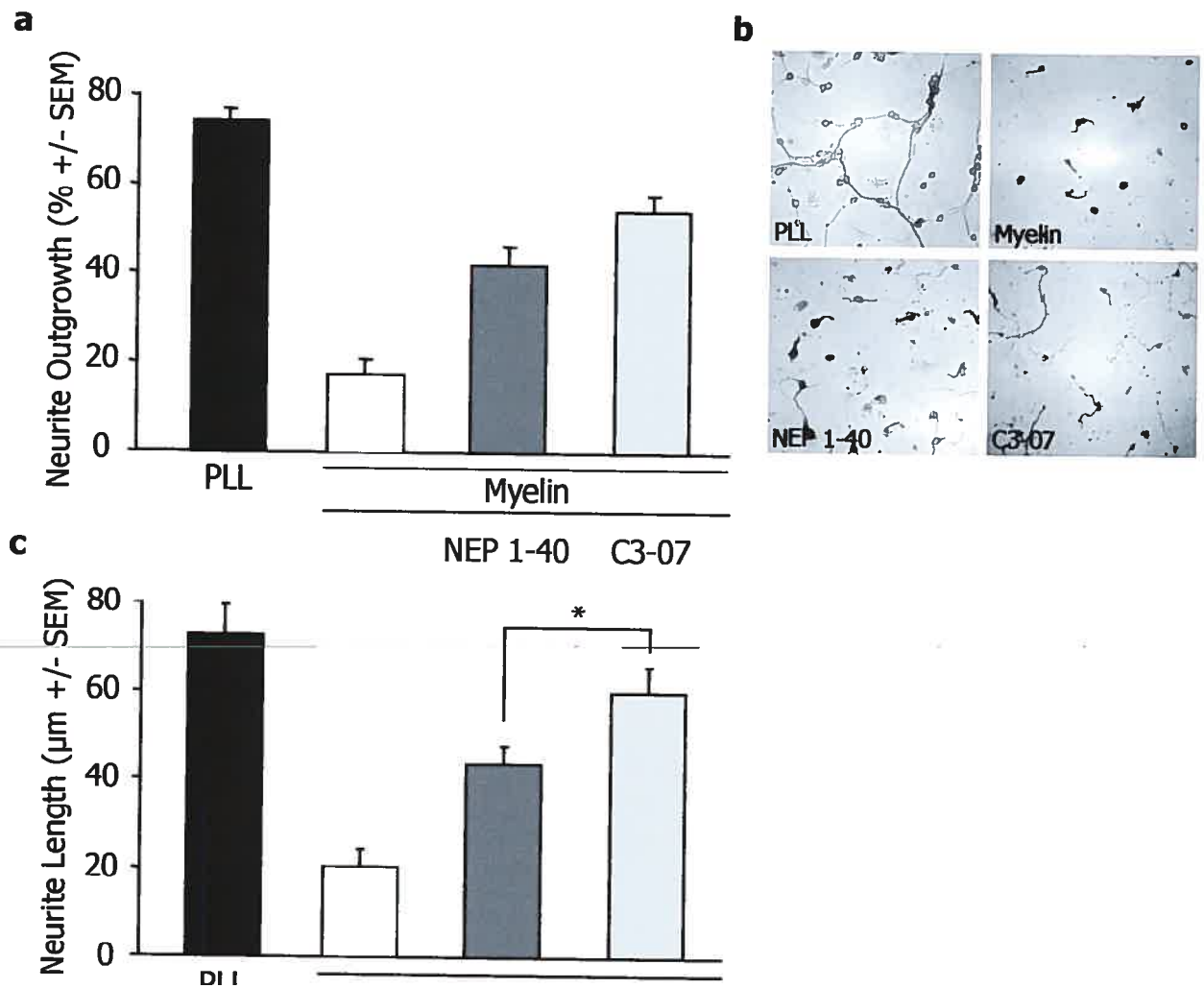
PLL
PLL+TNF
Myelin
Myelin+TNF
Dapi
TUNEL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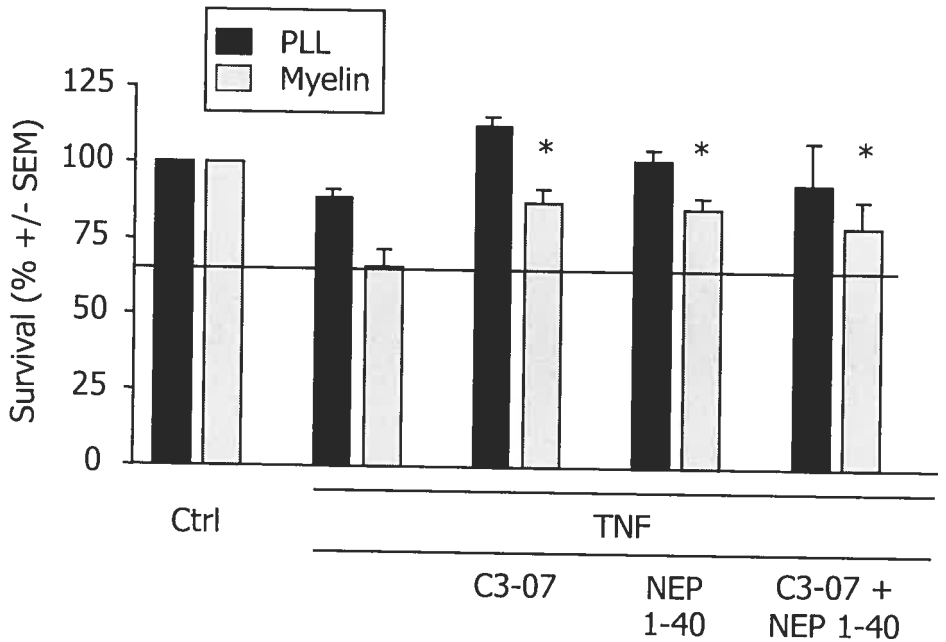
Neurite Outgrowth (% +/- SEM)
80
60
40
20
0
PLL
Myelin
NEP 1-40
C3-07
b
PLL
Myelin
NEP 1-40
C3-07
c
Neurite Length (µm +/- SEM)
80
60
40
20
0
*


PLL
Myelin
125
100
75
50
25
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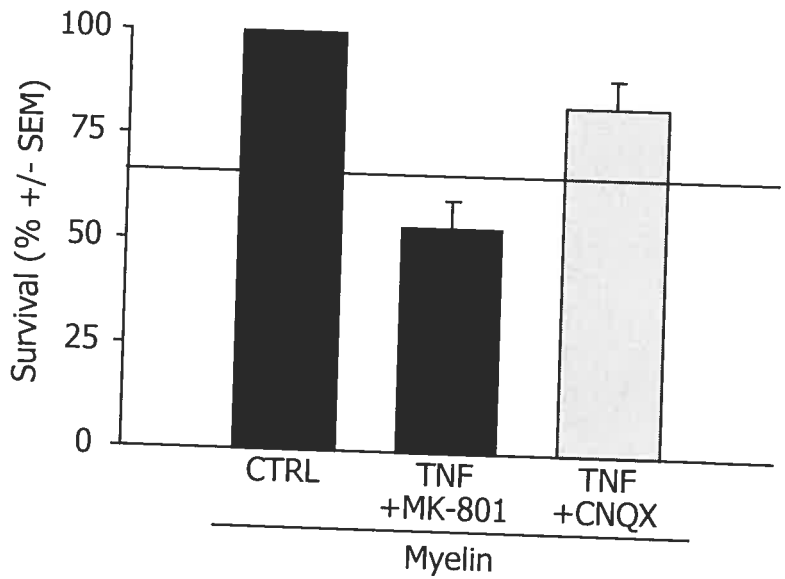
Survival (% +/- SEM)
*
*
*
Ctrl
TNF
C3-07
NEP
1-40
C3-07 +
NEP 1-40

a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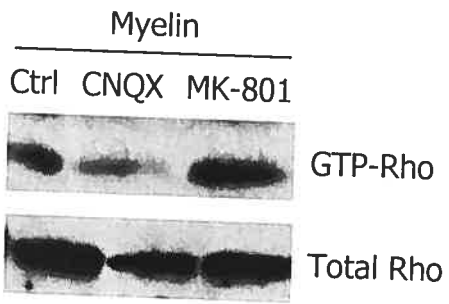
75
50
25
0
Survival (% +/- SEM)
CTRL
TNF
+MK-801
TNF
+CNQX
Myelin

b


Myelin
Ctrl
CNQX
MK-801
GTP-Rho
Total Rho

3.8 Figure Legends

Figure 1 The effect of myelin substrates on apoptosis of PC-12 cells. (a) Survival of PC-12 cells plated on test substrates. (b) Survival of PC-12 cells plated on test substrates and subjected to NGF withdrawal, staurosporine treatment (300 nm), or TNF-α (100 ng/ml) treatment. Data represent the average of four or more experiments performed in duplicate ± S.E.M; asterisk, statistically significant ($p \leq 0.05$).

Figure 2 The involvement of the Rho signaling pathway in the death of TNF-α treated PC-12 cells plated on myelin. (a) Pull down assay for GTP-bound Rho. GTP-Rho (top) and total Rho (bottom) were detected by Western blot with a RhoA antibody. (b) Measurement of GTP-Rho in TNF-α treated PC-12 cells plated on myelin substrates. (c) Survival of TNF-α treated V14 RhoA PC-12 cells (constitutively active) plated on test substrates. (d) Survival of TNF-α treated N19RhoA PC-12 cells (dominant negative) plated on test substrates. Data represent the average of four or more experiments performed in duplicate ± S.E.M.; asterisk, statistically significant ($p \leq 0.05$).

performed. White arrows show TUNEL-positive cells. Micrographs show cells representative of the total cell population.

Figure 4 Rho and NgR antagonists promote neurite outgrowth on inhibitory substrates. CGN treated with NEP1-40 (1 μM) (dark gray bar), or C3-07 (2.5 μg/ml) (light gray bar) and plated on test substrates. (a) The percentage of cells that extend neurites longer than one cell body diameter (neurite outgrowth). The average counts are shown for four or more experiments each performed in duplicate. Error bars represent the S.E.M. (b) Micrographs showing CGN treated with NEP 1-40 or C3-07. PLL and myelin controls were left untreated. (c) The length of the longest neurite per cell (neurite length). The average counts are shown for four or more experiments each performed in duplicate. Error bars represent the S.E.M; asterisk, statistically significant ($p \leq 0.05$).

Figure 5 NgR and Rho antagonists prevent cell death. Survival of CGN treated with NEP 1-40 (1μM), C3-07 (2.5 μg/ml), NEP 1-40 + C3-07, or left untreated (Ctrl) and exposed to TNF-α (100 ng/ml) on test substrates. The black like corresponds to the survival of TNF-α treated cells on

experiments performed in duplicate ± S.E.M. (b) Pull down assay for GTP-bound Rho in CGN treated with CNQX, MK-801, or left untreated (Ctrl) and plated on myelin. GTP-Rho (top) and total Rho (bottom) was detected by Western blot with a RhoA antibody.

Chapter 4

4 GENERAL DISCUSSION

4.1 Spinal cord injury

Traumatic SCI causes neurological impairment resulting in sensory and motor function deficits distal to the level of injury. At present, there are over 300,000 people in North America living with a SCI, and with an estimated 11, 000 new cases reported each year this number will continue to increase (The Canadian Paraplegic Association). Statistically, the majority of spinal cord injuries occur in males (81%) between the ages of 15 to 34 (78%). Causes of these injuries include vehicular accidents, falls, sports related injuries and violent trauma (eg. gun shot wounds) (The Canadian Paraplegic Association). The sudden physical and psychological effects that a SCI inflicts on an individual and their family are incalculable. Moreover, the societal cost of caring for individuals with SCI (i.e. medical, surgical, rehabilitative care, etc…) exceeds 7 billion dollars per year in the United States alone (Hulsebosch, 2002; Kwon et al., 2002).

SCI is an unmet medical need. Presently, there are no clinically approved treatments that promote SCI regeneration and restore nerve function. Current pharmalogical treatments focus on reducing the severity and extent of the vigorous inflammatory response that follows the initial injury. One can therefore see the urgent need for the development of

the negative factors present within the damaged CNS environment. In the pathology of SCI, there is evidence for both primary and secondary injury mechanisms. The effects from the primary injury are caused by the initial mechanical trauma, such as the direct contusion or compression of the spinal cord, whereas the secondary injury is initiated in response to the damage caused by the primary injury. The secondary wave of injury begins immediately after trauma and continues for weeks post injury (Beattie et al., 2000; Beattie et al., 2002b; Schwab and Bartholdi, 1996). Although nothing can be done to prevent the initial trauma, there is a crucial window of opportunity for therapeutic measures that could reduce secondary injury-induced celldeath and apoptosis.

To further understand the complex and multifunctional nature of CNS trauma, and in an attempt to promote regeneration after SCI, researchers have elicited a variety of scientific approaches and techniques. Several experimental models of SCI have been developed, which, in turn, have led to the development of many promising therapies. The present strategies used to promote spinal cord regeneration fall into 7 main groups: (i) cellular transplant and replacement; (ii) artificial substrates and bridges; (iii) cellular protection; (iv) trophic support; (v) modulation of the immune response; (vi) overcoming

re-connect with their appropriate targets. Axons and their targets must interact to re-form functional synapses. Finally, in order to be able to efficiently conduct action potentials, newly regenerated axons must undergo the process of re-myelination.

It is generally believed that an optimal therapy for human patients will require a series of combinatorial treatments that simultaneously addresses many of the complex factors that involved in the pathogenesis of SCI. It should be stressed, however that treatments need to be tested both individually, for their efficacy, and in combination for contraindications before any combinatory therapies reach the clinic. An optimal combinatory therapy for axonal regeneration should focus on a variety of targets and be implemented over the various post-injury stages. The initial focus of such a treatment should be aimed at limiting the extent of the secondary injury. The robust inflammatory response and secondary wave of cell death that follows SCI can be reduced by the administration of anti-inflammatory drugs, such as methylprednisolone (Coleman et al., 2000; Short et al., 2000), and of apoptotic inhibitors (Lu et al., 2000; Ozawa et al., 2002). In animal studies of SCI, rats receiving apoptosis-inhibiting drugs displayed increased motor function as compared to untreated controls (Yune et al., 2004). In cases where cell loss from the primary injury is

environment of the spinal cord favours glial differentiation. Therefore for this type of therapy to be more effective, it maybe necessary to pre-condition stem cells to differentiate into neurons prior to transplantation.

Since increased neuronal survival after injury does not necessarily correlate with increased regeneration (Goldberg and Barres, 2000), the second aim of an optimal therapeutic strategy should be to stimulate axonal regeneration. In the early 1900's, Ramón y Cajal elegantly documented the attempt of CNS neurons that survived traumatic injury to regrow. However, as he described it, this process was quickly aborted (Ramon y Cajal, 1928). Therefore, to promote regeneration, both intrinsic and extrinsic factors must be addressed. In a recent study, the Benowitz laboratory (Fischer et al., 2004) demonstrated that, in the optic nerve, blocking growth inhibitory signaling pathways is not enough to stimulate regeneration of the lesioned axons. In this study, regeneration was only observed when growth inhibitory signaling was blocked and the intrinsic growth programme of damaged axons was activated (Fischer et al., 2004). Thus, the addition of neurotrophins, or increased expression of regeneration associated genes (RAG), by such methods as viral infection, could be used to re-activate the intrinsic growth programme of injured CNS

guidance molecules, tissue grafts, or synthetic bridges can be used as directional scaffolding to encourage axonal growth in the proper direction (Schmidt and Leach, 2003).

4.2 Permeable Rho antagonists

It is now well documented that C3 specifically inactivates Rho, however this exoenzyme does not posses a natural cell-binding component allowing for efficient entry into cells. As a result, methods that disrupt the cell membrane, such are scrape loading and microinjection, are used to help aid the entry of C3 into cells. Such methods, however, are not efficient tools for delivery, and are effective only at high concentrations, limiting the use of C3 as a tool for biochemical studies on Rho signaling (Winton et al., 2002). The need for such high concentrations also prevents the use of C3 as a potential therapeutic treatment in humans, as high concentrations of this bacterial protein may be toxic. To overcome these limitations, we constructed and characterized five new cell-permeable C3-like chimeric proteins that are effective at substantially lower doses than those used in previous experiments with unmodified C3 (Winton et al., 2002). By increasing the

is the inverted micelle model (Derossi et al., 1996). The inverted micelle model was originally proposed as a possible mechanism responsible for cellular entry of the antennapedia homeodomain. It suggests that the positively charged homeodomain interacts with the negatively charged phospholipid membrane. This interaction destabilizes the lipid bilayer and results in the formation of inverted micelles, which shuttle the cell-penetrating protein and its cargo through the membrane, releasing them on the cytoplasmic side. Although this model may explain the mechanism of translocation of some cell-penetrating peptides, it does not explain the entry of other cell-penetrating peptides, such as Tat and poly-arginine peptides. Based on similarities with other fusogenic transport sequences (Du et al., 1998; Niidome et al., 1997; Pecheur et al., 1999; Rojas et al., 1998), we speculate that the presence of proline residues spaced approximately every 5 amino acids is important for the translocation of the cell-penetrating peptide used in C3-05 (Winton et al., 2002). Interestingly, it has been suggested that the proline residues may also restrict the chimeric protein to the cytoplasm, preventing entry into the nucleus (Prochiantz, 1999). The exact functional importance of proline residues in our cell-penetrating sequence could be directly tested through mutational analysis and screening. The proline residues could be replaced

To date, two other laboratories have developed permeable C3 fusion proteins. These two chimeric proteins were constructed using the B subunit of Diphtheria-toxin, (Boquet et al., 1995) and the binding components of C2 toxin (Barth et al., 1998), termed DC3B and C2IN-C3, respectively. Our three most effective C3-like chimeric proteins, C3-03, C3-05, and C3-06, all work at lower doses than DC3B (0.6 μg/ml) (Boquet et al., 1995) and C2IN-C2 (0.2-0.3 μg/ml) (Barth et al., 1998), being effective at 0.0025 μg/ml. In contrast to DC3B and C2IN-C3, it is believed that our C3-like chimeric proteins enter the cell by receptor-independent mechanisms. Therefore, upon cellular entry, these cell-permeable Rho antagonists are not contained inside endocytotic compartments. This may explain, in part, the lower effective doses of our C3-like proteins. In addition, C2IN-C3 is not independently cell permeable. C2II, the binding component of C2 toxin, must be present to induce the uptake of the C2IN-C3 fusion protein by endocytosis (Ohishi et al., 1980). Further, C2IN is a relatively large peptide consisting of 225 amino acids, which, when attached to C3-transferase, nearly doubles its molecular weight. All of the transport sequences we used to construct our chimeric proteins are under 50 amino acids, which may enhance uptake, as the total size of the protein is not significantly increased.

the Rho signaling pathway, several studies have examined the effects of manipulating Rho signaling in animal models of CNS injury (Dergham, 2002; Fournier et al., 2003; Hara et al., 2000a; Lehmann et al., 1999).

4.3.1 Targeting Rho

We have previously shown that the inactivation of Rho by treatment with C3 promotes long distance regeneration after micro-lesion of the optic nerve in adult rat (Lehmann et al., 1999) and both axonal regeneration and functional recovery after dorsal over-hemisection of the spinal cord in adult mice (Dergham et al., 2002). In the latter study, substantial improvements in behavior were observed as early as 24-hours post treatment (Dergham et al., 2002). This time point is too early to associate improvements in motor function with regeneration, and therefore suggests that the inactivation of Rho after SCI may have other beneficial effects. In concert with a growing body of literature that details an important role for Rho activation in apoptosis, Dubreuil *et al.* have provided direct evidence that the inactivation of Rho after SCI reduces the number of apoptotic cells (Dubreuil et al., 2003). They suggested that inactivation of Rho after SCI prevent-

effects of Rho inactivation on cell protection over time in models of ischemic or traumatic injury.

In contrast to our study, Fournier *et al.* (2002) recently demonstrated that slow-release treatment of C3 after SCI in adult rats fails to promote regeneration and functional recovery. In addition to differences in both the species and injury models used, other possible explanations for these conflicting results are the concentrations of C3 used and the methods by which it was delivered. Fournier *et al.* (2003) used an Alzet minipump to deliver a total dose of 500 μg, a ten fold higher concentration than used in our studies. This dose may have been toxic, resulting in the lethargy seen in the rats. Alternatively, this slow delivery of 0.75 μg / hour of C3 over a 3 week period may have not been sufficient to inactivate Rho; this cannot be confirmed, however, as this study did not examine the levels of Rho inactivation or the presence of C3 in the spinal cord, as we did in our studies (Dubreuil et al., 2003). In contrast, Dergham *et al.*(Dergham et al., 2002) administered a single, high dose of C3 (50 μg), contained in a fibrinogen matrix, directly into the lesion site. We demonstrated by immunocytochemistry that with this delivery method, C3-05 is able to enter neurons, astrocytes and oligodendrocytes, all of which show abnormal levels

inactivation of Rho kinase stimulates regeneration and functional recovery in both mouse and rat models of SCI (Dergham et al., 2002; Fournier et al., 2003). A third study using fasudil hydrochloride, an antivasospam drug that inhibits various protein kinases including Rho kinase, also induced rapid and extensive neurological recovery after spinal cord contusion injury in adult rats (Hara et al., 2000a). In addition, several studies have shown that treatment of cultured neurons with Y-27632 blocks growth cone collapse induced by growth inhibitory proteins (Borisoff et al., 2003; Dergham et al., 2002; Monnier et al., 2003). These results further implicate the manipulation of the Rho signaling pathway as a viable therapeutic intervention after SCI.

In recent studies from our laboratory, the ability of both Rho and Rho kinase antagonists to promote regeneration after SCI was directly compared. Although both C3 and Y-27632 stimulated regeneration, treatment with C3 promoted axonal growth over distances 4 times greater then observed after Y-27632 treatment. This difference in axonal re-growth between the two treatments suggests that in addition to Rho kinase, other downstream effectors of Rho, which are inactivated by C3 but not Y-27632, are involved in blocking axonal growth after SCI. It is also clear that Rho may appears to be a more

growth inhibitory protein-induced Rho activation are currently unknown, further studies are needed to identify and develop specific inhibitors towards GEFs involved in the growth inhibitory signaling pathway, before any conclusions can be drawn about the effectiveness of antagonizing proteins upstream of Rho.

4.3.4 cAMP

A number of studies examining growth inhibition (Cai et al., 2002; Cai et al., 2001) and spinal cord regeneration (Neumann et al., 2002b; Nikulina et al., 2004; Pearse et al., 2004; Qui et al., 2002) have described the beneficial effects of directly or indirectly increasing cAMP levels. Detailed examination of the cAMP-signaling pathway has shown that cAMP can regulate Rho activity (Forget et al., 2002; Manganello et al., 2003; Qiao et al., 2003). Experiments in non-neuronal cells demonstrated that,when activated by cAMP, PKA phosphorylates and inactivates Rho. This phosphorylation of Rho results in membrane dissociation and increased interaction with Rho GDI (Forget et al., 2002). Two independent studies have shown that treatment with Rolipram, a drug that prevents the breakdown of cAMP, prior to SCI results in an increase in the re-growth of injured axons

directly affects Rho activation levels in cells transfected with dominant negative PKA. As cAMP is known to act on many cellular targets, a possible combinatory therapy to promote spinal cord repair could involve the inactivation of Rho by C3-05, in concert with treatments that increase intracellular levels of cAMP.

4.3.5 Potential concerns associated with targeting the Rho signalling pathway

Even though some of the above-described results appear to be very promising, the development of C3-05 analogs and Rho kinase inhibitors for treatment of SCI in humans should still proceed with caution. Treatment with C3-like proteins results in the specific inactivation of RhoA, -B, and -C (Braun et al., 1989; Chardin et al., 1989; Kikuchi et al., 1988; Narumiya et al., 1988). The effects of RhoA in both neuronal and non-neuronal cells are well characterized. However, the same cannot be said for RhoB and RhoC and the indiscriminate inactivation of these proteins may have adverse effects. Further, RhoA mediates a wide spectrum of biological functions, from cytokinesis and guidance, to transcription regulation and endocytosis (Hall, 1998; Symons and Settleman, 2000; Takai et al., 2001). Therefore, flooding the body with Rho antagonists could result in potentially

4.4 Neuroinflammation after CNS injury

The human CNS has evolved to possess the unique property of 'immune privilege' and therefore, in contrast to other parts of the human body, lacks a normal immune function (Hausmann, 2003). The presence of the blood brain barrier (BBB) (Kniesel et al., 1996; Poduslo et al., 1994) and the absence of resident antigen presenting T-cells contribute to this CNS-specific deficit in local immune response (Schnell et al., 1999a). Following SCI, an immediate and vigorous immune response, is initiated, characterized by the activation of resident CNS microglia, the invasion of peripheral inflammatory cells and the release of cytokines and chemokines. (Dusart and Schwab, 1994; Popovich et al., 1997; Schnell et al., 1999b; Zhang et al., 1997). Although great progress has been made in characterizing this inflammatory response after CNS injury, the exact role played by the immune system in promoting repair and regeneration after CNS trauma has long been the subject of controversy. There is strong evidence for both a beneficial and detrimental role of the immune system in response to injury, suggesting a dual nature for post-traumatic inflammation (Jones et al., 2004; Popovich, 2000; Popovich and Jones, 2003; Schwartz, 2001; Schwartz and Hauben, 2002).

Interestingly, the inflammatory response induced by traumatic brain injury, has been demonstrated to be even less severe then that observed after SCI (Schnell et al., 1999b). Therefore scientists have attempted to experimentally increase the endogenous immune response in the CNS after injury. Following this strategy the Schwartz laboratory has developed promising new neuroprotective (Hauben et al., 2000; Hauben et al., 2001; Yoles et al., 2001) and regenerative therapies (Rapalino et al., 1998) using autoimmune T-cells and activated macrophages, respectively. The injection of T-cells that recognize myelin-associate self-antigens (i.e. autoimmune T-cells) after SCI increases neuronal survival (Hauben et al., 2000; Hauben et al., 2001; Yoles et al., 2001). Further, the transplantation of activated peripheral blood macrophages into the lesion site enhances myelin clearance, making the CNS environment more permissive to growth (Rapalino et al., 1998). To target the humoral branch of the immune system, Huang *et al.* (1999) developed a therapeutic vaccine, in which the immune system of adult mice was stimulated to produce antibodies against myelin. The production of such antibodies prior to SCI results in significantly increased axonal regeneration compared to control animals (Huang et al., 1999). A similar vaccine was also effective in promoting RGC regeneration in the adult optic nerve;

1987). Neutrophils are the first group of inflammatory cells to infiltrate the CNS after injury. They are detected at the lesion site as early as 3 hours post injury and remain present, at elevated levels, for several days (Carlson et al., 1998). The primary function of neutrophils is to remove tissue debris. However, these cells also release potentially damaging proteases and reactive oxygen species and therefore have also been implicated in secondary injury cell damage (Hausmann, 2003).

Under normal physiological, conditions microglia are present in the CNS in a dormant state (Schnell et al., 1999a). After SCI, microglia are 'activated', resulting in increased proliferation, changes in morphology and altered expression of cell surface proteins (Giulian et al., 1989; Stoll and Jander, 1999). The number of activated microglia increases during the first seven days post-injury and remain present at the lesion site for several weeks. Along with neutrophils and peripheral macrophages, activated microglia are also responsible for clearing cell debris from the injury site (Popovich et al., 1997; Schnell et al., 1999a; Stoll and Jander, 1999). Although debris clearance is a beneficial process that is necessary for wound healing, both macrophages and activated microglia secrete cytokines, such as interlukin-1 and TNF-α, and chemokines, such as leucotrienes and

Therefore, caution must be taken before using any treatment that enhances the immune response after CNS injury as even though they may be beneficial in the short-term, they can also reduce long-term recovery by increasing secondary damage and cell death.

We have recently shown that myelin-derived growth inhibitory proteins increase the cell death of TNF-α treated neurons in a Rho-dependent manner. Taking this study into account, we speculate that growth inhibitory proteins, in combination with the inflammatory response present after injury results in increased levels of cell death. To date, such increased in cell death has only been observed in tissue culture experiments. However, we are currently in the process of developing an experimental model to test our hypothesis *in vivo*. In this model, we plan to induce a rigorous TNF-α-mediated inflammatory response by microinjection of the bacterial endotoxin lipopolysaccharide into specific brain regions. The immune response induced by lipopolysaccharide injection is well documented and kwon to increase the release pro-inflammatory cytokines, such as TNF-α and interleukin-1β and interleukin-6 (Lacroix and Rivest, 1998; Laflamme et al., 1999). In addition to lipopolysaccharides, myelin-derived growth inhibitory proteins will also be injected into the brain and we will examine if the presence of myelin-derived growth

there has been increasing evidence suggesting a possible involvement for Rho in different classes of neurodegenerative diseases.

4.5.1 Genetic mutations

Some of the strongest indications of Rho's involvement in neurodegenerative diseases come from studies examining amyotrophic lateral sclerosis (ALS). ALS is a fatal neurological disorder characterized by the progressive degeneration of motor neurons. Recently, two groups independently demonstrated that loss of function mutations in the *alsin*, a Rho GEF, are present in familial forms of ALS and induce ALS-disease phenotypes (i.e. degeneration of motor neuron circuits). Using transgenic mice, Kobayahi *et al.* (2004) showed that the expression of dominant negative Rho or dominant negative Rho kinase increases apoptosis of spinal cord motor neurons at early development stages. These results suggest that Rho is involved in the survival of motor neurons and that the impairment of Rho signaling pathways may be a key step in the progression of neurodegeneration. Interestingly, increased expression of Nogo-A and decreased expression of Nogo-C are present in experimental models of ALS and human patients. In

4.5.2 Excitotoxicity

Recently, there has been increasing evidence suggesting a possible role of glutamate-mediated excitotoxicity in the pathology of chronic neurodegenerative diseases, such as ALS (Rao, 2004;Cluskey, 2001;Doble, 1999), HD (Doble, 1999), MS (Doble, 1999; Matute et al., 2001; Pitt et al., 2000), PD (Doble, 1999; Plaitakis and Shashidharan, 2000) and AD (Danysz and Parsons, 2003; Lue et al., 2001; Perry et al., 2001; Quadros et al., 2003). Glutamate is an important excitatory neurotransmitter in the mammalian CNS that can be neurotoxic at high levels. Accordingly, extracellular levels of glutamate are tightly regulated. Similar to CNS trauma, excitotoxicty in neurodegenerative disease is believed to occur in response to initial impairments caused by the disease and to result in further neuronal tissue degeneration (Doble, 1999). Several studies have demonstrated that abnormalities in glutamate release and uptake mechanisms contribute to the pathology of neurodegenerative diseases (Doble, 1999). Abnormally high concentrations of glutamate are detected in the cerebral spinal fluid of patients with ALS (Couratier et al., 1993) and MS (Matute et al., 2001), and a reduction in glutamate uptake has been observed in ALS (Cluskey and Ramsden, 2001) and AD (Danysz and Parsons, 2003). Although the exact

4.5.3 Inflammation

TNF-α has been implicated in the pathology of various inflammatory and neurodegenerative disorders, such as MS, PD and AD (Gonzalez-Scarano and Baltuch, 1999; Mayeux, 2003). In MS, TNF-α is detected at the site of demylinating lesions and is suggested to be responsible for myelin and oligodendrocyte damage (Ledeen and Chakraborty, 1998; Navikas and Link, 1996). In patients with Parkinson's disease, there is a higher density of TNF-α secreting glial cells and increased cytokine levels are detected. In AD activated astrocytes and microglia secrete TNF-α and are localized in large numbers around senile plaques (Gonzalez-Scarano and Baltuch, 1999). Therefore, as TNF-α has been previously shown to activate Rho (Neumann et al., 2002a) and to cause cell death (D'Souza et al., 1995; Haviv and Stein, 1999), we suspect that a role of TNF-α-induced Rho activation and apoptosis are implicated in these neurodegenerative diseases. It would be interesting to test if Rho activation by growth inhibitory proteins, in combination with TNF-α, contributes to cell death in neuroinflammatory disorders. This could be directly investigated by examining if treatment with NgR, or TNF-α antagonists affects cell death levels in animal models of neurodegeneration. Recently, Nogo-A was shown to be

features of AD is the accumulation of extracellular amyloid deposits located in multiple regions throughout the brain (Hardy and Selkoe, 2002; Selkoe and Schenk, 2003). Several studies have confirmed that the major components of these senile plaques are aggregated peptides of 40-42 amino acids, termed amyloid-β peptides (Selkoe and Schenk, 2003). These peptides are produced from the serial cleavage of the larger amyloid-β precursor protein and are the main focus of many AD studies (Esler and Wolfe, 2001; Wilson et al., 2003). The longer 42-residue amyloid-β peptide ($A\beta_{42}$), which is more hydrophobic and prone to aggregation, is associated with a pathological state and neurotoxicity. Another defining neuropathological characteristic of several neurodegenerative diseases, including AD, is the presence of filamentous inclusions of the microtubule-associated proteins Tau (Buee et al., 2000; Lee et al., 2001). Hyperphosphorylated forms of Tau aggregate are present in several neurodegenerative diseases, collectively termed tauopathies. Further, the aggregation of Tau has been linked to brain dysfunction and neurodegeneration (Lee et al., 2001).

4.6.1 Amyloid plaques

certain NSAIDs (slulindac sulfide, ibuprofen and naproxen) inactivates Rho in a concentration dependent manner, without affecting Rho expression levels. Second, they implicated both Rho and Rho kinase in the production of $A\beta_{42}$ and demonstrated that the inactivation of the Rho/Rho kinase-signaling pathway by NSAIDs reduces the production of $A\beta_{42}$ in both *in vitro* and *in vivo* models of AD (Zhou et al., 2003). Although this study is the first to provide direct evidence for an involvement of Rho in AD, the exact role of Rho in neurodegenerative diseases must be further investigated.

4.6.2 Neurofibillary tangles

Under normal conditions phosphorylation of the microtubule-associated protein Tau acts to negatively regulate the ability of this proteins to bind to and stabilize microtubules (Buee et al., 2000; Lee et al., 2001). The mechanisms responsible for the hyperphosphorylation and aggregation of Tau are currently unknown, however, numerous studies have identified Tau as a substrate for several kinases (Buee et al., 2000; Iqbal et al., 2003; Lau et al., 2002). Interestingly, the activity of two kinases known to phosphorylate Tau, glycogen synthase kinase (GSK-3β) (Sayas et al., 2002) and Rho kinase (Amano et

possible mechanisms by which Rho can regulate the phosphorylation state of Tau and participate in AD and potentially other tauopathies. Although much work needs to be done to determine Rho's possible involvement in neurodegenerative diseases, circumstantial evidence suggests a role for this GTPase in this pathogenesis.

4.7 General Conclusion

My doctoral research has focused on signaling mechanism involved in promoting regeneration after SCI. Many proteins in the CNS inhibit the regeneration of injured axons. We have shown that Rho GTPase is a key player in the signaling pathways triggered by these molecules. Over the course of my doctoral studies I constructed and characterized 5 permeable Rho antagonists that were shown to be able to freely cross the cell membrane and inactivate Rho at very lose doses (Winton et al., 2002). Making use of C3-05, the most effective antagonist constructed, I was able to demonstrate that Rho is involved in apoptotic cell death following SCI and that the inactivation of Rho is cell protective (Dubreuil et al., 2003). Further, I provide evidence that such Rho-dependent cell death is increased in the presence of the pro-inflammatory cytokine TNF-α and myelin derived

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Appendix A

Third Article

« Inactivation of Rho signaling in neuronal cell bodies promotes neurite growth on inhibitory substrates and regeneration of retinal ganglion cell axons in the optic nerve of adult rats. Johanne Bertrand, Matthew J. Winton, Robert B. Campenot and Lisa McKerracher. Manuscript in preperation to be submitted to the Journal of Neuroscience.»

Inactivation of Rho signaling in neuronal cell bodies promotes neurite growth on inhibitory substrates and regeneration of retinal ganglion cell axons in the optic nerve of adult rats

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Mature neurons in the central nervous system (CNS) fail to spontaneously regrow their axons following injury. Inhibitory proteins, present in CNS myelin and at the glial scar, are partly responsible for this lack of regeneration (reviewed by David and Lacroix 2003,(David and Lacroix, 2003)). Activation of the Rho GTPase is part of the signaling process of these inhibitors, and Rho activation leads to growth cone collapse and growth inhibition (Jalink et al., 1994; Jin and Strittmatter, 1997; Tigyi et al., 1996b).

In vitro, Rho inactivation by antagonist C3-transferase stimulates neurite growth on inhibitory substrates (Bito et al., 2000; Dergham et al., 2002; Lehmann et al., 1999; Monnier et al., 2003; Winton et al., 2002). Because cell bodies and neurites are equally exposed to the C3-containing culture media, it is not known whether C3 acts at the cell body or at the neurites to exert its growth promoting effect. *In vivo*, distance between cell bodies and axons often allows localized treatment application. Rho antagonists and most other treatments designed to promote regeneration by blocking the effect of inhibitory proteins have been used either at the lesion site (Dergham et al., 2002; Fournier et al., 2003; GrandPre et al., 2002; Lehmann et al., 1999; Weibel et al., 1994) or systemically (Li and Strittmatter, 2003; Merkler et al., 2001). However, cell bodies have been the preferred

To determine if treatment of the cell bodies with Rho antagonists can stimulate neurite growth on inhibitory substrates and promote axonal regeneration *in vitro* and *in vivo*, we used Campenot chambers and the optic nerve. Campenot chambers are a culture system that isolates neuronal cell bodies and distal axons into independent compartments, keeping culture media separated. This compartmentalization allows the exposure of cell bodies and distal axons to different test compounds. *In vivo,* the optic nerve provides a similar separation, with cell bodies located in the retina and axons located behind the ocular globe.

We used two modified versions of Rho antagonist C3. Both contain an added transport sequence to facilitate cell entry. C3-05 is affinity purified (Winton et al., 2002), while C3-07 is a more highly purified version that became available during the course of our experiments. We show that treatment with C3-07 at the cell body level can stimulate neurite growth on inhibitory substrates as effectively as a treatment applied to the axons. Also, using both *in vitro* and *in vivo* models, we show that axonal regeneration after axotomy is promoted by treatment with C3-like Rho antagonists independently of the application site, and that C3-07 is not confined to the cellular location where it is originally

RESULTS

Localized application of C3-07 increases axonal regrowth after axotomy

Inactivation of Rho by treatment with Rho signaling antagonists increases neurite growth on neutral or growth-permissive substrates (Lehmann et al., 1999), and stimulates neurite outgrowth on inhibitory substrates (Bito et al., 2000; Dergham et al., 2002; Lehmann et al., 1999; Monnier et al., 2003; Winton et al., 2002). Whether Rho antagonists promote growth by acting at the cell body or at the axon tips cannot be determined in a traditional culture system, because the whole neuron is exposed to the antagonist-containing media. We made use of the Campenot chamber cell culture system to examine if application of Rho antagonist C3-07 to specific cellular locations (i.e. cell body vs distal axons) promoted axonal growth after axotomy or in the presence of growth inhibitory substrate. This culture system allows cell bodies and distal axons to be exposed to different compounds by isolating them into separate compartments (Fig. 1A). Sympathetic neurons (superior cervical ganglia [SCG]) were maintained in Campenot chambers for 5 to 7 days, after which axons in the distal chambers were axotomized. Immediately after axotomy, media treated with C3-07 (2.5 μg/ml) was added to the cell body compartment, the 2 distal

After axonal regrowth was measured, lysates from cell body or distal axon compartments were prepared, pooled and separated by electrophoresis. Western blot analysis revealed the presence of C3-07 in distal axon lysates when C3-07 was applied only to the cell body compartment, and in cell body lysates when C3-07 was applied only to the distal axon compartment (Fig. 1C). In both cases however, the level of C3-07 present was substantially lower than detected in the chamber of initial application.

Localized application of C3-07 promotes axonal growth on MAG substrate

Next we chose to examine if C3-07 application to either the cell body compartment or the distal axon compartements was sufficient to overcome growth inhibition by myelin-associated protein (MAG). The MAG substrate was added to the side compartments of collagen-coated Campenot chambers. For technical reasons, approximately .5 mm separated the entrance to the side compartment and the edge of the MAG substrate. SCG were plated in the center compartment. After 1 day in culture, when axons are just entering the side compartments, C3-07 (2.5 μg/ml) was added to culture media of either the cell body compartment, the 2 distal axon compartments or all 3 compartments. Axonal length

mm, compared to 2.53 mm for treatment of the distal axons compartments and 2.52 for treatment of all compartments. At the same time point, control neurons plated on collagen did not grow significantly further (Fig. 2E). Therefore, C3-07 promotes axonal growth on inhibitory MAG substrate, irrespective of the site of application.

Intravitreal injection of C3-05 or C3-07 stimulates regeneration in the optic nerve.

Previous results from our group demonstrated that in the adult rat, an application of C3 at the site of an optic nerve lesion promotes regeneration (Lehmann et al., 1999). The optic nerve contains only the axons of the RGCs, while cell bodies are located in the retina, up to 5 mm away from the optic disc, where the optic nerve originates. To test if treatment of the RGCs cell bodies with Rho antagonists promoted regeneration *in vivo*, we specifically treated the retina with injection in the vitreous of the eye. Intravitreal injections allow delivery to the retina, with no direct access to RGC axons in the optic nerve.

We performed a microlesion of the optic nerve 1 mm behind the optic disc, and applied C3-05 to RGC cell bodies by intravitreal injection immediately after the microlesion (n = 4). Control animals received a PBS injection (n = 5), or no injection at all

significantly higher number of regenerating axons per section than controls, at distances of 50, 100 and 250 μm from the lesion site (Fig. 3C). We repeated these experiments with C3-07, a more highly purified version of C3-05, with the same enzymatic activity and transport sequence as C3-05. Regeneration results in animals injected with C3-07 (n = 7) (Fig. 3D) were similar to those of C3-05 treated animals, although we observed a greater number of longer axons in some animals (Fig. 3E).

Four weeks after microlesion, we observed significantly more axons growing past the lesion site in the C3-05 treated group (n = 5) than in the control groups (PBS n = 5, or no injection n = 5) at distances of 50, 100 and 250 μm (Fig. 4A). These results indicate that application of Rho antagonists C3-05/07 to RGC cell bodies can promote optic nerve regeneration after microlesion.

To examine if intravitreal treatment with C3-05 or C3-07 had an effect on axon length, we compared the average length of the longest axon growing into the optic nerve white matter after axotomy (Fig. 4B). At 2 weeks, the longest axons were not significantly longer in C3-05 treated animals than in PBS treated controls. Similar results were observed at 4 weeks, where no significant difference was found between a control group and C3-05

C3-07 is present in optic nerve following application to RGC cell bodies

We then examined if C3-07 could be found at the lesion site after its injection in the eye. After microlesion, 5 μg of C3-07 was injected in the vitreous and the optic nerve was examined 3 days later. Western blot analysis revealed the presence of intact C3-07 in optic nerve lysates, indicating that C3-07 was not confined to its original cellular location (Fig. 5).

Delayed treatment with C3-07 stimulates regeneration through the lesion scar

By 24 hours after microlesion, a CSPG positive scar is well formed (Selles-Navarro et al., 2001a). To determine if a delayed treatment with C3-07 stimulated regeneration of RGCs through the lesion scar, we injected C3-07 (n = 8) in the vitreous 4 days after microlesion of the optic nerve, and examined regeneration 10 days later. Control animals were injected with PBS (n = 5) 4 days after the microlesion. A large number of CTβ-positive axons could be seen past the lesion site in treated animals, while very few were observed in PBS controls (Fig. 6A). Animals treated with C3-07 had a significantly higher number of regenerating axons per section than controls, at distances of 50, 100, 250

Slow axonal transport is unchanged by C3-07 treatment

Axonal transport in normal and injured optic nerve of adult rat has been well described (ref). In uninjured optic nerve the rate of slow axonal transport of tubulin and neurofilaments is approximately 0,5 mm/day (McQuarrie et al., 1986). However, the transport rate decreases significantly when the optic nerve is crushed intracranialy, near the optic chiasma (McKerracher et al., 1990). To determine if C3-07 had an impact on slow axonal transport of tubulin and neurofilaments, RGC proteins were labeled by intraocular injection of ^{35}S-methionine. The optic nerve was crushed 6 days later and treated animal received an intraocular injection of C3-07 or PBS immediately after. Transport of labeled tubulin and middle neurofilament subunit (NF-M) was assessed 8 days later, 14 days after labeling. Optic nerves were removed and cut into 5 segments of 2 mm each (Fig. 7A). The distance traveled in the optic nerve was determined by identifying which of the 5 segments the front of NF-M and tubuline had reached.

The NF-M and tubuline fronts of uninjured control animals reached the fifth optic nerve segment 14 days after labeling (Fig. 7B-1). However, 6 days after labeling, the fronts had only reached the third segment (Fig. 7B-2). In animals whose nerve was crushed 6 days

tubulin fronts traveled no further in C3-07 treated animals that they did in PBS treated animals (Fig. 7B-5) or in animals that received no injection at all (Fig 7B-3). Therefore, C3-07 had no impact on slow axonal transport after injury. We tested if C3-07 affected slow axonal transport in uninjured nerve. The distance traveled by the protein fronts in uninjured animals 14 days after labeling was not impacted by a C3-07 injection on day 6 after labeling (data not shown).

DISCUSSION

Rho antagonists C3 or C3-05 have been shown to promote neurite outgrowth in the presence of inhibitory substrates (Bito et al., 2000; Dergham et al., 2002; Lehmann et al., 1999; Monnier et al., 2003; Winton et al., 2002) and stimulate axonal regeneration after optic nerve (Lehmann et al., 1999) or spinal cord injury (Dergham et al., 2002). In this study, we made use of C3-05 and a more highly purified version called C3-07 to further understand how C3-based Rho antagonists work to stimulate regeneration in the optic nerve. We show that treatment with Rho antagonists is as effective in promoting regeneration after optic nerve microlesion when applied to the RGCs cell bodies as it was

et al., 2003). Interestingly, although macrophages are also numerous at the lesion site (Berry et al., 1996; Selles-Navarro et al., 2001a), they do not elicit a regenerative response (Berry et al., 1996). Further, intravitreal grafting of acellular peripheral nerve has a small regenerative effect on RGCs (Berry et al., 1996) while acellular grafts connected to the cut end of the optic nerve do not (Berry et al., 1988; Hall and Berry, 1989) (verif référence). However, other experiments demonstrate, in Campenot chambers, that nerve growth factor (NGF) application to the axonal compartement is sufficient to promote axonal growth, while application to the cell body compartment is not (Campenot, 1994). Our findings are different; we observed the same growth promoting effect of Rho antagonists, independently of the application site. Our *in vitro* results in Campenot chambers suggest that treatment with a C3-based Rho antagonist has the same axonal growth promoting effect when applied to either the cell body or axonal compartment, or both. *In vivo*, our regeneration results with an intravitreal application of C3-05 or C3-07 were comparable to those obtained after a lesion site application of C3 (Lehmann et al., 1999), following a microlesion of the optic nerve. The similarity of results at either site can be explained, at least in part, by the fact that these C3-based molecules are not restricted to their original cellular location. We show

C3-05 or C3-07 to the cell body might improve regeneration primarily by increasing RGC survival after an optic nerve microlesion, while lesion site application would promote axonal regrowth mainly by directly blocking the effect of inhibitory proteins on the growth cone with local Rho inactivation. Preliminary results from our laboratory indicate that an intravitreal injection of C3-05 or C3-07 increased RGC survival following microlesion, while an application of C3 at the lesion site did not improve survival (unpublished observations). However, since C3-07 is present in axons even when applied only to the cell bodies, it is also possible that most of its regenerative effect is caused by its action at the growth cone. To our knowledge, we are the first to compare the effect of the same treatment at the cell body and the lesion site in the optic nerve. Understanding the molecular mechanisms involved at each site to promote axonal regrowth after injury will be important to identify which location is best suited for therapeutic application.

We have previously shown that CSPG, inhibitory molecules associated with the glial scar, are present at the site of an optic nerve microcrush lesion as early as 24 hours after the injury (Selles-Navarro et al., 2001a). The axonal regeneration observed with C3-07 injected intravitreally 4 days after the optic nerve injury thus indicates that Rho inactivation allows

regenerating axons are observed in control animals. Interestingly, the difference between treated and untreated animals was observed even 4 weeks after treatment. However, the number of regenerating axons decreased significantly between weeks 2 and 4, most likely reflecting the continuous death of RGCs observed after axotomy (refs). This decrease also indicates that while these Rho antagonists might provide some protection against cell death (see above and (Dubreuil et al., 2003)), they do not prevent it completely.

Regenerating axons did not grow for long distances after crossing the lesion site, a result consistent with the findings from most studies of regeneration in the rat optic nerve (Ellezam et al., 2003; Fischer et al., 2004; Lehmann et al., 1999; Leon et al., 2000; Sapieha et al., 2003). These results, along with the fact that treatments such as vaccination with a spinal cord homogenate or application of C3 to the lesion site can induce a much longer regeneration in the spinal cord (Dergham et al., 2002; Huang et al., 1999a) than in the optic nerve (Ellezam et al., 2003; Lehmann et al., 1999), could be explained in part by the findings of the Barres group that less than 1% of postnatal RGCs would retain the rapid axonal elongation ability they posessed in development (Goldberg et al., 2002c). However, axonal growth rates similar to what is observed in development was shown in recent

by C3-05/07 blocks a signalling pathway common to all the myelin inhibitors identified to date, namely MAG, OMgp, Nogo-66 and amino-Nogo, as well as CSPG. These findings suggest that the presence of myelin or glial scar inhibitory proteins is not the primary reason explaining the absence of spontaneous regeneration of RGCs. Our results confirm previous findings by other groups who suggest that other issues have to be adressed along with growth inhibitory proteins to ensure successful regeneration after optic nerve axotomy. The Barres group has shown that RGCs lose their trophic responsiveness following axotomy (Shen et al., 1999) and may therefore require priming by electrical activity or signals such as cAMP to increase their response to neurotrophic factors needed to induce axonal elongation (Goldberg et al., 2002a). More recently, experiments from the Benowitz group using dominant negative forms of NgR expressed in RGCs in vivo showed an almost total lack of regeneration unless RGCs were stimulated to grow by a lens injury (Fischer et al., 2004). Taken together, these results and ours strongly indicate that mature RGCs have to re-acquire a growth-promoting state before significant and extensive optic nerve regeneration can be expected. This will likely require a combination of trophic stimuli as well as factors to increase trophic responsiveness. Furthermore, slow axonal

glial scar and CNS myelin, blocking the effects of growth inhibitory proteins will be crucial to sustain and accelerate axonal elongation.

Our results show that C3-05/07 promote axonal regeneration in the inhibitory environment of the optic nerve, after microlesion injury. Because they can be applied intravitreally or at the lesion site, and because there is a therapeutic window of a few days after injury for their application, these C3-based Rho antagonists represent a promising alternative to promote regeneration as part of a combined treatment. Meanwhile, sustaining the effect of Rho antagonists could be an alternative. Even if growth inhibitory proteins are not the primary cause of all RGCs poor regeneration, C3-07 stimulated some axons to grow for relatively longer distances than C3-05. Additional studies could tell us if this stimulation is sustainable with multiple or continuous delivery of C3-07 and if some RGCs can be induced to grow for the whole distance of the optic nerve.

METHODS

Campenot chambers

Rat sympathetic neurons (superior cervical ganglion [SCG]) from postnatal day 0

side compartments. The cells were maintained in L15CO2 medium supplemented with 2.5 % rat serum 20 ng/ml NGF (Cedarlanes Laboratories, Hornby Ontario, Canada), vitamin C and 10 μM cytosine arabinside (CA) (Sigma, Oakville, Ontario, Canada).

Axotomy and axonal growth of Campenot cultures.

SCG were plated in Campenot chambers and maintained for 5 to 7 days. Distal axons were axotomized by spraying cold, sterile water through a 0.22-gauge needle into the two side chambers. This process was repeated approximately 3-4 times, until all distal axons had been axotomized. After axotomy, 2.5 μg/ml C3-07 was added to the various compartments (i.e. cell body compartment only, distal axon compartments only, or all 3 compartments) and axonal growth into side chambers was measured 3 days after axotomy. The media was changed every 48 hours and C3-07 was re-added to the fresh media in treated compartments. Axonal growth was measured as the distance from the beginning of the side chambers (i.e. the start of the grease barrier) to the longest axon present. Measurements were taken using an inverted microscope fitted with a digitizer that tracks stage movements to an accuracy of ±5 μm (Campenot, 1992). Experiments were completed

Campenot chambers with MAG substrates.

SCG were plated in Campenot chambers in which the two side chambers were pre-coated with MAG substrate (4 μg total protein). After 1 day in culture, C3-07 (2.5 μg/ml) was added to the various compartments (i.e. cell body compartment only, distal axon compartment only or both) and axonal growth into side chamber was measured for 3 consecutive days. The media was changed every 48 hours and C3-07 was re-added to the fresh media in treated compartments. Axonal growth was measured as discussed above. Experiments were completed in triplicate with a minimum of 10 tracks measured per culture chamber.

Western blots for Campenot chambers experiments

After axonal growth was measured, untreated and C3-07 treated Campenot cultures used in the axotomy studies were washed twice with cold Tris buffered saline (TBS) and lysed in modified RIPA buffer (50 mM Tris pH 7.2, 1% Triron X-100, 0.5% sodium deoxycholate, 0.1% SDS, 500 mM NaCl, 10 mM $MgCl_2$, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 mM phenylmethyl-sulfonyl fluoride (PMSF)). Pooled cell lysates from cell

Rho antagonists C3-05 and C3-07

C3-05 is a modified version of C3-ADP-rybosyltransferase from *Clostridium botulinum* with an added transport sequence that facilitates entry into cells. C3-05 was prepared as described (Ref Winton et al) by affinity purification and was determined to be approximately 95% pure. During the course of these experiments, a newer version of C3-05 became available, called C3-07. C3-07 differs from C3-05 because the DNA sequence for the GST tag used for affinity purification of C3-05 was removed before subcloning into a pET vector, and several N-terminal amino acids were introduced during subcloning. C3-07 was purified by fast-protein liquid chromatography (FPLC), as described (Han et al, 2001) The purified protein is approximately 99% pure, and is essentially endotoxin free.

Intraorbital microlesion surgery and C3-05/07 treatment

Intraorbital microlesions were performed on female Sprague-Dawley rats (180-200 g), under gas anesthesia with 2.5-3% isoflurane in oxygen. Microlesions effectively axotomize all RGC axons and provide a well defined lesion site (Selles-Navarro et al., 2001a). The surgery was performed as previously described (Selles-Navarro et al.,

were excluded from the study. The needle was slowly removed, and the injection site was sealed with surgical glue. The skin was sutured with 4-0 silk. The vascular integrity of the retina was verified by fundus examination, and animals whose retinas showed ischemic damage were excluded from the study. Rats were examined for regeneration 2 or 4 weeks later. All animals were given a dose of 0,015 mg/kg of buprenorphine (company, city) as analgesic right after surgery. Animals were kept in a controlled environment where they were exposed to a 12 hour light/dark cycle and had *ad libitum* access to food and water. All animal procedures followed guidelines from Canadian Council of Animal Care.

Anterograde labeling, immunohistochemistry and quantification of axon growth

Regenerating axons in the optic nerve were identified by anterograde labelling. Animals received an intravitreal injection of 5 µl of 1% cholera toxin β subunit (CTβ) 24 hours before sacrifice. Animals were deeply anesthesized with a 7% chloral hydrate overdose, followed immediately by intracardial perfusion with saline, then 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer, pH 7.2 (PB). The eyes were dissected in cold PBS, and the optic nerve was separated from the eye behind the globe. The nerves

antibody (1:200), and a 1 hour incubation with streptavidin conjugated to dichlorotriazinyl amino fluorescein DTAF (1:400).

Four to ten sections per animal were examined by epifluorescence microscopy, to detect CTβ-labeled axons, and photographed. The lesion site was identified using both the CTβ staining and dark field microscopy. Regeneration was measured by counting the number of CTβ-positive axons crossing a line parallel to the lesion site at distances of 50, 100, 250 and 500 µm. The longest axon was measured for each animal. Statistical significances were established using Student's *t*-test (*** $p < 0,001$, ** $p < 0,01$, * $p < 0,05$).

Verification of C3-07 cellular location in vivo

Microlesion of the left optic nerve was performed as described above, and 5 µg of C3-07 was injected intravitreally. Animals were sacrificed 3 days later by a 7% chloral hydrate overdose, followed by an intracardial perfusion with saline. Retinas and the first 2 mm segment of the optic nerve were removed, and stored at –80°C. Tissues were homogenized in 160 µl (retinas) or 20 µl (optic nerve segments) of Np40 lysis buffer

Radiolabeling and intracranial crush

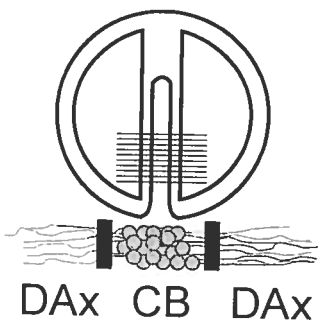
To radiolabel RGC proteins in the left eye, intraocular injection of 250 uCi of ^{35}S-methionine (ICN, Irvine,) in 5 µl saline was performed on female Sprague-Dawley rats (180-200 g), under gas anesthesia with 2.5-3% isoflurane in oxygen. Animals were separated in 6 groups. Two groups were sacrificed 6 (n = 3) or 14 (n = 3) days after labelling, without getting any surgery or treatment. Remaining groups were all sacrificed 14 days after labeling. An intracranial crush was performed, 6 days after labeling, on 3 groups, one receiving a C3-07 injection right after the lesion (n = 4), one a PBS injection (n = 3) and one getting no injection (n = 4). The last group had no crush surgery but received a C3-07 injection 6 days after labeling (n = 3). Intracranial crush were performed on the left optic nerve by aspirating a portion of the left frontal lobe. The RGC axons were severed just in front of the chiasma, approximately 9 mm from the optic disc, by crushing the optic nerve 2 to 3 times, for 5 to 10 seconds, with fine forceps. Treated animals were injected in the left eye with 1 µg of C3-07, diluted in 5 µl of PBS, while control animals were injected with 5 µl of PBS. In groups subjected to a crush and treatment, the injection was performed immediately after surgery. Intravitreal injections, skin suturing, verification of vascular

Tissue preparation, SDS gel electrophoresis and evaluation of slow axonal transport

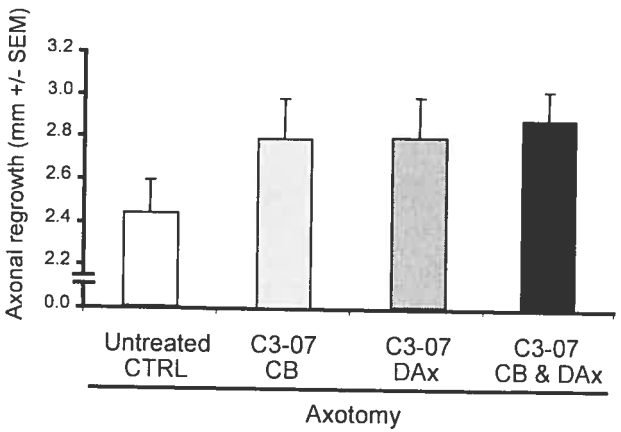
Six or 14 days after radiolabeling, animals used for slow axonal transport experiments were sacrificed by a 7% chloral hydrate overdose, followed immediately by intracardial perfusion with saline. The left optic nerve was removed, cut into 2 mm segments and stored at –80°C. Each 2 mm segment of optic nerve was homogenized in 60 ul of SDS-PAGE sample buffer using a motorized Teflon-in-glass homogenizer. The samples were clarified by high speed microcentrifugation for 5 minutes, and 40 ul of supernatant per sample were boiled for 3 minutes and clarified again by high speed microcentrifugation for 15 minutes. Samples were then loaded on 7.5% acrylamide gels for electrophoretic separation. The gels were stained with Coomassie blue, destained in 10% acetic acid, impregnated with Amplify dried and exposed to pre-flashed Hyperfilm MP for periods varying from 1 to 6 days.

Slow axonal transport speed was estimated from the distance traveled by labeled cytoskeletal proteins in the optic nerve. The distance traveled was determined by the position of the front of labeling in the optic nerve for tubulin and the 150 kDa middle neurofilament subunit (NF-M). NF-M comigrates with the other 2 neurofilaments subunits,

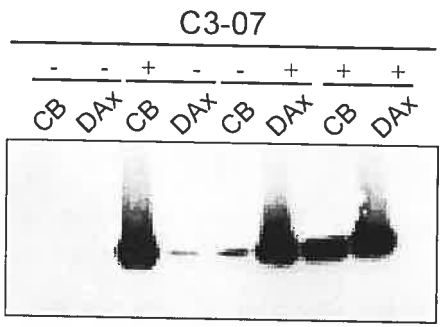
A

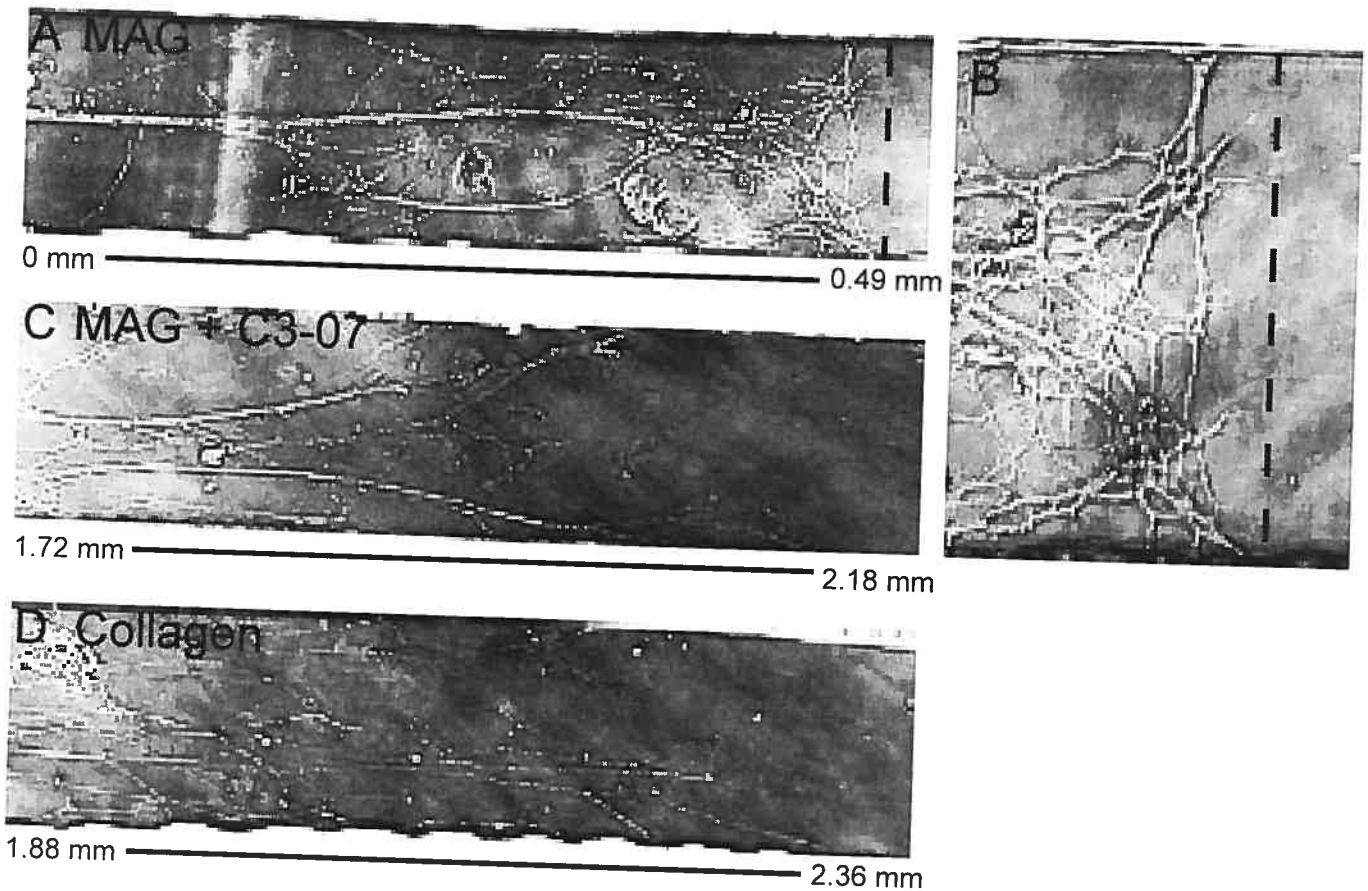

DAx CB DAx

B


Axonal regrowth (mm +/- SEM)
3.2
3.0
2.8
2.6
2.4
2.2
0.0
Untreated CTRL
C3-07 CB
C3-07 DAx
C3-07 CB & DAx
Axotomy

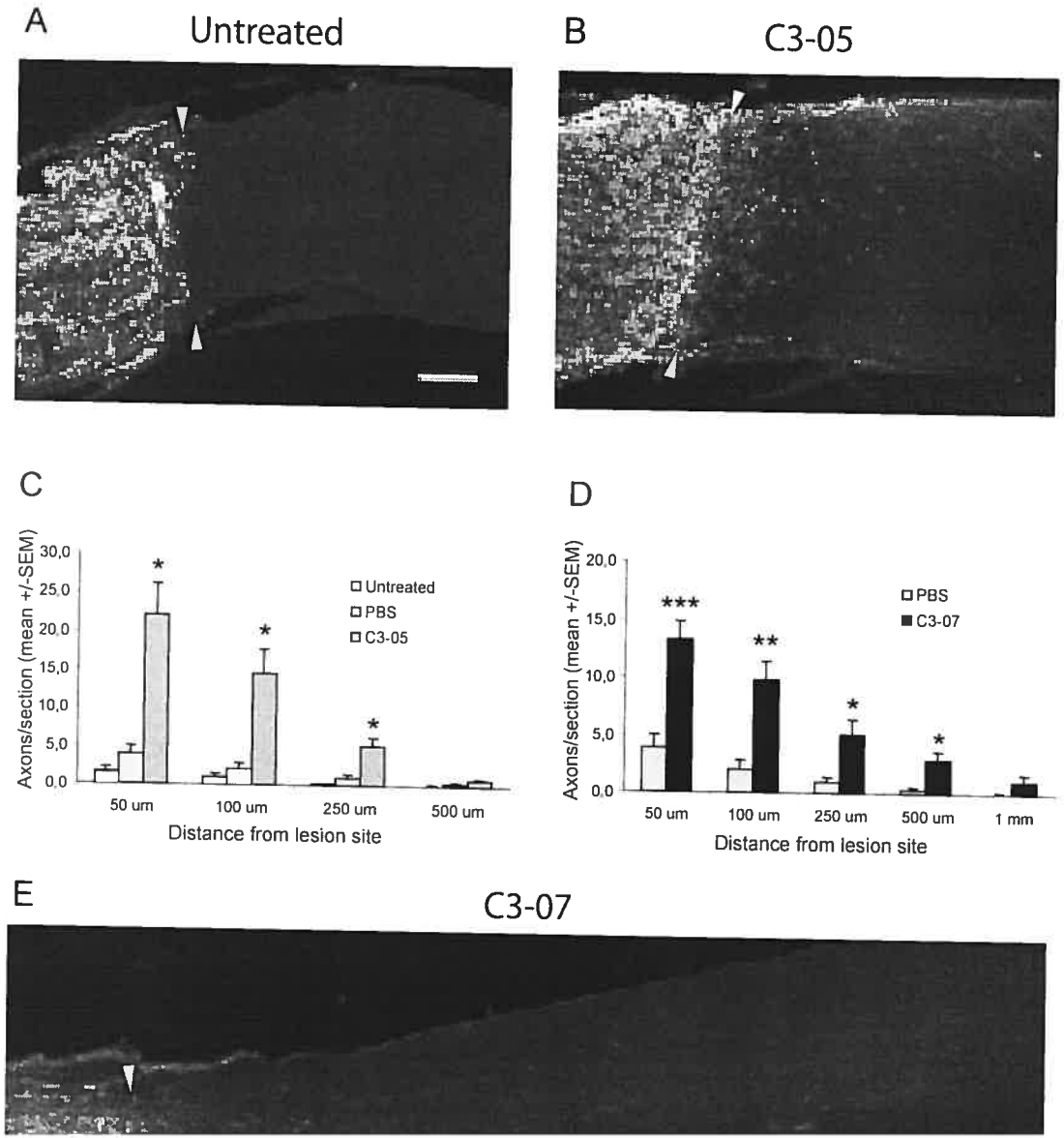
C


C3-07
- - + - - + + +
CB DAx CB DAx CB DAx CB DAx

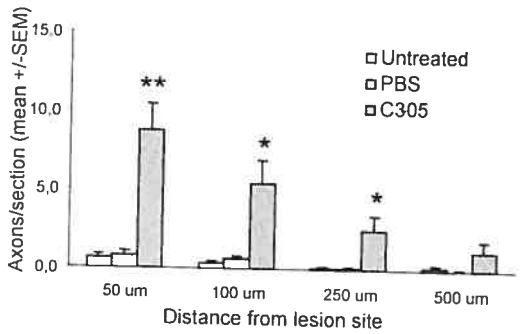

A MAG
0 mm
0.49 mm
B
C MAG + C3-07
1.72 mm
2.18 mm
D Collagen
1.88 mm
2.36 mm

E

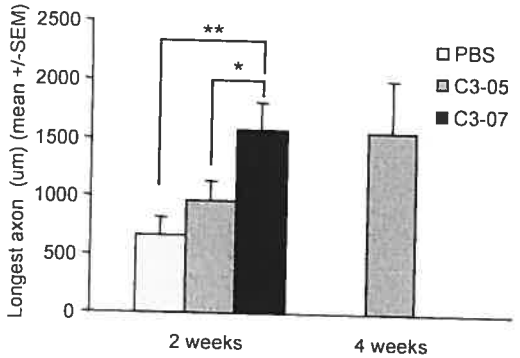

(mm +/- SEM)
4
3
Collagen CTRL
C3-07 CB + MAG
C3-07 DAx + MAG
C3-07 CB & DAx + MAG
MAG CTRL


A
Untreated
B
C3-05
C
Axons/section (mean +/-SEM)
30,0
25,0
20,0
15,0
10,0
5,0
0,0
*
*
*
Untreated
PBS
C3-05
50 um
100 um
250 um
500 um
Distance from lesion site
D
Axons/section (mean +/-SEM)
20,0
15,0
10,0
5,0
0,0
***
**
*
*
PBS
C3-07
50 um
100 um
250 um
500 um
1 mm
Distance from lesion site
E
C3-07

A

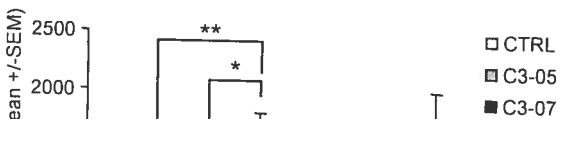


B

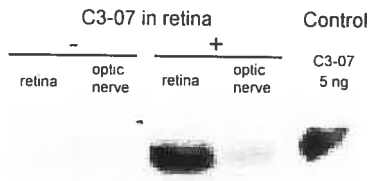


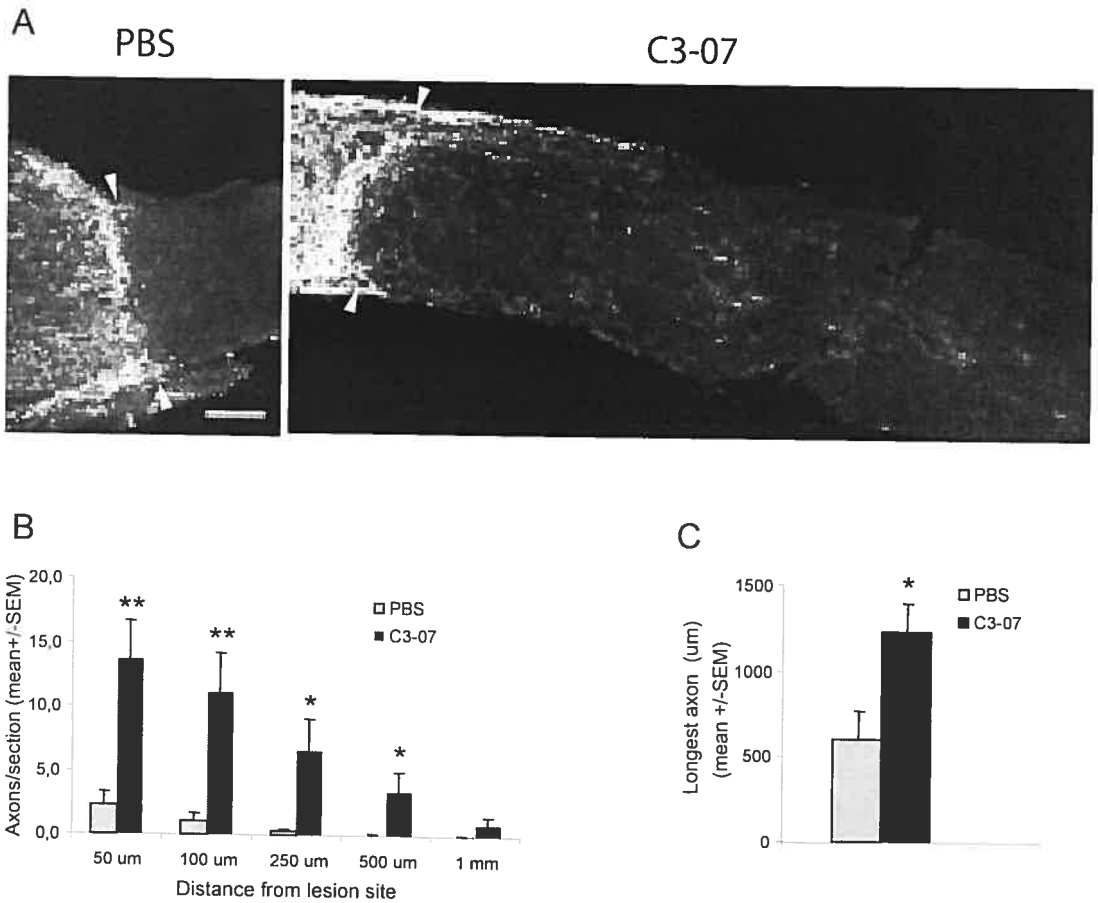
OR

B

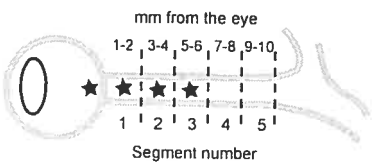


Old version


C3-07 in retina
Control
-
+
retina
optic nerve
retina
optic nerve
C3-07
5 ng


A
PBS
C3-07
B
Axons/section (mean+/-SEM)
20,0
15,0
10,0
5,0
0,0
**
**
*
*
PBS
C3-07
50 um
100 um
250 um
500 um
1 mm
Distance from lesion site
C
Longest axon (um)
(mean +/-SEM)
1500
1000
500
0
*
PBS
C3-07

A



B

	1	2	3	4	5
Day 0:	^{35}S-methionine	^{35}S-methionine	^{35}S-methionine	^{35}S-methionine	^{35}S-methionine
Day 6:	----	sacrifice	crush	crush + C3-07 injection	crush + PBS injection
Day 14:	sacrifice	----	sacrifice	sacrifice	sacrifice

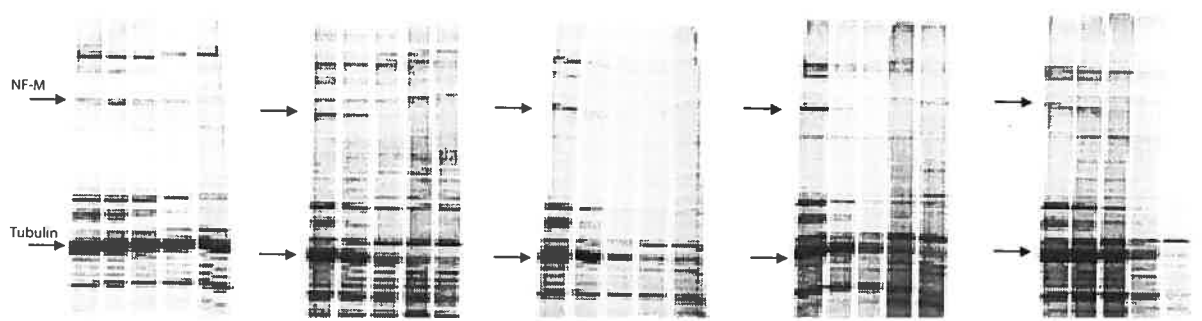


FIGURE LEGENDS

Figure 1. **C3-07 increases axonal regrowth *in vitro* after axotomy, independently of the cellular location of application.** (A) Model of a Campenot chamber. Campenot culture chambers isolate cells bodies (CB) and distal axons (DAx) of neuronal cells in separate compartments. Cells are plated in the center compartment and extend their axons into the distal chambers. This separation allows treatment of either cell bodies or distal axons with C3-07. (B) Axonal regrowth of axotomized axons following application of C3-07 to specific cellular locations: cell body compartment (CB), distal axon compartments (DAx), all compartments (CB & DAx). The longest axon per track was measured for a minimum of 10 tracks per culture chamber. Data represents the average of three or more experiments performed in triplicate. (C) Location of C3-07 3 days after it is applied to the cell body compartment (CB) or distal axon compartments (DAx) or all compartments. Western blot of pooled lysates from 3 chambers were probed with a polyclonal anti-C3 antibody.

Figure 2. **C3-07 promotes axonal growth on MAG substrates, independently of the**

measured for a minimum of 10 tracks per culture chamber. Data represents the average of four or more experiments performed in triplicate.

Figure 3. **Immediate treatment with intravitreal injection of C3-05 or C3-07 stimulates regeneration in the optic nerve after 2 weeks.** (A-B) Photographs of optic nerve sections immunostained with a CTβ antibody to reveal axons regenerating distally to the lesion site (arrowheads) 2 weeks after microlesion in untreated (A), or C3-05 (B) treated animal. (C-D) Quantification of regeneration in C3-05 (C) and C3-07 (D) treated animals, compared to controls. (E) Optic nerve section immunostained with a CTβ antibody to reveal axons regenerating distally to the lesion site (arrowheads) 2 weeks after microlesion in C3-07 treated animals. Scale bar (in A) A, B, E 100 um. *** $p < 0,001$, ** $p < 0,01$, * $p < 0,05$ Student's t-test.

Figure 4. **Immediate treatment with intravitreal injection of C3-05 stimulates regeneration in the optic nerve after 4 weeks.** (A) Quantification of regeneration in C3-05 treated animals compared to controls. (B) C3-05 and C3-07 differ in their impact on

Figure 5. ***In vivo,* C3-07 is not restricted to the cellular location of its original application.** C3-07 is detected in the optic nerve 3 days after 5 μg was injected in the vitreous, following a microlesion of the optic nerve. Lysates of retinas and corresponding optic nerves were processed on Western blots for C3 detection.

Figure 6. **Delayed treatment with intravitreal injection of C3-07 stimulates regeneration in the optic nerve after 2 weeks.** (A) Photographs of optic nerve sections immunostained with CTβ antibody to reveal axons regenerating distally to the lesion site (arrowheads) 2 weeks after microlesion in C3-07 treated animals and PBS controls. C3-07 or PBS were administered 4 days after the microlesion. Scale bar, 100 um. (B) Quantification of regeneration in C3-07 treated animals compared to controls. (C) Comparison of average longest axon in each treatment group, obtained from the longest axon measured for each animal in that group. ** $p < 0,01$, * $p < 0,05$ Student's t-test.

Figure 7. **C3-07 does not re-establish slow axonal transport after intracranial crush.** (A) At the time of sacrifice, the optic nerve was removed and cut into 5 segments of

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Appendix B

Fourth Article

«Rho activation patterns after spinal cord injury and the role of activated Rho in apoptosis. Catherine I. Dubreuil, Matthew J. Winton and Lisa McKerracher. J Cell Biol. 21; 162 (2): 233-43. 2003.»

Rho activation patterns after spinal cord injury and the role of activated Rho in apoptosis in the CNS

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Abstract

Growth inhibitory proteins in the CNS block axon growth and regeneration by signaling to Rho, an intracellular GTPase. It is not known how CNS trauma affects the expression and activation of RhoA. Here we detect GTP-bound RhoA in spinal cord homogenates and report that spinal cord injury (SCI) in both rats and mice activates RhoA over 10 fold in the absence of changes in RhoA expression. In situ Rho-GTP detection revealed that both neurons and glial cells showed Rho activation at SCI lesion sites. Application of a Rho antagonist (C3-05) reversed Rho activation and reduced the number of TUNEL-labeled cells by approximately 50% in both injured mouse and rat, showing a role for activated Rho in cell death following CNS injury. Next we examined the role of the p75 neurotrophin receptor ($p75^{NTR}$) in Rho signaling. After SCI, an upregulation of $p75^{NTR}$ was detected by western blot and observed in both neurons and glia. Treatment with C3-05 blocked the increase in $p75^{NTR}$ expression. Experiments with $p75^{NTR}$ null mutant mice showed that immediate Rho activation after SCI is $p75^{NTR}$ dependent. Our results indicate that blocking over-activation of Rho after SCI protects cells from $p75^{NTR}$ dependent apoptosis.

Introduction

Growth inhibitory proteins have long been known to inhibit axonal regeneration in the CNS (Schwab et al., 1993). These inhibitory proteins are enriched in myelin, and the three best characterized myelin-derived growth inhibitory proteins include myelin-associated glycoprotein (MAG), Nogo, and oligodendrocyte-myelin glycoprotein (OMgp) (McKerracher and Winton, 2002; Woolf and Bloechlinger, 2002). Recent data indicate that these three inhibitory proteins bind to the same neuronal receptor, the Nogo-66 receptor (NgR), but there are additional inhibitory proteins in the CNS that act through different receptors. More important, both NgR and NgR-independent inhibitory proteins signal to activate Rho, a small intracellular GTPase (Niederost et al., 2002).

Early experiments demonstrated that lysophosphatydic acid causes neurite retraction and cell rounding by activating Rho (Jalink et al., 1994; Tigyi et al., 1996a). The use of C3 transferase to inactivate Rho in primary neurons plated on various types of inhibitory proteins and dominant negative Rho expressing PC-12 cells provides direct evidence that the inactivation of Rho results in neurite outgrowth on inhibitory substrates. In vivo experiments in rats and mice have shown that inactivation of Rho or of Rho kinase

signaling by MAG (Yamashita et al., 2002). Also, Rho binds to $p75^{NTR}$ (Yamashita et al., 1999) and Rho is likely to form part of the membrane raft receptor complex responsible for growth inhibitory signaling (McKerracher and Winton, 2002; Woolf and Bloechlinger, 2002). While $p75^{NTR}$ has been implicated in apoptosis after SCI (Casha et al., 2001) it is not known to what extent Rho signaling by $p75^{NTR}$ participates in apoptotic events after SCI.

Isoforms of Rho exist, and in neurons RhoA is expressed at higher levels than RhoB and RhoC (Lehmann et al., 1999). Therefore, we have focused on RhoA for our studies in neurons. In non-neuronal cells Rho family GTPases are best characterized for their effects on organization and regulation of the actin cytoskeleton (Ridley, 2001), but they have also been shown to play a role in the regulation of apoptosis (Aznar and Lacal, 2001; Coleman and Olson, 2002; Jimenez et al., 1995). The extent to which Rho may participate in apoptotic pathways in neuronal cells has yet to be determined. In neurons, Rho is activated in response to chemorepulsive molecules (Jin and Strittmatter, 1997; Wahl, 2000), and is important in axon guidance during development. In adult neurons, inhibitory substrates (Lehmann et al., 1999; Niederost et al., 2002; Winton et al., 2002) and secreted factors such

homogenates following SCI. We show that SCI causes an increase in active Rho without affecting RhoA expression levels. We made use of an *in situ* pull down assay (Li, 2002) to determine that neurons and glia in the spinal cord show Rho activation. To test the use of a Rho antagonist to reverse Rho activation, we used a cell permeable form of C3 transferase (C3-05) that has a short transport sequence added to the carboxy terminal to help entry into cells (Winton et al., 2002). We show that C3-05 specifically inactivates Rho *in vivo*, and prevents up-regulation of $p75^{NTR}$. Treatment of injured spinal cord with C3-05 not only effectively reversed Rho activation but also had cell protective effects.

Results

Rho is activated by inhibitory substrates

To examine the effect of growth inhibitory proteins on Rho activation we plated PC-12 cells on myelin, MAG, or poly-L-lysine (PLL) substrates. We measured amounts of GTP-Rho in cell lysates by precipitation with RBD from rhotekin that binds only GTP bound Rho (Reid et al., 1996a). Cells plated on inhibitory substrates had high endogenous Rho-GTP levels compared to PLL controls (Figure 1A). The activation of Rho in cells plated on myelin or MAG was reversed by treatment with the Rho antagonist C3-05 (Figure 1B). The RBD beads incubated without lysate (buffer only) show no active Rho, when overexposed GST-RBD is detectable (Figure 1C) showing the specificity of the assay for Rho. Treatment of neuronal cells with C3-05 promotes neurite outgrowth on MAG or myelin substrates (Winton et al., 2002). Our results with MAG and myelin are consistent with recent studies showing Rho activation in the presence of Nogo (Niederost et al., 2002) or upon activation of Nogo receptor (Wang et al., 2002b).

Rho is activated after traumatic SCI injury

pooled, and each gel lane represents results from one animal. In uninjured CNS tissue, GTP-Rho levels were consistently low (Figure 2, controls). By contrast, Rho activation is dramatically increased after injury (Figure 2A and 2B), increasing over 10 fold (Figure 2C). Expression levels of total RhoA, as detected by western blots from tissue homogenates used for isolation of GTP-Rho, did not change (Figures 2A and 2B). These results show that Rho is massively activated in CNS tissue of rats and mice following spinal cord injury as compared to uninjured spinal cord. To examine if the activation of Rho after SCI injury was sustained or transient, we prepared homogenates from transected spinal cord 1.5 hours, 24 hours, 3 days and 7 days after lesion. Interestingly, we found that Rho was active as early as 1.5 hours after injury. The significant increase in activation observed by 24 hours was sustained for at least 7 days (Figures 2B and 2C).

In neuronal like cells (PC-12), Rho becomes activated when cells are plated on substrates of MAG or myelin (Figure 1A). It has also been shown that MAG, is not only present in myelin, but is released from damaged white matter after injury (Tang et al., 2001). To confirm that growth inhibitory proteins were present in the lesion sites after SCI, we examined MAG expression levels after injury. The same tissue homogenates used for

Treatment with C3-05 reverses Rho activation after injury

To test if we could reverse the increase in Rho activation in injured spinal cord, we made use of the Rho antagonist C3-05 (Winton et al., 2002). We injected C3-05 in a fibrin matrix into the lesion site after spinal cord transection, or C3-05 alone into contused spinal cord, and the lesion sites were removed 24 hours later. Treatment with C3-05 inactivated Rho, bringing the RhoA activation levels back to the normal basal state (Figures 3A and 3B). To determine if the reversal of Rho activation was sustained after a single injection of the compound, we examined rats 7 days after transection injury and treatment. Even 7 days after C3-05 treatment, Rho activation still remained at basal levels (Figures 3A and 3B). Next, we asked if C3-05 remained at the lesion site after treatment. Probing the homogenates with a polyclonal antibody raised against C3 (Winton et al., 2002) demonstrated that C3-05 was detected at the lesion site at all of the time points tested (Figure 3A). To determine if endogenous cells in the spinal cord were able to take up and retain C3-05 after treatment, we examined sections of rat spinal cord double-labeled with an antibody specific for C3 and with cell type specific markers. We detected intracellular C3 immunoreaction in neurons, astrocytes and oligodendrocytes after injection of C3-05

endogenous Rho activation levels. We incubated sections with GST-RBD and cells that bound high levels of RBD were detected with an anti-GST antibody. Active Rho was detected in many cells in both the grey (Figure 4, pannels 1 and 2) and white matter (Figure 4, panel 3) of injured spinal cord. We also found that Rho was activated both rostral (Figure 4, panel 1) and caudal (Figure 4, panel 2) to the lesion site. At further distances from the lesion site, staining for active Rho was very faint or absent (Figure 4, panel 4). Rho-GTP was not detected in uninjured spinal cord (Figure 5A left) or after C3-05 treatment was used to reverse the increase in Rho activation after SCI (Figure 5A middle). To asses the specificity of the technique, we incubated sections with GST without RBD and no positive cellular active Rho staining is visible, (Figure 5B, right).

To specifically determine which cell types express active Rho, sections were double-labeled with cell type specific markers. We detected active Rho in neurons, astrocytes and oligodendrocytes 24 hours, 3days and 7 days after injury (Figure 5B). Therefore, the activation of Rho observed after injury (Figure 2 and 3) is an endogenous cellular response (Figure 5B). The ability to detect GTP-Rho without prior transfection (Li, 2002) confirms the high level of endogenous active Rho in neurons and glia after SCI.

(TUNEL) (Figure 6A, top panel), similar to that observed after rat SCI (Figure 6A, bottom panel). Importantly, in both mice and rats treated with C3-05, the number of TUNEL-labeled cells was significantly reduced by approximately 50% after SCI (Figure 6B). Not only was C3-05 present in neurons, astrocytes and oligodendrocytes (Figure 3C), but most cells containing C3-05 were not TUNEL positive (Figures 6C and D). The small number of cells double-labeled with C3 and TUNEL (16%) suggest that C3-05 penetrated into some cells that had progressed too far into the apoptotic cascade to be rescued from death. Together our results indicate that inactivation of Rho after SCI protects cells from apoptosis. These findings have clinical relevance because neuroprotective treatments after spinal cord injury lead to improved functional recovery (Liu et al., 1997b).

Inhibitory substrates regulate Rho activation after injury by a p75 dependant mechanism

It has recently been shown that MAG activates Rho in the presence of $p75^{NTR}$ (Yamashita et al., 2002) and that MAG interacts with neuronal lipid rafts containing NgR, GT1b, $p75^{NTR}$ and Rho (Vinson et al., 2003). We have shown that after SCI, MAG is

(Figure 7C). These results indicate that Rho is activated through a $p75^{NTR}$ dependant mechanism early after SCI but at later time points $p75^{NTR}$ independent activation occurs.

The $p75^{NTR}$ has been implicated in the regulation of apoptosis after injury in the nervous system (Cheema et al., 1996; Dechant and Barde, 2002; Frade and Barde, 1999). In addition to counting TUNEL cells after SCI (Figure 6B), we counted $p75^{NTR}$ labeled cells and double labeled cells. Many cells express $p75^{NTR}$ after SCI (Figure 8B) and 74% of these cells were also TUNEL positive. Treatment with C3-05 resulted in a decrease in $p75^{NTR}$ alone and in $p75^{NTR}$ and TUNEL (Figure 8A and B). These results suggest a correlation between Rho activation, $p75^{NTR}$ expression, and cell death. To further investigate the involvement of $p75^{NTR}$ in Rho activation after spinal cord injury, we probed the homogenates of transected spinal cord with $p75^{NTR}$-specific antibodies, using the same homogenates as shown in bottom panel (Figure 8C, bottom panel). There was very low $p75^{NTR}$ detectected in western blots in the adult spinal cord (Figures 7B and 8C, controls). Levels of $p75^{NTR}$ increased as early as 24 hours after SCI and high levels were detected 3 and 7 days post injury a finding consistent with previous reports of $p75^{NTR}$ upregulation after SCI (Beattie et al., 2002a; Casha et al., 2001; Widenfalk et al., 2001). Treatment with

Discussion

It is now well established that neurons in the CNS respond to negative growth inhibitory cues, as well as positive growth-promoting signals. Much work has underlined the importance of myelin-derived growth inhibitory proteins expressed by oligodendrocytes and present in white matter (Schwab, 2002; Schwab et al., 1993). The consequence of a growth inhibitory environment in the CNS is that injured neurons fail to regrow their transected axons, even though they have an inherent capacity to regenerate. The molecular mechanisms of neuronal growth after injury have been widely studied in order to promote regeneration after SCI (David and Lacroix, 2003). Work from our lab and others has established that inactivation of Rho signaling is sufficient to promote axon growth on growth inhibitory substrates (Dergham et al., 2002; Fournier et al., 2003; Jin and Strittmatter, 1997; Lehmann et al., 1999; Niederost et al., 2002) and to stimulate axon regeneration after spinal cord injury (Dergham et al., 2002; Fournier et al., 2003; Hara et al., 2000b). We have shown here that Rho is activated when neuronal like cells are plated on myelin, and that Rho is significantly activated in neurons and glia *in vivo* after SCI. We report that administration of C3-05 reverses Rho inactivation *in vivo*, and protects cells

contributes to increased Rho activation after SCI. First, we showed that neuronal Rho is activated by MAG and myelin when PC12 cells are plated on inhibitory substrates (Figure 1). Soluble Nogo fusion proteins can also activate neuronal Rho (Fournier et al., 2003; Niederost et al., 2002). Other evidence indicates that Rho is activated by NgR independent growth inhibitory proteins. Inactivation of Rho promotes neurite growth on chondroitin sulfate proteoglycans (Dergham et al., 2002) that are present at glial scars. Also, collapsin and ephrins, chemoreplusive factors that act through different receptors, respectively, both activate Rho (Jin and Strittmatter, 1997; Wahl, 2000). Preliminary evidence from our lab indicates that astrocytes plated on inhibitory substrates show Rho activation (Dergham, Dubreuil, and McKerracher, unpublished results). Therefore, inhibitory proteins may activate Rho in both neurons and glial cells by NgR and NgR-independent mechanisms.

The inflammatory environment may contribute to Rho activation after SCI. Reactive astrocytes secrete tumor necrosis factor (TNF) and TNF has been shown to activate Rho in neurons expressing TNF receptors (Neumann et al., 2002a). Inflammation after injury is considered to cause secondary damage because it progresses with time, and causes continued cell death after the primary traumatic insult (Popovich and Jones, 2003;

activation is interesting because both thrombin and TNF, known to activate Rho are $p75^{NTR}$ independent and are present early after SCI (Citron et al., 2000; Donovan et al., 1997; Lee et al., 2000). Our experiments with the $p75^{NTR}$ knock out mice were with whole tissue homogenates, and do not address significant changes in individual cell types early after injury. The massive Rho activation we observe in normal mice and rats after SCI likely represents the combined effects of the many different Rho activating factors. Secondary damage by inflammation may also contribute to activation of Rho, and if this is the case, then Rho may be an important target to prevent secondary inflammatory damage. Our results following treatment with C3-05 show that inactivation of Rho reduces cell death that follows injury. Further, the massive activation of Rho that we observed after injury was sustained for at least 7 days. Therefore, multiple local signals may activate Rho in CNS cells. We speculate that continued presence of growth inhibitory molecules at the site of a CNS lesion contributes to sustained activation of Rho in neurons and glia after SCI.

Rho activation leads to apoptosis after SCI

Rho GTPases are known regulators of apoptosis in various cell types. In non-

is critical in the effect of Rho signalling and cell death. In PC-12 cells, a neural cell line, Rho proteins have been shown to induce Rho- dependant membrane blebbing (Mills et al., 1998), a morphological characteristic of apoptosis. In cultured astrocytes and hippocampal neurons, treatment with thrombin, a protease found after CNS trauma, causes Rho dependant apoptosis. Treatment with C3 reversed the thrombin-induced apoptosis of astrocytes and neurons by approximately 50% (Donovan et al., 1997). These data support our direct evidence that inactivation of Rho by C3-05 is cell protective to neurons and glia. Although Rho activation in neurons alone may not be sufficient to cause cell death, our data supports the hypothesis that Rho activation enhances apoptotic signaling cascades. We hypothesise that the combination of Rho activating factors released after SCI, including the myelin growth inhibitory proteins, leads to Rho dependant apoptosis. This suggests that Rho activation is required but not necessary to mediate apoptosis. Further, TNF activates Rho in neurons (Neumann et al., 2002a) and antibody mediated blocking of TNF reduces apoptosis after SCI (Lee et al., 2000). These findings also support our direct demonstration that inactivation of Rho is protective for cells in the damaged CNS.

apoptotic cascades (Aznar and Lacal, 2001; Huang and Reichardt, 2001). Therefore, we speculate that treatment with C3-05 to block Rho activation after injury suppresses apoptosis by preventing the synthesis of pro-apoptotic proteins such as $p75^{NTR}$.

The $p75^{NTR}$ contributes to initial apoptotic cascades that follow injury in the CNS. In oligodendrocytes, an increase the expression of $p75^{NTR}$ after SCI leads to apoptotic cell death (Beattie et al., 2002a; Casha et al., 2001). In astrocytes, $p75^{NTR}$ expression is observed after exposure to inflammatory cytokines (Hutton et al., 1992; Semkova and Krieglstein, 1999), also known to activate Rho (Neumann et al., 2002a). In neurons, apoptosis following growth factor deprivation is mediated by $p75^{NTR}$ (Kaplan and Miller, 2000), and reducing $p75^{NTR}$ levels prevents death of axotomized neurons (Cheema et al., 1996). Reducing $p75^{NTR}$ levels reduces apoptosis in contused spinal cord (Brandoli et al., 2001). In $p75^{NTR}$ -/- mice there is a decrease in apoptosis in the spinal cord (Frade and Barde, 1999). We have shown that both neurons and glia have unusually high levels of active Rho after SCI (Figure 4A). Moreover, reversal of Rho with C3-05 reduced apoptosis (Figure 6B) and prevented $p75^{NTR}$ up-regulation (Figure 8B and C). Together these results indicate that Rho activation induces apoptosis in a $p75^{NTR}$ dependant manner early on after

Materials and methods

Surgical Procedures

Rats were anaesthetised under 2-3% isoflurane. For spinal cord injury, adult female Long-Evans rats (200-250 g) underwent laminectomy at thoracic level T10-T11 for SCI at T10. Control animals were sham operations with laminectomy only. Dorsal over-hemisections were done at a depth of 1.6 mm. For contusion experiments, the NYU impactor device was used with 10 g at 25 mm. From 24 hours to 7 days after SCI, animals received an overdose of chloral hydrate anaesthetic, were perfused with saline, and the spinal cords removed. Approximately 5 mm surrounding the injured area was isolated and frozen at –80 °C. Balb/c female mice (20-22 g) and p75 knockout mice (Lee et al., 1992) (Jackson mice, stock number 002213, Bar Harbor, Maine) were anaesthetised with hypnorm (20 ml/Kg) and diazepam (1 mg/Kg). Dorsal over-hemisections were performed at T8; n=5 at 24h, n=5 at 3d. After perfusion with saline, 2-3 mm of spinal cord from the lesion site was removed for analysis. After SCI, bladders of all animals were expressed 2-3 times per day. Rats were given 5 ml of 0.9% saline subcutaneously twice a day for 1 week and received daily subcutaneous injections of baytril (10 mg/Kg).

To treat rats with a Rho antagonist, 50 μg of C3-05 was injected in a fibrin matrix (Tisseel kit, Baxter, Mississauga, Canada) into transected spinal cord, as described (Dergham et al., 2002). In mice, 10 μg of C3-05 in fibrin was injected, except for the experiment for TUNEL labeling where 1 μg in fibrin was injected. C3-05 (50 μg) in phosphate-buffered saline without fibrin was injected into rat contusion injury sites. All animal procedures followed guidelines from the Canadian Council of Animal Care.

Cell Culture

PC-12 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) with 10% horse serum (HS), 5% fetal bovine serum (FBS), 1% penicillin-streptomycin (P/S). PC-12 cells were grown on poly-L-lysine (0.1 μg/ml) (Sigma, Oakville, Ontario, Canada) or myelin (8 μg per well) or MAG (8 μg) coated 6 well culture dishes. After the cells settled (3-6 hours at 37°C), the media was aspirated and fresh media containing the C3-05 (1 μg/ml) was added to the undifferentiated cultures. The cells were harvested 24 hours later, they were washed with ice cold Tris buffered saline (TBS) and lysed in modified RIPA buffer (50 mM Tris pH 7.2, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 500 mM NaCl, 10 mM $MgCl_2$, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM phenylmethyl-sulfonyl fluoride (PMSF)). Cell lysates were clarified by centrifugation at 13,000 g for 10 minutes at 4°C and kept at -80°C.

Pull down assays and immunoblotting

Purification of GST-Rho Binding Domain (GST-RBD) was performed as previously described (Ren and Schwartz, 2000). Bacteria expressing GST-RBD in a pGEX vector (a gift from John Collard, Division of Cell Biology, Netherlands Cancer Institute) were grown in L-broth (LB) with 100 μl/ml amplicillin. Overnight cultures were diluted 1:10 into 3600 ml LB and incubated in a shaking bacterial incubator at 37°C for 2 hours. Isopropyl-β-D-thiogalactopyranoside (0.5 mM) was then added to the incubating cultures for 2 hours. Bacteria were collected by centrifugation at 5,000 g for 15 minutes. The pellets were resuspended in 40 ml lysis buffer (50 mM Tris pH 7.5, 1% Triton-X, 150 mM NaCl, 5mM $MgCl_2$, 1mM DTT, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM PMSF). After

sonication, the lysates were spun at 14,000 rpm for 30 minutes at 4°C. The clarified bacterial lysate was then incubated with glutathion agarose beads (0.6 ml wet volume; preswelled with water) (Sigma, Oakville, Canada) for 60 minutes at 4°C. The coupled beads were then washed 6 times in wash buffer (50 mM tris pH 7.5, 0.5% Triton X-100, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 1 μg /ml aprotinin, 1 μg/ml leupeptin and 0.1 mM PMSF) and once in wash buffer containing 10% glycerol. Beads were then resuspended in 8 mls of the wash buffer containing 10% glycerol and stored overnight at -80°C. Frozen tissue was homogenized in RIPA buffer (50 mM Tris pH 7.2, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 500 mM NaCl, 10 mM $MgCl_2$, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM PMSF). The homogenates and cell lysates were clarified by two 10 minute centrifugations at 13,000 g at 4 °C. They were then incubated for 50 minutes at 4 °C with GST-RBD (a gift from John Collard, Division of Cell Biology, Netherlands Cancer Institute) coupled beads (20-30 μg/sample). The beads were then washed 4 times and eluted in sample buffer. GTP- bound Rho and total Rho present in tissue homogenates were detected by western blot. The proteins were transferred to nitrocellulose and probed using a monoclonal RhoA antibody (Santa Cruz, Santa Cruz, California). Bands were visualized with peroxidase-linked secondary antibodies (Promega, Madison, Wyoming) and an HRP based chemiluminescence reaction (Pierce, Rockford, Illinois). C3-05 was detected using a C3-specific polyclonal antibody (Winton et al., 2002). $P75^{NTR}$ was detected with a polyclonal antibody raised against $p75^{NTR}$ (Promega, Madison, Wyoming). For all blots, 20 micrograms of protein was loaded into each lane. Blots were scanned for densitometry using an Epson perfection 1200U scanner, transferred to Adobe photoshop 6.0 and the images were the analysed with the densitometry IQ MAC 1.2

software (Molecular Dynamics, Sunnyvale, California). The software measures the pixel density in the band image after background subtraction, and the densitometry value is in arbitrary units. Statistical tests were performed using In Stat (Graph Pad, San Diego, California).

For *in situ* pull down assays, rat spinal cord cryosections (16 μm thickness, fresh) were post fixed with 4% PFA and incubated with the clarified bacterial lysate, prepared from bacteria expressing GST-RBD or GST alone as described above, overnight at 4°C. The sections were then washed 3 times is TBS, blocked in 3% BSA for 1 hr at room temperature and incubated with anti-GST antibody (Cell signalling, New England Biolabs, Mississauga, Canada) and with cell-type specific antibodies (NeuN, GFAP and MAB328; Chemicon, Temecula, California) or with antibody raised against $p75^{NTR}$ (Promega, Madison, Wyoming) overnight at 4°C. Sections were washed in TBS and incubated for 2 hr at room temperature with FITC, Texas Red or Rhodamine conjugated secondary antibodies (Jackson ImmunoResearch, Mississauga, Canada).

TUNEL labeling and immunohistochemistry.

Spinal cord samples of 3 mm and 4 mm spanning the lesion sites of mice and rats, respectively, were dissected. Normal spinal cord was a 4 mm section from sham control cords. All spinal cord peices were post-fixed in 4% paraformaldehyde, washed and embedded in paraffin. Transverse sections of 6 μm thickness were cut, deparaffinized in xylene, and rehydrated by ethanol washes. TUNEL labeling was carried out using the Fluorescein-FragEL DNA Fragmentation Kit (Oncogene, Boston, Massachusetts). The sections were co-stained with Hoechst 33342 (Sigma) and only TUNEL positive cells that

correlated with Hoechst 33342 stained nuclei were counted. To quantitatively examine the numbers of apoptotic cells, TUNEL positive cells were counted on sections from control, lesion and C3-05 treated animals. A blinded researcher counted the total number of TUNEL positive cells located in the entire transverse section. The average number of TUNEL positive cells per section was calculated from values obtained by counting 40-50 random sections throughout the lesion site of each animal, with 3 animals examined per group. The TUNEL positive cells (green) were distinguished from autofluorescent macrophages (red) through the use of a merge red/green filter. Cells labeled with both TUNEL (green) and p75 (red) were counted in a merge red/green filter, after verifying colocalization with Hoechst stain. Values were obtained by counting 20 random sections throughout the lesion site of each animal, with 3 animals examined per group. Immunohistochemistry with cell-type specific antibodies (NeuN, GFAP and MAB328; Chemicon, Temecula, California), or with a polyclonal C3 antibody (Winton et al., 2002) was performed on paraffin sections. After deparafinization, transverse sections were treated with 2x saline sodium citrate at 80 °C for 20 minutes. Sections were blocked in Tris-buffered saline (TBS) containing 3% BSA and 2% goat serum and incubated overnight with primary antibody at 4°C. Followed by a 2 hr incubation with FITC or Texas Red conjugated secondary antibodies (Jackson ImmunoResearch, Mississauga, Canada). All pictures were taken with northern eclipse software and transferred to Adobe illustrator 9.0.

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Figure legends

Figure 1. **RhoA is activated when cells are plated on growth inhibitory substrates.** (A) Rho activation levels were examined *in vitro* in PC-12 cells plated on either poly-L-lysine, myelin (8 μg) or MAG (8 μg). Active GTP bound RhoA was isolated by pull down assay 24 after the cells were plated on substrates and detected by immunoblotting with anti-RhoA antibody. Total Rho levels were determined from whole cell lysates as shown in bottom panel. (B) Reversal of Rho activation by treatment of cells with C3-05. PC-12 cells plated on myelin were treated with C3-05 (1 μg/ml) and Rho-GTP levels were detected by pull down assay. The middle panel shows total Rho levels and the bottom panel shows whole cell lysates probed with an anti-C3 antibody. Samples for pull downs, total Rho and C3 blots were from the same homogenates. (C) Pull down assay with GST-RBD without lysate. Beads incubated with buffer only show no active Rho, only GST-RBD band is detected when blot is overexposed.

Figure 2. **RhoA activation in normal and injured spinal cord tissue.**
(A) RhoA was examined in normal rat and mouse spinal cord homogenates (control) and homogenates prepared 24 hours after transection or contusion injury, as indicated (lesion). Active GTP-RhoA was isolated by pull down assay and detected by immunoblot with anti-RhoA antibody. Total Rho in the tissue homogenates from the same animals was detected by immunoblot with anti-RhoA antibody. (B) RhoA is activated as early as 1.5 hr post injury and activation was sustained for at least 7 days after injury. (C) Quantitative analysis by densitometry of GTP-RhoA after transection of rat spinal cord shown as mean +/- S.E.M for all animals examined; 24 hours ($n = 5$), 3 days ($n = 3$) and 7 days ($n = 3$). n

represents the number of animals. *, $P < 0.05$, as compared with uninjured control; P value determined by unpaired t-test. (D) Western blot showing MAG at the lesion sites at all time points tested; second panel shown shows overexposed blot to see MAG degradation present after injury (5 μg of protein was loaded per lane). Immunoreaction to purified MAG and myelin with the MAG antibody are shown in the last panel.

Figure 3. **Treatment with the Rho antagonist C3-05 after contusion or transection of the spinal cord reverses RhoA activation after injury.**

(A) Injection of C3-05 into the injury site reversed RhoA activation to basal levels after SCI. Active GTP-RhoA was isolated by pull down assay and detected with antibodies specific for RhoA. Total RhoA from the same animals was detected by immunoblot. Anti-C3 antibody immunoblot of the same homogenate showed C3-05 was detected at the lesion site for 7 days (C3-05). The same homogenates were used to determine levels of Rho and C3. (B) Densitometric analysis of the reversal of Rho activation by C3-05 after mouse hemisection ($n = 2$); rat contusion, ($n = 3$); rat transection after 24 hr ($n = 3$); rat transection after 3 d ($n = 3$) and rat transection after 7 d ($n = 2$). n represents the number of animals. *, $P < 0.05$ compared to lesion without treatment; P value determined by unpaired t-test. (C) Double immunocytochemistry with cell-type specific markers (red) and a specific antibody against C3 (green). Neurons (NeuN), astrocytes (GFAP) and oligodendrocytes (MAB328) show C3 immunoreactivity within cells in injured rat spinal cord treated with C3-05. Scale bar, 50 μm.

Figure 4. **Rho is activated in a large population of cells rostral and caudal to the lesion site.**

Top panel shows Nissel stained longitudinal section of rat spinal cord, 24 hr after dorsal over-hemisection; scale bar 1 mm. Higher magnification of areas spanning the section are shown boxed and numbered. Magnified panels 1-4 show active Rho (GST-RBD detection) in a large number of cells spanning the lesion site. Panel 1 shows active Rho in grey matter rostral to the lesion, panel 2 shows active Rho in grey matter caudal to the lesion, panel 3 shows active Rho in white matter ventral to the lesion and panel 4 shows an absence of GST-RBD detection distal to the lesion. Scale bar, 100 μm.

Figure 5. **Rho is active in neurons and glial cells after SCI, detected by *in situ* pull down assay.**

(A) Control uninjured animals (control+GST-RBD) and C3-05 treatment of animals after SCI (C3-05+GST-RBD) probed with GST-RBD show no active Rho. Sections of animals after SCI incubated with lysate from empty pGEX vector expressing only GST protein (lesion+GST-pGEX) show only background levels of GST in the spinal cord and no active Rho. (B) Double labeling of spinal cord with cell type specific markers (red) and GST antibody to detect GST-RBD (green). At all time points tested (24 hr, 3 d and 7d), GTP-Rho was detected in neurons (NeuN), astrocytes (GFAP) and oligodendrocytes (MAB328) after SCI.

Figure 6. **Inhibition of Rho activation with C3-05 protects cells from apoptosis.**

(A) Sections of injured spinal cords from mouse (top panel) and rat (bottom panel) were double labeled with specific cell markers NeuN, GFAP, or MAB328 (red) and by TUNEL (green) to detect apoptotic cells. Scale bar, 50 μm. (B) Treatment of injured spinal cord with C3-05 significantly decreased the number of TUNEL positive cells counted in both mice (right) and rats (left). TUNEL positive cells were counted in 40-50 sections per animal, taken from a 3 or 4 mm segment of the lesion site in mice and rats, respectively, with 3 animals examined per group. *, $P < 0.05$ as compared to lesion without treatment; P value determined by unpaired t-test.

(C) Sections from rat spinal cord showing that most C3 immunostained cells were not TUNEL positive. Arrow, one doubled cell. Scale bar, 50 μm. (D) C3 labeled cells are less likely to be TUNEL positive. C3 and TUNEL cells were counted and compared to the number of TUNEL labeled cells in C3-05 treated animals.

Figure 7. **Rho is activated after SCI by a $p75^{NTR}$ dependant mechanism.**

(A) $p75^{NTR}$ labeled cells colocalize with active GTP-Rho in injured spinal cord. Cells labeled with $p75^{NTR}$ (red) and GST-RBD (green) in grey matter (top panel) and in white matter (bottom panel). Scale, 100μM. (B) Rho activation after SCI in normal and $p75^{NTR}$-/- mice. Active GTP-RhoA was isolated by pull down assay and detected by immunoblotting with anti-RhoA antibody (top panel). In $p75^{NTR}$ -/- mice 24 hr after injury only basal levels of active Rho are detected as compared to normal mice. Paired samples were run on the same gel and blots were developed under the same conditions. Total Rho in the tissue homogenates from the same animals was detected by immunoblotting with anti-RhoA

antibody. MAG was detected in the same homogenates by western blot (apparent MW 100 Kda). The $p75^{NTR}$ levels (apparent MW 75 Kda) are shown in bottom panel. In control uninjured animals low levels of $p75^{NTR}$ are detected, with $p75^{NTR}$ only being upregulated after injury. (C) Active Rho is detected in $p75^{NTR}$

-/- mice 3 days after SCI.

Figure 8. **Rho regulates $p75^{NTR}$ expression after injury**

(A) Transverse sections throughout rat lesion sites show cells double labeled with $p75^{NTR}$ specific antibody (red) and with TUNEL (green). (B) The number of $p75^{NTR}$ labeled cells (left) and $p75^{NTR}$ cells positive for TUNEL (right) in transverse sections of rat spinal cord after injury. (C) The $p75^{NTR}$ protein levels increase after SCI, but not after treatment with C3-05. Detection of $p75^{NTR}$ by western blot after SCI and treatment with C3-05. The same tissue homogenates used to show asctive Rho, shown in bottom panel, were probed with a $p75^{NTR}$ specific polyclonal antibody (top) and an anti-C3 antibody (panel 2). RhoA in whole tissue homogenate from the same animals is also shown (panel 3). Last panel shows GTP bound active Rho.

Figure 9. **Schematic diagram showing possible apoptotic cascade mediated by Rho after SCI.**

Both myelin-derived growth inhibitory proteins (Figure 1) and TNF (Neumann et al., 2002a) directly activate Rho. $P75^{NTR}$ activates Rho in the absence of neurotrophin binding (Yamashita et al., 1999). The inactivation of Rho by C3-05 after SCI blocks the increase of

$p75^{NTR}$ protein levels (Figure 8C) and inhibits apoptosis (Figure 6B and C). Inactivation of Rho with C3-05 both prevents apoptosis, as shown in this paper, and stimulates regeneration (Dergham et al., 2002; Lehmann et al., 1999). Gray lines indicate C3-05 treatment and inactivation of Rho, black lines indicate the effects of active GTP bound Rho.

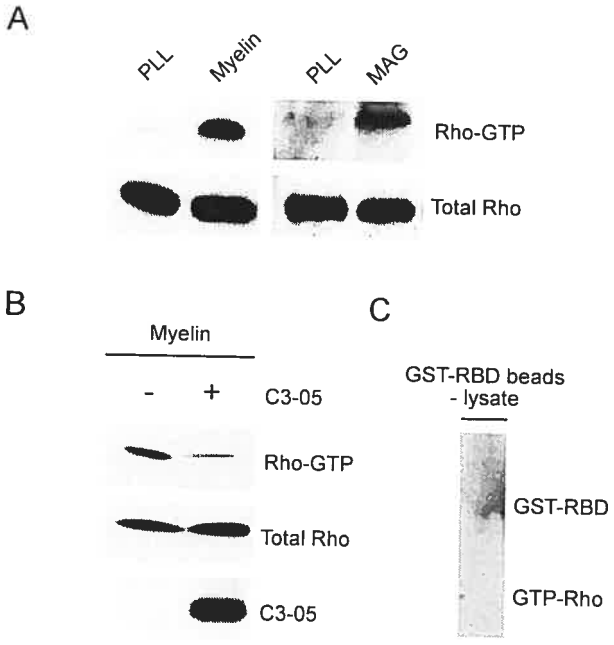

A
PLL
Myelin
PLL
MAG
Rho-GTP
Total Rho
B
Myelin
-
+
C3-05
Rho-GTP
Total Rho
C3-05
C
GST-RBD beads
- lysate
GST-RBD
GTP-Rho

Figure 1

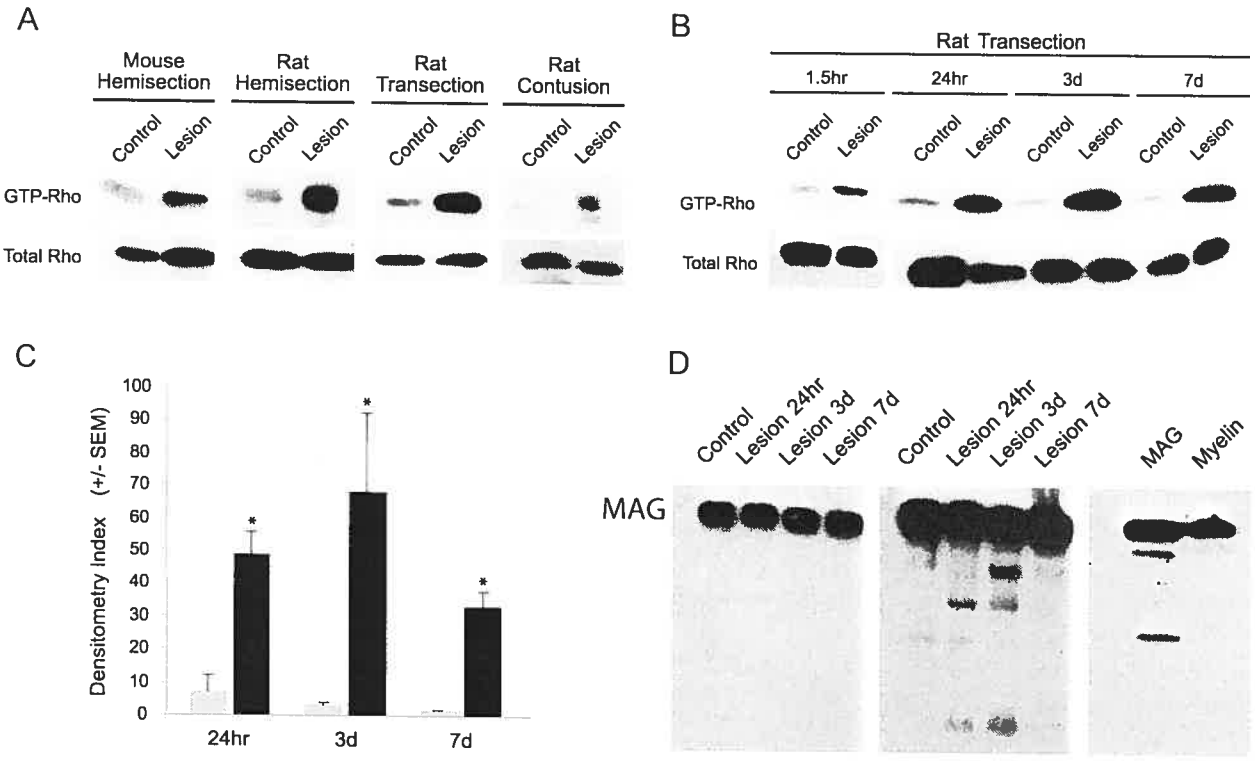

A
Mouse Hemisection
Rat Hemisection
Rat Transection
Rat Contusion
Control
Lesion
GTP-Rho
Total Rho
B
Rat Transection
1.5hr
24hr
3d
7d
Control
Lesion
GTP-Rho
Total Rho
C
Densitometry Index (+/- SEM)
100
90
80
70
60
50
40
30
20
10
0
24hr
3d
7d
D
Control
Lesion 24hr
Lesion 3d
Lesion 7d
MAG
Myelin

Figure 2

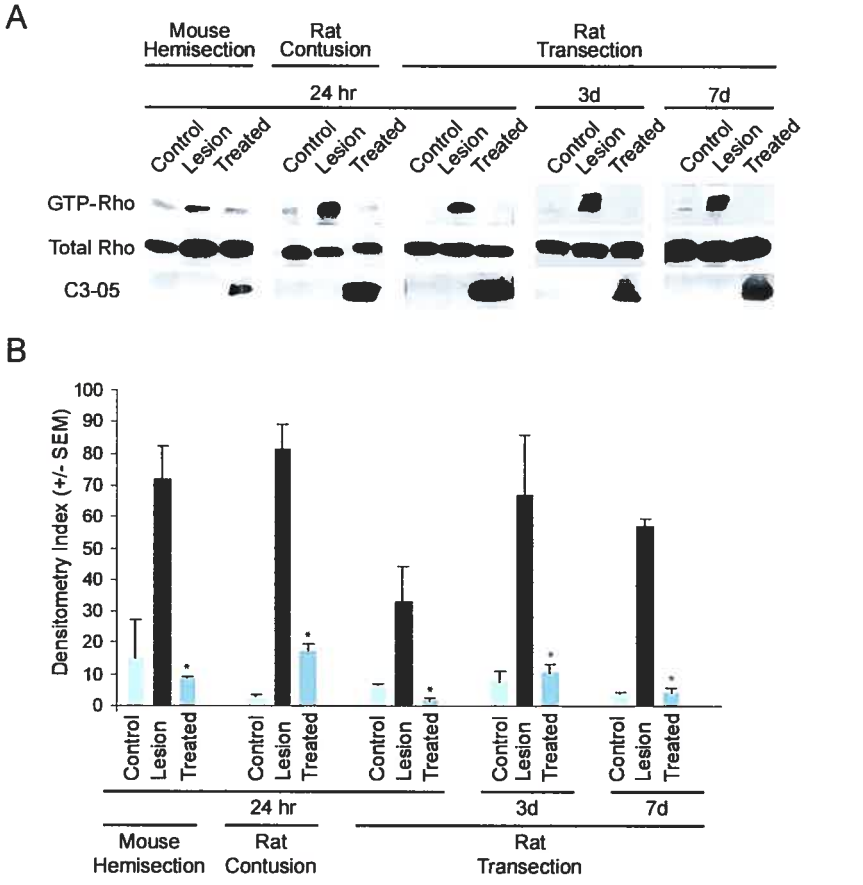

A
Mouse Hemisection
Rat Contusion
Rat Transection
24 hr
3d
7d
Control
Lesion
Treated
GTP-Rho
Total Rho
C3-05
B
Densitometry Index (+/- SEM)
100
90
80
70
60
50
40
30
20
10
0
Control
Lesion
Treated
24 hr
3d
7d
Mouse Hemisection
Rat Contusion
Rat Transection
C
NeuN
C3
GFAP
C3
MAB328
C3

Figure 3

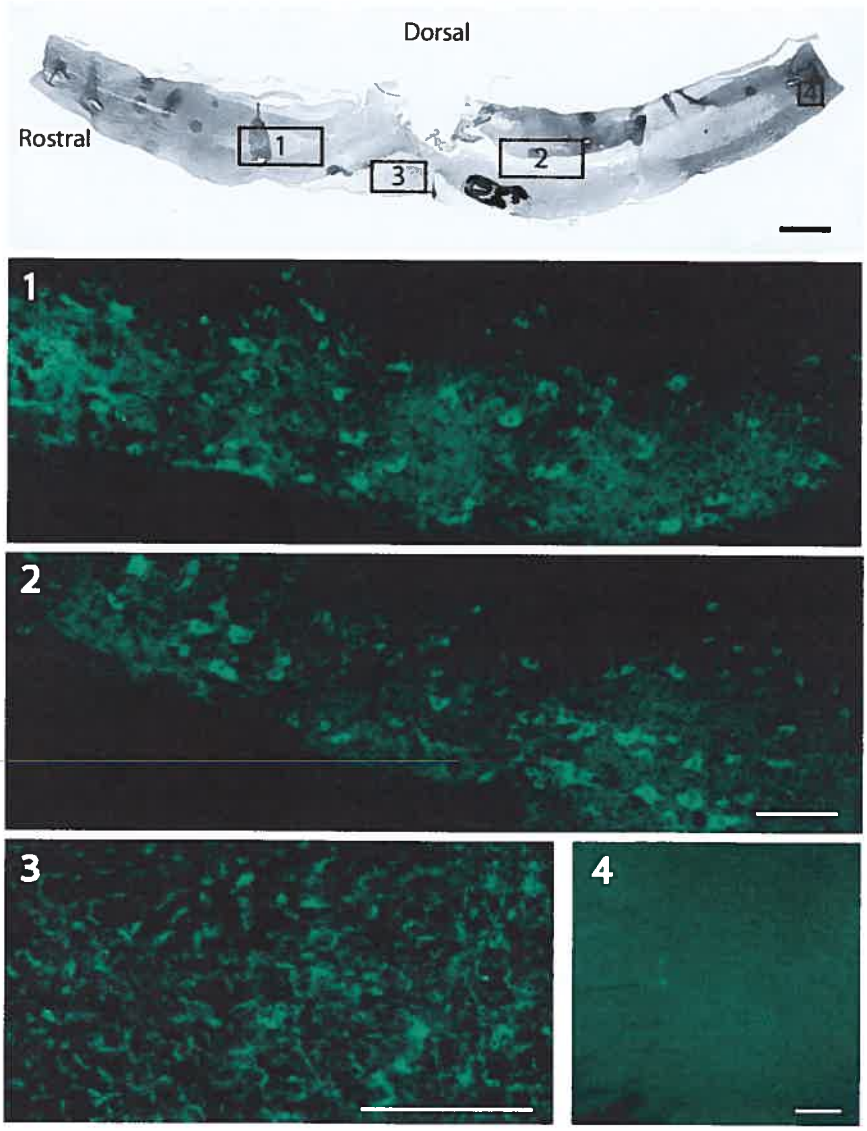
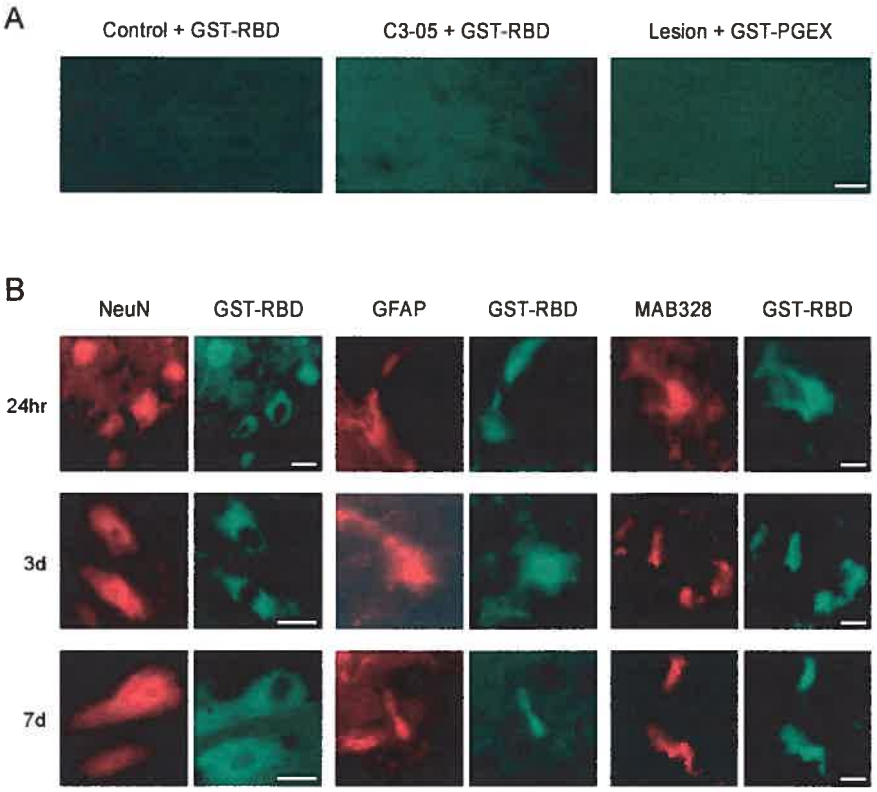


Figure 4


A
Control + GST-RBD
C3-05 + GST-RBD
Lesion + GST-PGEX
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NeuN
GST-RBD
GFAP
GST-RBD
MAB328
GST-RBD
24hr
3d
7d

Figure 5

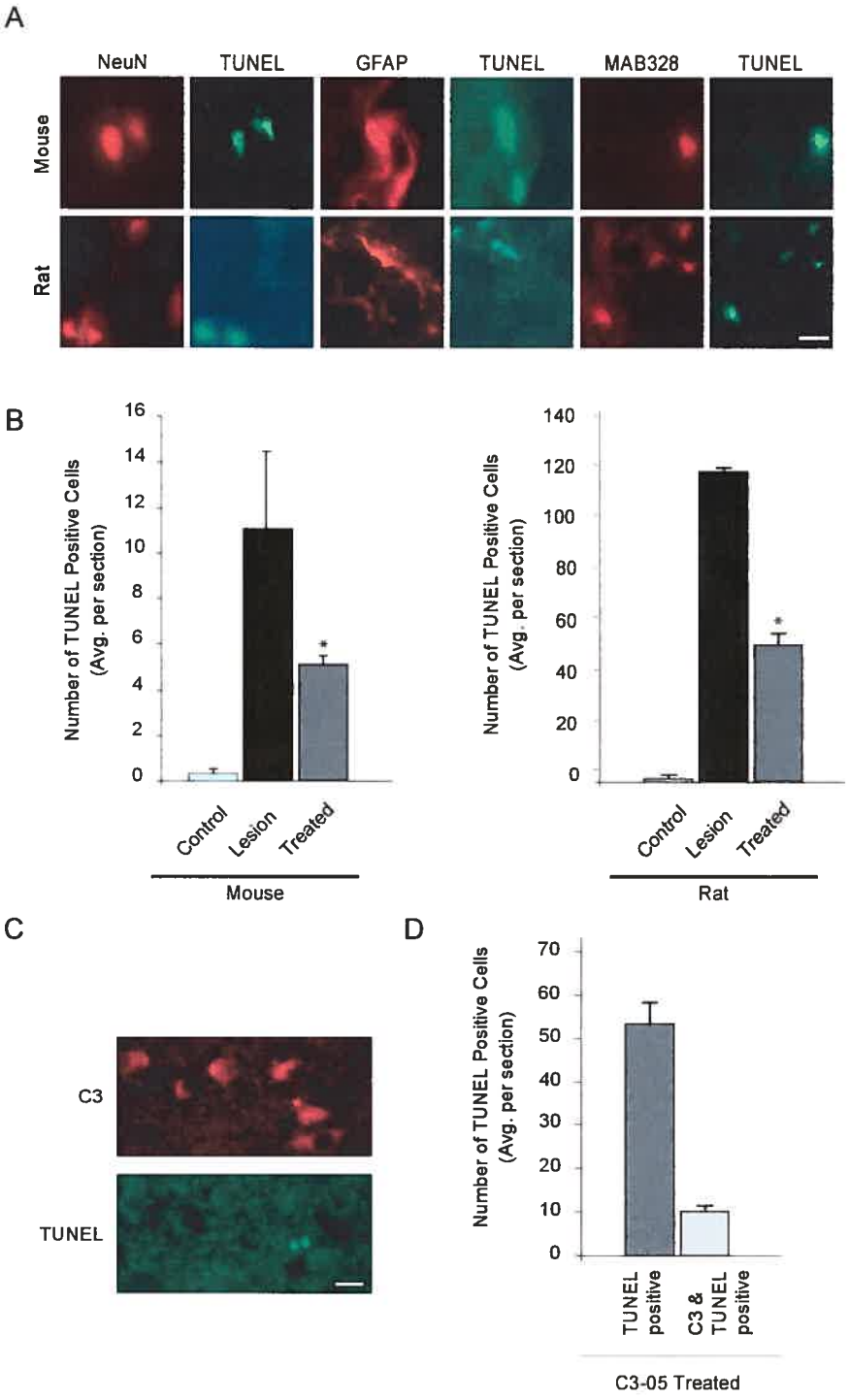
A
NeuN
TUNEL
GFAP
TUNEL
MAB328
TUNEL
Mouse
Rat
B
Number of TUNEL Positive Cells (Avg. per section)
16
14
12
10
8
6
4
2
0
Control
Lesion
Treated
Mouse
140
120
100
80
60
40
20
0
Rat
C
C3
TUNEL
D
70
60
50
40
30
20
10
0
TUNEL positive
C3 & TUNEL positive
C3-05 Treated

Figure 6

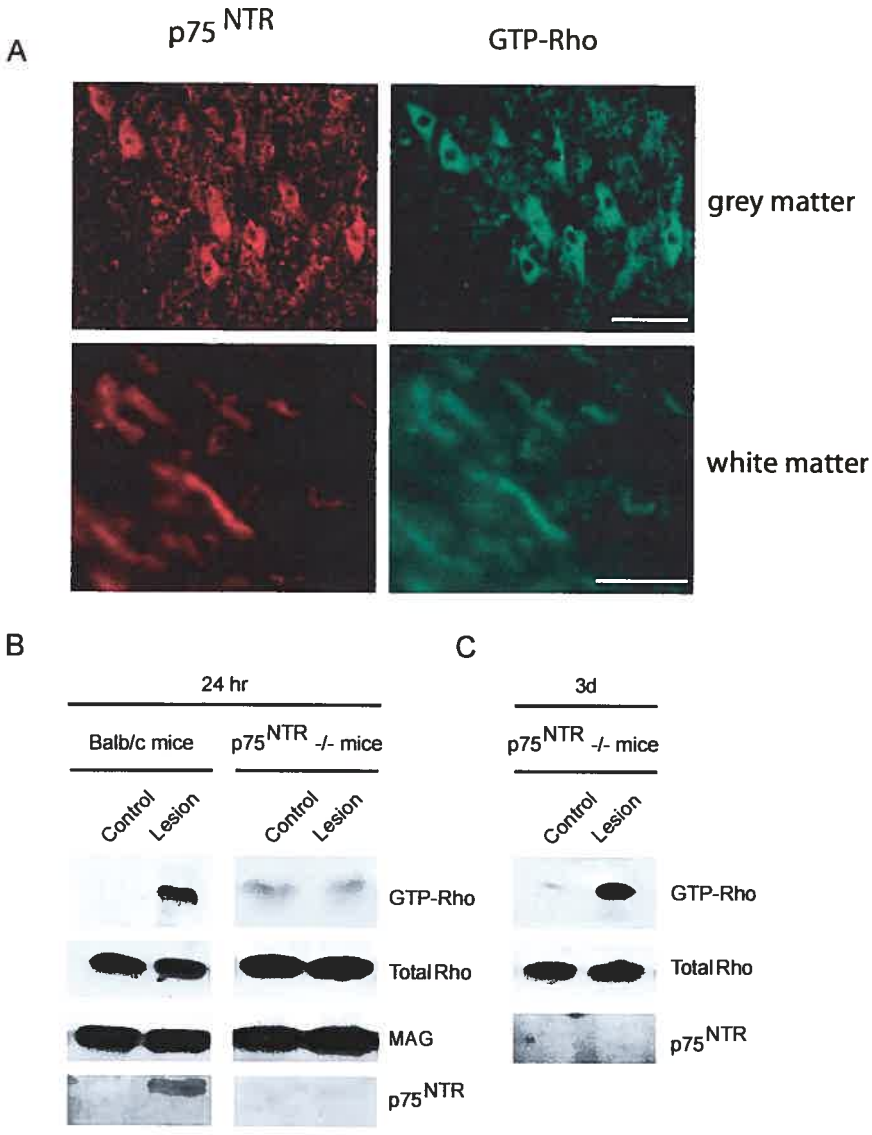
A
p75 NTR
GTP-Rho
grey matter
white matter
B
24 hr
Balb/c mice
p75NTR -/- mice
Control
Lesion
Control
Lesion
GTP-Rho
Total Rho
MAG
p75NTR
C
3d
p75NTR -/- mice
Control
Lesion
GTP-Rho
Total Rho
p75NTR

Figure 7

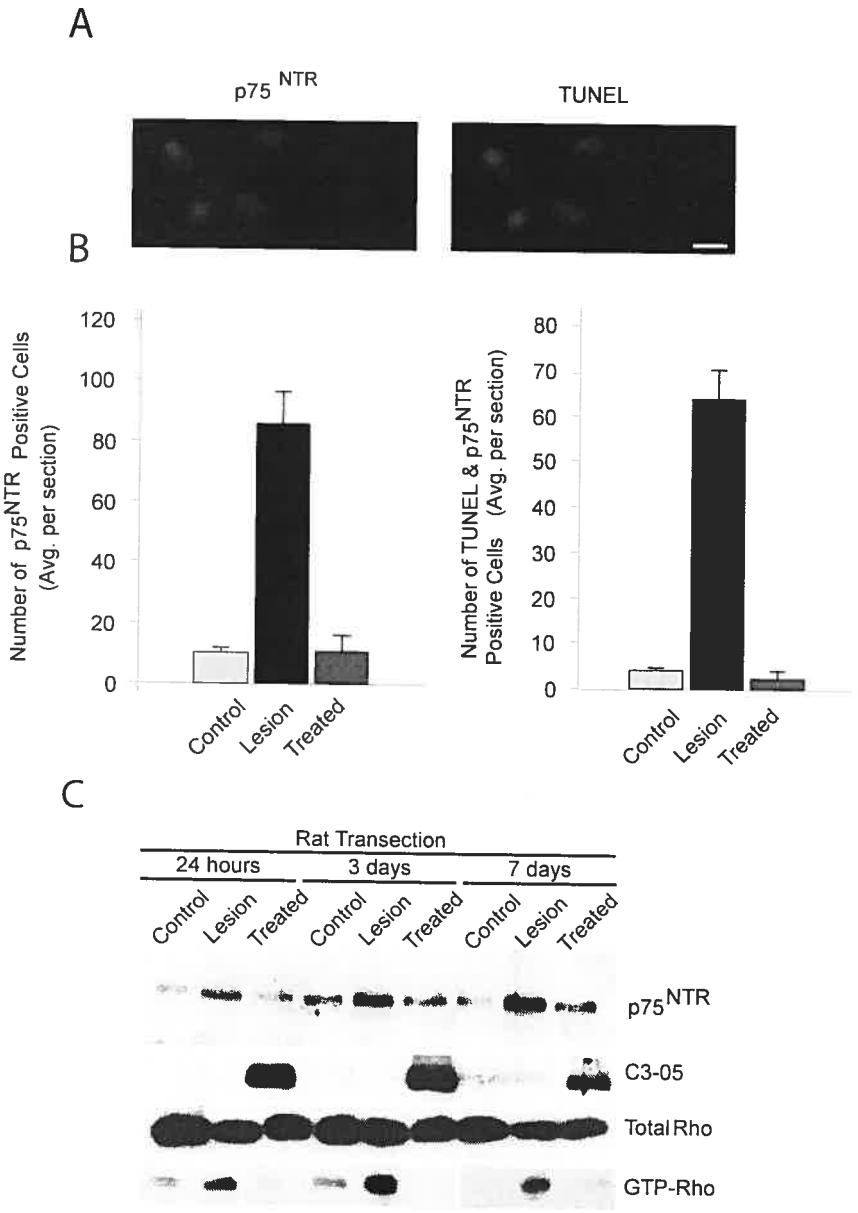
A
p75 NTR
TUNEL
B
Number of p75NTR Positive Cells (Avg. per section)
120
100
80
60
40
20
0
Control
Lesion
Treated
Number of TUNEL & p75NTR Positive Cells (Avg. per section)
80
70
60
50
40
30
20
10
0
Control
Lesion
Treated
C
Rat Transection
24 hours
3 days
7 days
Control
Lesion
Treated
Control
Lesion
Treated
Control
Lesion
Treated
p75NTR
C3-05
Total Rho
GTP-Rho

Figure 8

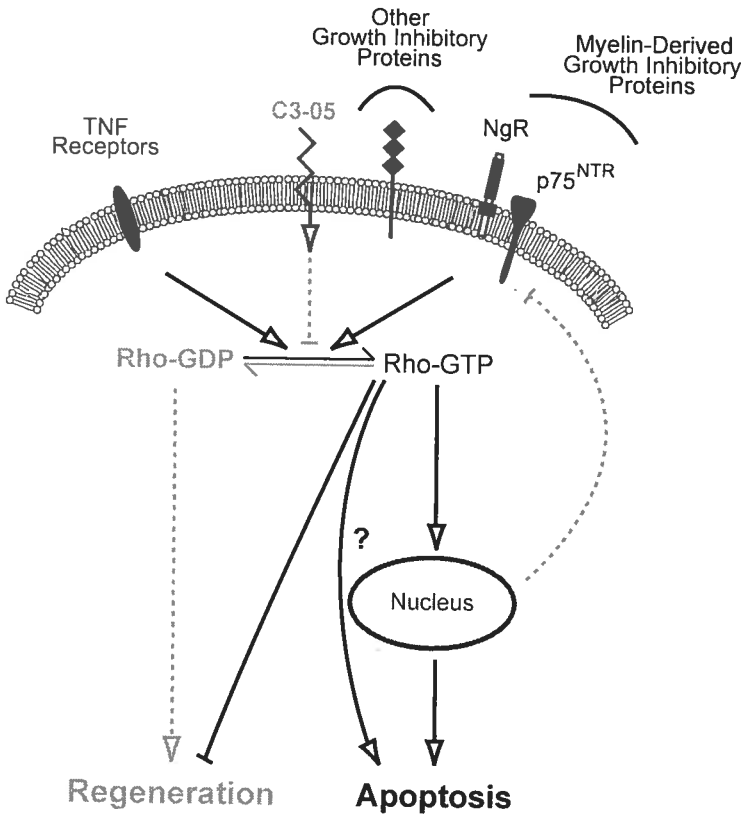
Other
Growth Inhibitory
Proteins
Myelin-Derived
Growth Inhibitory
Proteins
C3-05
TNF
Receptors
NgR
p75NTR
Rho-GDP
Rho-GTP
?
Nucleus
Regeneration
Apoptosis

Figure 9

Appendix C

FIFTH ARTICLE

« Nogo on the go. Lisa McKerracher and Matthew J. Winton.
Neuron 36: 345-348, 2003.»

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Nogo on the go

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Abstract

Growth inhibition in the central nervous system (CNS) is a major barrier to axon regeneration. Recent findings indicate that three distinct myelin proteins, myelin-associated glycoprotein (MAG), Nogo, and oligodendrocyte-myelin glycoprotein (OMgp), inhibit axon growth by binding a common receptor, the Nogo-66 receptor (NgR). NgR binds p75 neurotrophin receptor ($p75^{NTR}$) to inhibit neurite growth by a Rho-dependent pathway.

Growth inhibitory proteins in myelin

It is now well established that the axons adult CNS are capable of only a limited amount of regrowth after injury and that an unfavorable growth environment plays a major role in this lack of regeneration. Much of the axon growth inhibitory activity in the CNS is associated with myelin, and a number of individual proteins that inhibit axon growth have been identified. To date three inhibitory components of myelin have been identified: myelin-associated glycoprotein (MAG), Nogo, and most recently oligodendrocyte-myelin glycoprotein (OMgp) (Kottis et al., 2002; Wang et al., 2002). Although distinct in molecular structure, these proteins share a number of common attributes, including their expression and localization in the myelin membrane directly adjacent to the axon. Together with two additional reports on MAG (Domeniconi et al., 2002; Liu et al., 2002), these studies show that MAG, Nogo, and OMgp are all important myelin-derived growth inhibitory proteins that bind a common receptor, NgR, to block neurite growth.

MAG was identified as the first myelin-derived growth inhibitory protein by two distinct experimental strategies. MAG inhibitory activity was detected in myelin after extraction with octylglucoside, fractionation by ion exchange chromatography, and screening for inhibitory activity (McKerracher et al., 1994), and by a different approach, the Filbin group showed that CHO cells transfected with MAG inhibited neurite growth (Mukhopadhyay et al., 199). Although the identification of MAG as a growth inhibitory protein was at first controversial, it is now well established that MAG can function as an inhibitor of neurite growth. Further, it has also been shown that MAG is bifunctional; in addition to inhibiting regrowth of adult axons, MAG can stimulate axon regrowth from

young neurons, and changes in the endogenous levels of cAMP may account for these developmental changes in activity.

Nogo was reported independently by three groups as the long sought after high molecular weight myelin inhibitor first characterized by the Schwab group (reviewed in Brittis and Flanagan, 2001). Somewhat surprisingly, Nogo was found to have three different splice variants: NogoA, NogoB, and NogoC, the latter two of which are widely expressed outside the CNS. Only NogoA possesses a unique N-terminal region not shared by NogoB and NogoC, and NogoA is specifically expressed in the CNS and is present on the cell surface of oligodendrocytes. NogoA has two functional domains: Nogo-66 and Amino-Nogo. Nogo-66 is the transmembrane loop region shared by all Nogo isoforms, while Amino-Nogo is the unique N-terminal domain.

While both domains are thought to contribute to myelin-derived inhibition of neurite growth, Nogo-66 specifically inhibits neurite growth, while the Amino-Nogo fragment inhibits spreading and migration of non-neuronal cells, as well as blocking neurite growth (Fournier et al., 2001). The findings that the amino-terminal fragment possesses inhibitory activity is somewhat perplexing, given that structural studies suggest Amino-Nogo is located on the cytoplasmic side of the membrane. Although this apparent paradox remains to be resolved, possibilities include potential conformational changes in Nogo that might expose the amino-terminal domain or potentially that injury in the CNS might result in a disruption of myelin such that both domains are exposed. The Strittmatter group followed up on the initial cloning of Nogo to identify the receptor for the Nogo-66 domain, using an expression cloning strategy to isolate binding proteins. A single receptor, NgR was identified and found to encode a protein which is associated with the cell membrane by

a glycosylphosphatidylinositol (GPI) linkage (Fournier et al., 2001). Mutated forms of the receptor eliminated growth inhibition by Nogo-66, supporting its importance as a receptor for Nogo. Structure-function analyses suggested that that a leucine-rich repeat domain is important for Nogo binding. NgR is highly expressed in brain, and in situ hybridization experiments show that NgR is predominantly expressed by neurons (Fournier et al., 2001).

OMgp is the most recently identified of the inhibitory components of myelin, and was identified independently by two groups. In the experiments to purify myelin inhibitors where MAG was identified, it was noted that there were two major peaks of inhibitory activity, with MAG present in the first peak (McKerracher et al., 1994). The Braun group further separated inhibitory proteins in the second peak by PNA-agarose chromatography and identified OMgp as a potent inhibitor, naming it first Arretin based on its growth inhibitory properties (Kottis et al., 2002). Coming from another angle, the He group identified OMgp by testing whether any GPI-anchored myelin proteins could act as regeneration inhibitors (Wang et al., 2002a). OMgp was found to be highly enriched in Phospholipase C-released fractions of myelin and shown to have potent growth cone collapsing and neurite outgrowth inhibitory activities. The inhibitory activity of OMgp in vitro appears to be as potent as that of MAG and Nogo and all three proteins have a similar distribution in the myelin sheath, suggesting that all likely contribute to growth inhibition in the adult CNS.

While recent years have witnessed a spurt in information about the molecular components of myelin and their activities, still relatively little is known about how they mediate their inhibitory effects on growing axons. As for any extracellular molecule, understanding the mechanisms by which a cell or in this case, an axon, reads this signal

requires knowledge of the signal transduction machinery involved. In this respect, identification of NgR as the receptor for Nogo represented a critical contribution, it was thought, at least for understanding the mechanisms underlying the effects of Nogo and its particular contribution to the inhibitory activities of myelin. The surprise came when it was shown that in addition to binding Nogo-66, the NgR could also bind and mediate the inhibitory activities of OMgp (Wang et al., 2002a) and MAG (Domeniconi et al. 2002; Liu et al., 2002). These studies bring these various molecules to an intersection, at the level of the NogoR, and suggest that these seemingly distinct proteins might have more in common than initially anticipated.

MAG, Nogo, and OMgp share a common receptor that mediates growth inhibition

Wang et al. identified the NgR as an OMgp binding protein via an expression cloning strategy and confirmed a functional role for the NgR in growth inhibition by OMgp by showing that NgR transfected into neurons which would normally not be inhibited by OMgp can make these neurons sensitive to OMgp (Wang et al., 2002a). Following closely on the heals of this report, two additional studies thrust the Nogo-66 receptor into center stage by demonstrating that in addition to binding Nogo-66 and OMgp, the NgR also binds MAG (Liu et al., 2000; Domeniconi et al., 2002). The Strittmatter group used an alkaline phosphatase-NgR fusion protein to attempt to identify NgR co-receptors by expression cloning and, in the process, identified MAG (Liu et al., 2002). Independently, the Filbin group honed in on the NgR receptor as candidate for MAG binding based on a similarity in molecular weight to candidates revealed in a previous characterization of MAG binding

proteins. Both studies show that NgR signals growth inhibition by MAG, and that NgR is a required component of MAG inhibitory signaling.

Therefore, NgR mediates growth inhibition by MAG, Nogo, and OMgp, three structurally different proteins. Even more surprising is that all three myelin-derived growth inhibitory proteins bind NgR with high affinity of approximately 5 nM (OMgp, 5 nM [Wang et al., 2002a]; Nogo-66, 7 nM [Fournier et al., 2001]; MAG, 8 nM [Domeniconi et al., 2002]). Also, all three proteins are inhibitory in either substrate bound or soluble form, and dominant-negative NgR removes sensitivity to all three growth inhibitory proteins. The challenge now is to elucidate the signaling mechanisms.

Rho GTPase and growth inhibition

NgR is GPI-linked to the cell surface and does not have an intracellular signaling domain, so it is assumed that it must function as a part of a signaling complex. One clue to understanding signal transduction mechanisms was the demonstration that the small GTPase Rho is key intracellular effector for growth inhibitory signaling by myelin. Rho GTPases are a family of highly related proteins that are best characterized for their effect on the actin cytoskeleton. The major members of the Rho family include Rho, Rac and Cdc42. Isoforms of Rho exist, and in neurons RhoA is expressed at higher levels than RhoB and RhoC (Lehmann et al., 1999). GTPases have two conformations: a GDP-bound inactive state and a GTP-bound active state. In neurons, myelin and MAG inhibit growth by Rho-dependent mechanisms (Lehmann et al., 1999), and more specifically, it has been shown that Rho is activated in contact with myelin (Winton et al., 2002). It has not yet been tested whether Nogo or OMgp ligands acting via NgR can also activate Rho.

The first indication that Rho family GTPases might play a key role in growth inhibition came from studies of lysophophatidic acid (LPA) and neurite retraction. LPA is known to activate Rho, and the ability of LPA to cause neurite retraction suggested that Rho might also be involved in growth cone collapse. Lehmann et al. (Lehmann et al., 1999) showed that cells transfected with dominant negative Rho could grow neurites on myelin substrates, and that treatment with C3 transferase to inactivate Rho could also allow neurite growth on inhibitory substrates. C3 transferase is a bacterial endotoxin which inactivates Rho by ADP ribosylation. Similarly, it has now been shown that treatment of neurons with C3 transferase allows neurite growth in the presence of chemorelpulsive guidance factors, and moreover, inactivation of Rho with cell permeable C3 analogs reverses Rho activation concomitantly with their ability to promote growth on myelin substrates.

More recently, a Rho kinase inhibitor called Y-27632 has been used to probe the role of Rho in growth inhibitory signaling. Treating neurons with C3 transferase or with Y-27632 have similar effects – both compounds promote growth on inhibitory substrates. Moreover, both compounds not only override growth inhibition by myelin, but also by the chondroitin sulfate proteoglycans (Dergham et al., 2002), a family of growth inhibitory proteins concentrated at regions of the glial scar. A number of laboratories have shown that treatment of neurons with Y-27632 stimulates neurite growth. More specifically growth cone collapse by chemorepulsive factors important in development can be blocked by treatment with Y-27632. Therefore, Rho may be a convergent point for signaling by different inhibitory receptors, not just NgR.

Signal transduction by NgR and $p75^{NTR}$

So what is the identity of the NgR's signaling partner? A recent study has resolved this question in part by showing that NgR binds to the extracellular domain of p75NTR (Wang et al, 2002b). Disrupting the interaction between NgR and $p75^{NTR}$, by either NgR dominant negative mutation or in a $p75^{NTR}$ null mutant mouse, makes neurons unresponsive to myelin. These findings help explain why growth inhibition by MAG activates Rho in a $p75^{NTR}$ dependent manner. In the search for $p75^{NTR}$ –interacting proteins, Barde's group first detected RhoA binding to $p75^{NTR}$ (Yamashita et al., 1999). The other isoforms of Rho, RhoB and RhoC do not bind $p75^{NTR}$. Co-localization of $p75^{NTR}$ and MAG binding suggested that $p75^{NTR}$ clusters with MAG receptors (Yamashita et al., 2002), now known to be NgR. Moreover, neurons isolated from $p75^{NTR}$ null mutant mice were not inhibited by MAG, showing a key role of $p75^{NTR}$ in growth inhibitory signaling by MAG (Yamashita et al., 2002). Together with the findings that NgR binds $p75^{NTR}$, and that NgR binds MAG, Nogo and OMgp, our new understanding is that neurite outgrowth is blocked by myelin inhibitors by when NgR and $p75^{NTR}$ interact upon ligand binding to NgR. Ligand binding then activates Rho in a $p75^{NTR}$ –dependant manner (Figure 1).

How might such an inhibitory signaling complex function in the context of the cell? Growth inhibitory signaling complexes may be located within specialized lipid microdomains of the growth cone membrane, as membrane rafts. Such membrane rafts are rich in cholesterol, sphingomyelin and gangliosides, and NgR, as a GPI-anchored protein, could potentially link to sphingomyelin. In addition, Rho has been shown to associate with lipid rafts. Intriguingly, the major brain gangliosides GD1 and GT1b had been previously suggested as candidate MAG receptors. (Vinson et al., 2001; Vyas et al., 2002). Neurite

growth is reduced by addition of soluble GD1a and GT1b to neurons plated on MAG CHO cells (Vinson et al., 2001), and multivalent clustering of gangliosides completely blocks neurite growth in the absence of inhibitory molecules (Vyas et al., 2002). In addition, neurons from transgenic mice engineered to lack gangliosides are not inhibited by MAG (Vyas et al., 2002). Yamashita and colleagues also found that the ganglioside GT1b associated with $p75^{NTR}$, as detected by immunoprecipation, suggesting that gangliosides participate in the MAG receptor-signaling complex (Yamashita et al., 2002). Together with the new data on NgR, these data suggest a model whereby ganglioside clustering in membrane rafts might help form NgR receptor complexes with $p75^{NTR}$ upon MAG binding to NgR (Figure 1).

The evidence in support of an involvement of gangliosides and Rho in inhibitory signaling complexes is strengthened by studies in which the inhibition of neurite growth by anti-GT1b antibodies was reversed by treatment with Y27632, the inhibitor to Rho kinase (Vinson et al., 2001). While NgR is the binding receptor for MAG, Nogo, and OMgp, at least in the case of MAG signaling, gangliosides may act to modulate the formation of inhibitory signaling complexes in response to ligand binding. It has not been investigated if gangliosides might also modulate growth inhibition by OMgp and Nogo. Although as yet untested, it is tempting to speculate that the role of gangliosides may be to help cluster and stabilize receptor complexes once the ligand binds to NgR (Figure 1). This would be consistent with sustained activation of Rho in response to inhibitory signaling (Winton et al., 2002).

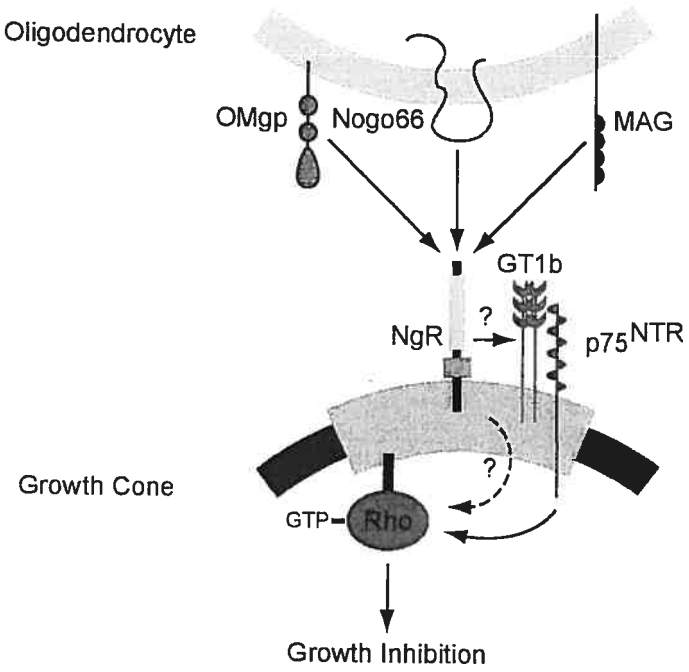

Figure 1. **Schematic Diagram Showing Signaling by Myelin-Derived Growth Inhibitory Proteins**

MAG, Nogo66 and OMpg all bind with high affinity to the Nogo receptor (NgR), likely located in membrane rafts. We suggest that binding of inhibitory ligands causes clustering of gangliosides (GT1b) in membrane rafts. It is not known if additional NgR binding partners may be present (indicated by a straight, dotted arrow and question mark). The $p75^{NTR}$ also participates in inhibitory signaling, and likely forms part of the receptor complex in the membrane rafts. Rho is activated by growth inhibitory proteins, likely by $p75^{NTR}$. The activation of Rho results in growth inhibition.

What is the role of neurotrophin signaling in growth inhibition? Although neurotrophins typically promote neurite growth and survival, they appear to be relatively ineffective in overcoming growth inhibition (Cai et al., 1999). An interesting exception to this is that neurotrophins can override growth inhibition if neurons are exposed to the neurotrophin before plating on growth inhibitory substrates (Cai et al., 1999). These findings are consistent with the observations that in the presence of neurotrophin binding, $p75^{NTR}$ inactivates Rho (Yamashita et al., 1999) and perhaps suggests that once Rho is activated, neurotrophin signaling is not sufficient to reverse Rho activation. Other cellular signaling pathways, such as integrin-based signaling, are also well known to reverse Rho activation. Moreover, laminin, which binds integrins, is known to reverse myelin-derived growth inhibition and suggest that extracellular matrix interactions may play a role in modulating growth inhibition as well.

The role of cAMP in a common signaling pathway in growth inhibition

A number of different studies have described the importance of cyclic nucleotide levels in the neuronal response to myelin-derived inhibitors of axon growth (reviewed by (Snider et al., 2002). The levels of cAMP and cGMP can alter the effects of neurotrophic and guidance factors, with low levels of cyclic nucleotides promoting chemorepulsion, and high levels supporting chemoattraction. Therefore, the level of cAMP acts as a switch for inhibitory signaling. Elevation of cAMP in the growth cones can convert repulsion by MAG to attraction. Although as yet untested, it seems likely that cyclic nucleotide levels will also be found to be important in Nogo and OMgp signaling.

The relationship between cAMP and Rho signaling to overcome growth inhibition has not been well studied in neurons, but experiments in other systems have shown that Protein kinase A can phosphorylate Rho preventing its activation. Phosphorylation of Rho also causes it to dissociate from the membrane. Thus, one possibility is that increased levels of cAMP may disrupt the NgR signaling complex, such that consequently the signaling effort Rho is no longer able transmit inhibitory signals. Further work will be required to test this model.

Convergent Progress in Spinal Cord Injury Research

In the last several years, exciting progress has been made in elucidating new ways to stimulate regeneration. Many of the successful strategies either block inhibitory proteins or block signaling by inhibitory proteins. Altering the inhibitory environment of the CNS with antibodies raised against inhibitory proteins has been tested in many different injury models. For instance, the IN-1 antibody promotes axon regeneration on myelin in many different regions of the CNS (for review, Brittis and Flanagan, 2001). Likewise, a peptide antagonist of the NgR, called NEP1-40, which binds the NgR but does not activate it, was also shown to be able to reverse inhibition by Nogo-66 (GrandPre et al., 2002). Like the IN-1 antibody, NEP1-40 promotes regeneration in the injured spinal cord, underlining the importance of NgR inhibition in blocking regeneration. Animals treated with NEP1-40 showed significant functional recovery, demonstrating its potential for therapeutic use.

NEP1-40 did not, however, completely block myelin inhibition in vitro, which might be explained by the activities of inhibitory proteins that do not bind NgR, such as Amino-Nogo, as well as other inhibitory proteoglycans also present in myelin. In

retrospect, a somewhat surprising aspect of this study is the finding that NEP1-40 does not reduce MAG inhibition of neurite growth (Liu et al., 2002), and further, it is not known if neutralizing all myelin inhibitors would promote an even more robust regeneration.

Blocking components of the intracellular inhibitory signaling cascade is yet another new strategy to promote regeneration in the spinal cord, and three new studies now show that blocking inhibitory signaling can promote regeneration in the CNS. One of the new studies shows that inactivation of Rho promotes regeneration in injured spinal cord (Dergham et al., 2002). The use of C3 as an antagonist of Rho, or Y27632 to block Rho kinase were compared, and both in inhibitors of the Rho signaling pathway promote axon regeneration after spinal cord injury. Further, hindlimb movement also was recovered in both C3 and Y27632-treated animals. Two other studies show that raising cAMP levels in advance of injury can overcome growth inhibition and promote regeneration after spinal cord injury (Neumann et al., 2002; Qiu et al., 2002) Although it has not yet been shown that subsequent application of cAMP can promote regeneration or that such treatment can lead to functional recovery, together these new studies show that modulating intracellular signaling pathways to promote regeneration is an attractive strategy to promote repair after SCI.

While enormous progress has been made in promoting regeneration after spinal cord injury, more work is needed to understand the mechanisms of repair. It is known that sprouting of uninjured collaterals and reorganization of intact pathways may contribute importantly to spontaneous recovery after spinal cord injury. Strategies that promote axonal regeneration likely stimulate the spontaneous intrinsic repair processes as well. The

rapid progress made in this field gives real hope for therapeutic interventions to promote repair after spinal cord injury.

Conclusion

Our new understanding of MAG, Nogo and OMgp as myelin-derived growth inhibitory proteins provide a basis for studies of growth inhibitory signaling in the CNS. The findings that three protein inhibitors with no structural relationship bind to a common receptor, NgR, and that NgR binds $p75^{NTR}$, are surprising and important. MAG, Nogo and OMgp all share a common localization in myelin adjacent to the axon membrane where they can interact with NgR. These new findings indicate that myelin-derived growth inhibitory proteins are not only important for regeneration, but have an important role in regulating plasticity and axon – glial interactions. The novel findings provide new avenues to explore the neuronal response to growth inhibitory molecules and lead to further understanding of the barriers to axon regeneration after injury in the CNS.

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Appendix D

SIXTH ARTICLE

« Targeting Rho to stimulate repair after spinal cord injury. Lisa McKerracher and Matthew J. Winton. TISCR 8 (4): 69-75, 2003.»

Targeting Rho to Stimulate Repair After Spinal Cord Injury

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Running Title: Targeting Rho to Stimulate Repair

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Summary

Growth inhibitory proteins block axon regeneration in the CNS. Many growth inhibitory proteins have been identified, and the Nogo66 receptor (NgR) binds the three major myelin-derived inhibitors. Many studies now indicate that NgR signals to the small GTPase Rho. Our studies have shown that Rho is an important intracellular target for overcoming growth inhibition and promoting axon regeneration after injury. The inactivation of the Rho signaling pathway promotes neurite outgrowth of primary neuronal cell cultures plated on growth inhibitory substrates. To inactivate Rho signaling C3 transferase, C3-05 (Cethrin™), a more potent C3 transferase derivative, or Y-27632, an inhibitor of Rho kinase, have been tested. In vivo, we have documented the regeneration of transected axons after treatment with C3 transferase, C3-05 and Y-27632. C3 transferase and C3-05 have been tested in two different animals models, microcrush lesion of the adult rat optic optic nerve, over-hemisection of adult mouse spinal cord. Treatment with C3-05 gave similar results to C3 transferase, except that the required doses are much lower. Animals treated with C3 transferase, C3-05, or Y-27632 after SCI all showed axon regeneration and impressive functional recovery. Inactivation of Rho to promote regeneration and functional recovery after SCI is simple, and our studies reveal the potential for a new, straightforward technique to promote axon regeneration.

Introduction

Injury in the spinal cord disrupts or breaks axons, which are the fine projections of neurons that act as fibres of communication. Neurons in the central nervous system (CNS) have a remarkable capacity to regenerate their transected axons when provided with an appropriate growth environment. Advances in our understanding of axon regeneration have allowed the development of different experimental strategies to stimulate axon regeneration in animal models of spinal cord injury (SCI). By replacing the CNS environment with a peripheral nerve graft, the Aguayo group clearly demonstrated that damaged axons once thought to lack the intrinsic ability to regenerate could re-grow long distances in a permissive environment(Aguayo et al., 1991). The inability of the CNS environment to support growth was initially thought to be solely due to a lack of growth promoting signals, however, Schwab and colleagues demonstrated that the CNS environment contains growth inhibitory proteins that block regeneration(Schwab et al., 1993). Antibodies raised specifically against growth inhibitory proteins, or complete myelin have been used to promote axon regeneration in the spinal cord. Many potent growth inhibitory molecules are concentrated in myelin, the white matter territory where projection neurons extend long axons. Other inhibitory proteins such as proteoglycans are expressed by cells that form the scar directly at the lesion site. Therefore, the challenge is to find a strategy that blocks all the CNS growth inhibitory activity. A growing body of evidence suggests that the Rho signaling pathway regulates the neuronal response to diverse growth inhibitory proteins. Therefore, Rho is a new and potentially very powerful target to promote repair after spinal cord injury.

Growth inhibitory proteins

Many different groups have now documented an important growth inhibitory activity present in myelin. The inhibitory role of myelin was for a time questioned because several studies showed that transplanted neurons grew axons on white matter (reviewed by Brittis and Flannagan). (Brittis and Flanagan, 2001)More recent experiments on conditioning lesions and priming of neurons have helped to explain why some adult neurons grow on inhibitory substrates, (Snider et al., 2002)however, it is clear that injured neurons do not regenerate their axons spontaneously on white matter in the absence of experimental interventions. Moreover, another new study suggests that the balance between negative inhibitory cues and positive growth cues may be a critical determinant for regeneration. Chondroitinase ABC used to remove inhibition by chondroitin sulfate proteoglycans, not myelin, has also been a successful strategy to promote regeneration(Bradbury et al., 2002). In this study axons grew around the lesion area, which typically becomes demyelinated. It will be important to learn to if chondroitinase treated axons grow on myelin. However, a wealth of data shows axons do not grow well on myelin, and myelin inhibitors remain an important field of investigation for studies of regeneration in the CNS.

The first myelin-derived inhibitory activity to be identified was myelin-associated glycoprotein (MAG), a well-characterized myelin component. The inhibitory activity of MAG was discovered independently by our laboratory(McKerracher et al., 1994a) and the Filbin group(Mukhopadhyay et al., 1994). Several inhibitory proteoglycans that associate with myelin were also identified, [8, 9]although their role in myelin biology is less understood. Another growth inhibitory protein, called Nogo was reported next. Different splice variants of Nogo exist and Nogo contains two different inhibitory domains, Nogo66

and Amino-Nogo(Chen et al., 2000). Most recently, another myelin-derived growth inhibitory protein, oligodendrocyte mylelin glycoprotein (OMgp) was identified independently by two groups. (Kottis et al., 2002; Wang et al., 2002b)

New evidence suggests that the three major myelin derived growth inhibitory proteins, MAG, Nogo66 and OMgp all bind to the same neuronal receptor, the Nogo66 receptor (NgR) (reviewed by McKerracher and Winton(McKerracher and Winton, 2002)). NgR is expressed in neurons and located on the cell surface by glycosylphosphatidylinositol (GPI) linkage. This means that NgR does not have an intracellular signalling domain, and must function as part of a signalling complex. Amino-Nogo does not bind NgR, and therefore additional neuronal receptors are likely expressed by neurons to mediate growth inhibition by Nogo as well.

Growth inhibitory proteins present at the glial scar near an injury site are another important barrier of axon growth. Reactive astrocytes that form the glial scar express many different types of chondroitin sulfate proteoglycans (CSPG). CSPG core proteins that inhibit axon growth include versican, phosphocan, NG2 and neurocan. The full complement of proteoglycans expressed at the glial scar, and their time course of expression, has not been thoroughly explored. However, in tissue culture, reactive astrocytes block axon growth and express inhibitory proteoglycans(Fawcett and Asher, 1999; McKeon et al., 1995). Other cell types also contribute to scar formation. For example, meningeal cells express growth inhibitory proteins on their cell surfaces and invade the scar region to line the lesion cavity(Fawcett and Asher, 1999). The neuronal receptors for the proteoglycans expressed at the glial scar are not known.

Recent evidence from our lab and others suggests that all of the growth inhibitory proteins share a common signaling pathway in neurons, and the key-signaling component involved is Rho GTPase(Bito et al., 2000; Dergham et al., 2002; Lehmann et al., 1999; Vinson et al., 2001; Winton et al., 2002). Rho is important in development for its role in regulating the neuronal response to chemorepulsive guidance molecules(Wahl et al., 2000). Active Rho is known to cause growth cone collapse and neurite retraction(Jalink et al., 1994). The inactivation of Rho, however, allows neurons to extend axons on growth inhibitory myelin and proteoglycan substrates(Dergham et al., 2002). Moreover, it is suggested that the NgR signalling complex is linked to the Rho signalling pathway (see McKerracher and Winton(McKerracher and Winton, 2002)). Therefore, the binding of MAG, Nogo or OMgp ligands to NgR results in the activation Rho, and this has only been directly shown for MAG. The activated form of Rho then signals downstream growth inhibitory effects. Therefore, targeting Rho should block growth inhibitory signalling by both NgR mediated mechanisms, as well as those that do not act through the NgR, such as Amino-Nogo, chemorepulsive molecules, and proteoglycans.

Rho GTPases

Rho GTPases are a family of highly related proteins that are present in all cells as important signalling switches. Rho is best characterized for its effect on the actin cytoskeleton and the regulation of motility, but recent evidence also implicated Rho in the regulation of apoptosis[23]. In neurons, inactivation of Rho is necessary for axonal growth and extension, both in development and after injury. GTPases have two conformations: a GDP-bound inactive state and a GTP-bound active state. We discovered that in neurons,

Rho is activated in response to contact with myelin(McKerracher and Winton, 2002). Therefore, when neurons contact a growth inhibitory substrate, the activation of Rho prevents regeneration. In contrast, the inactivation of Rho promotes axon growth on myelin and other inhibitory substrates.

Antagonists of Rho signaling

Various strains of bacteria produce proteins that act as toxins by causing functional changes to target proteins. The bacteria *Clostridium botulinum* produces several toxins, one of which, C3 transferase, specifically inactivates Rho, but does not posses a natural cell-binding component that would allow it to efficiently enter cells. Methods that disrupt the cell membrane help aid the entry of C3 into cells. Such methods, however, are not efficient tools for delivery, and are only effective when high concentrations of C3 are used(Winton et al., 2002). To overcome this limitation, we have constructed a permeable form of C3 transferase, C3-05 (Cethrin^{TM}), which can freely cross the cell membrane to inactivate Rho(Winton et al., 2002). C3-05 promotes growth when cultured neuronal cells are plated on growth inhibitory substrates at concentrations 10,000 times lower than unmodified C3. By increasing the efficiency of C3 transferase, doses are now in the range feasible for clinical development.

When Rho is active it binds to and activates several protein kinases. One downstream target of Rho, Rho Kinase (ROK) is a key regulator of neurite growth(Bito et al., 2000). When ROK is activated it induces neurite retraction. A Rho kinase inhibitor, Y-27632, has been shown to specifically inactivate ROK, and it promotes growth of neurites, both on

permissive substrates(Bito et al., 2000) and on growth inhibitory substrates(Dergham et al., 2002). We have shown that when ROK activity is inhibited with Y-27632, primary neuronal cultures extend neurites on myelin, proteoglycan or mixed substrates(Dergham et al., 2002). The importance of Rho kinase in growth inhibition is underlined by studies with a second Rho kinase inhibitor, fasudil hydrochloride. Fasudil improves the motor function recovery of rats after spinal cord injury(Hara et al., 2000b), as does treatment with C3, Y-27632(Dergham et al., 2002), or C3-05 (unpublished).

Inactivation of Rho promotes regeneration on white matter in the optic nerve

The optic nerve is a well-established experimental model of axon regeneration in the CNS. The neurons in the retina that project to the brain are the retinal ganglion cells (RGCs). RGCs send a long axon projection into the optic nerve, a pure while matter environment. The simple anatomy of the optic nerve and ease of studying RGC axons makes injury of the optic nerve an attractive model to study regeneration in the CNS. We used a microcrush lesion to examine the role of Rho in the regeneration of injured CNS axons. Typically after transection, cut axons in the optic nerve stop growing abruptly at the lesion site. However, when Rho is inactivated by C3, cut axons were able to extend past the lesion site into the myelin rich white matter(Lehmann et al., 1999), and these studies have been confirmed with C3-05 (unpublished). Moreover, axons visible past the lesion site had a twisted path of growth, characteristic of regenerating axons. These results demonstrate that the small GTPase Rho has a crucial role in regulating the growth of CNS axons.

Inactivation of Rho signaling pathway promotes regeneration after spinal cord injury

These successful results in promoting regeneration of cut RGC axons in the optic nerve lead us to examine the inactivation of Rho after spinal cord injury as a strategy to promote repair. To study spinal cord injury in adult mice, we used a dorsal over-hemisection at vertebral level T7(Dergham et al., 2002). Immediately after injury, C3 transferase or Y-27632 was delivered directly into the lesion in a fibrin matrix. Fibrin is a protein clot formed when fibrinogen is cleaved by thrombin. Fibrinogen, thrombin and C3 were mixed together, the solution was injected on the site of spinal cord injury, and immediately after injection the liquid polymerized into a clot holding the C3 transferase or Y-27632 in place at the site of lesion. Studies where we have removed the spinal cord tissue 24 hours or one week after application of C3-05 in fibrin indicate that this method successfully retains the Rho antagonist at the site of injury where it is needed.

After SCI the cortico-spinal tract (CST) was anterogradely labeled to study if the treatments with C3 transferase elicited regeneration. The CST is a descending pathway in the dorsal spinal cord of mice. One month after SCI, wheat germ agglutinin horseradish peroxidase (WGA-HRP) was injected into several sites of the motor cortex. Two days after the WGA-HRP injections, the animals were fixed by perfusion, and longitudinal cryostat sections of the spinal cord were obtained and reacted for HRP enzymatic activity. The sections were also counter-stained with neutral red, allowing confirmation that the lesion scar extended past the central canal. In control animals, the bundle of CST axons had retracted from the lesion site, as previously observed. Treatment of the injured spinal cord with C3 transferase or Y-27632 caused extensive axonal sprouting, as well as the

regeneration of axons. After treatment with C3 transferase, axons were observed as far as 12 mm past the lesion site. Treatment of spinal cord injured adult mice with Y27632 resulted in the regeneration of axons to distances of up to 3 mm past the lesion site(Dergham et al., 2002).

To successfully regenerate, injured axons must express the genes necessary to promote and sustain growth. One such growth-associated gene, the GAP-43 gene, is expressed in all neurons during development when neurons are extending axons. This gene is also re-expressed in regenerating axons after injury. To confirm the presence of long distance regeneration in C3 transferase treated animals, we examined the expression levels of GAP-43 protein in the neuronal cells bodies of CST axons. In contrast to untreated animals, a high level of GAP-43 expression was observed in the motor cortex of C3-treated animals(Dergham et al., 2002). These results indicate that the inactivation of Rho by C3 transferase treatment results in the expression of growth-associated genes required for axon regeneration.

Functional recovery after spinal cord injury and treatment with Rho antagonists

Growth of cortico-spinal fibers does not necessarily correlate with functional recovery. Therefore to assess the functional recovery of animals after treatment with C3 transferase, we used a rating scale that has been developed to measure and evaluate the motor performance of injured animals(Dergham et al., 2002). Both treated and control mice were followed for one month. A continuous improvement in walking skills was observed in the treated mice while control animals did not recover beyond a plateau at 1-2 weeks.

Unexpectedly, mice treated with C3 transferase, or Y27632 showed a dramatic behavioral recovery after 24 hours. These results occur at too early of a time point to be explained by long distance regeneration. New results indicate that treatment with C3-05 has neuroprotective properties (unpublished), and it is likely that early neuroprotection limits the extent of injury. These finding on functional recovery after C3 transferase or Y27632 are in agreement with the functional recovery reported after use of the Rho kinase inhibitor fasudil after SCI in rats(Hara et al., 2000b).

While inactivation of Rho after spinal cord injury stimulates long-distance axon regeneration, we cannot correlate the observed functional recovery with axon regeneration. Improvement in locomotor tests cannot be correlated with the regeneration of specific tracts. Also, it is possible our treatments enhance the spontaneous recovery process that occurs after SCI. In our studies, the neuroprotective effects of C3 could play an important role in improved recovery. While the late recovery of hindlimb-forelimb coordination observed at one month was consistent with regeneration of cut fibres, it is well documented that reorganization of collateral fibres of cut axons occurs after incomplete SCI(Weidner et al., 2001). C3 treatment may enhance spontaneous plasticity of axons and dendrites. Additionally, it has been shown that sparing of a few ventrolateral fibers may translate into significant differences in locomotor performance (reviewed by Rossignaol(Rossignol, 2000)). Therefore, many factors may contribute to functional recovery. Nonetheless, treatments that stimulate functional recovery in animal models give hope that effective treatment for spinal cord injury will be developed in the foreseeable future. Towards this end, we are continuing our studies with the new cell permeable Rho antagonists.

Conclusions

Our studies demonstrate the importance of Rho GTPase as intracellular target for promoting axon regeneration after CNS injury. In tissue cultures studies the direct inactivation of Rho, or the inhibition of Rho kinase was effective in promoting axon growth on myelin-derived growth inhibitory proteins and on inhibitory proteoglycan substrates. Using two different animal models of CNS injury we show that the inactivation of the Rho signaling pathway is a simple and effective method to promote the regeneration of transected axons. Furthermore, mice treated with C3 transferase, C3-05 or Y27632 after spinal cord injury showed increased improvements in functional recovery. These studies show the potential of targeting the Rho signaling pathway to promote functional recovery after spinal cord injury. Inactivation of neuronal Rho allows axons to regenerate directly in the native environment of the CNS by over coming growth inhibition.

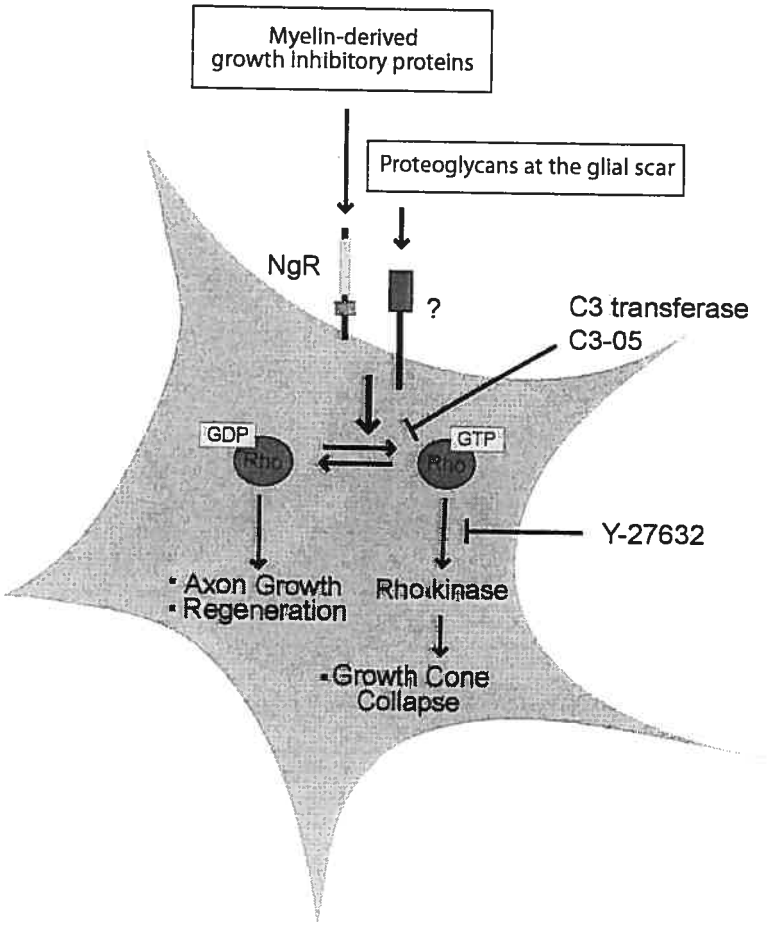

Figure 1. Inhibitory proteins block regeneration by activating Rho.

Growth inhibitory proteins in myelin and inhibitory proteins located at the glial scar after injury interact with neurons through receptors. The Nogo-66 receptor (NgR) binds three myelin inhibitors (MAG, Nogo-66 and OMgp), whereas receptors for proteoglycan inhibitors have not been well characterized. All growth inhibitory proteins share a common signaling pathway to Rho. Inactivation of Rho with C3 transferase or with C3-05, or inhibiting Rho kinase with Y-27632 blocks inhibitory signaling. Blocking activation of Rho signaling promotes axon growth and regeneration.

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Appendix E

SEVENTH ARTICLE

« Inactivation of intracellular Rho to stimulate axon growth and regeneration. Benjamin Ellezam, Catherine Dubreuil, Matthew Winton, Leanna Loy, Pauline Dergham, Inmaculada Sellés-Navarro, Lisa McKerracher. Prog Brain Res. 137: 371-380, 2002 .»

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Inactivation of Intracellular Rho to Stimulate Axon Growth and Regeneration

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Key words : GTPase, signaling, optic nerve, growth inhibition, spinal cord injury.

Summary

Our studies indicate that the small GTPase Rho is an important intracellular target for promoting axon regrowth after injury. In tissue culture, inactivation of the Rho signaling pathway is effective in promoting neurite growth on growth inhibitory CNS substrates by two different methods: inactivation of Rho with C3 transferase, and inactivation by dominant negative mutation of Rho. *In vivo*, we have documented the regeneration of transected axons after treatment with C3 in two different animals models, microcrush lesion of the adult rat optic optic nerve, and over-hemisection of adult mouse spinal cord. Mice treated with C3 after SCI showed impressive functional recovery although it important to point out that mice differ from rats in their response to spinal cord injury, especially in the extent of cavitation at the lesion site (Steward, 1999). It remains to be determined to what extent the regeneration of specific descending and ascending spinal axons contribute to the recovery, and whether inactivation of Rho enhances the spontaneous plasticity of axonal and dendritic remodeling after SCI. Inactivaction of Rho with C3 to promote regeneration and functional recovery after SCI is simple, and our studies show the potential for a new, straightforward technique to promote axon regeneration.

Introduction

Damage to neuronal function following spinal cord injury (SCI) arises from a complex series of reactions. A key determinant of functional loss after SCI is axon injury at the lesion site. Projection neurons that extend long axons within the spinal tracts are crucial for motor and sensory function, and their axons do not regenerate following transection, even though their cell bodies may remain alive for many years. This regenerative failure is explained in part by

the presence of growth inhibitory proteins. These molecules repress axon regeneration by severely limiting the ability of growth cones to extend. Most known growth inhibitory molecules are concentrated in myelin, the white matter territory where projection neurons extend long axons. Other inhibitory proteins such as proteoglycans are expressed by cells that form the scar directly at the lesion site. Therefore, one challenge to stimulate axon regeneration after injury is to overcome the neuronal response to the diverse types of inhibitory proteins that are expressed in the CNS.

As in development, growing axons in regeneration require the formation of a growth cone, the sensorimotor apparatus that forms at the proximal tip of a cut axon soon after injury. Regrowth of a cut axon depends on the coordinated assembly, disassembly and contraction of the actin cytoskeleton in the growth cone, and this process is responsible for the extension and retraction of the axon in response to positive and negative extracellular cues. In the mammalian CNS, it is thought that negative cues that limit regeneration have a stronger influence or are in greater abundance than the positive cues, which explains why growth cones fail to extend very far. In tissue culture, the response of growth cones to inhibitory molecules is to collapse, and growth cone collapse depends on the balance of inhibitory to growth promoting cues (David *et al.*, 1995; Wenk *et al.*, 2000).

Actin-mediated cell motility is regulated in all cells by the Rho family of GTPases. In neurons, intracellular Rho GTPases regulate the response of growth cones to both chemorepulsive guidance cues and growth inhibitory proteins (Dickson, 2001; Jin and Strittmatter, 1997; Kuhn *et al.*, 1999; Lehmann *et al.*, 1999; Wahl *et al.*, 2000). Growth inhibitory proteins that induce growth cone collapse activate Rho, and molecules that promote neurite growth inactivate Rho (Lehmann *et al.*, 1999; Wahl *et al.*, 2000; Wenk *et al.*, 2000).

We have investigated whether targeting Rho GTPase activity in neurons can allow them to ignore growth inhibitory signalling and grow directly on inhibitory substrates. The inactivation of Rho not only allows axon growth on myelin and chondroitin sulfate proteoglycan (CSPG) substrates, but also allows axon regeneration after injury in the CNS. Moreover, recent studies suggest that the inactivation of Rho may also have neuroprotective effects (Trapp, 2001).

Regulation of Rho GTPases

GTPases bind and hydrolyse GTP and cycle between active and inactive states. They are active when bound to GTP and lose their activity upon hydrolysis to GDP. (Bishop and Hall, 2000;Schwartz and Shattil, 2000). To date, more than ten mammalian Rho family members have been identified, and each Rho family member has several isoforms. Rho, Rac and Cdc 42 were the first identified and are the best characterised of the Rho family GTPases. Isoforms of the Rho group include Rho A, Rho B and Rho C (Takai et al., 2001). PC12 cells express Rho A, Rho B, Rho C and one unidentified Rho isoform (Lehmann et al., 1999) that may be Rho E, a Rho family protein that shares the effector domain of Rho A, B and C and promotes motility through actin reorganization. Unlike Rho A and Rho B, Rho E is not affected by C3-transferase, an inhibitor of Rho activity (Guasch, 1998; Wilde *et al.*, 2001). In neurons, Rho and Rac have opposing effects: active Rho inhibits growth and active Rac stimulates it (Lin *et al.*, 1994; van Leeuwen *et al.*, 1997). Interestingly, it was shown that the effect of neurotrophins on promoting neurite outgrowth is mediated by the p75 receptor by Rho inactivation (Yamashita *et al.*, 1999). Other studies examining the cross talk between different GTPases show that neurotrophins also activate Rac (Yamaguchi et al., 2001). *In vivo*, GTPases may affect both axons and dendrites differently (Luo *et al.*, 1996; Ruchhoeft, 1999), and one

consistent finding is that Rho is important in regulating growth cone motility. In the CNS, there is recent evidence that one isoform of Rho, Rho B, is up-regulated after ischemia suggesting that this GTPase may play a role in the neuronal response to injury (Trapp, 2001). Moreover, we have preliminary evidence that an imbalance in Rho expression and activity occurs after SCI, a change that could contribute to the failure of axons to regrow after injury. While the coordinated regulation of the different GTPases remains to be elucidated in regenerating axons, it is clear that the different Rho family GTPases regulate the initiation, growth and guidance of both axons and dendrites by acting on the actin cytoskeleton in response to diverse extracellular signals.

While Rho GTPases act as molecular switches cycling between active GTP bound and inactive GDP bound states, this switching is catalyzed by other proteins. The guanine exchange factors (GEFs) promote GTP binding to small GTPases. The GTPase activating proteins (GAPs) hydrolyse GTP, pushing the GTPase into the inactive GDP bound state. While Rho is expressed in all cell types, GEFs may exhibit cell-type specificity. Several GEFs that are known to play an important role in axon formation and guidance include Trio, a GEF for Rac, Rho and Cdc42 (Lin and Greenberg, 2000) and Tiam1, a GEF specific for Rac (Kunda et al., 2001). Once activated, the Rho GTPases bind and activate different effector proteins. A principal effector of activated Rho is Rho-associated kinase (ROK), a serine threonine kinase that is activated by Rho-GTP (Matsui et al., 1996). Microinjection of the catalytic domain of ROK into neurons induces neurite retraction, and inhibition of ROK with Y27632, a specific ROK inhibitor, promotes neurite outgrowth (Katoh et al., 1998). Therefore, inhibiting either Rho or its effector ROK is sufficient to promote neurite outgrowth in tissue culture.

Rho GTPases play an important role in integrating different signaling pathways that influence growth cone morphology and collapse (Fig. 1). Recently, Wahl *et al.* (Wahl et al., 2000) demonstrated that ephrin-A5, a known inhibitory molecule and ligand of the Eph tyrosine kinase receptors, causes the collapse of growth cones by activating Rho A and down-regulating Rac1. This induced collapse was significantly reduced when the cultures were pretreated with the Rho inhibitor C3-transferase, or the ROK inhibitor Y27632. Consistent with these findings, a newly discovered Rho family GEF, ephexin, links Eph/ ephrin receptor complex to intracellular signaling by Rho GTPases to influence growth cone collapse. Ephexin can strongly activate both Rho A and Cdc42, but can only weakly activate Rac1. These studies provide strong evidence for a direct link between extracellular growth inhibitory cues and Rho GTPases. Although we are just beginning to understand how extracellular guidance cues control cytoskeleton dynamics and growth cone motility in neurons, there is growing evidence that GTPases in non-neuronal cells are directly modulated by many different extracellular cues. A link between integrin signaling and Rho has been well established in fibroblasts (Adams and Schwartz, 2000; Wenk *et al.*, 2000), and integrin binding to laminin is well known to promote neurite outgrowth (David et al., 1995). Therefore, Rho appears to integrate diverse positive and negative signals in axon regeneration.

Extracellular cues can affect many other aspects of cellular regulation, particularly the levels of intracellular cAMP. There is an interresting link between Rho signalling and cAMP levels. Increased cAMP levels allow neurons to extend neurites on inhibitory substrates (Cai et al., 1999) and it is thought that endogenous cAMP levels determine the regenerative capacity of a neuron (Qiu, Cai and Filbin, this volume). Increases in cAMP levels are known to inactivate Rho (Lang et al., 1996), and changes brought about by increasing cAMP levels can

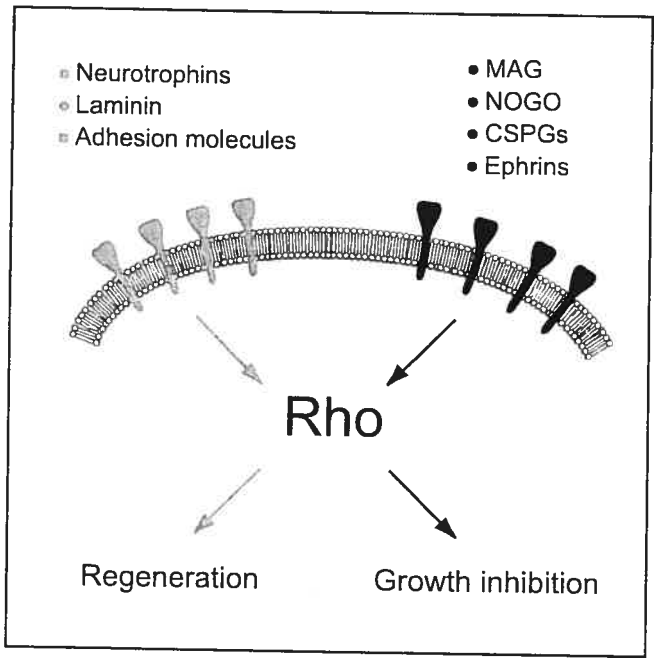

Figure 1. Schematic diagram to show that multiple growth signals converg to Rho. The activity state of Rho is influenced by extracellular cues through both positive (gray) and negative (black) receptor-mediated signaling.

be counteracted by Rho activation and by ROK (Dong et al., 1998). Either cAMP or Rho can be manipulated to promote neurite outgrowth in the presence of growth inhibitory molecules (Lehmann et al., 1999; Song et al., 1998). Thus, the Rho pathway appears to act downstream of cAMP, and Rho represents a specific and important target to promote axon growth.

While Rho is best known for regulating the actin cytoskeleton (Mackay and Hall, 1998), more recent evidence implicates activation of Rho with apoptosis. An upregulation of Rho B occurs in ishemia-injured neurons, and stabilization of the actin cytoskeleton helps protect neurons from ishemic cell death (Trapp, 2001). In non-neuronal cells, Rho B is required for apoptosis and regulates the apoptotic response of neuroplastic cells to DNA damage (Liu and Strittmatter, 2001). Therefore, growing evidence suggests that the inactivation of Rho should not only promote axon regeneration but also limit cell death after injury.

Inactivation of Rho by C3-transferase promotes neurite growth

C3-ADP ribosyltransferase is a 24kD exoenzyme synthesized by *Clostridium botulinum* which specifically ADP ribosylates the Rho A, B, and C isoforms, but not any other members of the Rho families (Wilde and Aktories, 2001). We used recombinant C3-transferase to test if the inactivation of Rho would allow axons to grow on complex myelin and proteoglycan growth inhibitory substrates. The cDNA encoding C3 was cloned into a pGex2T vector (Amersham Parmacia, Quebec Canada) that has a glutathione-S-transferase (GST) tag. The recombinant C3 protein was produced in E.coli and purified by affinity purification with glutathione-agrose beads. Thrombin was used to cleave the GST tag from the purified protein, and p-aminobenzamidine agrose beads were used to remove thrombin. Purified C3 was then centrifuged to remove the beads, concentrated, desalted, and stored at –80°C.

To test the ability of C3 to promote neurite growth on inhibitory substrates we have examined two types of primary neuronal cells : retinal neurons and cerebellar granule cells that were isolated from early postnatal rats. Retinal neurons were dissociated and plated on inhibitory substrates made of either MAG, myelin or chondroitin sulfate proteoglycans. Addition of C3 allowed retinal neurons to grow neurites on all three inhibitory substrates, and brought an increase in both the number of neurons extending neurites and the average neurite length. Cerebellar neurons were dissociated, prelabelled with DiI, and triturated with C3 (25-50μg/ml) or buffer, then plated on laminin as a growth promoting substrate (Fig. 2, A and B) or myelin-associated glycoprotein (MAG) as a growth inhibitory substrate (Fig. 2, C and D). With cerebellar neurons, C3 treatment allowed neurite outgrowth on MAG (Fig. 2D) and potentiated neurite outgrowth on laminin (Fig. 1B). Untreated cerebellar neurons plated on MAG did not extend neurites (Fig. 2C).

Although C3 is a very effective way to inactivate Rho and stimulate neurite outgrowth, it is not very cell permeable. Thus, trituration of primary neurons was necessary to enable C3 to enter the cell and allow neurites to grow on inhibitory substrates. However, to improve delivery of C3, we have developed permeable forms that allow us to simply add it to the cell culture medium. Experiments using our new C3-like constructs give the same results as those with C3, the advantage being that lower doses are required.

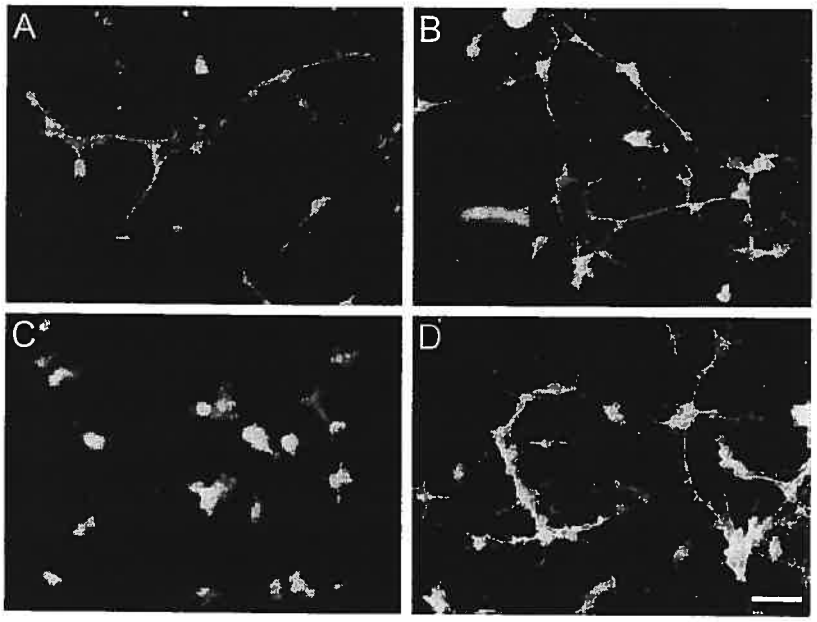

Figure 2. Treatment of cerebellar granule cells with C3 promotes neurite outgrowth. (A and B) Dissociated cerebellar neurons treated with (A) buffer or (B) C3 and plated on laminin. (C and D) Neurons treated with (C) buffer or (D) C3 and plated on MAG. Treatment of neurons with C3 potentiated neurite growth on laminin, and allowed neurons to extend neurites on a growth inhibitory substrate.

Inactivation of Rho is sufficient to promote neurite growth on inhibitory substrates.

To verify that inactivation of Rho was sufficient to allow neurons to extend neurites on inhibitory substrates, we examined the ability of PC12 cells transfected with dominant negative RhoA (N19TRhoA) to grow on recombinant MAG substrates. Transfected N19TRhoA cells and mock-transfected PC12 cells were examined for their ability to extend neurites when plated on inhibitory MAG substrates. Inactivation of RhoA by dominant negative mutation was sufficient to allow N19TRhoA cells plated on MAG substrates to extend neurites, and by contrast, mock transfected cells were unable to grow on MAG substrates (Lehmann et al., 1999). It is not known if dominant negative mutations of Rho expressed *in vivo* by gene therapy techniques would allow regeneration after axonal injury. In this case, the dominant negative Rho expressed in the cell body would have to be transported to the tip of the transected axon to be effective. It would be useful to know if this transport occurs at the slow or fast rates of axonal transport, and if direct delivery of dominant negative Rho to a neuronal cell body could stimulate the growth of an axon cut many centimeters away.

The optic nerve microcrush model to study axon regeneration

Aguayo and colleagues first showed adult RGC axons could regenerate if they were provided with an alternative environment such as a peripheral nerve graft (So and Aguayo, 1985; Vidal-Sanz *et al.*, 1987). It is apparent now that RGCs can regenerate in their native optic nerve environment if given trophic support (Berry *et al.*, 1996; Berry *et al.*, 1999; Leon *et al.*, 2000), if the optic nerve environment is modified by cell transplantation (Lazarov-Spiegler *et al.*, 1996), or if growth inhibitory myelin proteins are blocked (Ellezam and McKerracher,

2000; Weibel *et al.*, 1994). To develop those strategies, investigators have relied on optic nerve crush to unequivocally axotomize all RGCs yet preserve tissue integrity between proximal and distal segments. Standard crush lesions cause less cell death and optic nerve damage than complete transection of the optic nerve (Berkelaar *et al.*, 1994;Kiernan, 1985). However, crushing the nerve with forceps creates an area of cavitation with a poorly delimited injury site that makes quantitation of axon regeneration difficult (Berry *et al.*, 1996; Giftochristos and David, 1988; Weibel *et al.*, 1994; Zeng *et al.*, 1994). Therefore, we developed a new type of lesion where complete axotomy is achieved by constricting the optic nerve with 10-0 sutures for 60 seconds (Lehmann et al., 1999). This microcrush lesion results in a clear and defined injury site that is suitable for precise anatomical studies of axon retraction and regeneration (Selles-Navarro et al., 2001a).

To characterize the microlesion model we have examined the neuronal and non-neuronal responses to a microcrush lesion. We have found that as early as 6 hr following microcrush of the optic nerve, anterogradely labeled RGC axons retract up to 200 μm from the lesion site, and in the following week they sprout back toward the site of lesion where they abruptly stop (Selles-Navarro et al., 2001b). This initial growth response is consistent with the early sprouting observed by Zeng *et al.* (1994) using electron microscopy. At 2 weeks, still very few axons grow past the injury site, most of them still remaining on the proximal side (Figure 3C). As for the non-neuronal response, it is similar to that observed after typical optic nerve crush made with forceps (Berry et al., 1996), although constrained to a smaller lesion area. Immediately after injury (24 hr) there is a GFAP-negative region, while CSPGs detected with CS56 antibody are expressed along a discrete injury line (Selles-Navarro et al., 2001a). CSPG immunoreactivity remains detectable for at least 2 weeks, indicating formation of a persistent

glial scar. In fact, it might be more appropriate to refer to the glial scar as the lesion scar since invading meningeal cells contribute importantly to its formation (Selles-Navarro et al., 2001a). Indeed, a network of newly formed blood vessels quickly fills the injury site and appears to form of a tight physical barrier.

Studying the microcrush lesion model has provided us with some insight on the reason for RGC regeneration failure. Anterograde tracing examination clearly shows that early after optic nerve lesion adult RGCs have retained their potential to regenerate, since after the initial retraction they can regrow for up to 200 μm. However, without any treatment, the vast majority of axons stop abruptly at the C556 immunoreactive lesion barrier. Yet, despite the presence of a barrier, some axons do cross the lesion site, but only grow within the myelin-rich white matter for very short distances. More often these axons will either be contained outside the nerve and along the sheath or in the first 50 μm past the lesion site, within the limits of the myelin-free zone (Lehmann et al., 1999; Selles-Navarro et al., 2001a) suggesting the importance of myelin inhibitors in blocking regeneration. Moreover, when myelin inhibitors are blocked without any additional intervention, significant regeneration is observed (Weibel et al., 1994), a regrowth most likely driven by the initial sprouting reaction seen after injury. Thus, both the lesion scar and myelin inhibitors contribute to the lack of significant RGC regeneration after optic nerve injury.

Another possible explanation for RGC regenerative failure is the delayed apoptosis that follows axotomy. Indeed, five days after optic nerve injury RGCs rapidly start to die (Berkelaar *et al.*, 1994). However, one week after microlesion about 60% of RGCs still survive (Selles-Navarro et al., 2001a) and their axons can still actively transport the anterograde tracer

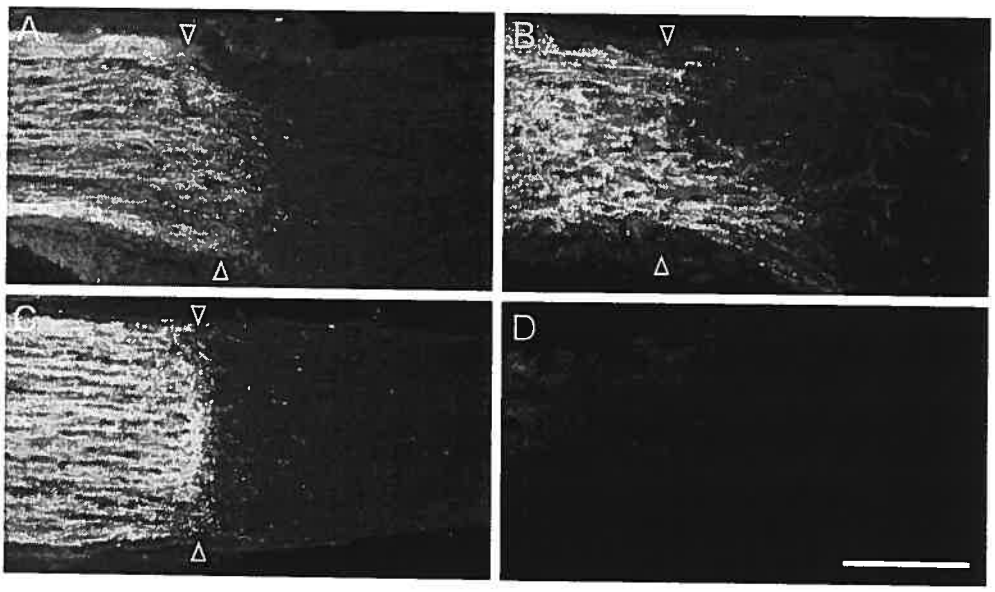

Figure 3. Regeneration of retinal ganglion cells axons after injury and treatment with C3. (A) Seven days after injury and C3 treatment many axons cross the lesion site as compared to animals treated with buffer alone where axons stop abruptly at the lesion site. (B) Two weeks after C3 treatment many axons cross the lesion scar (arrow heads) to grow in the distal white matter. (C) Control animal treated with buffer in the Gelfoam and Elvax 7 days after injury and treatment. (D) Normal optic nerve showing that cholera toxin B subunit, used as the anterograde tracer in these studies, does not accumulate when there is no lesion.

cholera toxin B. Moreover, one week after optic nerve injury, most surviving cells have normal morphology when observed after Fluorogold labelling and do not show signs of impending apoptosis (Kikuchi *et al.*, 2000). Yet, all those surviving RGCs typically do not extend an axon farther than the lesion site. Therefore, the poor regeneration observed at 7 days cannot be accounted for by poor cell survival alone, making growth inhibition an essential target for new regeneration strategies.

C3 promotes axon regeneration of retinal ganglion cell axons

To test if blocking Rho signaling could promote axon regeneration *in vivo*, we studied the effect of C3 on RGC regeneration after optic nerve microcrush (Lehmann et al., 1999). We applied C3 to the lesion site as a 2 mg/ml solution absorbed in Gelfoam, and then wrapped the Gelfoam around the nerve at the site of lesion. In addition, two 3 mm long tubes of Elvax, a slow release polymer loaded with 20 μg C3, were inserted in the Gelfoam near the nerve for continued slow release of C3. For controls, phosphate buffered saline (PBS) was used in the Gelfaom and Elvax implants. Seven days or two weeks after optic nerve crush, RGC axons were anterogradely labeled with cholera toxin β subunit injected into the vitreous, then the animals were fixed by perfusion with 4% paraformaldehyde and longitudinal cryostat sections of the optic nerve were processed for immunoreactivity to cholera toxin β subunit. For a quantitative analysis the numbers of axons per section were counted at distances of 100 μm, 250 μm, and 500 μm, and at least 4 sections per animal were analyzed. Two C3 treated animals and four controls were examined 7 days after lesion. For examination 2 weeks post lesion, 16 animals were treated with C3, 10 animals were treated with buffer as controls, and 4 animals received microcrush lesion only.

After treatment of crushed optic nerve with C3, large numbers of axons extended through the site of the crush to grow in the distal optic nerve. Seven days after lesion, many axons had grown past the region of the lesion scar (Fig. 2A). In control animals, axons had sprouted to the lesion site by 7 days, but the vast majority stopped abruptly at the lesion site (Fig. 3C). Most of our observations were made two weeks after lesion where the results were even more dramatic. In C3-treated animals, many axons extended 500 μm past the lesion site by two weeks, and axons that extended in the distal optic nerve showed a twisted path of growth, supporting their identification as regenerating axons (Fig. 3B). In control animals, only a few axons were able to grow past the lesion at 1 week, and growth was not observably further at the two week time point. Finally, to rule out the possibility that some of the fibres observed past the lesion site are actually axons that were spared at the time of injury, we injected the antergrade tracer in the vitreous of untreated animals with intact optic nerves and allowed the same amount of time for the tracer to be transported before perfusion. Longitudinal sections of those nerves did not show axon profiles past the lamina cribosa (Fig. 3D). Although RGC somas showed accumulation of the tracer (data not shown), axons in turn did not retain it, most likely because the tracer accumulated at their synaptic endings. These experiments rule out the possibility that spared fibers were detected after microlesion and C3 treatment.

To quantitatively examine the differences between C3 and buffer-treated animals, we counted the number of axons in each section at distances of 100 μm, 250 μm, and 500 μm past the lesion site. Significantly more axons extended past the lesion in the C3-treated animals than in the microcrush lesion or buffer-treated controls at distances of 100 μm and 250 μm. Therefore, C3 applied to injured RGC axons can enter axotomized axons and promote robust axon regeneration in the inhospitable growth environment of the optic nerve. We speculate that

C3, which is not readily cell permeable, was effective in eliciting regenertion because it was able to enter injured axons. It is known that injured axons readily take up substances from their environment. Our current investigations are to determine to what extent the new permeable forms of C3 that we have made can further augment the regeneration response after injury.

In vivo Spinal Cord Injury Experiments

We have begun a series of experiments to test the ability of C3 to promote regeneration and functional recovery after spinal cord injury. For these studies, we have used a dorsal over-hemisection of the mouse spinal cord. We chose this model because we have previously used it to test a therapeutic vaccine to promote axon regeneration (Huang et al., 1999a) and because it is highly suitable to study by anterograde tracing the regeneration of fibres in the corticospinal tract. In considering the various *in vivo* models of spinal cord injury, it is important to keep in mind that each model has distinct advantages and disadvantages. The contusion model is believed to most closely resemble human SCI (Bresnahan *et al.*, 1987; Gruner, 1992; Wrathall *et al.*, 1985), and recovery of hindlimb movement can be measured with the BBB scale (Basso *et al.*, 1996). After contusion there is a rim of spared tissue, and therefore, this model is unsuitable for the unequivocal histological determination of successful axon regeneration.

To study the ability of C3 to promote axon regeneration in the injured spinal cord, we lesioned the spinal cord at the T7 level by cutting past the central canal with microscissors, then re-cutting with a surgical knife. One month later, wheat germ agglutinin horse radish peroxidase (WGA-HRP) was injected into several sites of the motor cortex. Two days later, the animals were fixed by perfusion, longitudinal cryostat sections obtained and reacted for HRP enzymatic activity, then counter-stained with neutral red. This staining allowed us to confirm

that the lesion scar extended past the central canal. In control animals, the bundle of CST axons retracted from the lesion site, as previously observed (Huang, 1999; Li *et al.*, 1996). In mice treated with C3, many axons extended into the lesion site and some axons were able to grow distances of up to 10 mm. Therefore, as in injured optic nerve, C3 used to inactivate Rho can promote axon regeneration in the injured adult spinal cord.

We have also studied recovery of hindlimb movement and walking in treated mice. Treated mice showed an improvement in locomotion within 24 hours. This rapid recovery may be due to neuroprotection because neuroprotection improves functional outcomes (Giménez y ribotta, Gaviria, Menet, and Privat, this volume). The activation of Rho has been implicated in cell death after ischemia in the CNS (Trapp, 2001), and thus, the inactivation of Rho by C3 may be neuroprotective. Moreover, treated mice showed recovery of walking with hind-limb-forelimb coordination. Control mice recovered walking, but did not recover coordination between hindlimbs and forelimbs.

At this stage of the research, we cannot correlate the functional recovery we observe with axon regeneration. The BBB openfield locomotor test cannot be correlated with the regeneration of specific tracts, and adaptive plasticity of preserved tracts is likely to contribute to recovery. In our studies, the potential neuroprotective effect of C3 could play an important role in improved recovery, and therefore, it is not clear to what extent improvements in the BBB score reflect the observed regeneration. While the late recovery of hindlimb-forelimb coordination we observe at one month is consistent with regeneration of cut fibres, it is well documented that reorganization of collateral CST fibres occurs after SCI (Weidner, 2001) and this process could be enhanced by C3 treatment that might enhance spontaneous plasticity of axons and dendritic remodelling. After incomplete SCI, there is plasticity of motor systems

attributed to cortical and subcortical levels, including spinal cord circuitry (reviewed by (Raineteau and Schwab, 2001). This plasticity may be attributed to axonal or dendritic sprouting of collaterals and synaptic strengthening or weakening. Additionally, it has been shown that sparing of a few ventrolateral fibers may translate into significant differences in locomotor performance (Brustein and Rossignol, 1998) since these fibers are important in the initiation and control of locomotor pattern through spinal central pattern generators (reviewed by (Rossignol, 2000). Moreover, the SC devoid of supraspinal input but with peripheral afferents is in and of itself capable of generating hindlimb locomotion through central pattern generators (Rossignol, 2000). Therefore, many factors may contribute to functional recovery. Nonetheless, treatments that stimulate functional recovery in animal models give hope that effective treatment for spinal cord injury will be developed in the foreseeable future. Towards this end, we are developing more effective recombinant Rho antagonists that have increased cell permeability and that can stimulate spinal cord repair at lower doses. This strategy to improve recovery after spinal cord injury is simple: a single recombinant protein given once soon after injury. Further research with different animal models is needed to more directly compare C3-induced functional recovery with the recovery observed with other strategies described in this volume.

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Appendix F

ARTICLE CONTRIBUTIONS

Publication:

Characterization of new cell permeable C3-like proteins that inactivate Rho and stimulated neurite outgrowth on inhibitory substrates. Matthew J. Winton, Catherine I. Dubreuil, Dana Lasko, Nicole Leclerc and Lisa McKerracher. J Biol Chem. 277(36): 32820-9; 2002.

Contribution:

For this publication I completed all experiments with the exception of the Rho pulldown assays and was the main author of the text. During the course of this project I learned a series of molecular and cellular biology techniques that allowed me to construct and purify the new chimeric proteins. Once purified I tested and characterized the constructs in tissue culture using PC-12 cells, fibroblast cells (NIH 3T3, CHO) and primary neurons (retinal ganglion cells).

Publication:

Growth inhibitory proteins contribute to Rho-dependent apoptosis of TNF-α. Matthew J. Winton, Catherine I. Dubreuil and Lisa McKerracher. Submitted.

Contribution:

For this publication I completed all experiments with the exception of the Rho pulldown assays and was the author of the text. During the course of this project I learned to culture cerebellar granule neurons and examined their response to growth inhibitory proteins.

Publication:

Inactivation of Rho signaling in neuronal cell bodies promotes neurite growth on inhibitory substrates and regeneration of retinal ganglion cell axons in the optic nerve of adult rats. Johanne Bertrand, Matthew J. Winton, Robert B. Campenot and Lisa McKerracher. Manuscript in preparation.

Contribution:

This study was completed as part of Johanne Bertrand's doctoral studies. As second author of this publication I was responsible for all *in vitro* studies. For the completion of these studies I collaborated with Dr. Robert Campenot at the University of Alberta and traveled to Edmonton on two separate occasions to complete this study. I also help correct and edit the manuscript prior to submission.

Publication:

Rho activation patterns after spinal cord injury and the role of activated Rho in apoptosis. Catherine I. Dubreuil, Matthew J. Winton and Lisa McKerracher. J Cell Biol. 21; 162 (2): 233-43. 2003.

Contribution:

This study was completed as part of Catherine Dubreuil's doctoral studies. As second author of this publication I was responsible for studies that examined, by immunohistochemistry, the cellular location of C3-05 after *in vivo* treatment and apoptosis quantification by TUNEL labeling. I also corrected and edited the manuscript prior to submission.

Publication:

Nogo on the go. Lisa McKerracher and Matthew J. Winton. Neuron. 36(3): 345-8; 2002.

Contribution:

This review article was written together with my supervisor, Dr. Lisa McKerracher. For this review article I wrote the section entitled *Signal Transduction by NgR and p75 ^{NTR}*. In addition I designed and drew the schematic figure (Fig.1), and helped to correct and edit the manuscript prior to submission.

Publication:

Targeting Rho to stimulate repair after spinal cord injury. Lisa McKerracher and Matthew J. Winton. Topics in Spinal Cord Injury Rehabilitation. 8(4): 69-75; Spring 2003.

Contribution:

This review article was written together with my supervisor, Dr. Lisa McKerracher. This review article is published in a non-scientific journal that services the spinal cord injury community and therefore was written for a lay audience. Dr. McKerracher provided me with a rough outline of the specific topics that were to be covered by this review and taking that into account I wrote the initial draft of the manuscript. In addition, I designed and drew the schematic figure (Fig.1) and helped to correct and edit the manuscript prior to submission.

Publication:

Inactivation of intracellular Rho to stimulate axon growth and regeneration. Benjamin Ellezam, Catherine Dubreuil, Matthew Winton, Leanna Loy, Pauline Dergham, Inmaculada Sellés-Navarro, Lisa McKerracher. Prog Brain Res. 137: 371-380, 2002:

Contribution:

This review article was written together with other members of the laboratory. For this review article I contributed to the sections regarding Rho GTPases and the *in vitro* effects of C3-transferase.